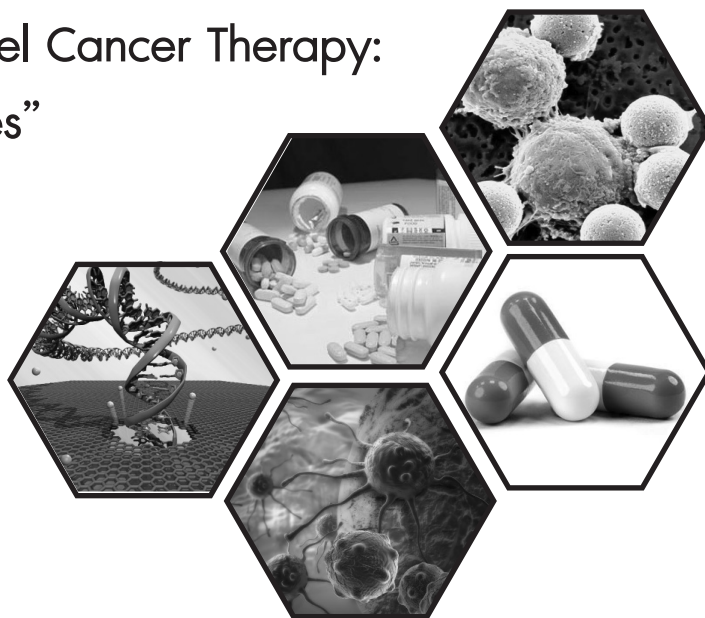การประชุมวิชาการสมาคมเภสัชวิทยาแห่งประเทศไทยครั้งที่ 37

37th

Proceedings of the Congress on Pharmacology of Thailand

"Genomic Medicine and Novel Cancer Therapy:
Challenges and Opportunities"

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Sunee Grand Hotel & Convention Center, Ubon Ratchathani

Organized by

The Pharmacological and Therapeutic Society of Thailand
Faculty of Pharmaceutical Sciences, Ubon Ratchathani University

THE 37th CONGRESS ON PHARMACOLOGY OF THAILAND

Genomics Medicine and Novel Cancer Therapy: Challenges and Opportunities

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At Tubtim Siam Room 4 - 5, the 5th Floor, Sunee Grand Hotel & Convention Center, Ubon Ratchathani

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สารบัญ

ผลงานวิชาการ

บทคัดย่อ

บทความฉบับเต็ม

สารจากอธิการบดีมหาวิทยาลัยอุบลราชธานี

      มหาวิทยาลัยอุบลราชธานี รู้สึกเป็นเกียรติอย่างยิ่งที่มีโอกาสต้อนรับผู้เข้าร่วมประชุมวิชาการ สมาคมเภสัชวิทยาแห่งประเทศไทย ครั้งที่ 37 ซึ่งจัดขึ้นในระหว่างวันที่ 28 – 30 พฤษภาคม 2558 ณ โรงแรม สุนีย์แกรนด์แอนด์คอนเวนชั่น เซ็นเตอร์ จังหวัดอุบลราชธานี ในปีนี้คณะเภสัชศาสตร์ มหาวิทยาลัย อุบลราชธานีเป็นเจ้าภาพร่วมกับสมาคมเภสัชวิทยาแห่งประเทศไทยจัดการประชุมในเรื่อง "Genomics Medicine and Novel Cancer therapy: Challenges and Opportunities" โดยมีวัตถุประสงค์เพื่อเป็นเวทีให้ นักเภสัชวิทยา บุคลากรทางด้านวิทยาศาสตร์สุขภาพ นิสิตนักศึกษาและผู้สนใจสามารถติดตามความก้าวหน้าทาง วิชาการจากวิทยากรผู้ทรงคุณวุฒิหลายท่าน ซึ่งปัจจุบันการศึกษาวิจัยและเทคนิคการพัฒนายาและผลิตภัณฑ์ส่งเสริม สุขภาพมีความซับซ้อนมากขึ้น มีการนำเทคโนโลยีใหม่ๆ มาบูรณาการเพื่อให้ได้ยาที่ตรงกับกลุ่มโรคเป้าหมายมาก ยิ่งขึ้น นอกจากนี้ยังเป็นเวทีให้ผู้เข้าร่วมประชุมได้มีโอกาสแสดงผลงานทางวิชาการ สามารถแลกเปลี่ยนความคิดเห็น และเรียนรู้ประสบการณ์ร่วมกันทั้งในด้านวิจัยและการสร้างเสริมองค์ความรู้ทางเภสัชวิทยาเพื่อเป็นประโยชน์ในการ ประยุกต์ใช้ในการปฏิบัติหน้าที่ให้ดียิ่งขึ้นอันจะส่งผลดีต่อสุขภาพของประชาชนในอนาคตอีกด้วย

      มหาวิทยาลัยอุบลราชธานี หวังเป็นอย่างยิ่งว่าผู้เข้าร่วมประชุมคงได้รับความรู้และความประทับใจจาก งานประชุมวิชาการในครั้งนี้ หวังว่ามหาวิทยาลัยอุบลราชธานีคงได้มีโอกาสต้อนรับท่านอีกในการประชุม ที่มหาวิทยาลัยจัดขึ้นในครั้งต่อไป

รองศาสตราจารย์นงนิตย์ ธีระวัฒนสุข
อธิการบดีมหาวิทยาลัยอุบลราชธานี

สารจากนายกสมาคมเภสัชวิทยาแห่งประเทศไทย

เรียน   สมาชิกสมาคมเภสัชวิทยาฯ และผู้เข้าร่วมประชุมทุกท่าน

สมาคมฯได้จัดให้มีการประชุมวิชาการของสมาคมฯเป็นประจำทุกปีนับตั้งแต่ปี พ.ศ. 2521 ทั้งนี้ เพื่อเป็นเวทีสำหรับ อาจารย์ นักวิจัย ตลอดจน นิสิตนักศึกษาในระดับบัณฑิตศึกษา เพื่อเผยแพร่ แลกเปลี่ยน ประสบการณ์ในงานวิจัย และก้าวทันวิทยาการใหม่ ๆ ทางเภสัชวิทยาและสาขาที่เกี่ยวข้อง

ในนามของสมาคมเภสัชวิทยาฯ ดิฉันขอถือโอกาสนี้แสดงความชื่นชมและขอบคุณต่อคณะเภสัชศาสตร์ มหาวิทยาลัยอุบลราชธานี ที่ได้ให้เกียรติในการเป็นเจ้าภาพในการจัดงานด้วยความมุ่งมั่นและทุ่มเทจนทำให้ งานสำเร็จลุล่วงไปด้วยดี โดยการประชุมครั้งนี้นับเป็นครั้งที่ 37 ของการประชุมวิชาการประจำปีของสมาคมฯ ในหัวข้อเรื่อง "Genomics Medicine and Novel Cancer Therapy: Challenges and Opportunities" จัดขึ้นใน ระหว่างวันที่ 28-30 พฤษภาคม 2558 ณ โรงแรมสุนีย์แกรนด์แอนด์คอนเวนชั่น เซ็นเตอร์ จังหวัดอุบลราชธานี วาระสำคัญซึ่งนับเป็นจุดเด่นของงานคือ"ปาฐกถาเกียรติยศ รองศาสตราจารย์ ดร. จิรวัฒน์ สดาวงศ์วิวัฒน์" และ "กิตติคุณประกาศ ศาสตราจารย์ นายแพทย์ อวย เกตุสิงห์"   เพื่อเป็นเกียรติและแสดงความกตัญญู กตเวทิตาแก่ทั้งสองท่านซึ่งถือว่าเป็นบรมครูและผู้มีคุโณปการอย่างสูงต่อวงการเภสัชวิทยาของประเทศไทย และเป็นแบบอย่างที่ดีแก่นักวิจัยรุ่นหลัง โดยสมาคมฯได้จัดให้มีการมอบรางวัลศาสตราจารย์ นายแพทย์ อวย เกตุสิงห์ แก่นักเภสัชวิทยาดีเด่นและผลงานวิจัยทางเภสัชวิทยาดีเด่น เป็นประจำทุกปี ตั้งแต่ปี พ.ศ. 2557

ดิฉันหวังเป็นอย่างยิ่งว่าทุกท่านจะได้รับความรู้จากการประชุมครั้งนี้ รวมทั้งความสุขใจ ในมิตรภาพอันดีระหว่างสมาชิกสมาคมฯ ผู้เข้าร่วมประชุม และสถานที่ท่องเที่ยวและธรรมชาติอันสวยงามของ จังหวัดอุบลราชธานี ขอขอบคุณวิทยากรทุกท่านที่ให้เกียรติและเสียสละเวลามาบรรยายในการประชุม และ ผู้สนับสนุนการประชุมจากทั้งภาครัฐและเอกชน

ในโอกาสนี้ดิฉันขอถือโอกาสประชาสัมพันธ์การประชุมวิชาการ "The 13th Asia Pacific Federation of Pharmacologist (APFP) Meeting" ซึ่งประเทศไทย โดยสมาคมเภสัชวิทยาฯ ได้รับเกียรติจาก สมาพันธ์นักเภสัชวิทยาแห่งเอเชียและแปซิฟิก "Asia Pacific Federation of Pharmacologists (APFP)" ให้เป็นเจ้าภาพในการจัดประชุม ในหัวข้อ "New Paradigms in Pharmacology for Global Health" ในระหว่าง วันที่ 1-3 กุมภาพันธ์ 2559 ณ โรงแรม เดอะ เบอร์เคลีย์ กรุงเทพมหานคร หวังเป็นอย่างยิ่งว่าจะได้รับความ ร่วมมือจากท่านสมาชิกและผู้เข้าร่วมประชุมในการสนับสนุนการประชุมครั้งนี้ให้สำเร็จลุล่วงไปด้วยดี ซึ่งจะเป็น การแสดงศักยภาพของนักวิจัยของไทยทางสาขาวิชาเภสัชวิทยา

ศาสตราจารย์เกศรา ณ บางช้าง

นายกสมาคมเภสัชวิทยาแห่งประเทศไทย

สารจากคณบดีคณะเภสัชศาสตร์ มหาวิทยาลัยอุบลราชธานี

คณะเภสัชศาสตร์ มหาวิทยาลัยอุบลราชธานีได้รับเกียรติเป็นเจ้าภาพร่วมกับสมาคมเภสัชวิทยา
แห่งประเทศไทย จัดประชุมวิชาการสมาคมเภสัชวิทยาแห่งประเทศไทย ครั้งที่ 37 ระหว่างวันที่ 28 –30
พฤษภาคม 2558 ณ โรงแรมสุนีย์แกรนด์แอนด์คอนเวนชั่น เซ็นเตอร์ จังหวัดอุบลราชธานี ในเรื่อง "Genomics
medicine and Novel cancer therapy: Challenges and opportunities" ซึ่งเป็นองค์ความรู้ใหม่ในการรักษา
โรคมะเร็งด้วยวิทยาการด้านจีโนม การจัดประชุมวิชาการครั้งนี้มีวัตถุประสงค์เพื่อเป็นเวทีให้นักเภสัชวิทยา
บุคลากรด้านวิทยาศาสตร์สุขภาพ นิสิตนักศึกษาและผู้สนใจสามารถติดตามความก้าวหน้าทางวิชาการจาก
วิทยากรผู้ทรงคุณวุฒินอกจากนี้ยังเป็นเวทีให้นักวิจัยได้แสดงผลงานทางวิชาการและแลกเปลี่ยนเรียนรู้
ประสบการณ์ร่วมกันทั้งในด้านการวิจัยและองค์ความรู้ทางเภสัชวิทยา เป็นที่น่ายินดีเป็นอย่างยิ่งที่การจัด
ประชุมวิชาการในปีนี้ ได้รับความสนใจจากนักวิจัยส่งผลงานทางวิชาการเพื่อร่วมนำเสนอในรูปแบบโปสเตอร์
จำนวนทั้งสิ้น 74 ผลงาน โดยเป็นรายงานสืบเนื่องการประชุมวิชาการ (Proceeding) จำนวน 31 ผลงาน และ
เป็นบทคัดย่อ (Abstract) จำนวน 43 ผลงาน

ในโอกาสนี้ ในนามของคณะผู้จัดงาน ใคร่ขอขอบพระคุณคณะกรรมการของสมาคมเภสัชวิทยา
แห่งประเทศไทยเป็นอย่างสูงที่กรุณาให้โอกาสคณะเภสัชศาสตร์ มหาวิทยาลัยอุบลราชธานีในการเป็นเจ้าภาพร่วม
ขอขอบพระคุณคณะกรรมการพิจารณาผลงานทางวิชาการ และคณะกรรมการตัดสินผลงานทางวิชาการทุกท่าน
ที่กรุณาเอื้อเฟื้อพิจารณาคุณภาพของผลงานวิจัย ขอขอบพระคุณวิทยากรผู้ทรงคุณวุฒิที่กรุณาสละเวลาให้
เกียรติมาบรรยาย และขอขอบคุณคณะกรรมการผู้จัดงานทุกท่านที่ให้ความร่วมมือ ช่วยเหลือ เกื้อกูลกันจนทำให้
งานประชุมสำเร็จลุล่วงเป็นอย่างดี

คณะเภสัชศาสตร์ มหาวิทยาลัยอุบลราชธานี หวังเป็นอย่างยิ่งว่าผู้เข้าร่วมประชุมคงได้รับความรู้
และความประทับใจจากงานประชุมวิชาการในครั้งนี้ และหวังว่าคณะเภสัชศาสตร์ มหาวิทยาลัยอุบลราชธานี
คงได้มีโอกาสต้อนรับท่านอีกในครั้งต่อไป

ผู้ช่วยศาสตราจารย์ชุตินันท์ ประสิทธิ์ภูริปรีชา
คณบดีคณะเภสัชศาสตร์ มหาวิทยาลัยอุบลราชธานี

หลักการและเหตุผล

การศึกษาวิจัยทางด้านวิทยาศาสตร์สุขภาพและด้านยามีการดำเนินไปอย่างต่อเนื่อง เพื่อพัฒนาคุณภาพชีวิต และสุขภาพของทุกคนให้ดีขึ้น ปัจจุบันการศึกษาวิจัยและเทคนิคการพัฒนายาและผลิตภัณฑ์ส่งเสริมสุขภาพมีความ ซับซ้อนมากขึ้น มีการนำเทคโนโลยีด้านต่างๆ มาบูรณาการเพื่อให้ได้ผลิตภัณฑ์สุขภาพที่ตรงกับกลุ่มโรคเป้าหมาย และได้ยาที่มีความปลอดภัยสู่ท้องตลาด ทันต่อภาวะโรคที่เปลี่ยนแปลงอย่างรวดเร็ว นอกจากนี้ ในสังคมไทยปัจจุบัน แนวโน้มพฤติกรรมการดูแลสุขภาพโดยการเสริมสร้างสุขภาพและป้องกันโรคมีมากขึ้น ผลิตภัณฑ์เพื่อการเสริมสร้าง สุขภาพและความงามจึงมีการผลิตออกมามากมาย รวมทั้งมีการแข่งขันทางการตลาดอย่างสูง จึงมีความสำคัญเป็น อย่างยิ่ง ที่เภสัชกรและบุคลากรทางการแพทย์ ต้องติดตามและพัฒนาตนเองอย่างต่อเนื่องในองค์ความรู้ใหม่ที่เกี่ยวข้อง ทั้งการพัฒนาผลิตภัณฑ์ชีวภาพ คุณภาพของผลิตภัณฑ์ ความปลอดภัยของผลิตภัณฑ์ การเฝ้าติดตามอาการไม่พึง ประสงค์ ตลอดจนการใช้ยาอย่างเหมาะสม สมาคมเภสัชวิทยาแห่งประเทศไทย ร่วมกับคณะเภสัชศาสตร์ มหาวิทยาลัย อุบลราชธานี จึงจัดประชุมวิชาการประจำปี ครั้งที่ 37 เพื่อเป็นเวทีให้นักเภสัชวิทยา บุคลากรทางด้านวิทยาศาสตร์ สุขภาพ ศิษย์เก่า นิสิตนักศึกษา และผู้สนใจสามารถติดตามความก้าวหน้าทางวิชาการ สามารถแลกเปลี่ยน ความคิดเห็น และเรียนรู้ประสบการณ์ร่วมกัน ทั้งในด้านวิจัยและการสร้างเสริมองค์ความรู้ทางเภสัชวิทยา เพื่อให้สามารถนำไปใช้ในการปฏิบัติหน้าที่ให้ดียิ่งขึ้นอันจะส่งผลดีต่อสุขภาพของประชาชนในอนาคต

วัตถุประสงค์การจัดการประชุมวิชาการฯ

1. เพื่อเป็นเวทีให้นักเภสัชวิทยา บุคลากรทางด้านวิทยาศาสตร์สุขภาพ ศิษย์เก่า นิสิตนักศึกษา และผู้สนใจสามารถติดตามความก้าวหน้าทางวิชาการ สามารถแลกเปลี่ยนความคิดเห็น และ เรียนรู้ประสบการณ์ร่วมกัน ทั้งในด้านวิจัยและการสร้างเสริมองค์ความรู้ทางเภสัชวิทยา
2. เพื่อเพิ่มพูนความรู้แก่เภสัชกร บุคลากรทางกาสาธารณสุข ศิษย์เก่าและผู้ที่สนใจ ในด้าน ความก้าวหน้าทางเภสัชวิทยา และองค์ความรู้ทางด้านวิทยาศาสตร์สุขภาพที่ทันสมัยเป็นปัจจุบัน ตลอดจนแนวโน้มในอนาคต
3. เพื่อส่งเสริมและสนับสนุนการพัฒนาเครือข่ายทางวิชาการสำหรับคณะเภสัชศาสตร์ นักเภสัชวิทยา ศิษย์เก่า และบุคลากรทางสาธารณสุข เป็นผลให้เกิดการประสานงานกิจกรรมเชิงวิชาชีพที่มี ประสิทธิภาพและเข้มแข็ง เช่น การฝึกปฏิบัติงานวิชาชีพของนักศึกษามหาวิทยาลัยอุบลราชธานี เป็นต้น
4. เพื่อเผยแพร่ชื่อเสียงและบทบาทของคณะเภสัชศาสตร์ มหาวิทยาลัยอุบลราชธานี

ผู้ทรงคุณวุฒิ

ศ. ดร. เกศรา ณ บางช้าง	มหาวิทยาลัยธรรมศาสตร์
ผศ. ดร. วรรณา ชัยเจริญกุล	มหาวิทยาลัยธรรมศาสตร์
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รศ. ดร. ศรีจันทร์ พรจิราศิลป์	มหาวิทยาลัยมหิดล
ผศ. ดร. พรพรรณ วิวิธนาภรณ์	มหาวิทยาลัยมหิดล
ผศ. ดร. สัณหภาส สุดวิลัย	มหาวิทยาลัยมหิดล
ผศ. ดร. อาทิตย์ ไชยร้องเดื่อ	มหาวิทยาลัยมหิดล
ผศ. ดร. ศรินทร์ ฉิมณรงค์	มหาวิทยาลัยมหิดล
ดร. ศิวนนท์ จิรวัฒโนทัย	มหาวิทยาลัยมหิดล
ดร. กฤษฎา ศักดิ์ชัยศรี	มหาวิทยาลัยมหิดล
ภก. อนันต์ชัย อัศวเมฆิน	มหาวิทยาลัยมหิดล
ศ. ดร. วีรพล คู่คงวิริยพันธุ์	มหาวิทยาลัยขอนแก่น
รศ. ดร. จินตนา สัตยาศัย	มหาวิทยาลัยขอนแก่น
รศ. ดร. วิจิตรา ทัศนียกุล	มหาวิทยาลัยขอนแก่น
รศ. ดร. สมทรง ลาวัณย์ประเสริฐ	จุฬาลงกรณ์มหาวิทยาลัย
รศ. ดร. ปิติ จันทร์วรโชติ	จุฬาลงกรณ์มหาวิทยาลัย
รศ. ดร. สุพีชา วิทยเลิศปัญญา	จุฬาลงกรณ์มหาวิทยาลัย
ผศ. ดร. พรพิมล กิจสนาโยธิน	จุฬาลงกรณ์มหาวิทยาลัย
ผศ. ดร. ภัสราภา โตวิวัฒน์	จุฬาลงกรณ์มหาวิทยาลัย
ดร. ปิยนุช วงศ์อนันต์	จุฬาลงกรณ์มหาวิทยาลัย
ดร. สพ. ญ. วัชราภรณ์ ติยะสัตย์กุลโกวิท	จุฬาลงกรณ์มหาวิทยาลัย
ผศ. ดร.กล่าวขวัญ ศรีสุข	มหาวิทยาลัยบูรพา
รศ. ดร. สุพัตรา ศรีไชยรัตน์	มหาวิทยาลัยรังสิต
ดร. วัชราภรณ์ เทวกุล ณ อยุธยา	มหาวิทยาลัยรังสิต
พ.อ. หญิง รศ. ภญ. นิสามณี สัตยาบัน	วิทยาลัยแพทยศาสตร์พระมงกุฎเกล้า
ผศ. ดร. ปวิตรา พูลบุตร	มหาวิทยาลัยมหาสารคาม
ผศ. ดร. อรพิณ วงศ์สวัสดิ์กุล	มหาวิทยาลัยศรีนครินทรวิโรฒ
ผศ.ดร.ตุลยา โพธารส	มหาวิทยาลัยศรีนครินทรวิโรฒ
ดร. สถาพร พฤฒิพรรลาย	มหาวิทยาลัยสงขลานครินทร์
ดร. วันดี อุดมอักษร	มหาวิทยาลัยสงขลานครินทร์
รศ. ดร. วันทนา เหรียญมงคล	มหาวิทยาลัยสงขลานครินทร์
ดร. ปิยจิต วัชรศิษย์	สถาบันวิจัยจุฬาภรณ์
รศ. ดร. รัตติมา จีนาพงษา	มหาวิทยาลัยนเรศวร
ผศ. ดร. สุริศักดิ์ ประสานพันธ์	มหาวิทยาลัยนเรศวร
ผศ. สพ. ญ. กาญจนา อิ่มศิลป์	มหาวิทยาลัยเกษตรศาสตร์
ผศ. ดร. ชุตินันท์ ประสิทธิ์ภูริปรีชา	มหาวิทยาลัยอุบลราชธานี
ผศ. ดร. ปรีชา บุญจูง	มหาวิทยาลัยอุบลราชธานี
ดร. เพียงเพ็ญ ธิโสดา	มหาวิทยาลัยอุบลราชธานี
ดร. ธนวดี ปรีเปรม	มหาวิทยาลัยอุบลราชธานี

กำหนดการ

THE 37th CONGRESS ON PHARMACOLOGY OF THAILAND
GENOMIC MEDICINE AND NOVEL CANCER THERAPY: CHALLENGES AND OPPORTUNITIES
28-30 MAY 2015
TUBTIM SIAM ROOM 4 - 5, The 5 FLOOR, SUNEE GRAND HOTEL & CONVENTION CENTER, UBON RATCHATHANI

วันที่ 28 พฤษภาคม 2558		
เวลา	กิจกรรม / หัวข้อ	วิทยากร
08.00-08.45 น.	ลงทะเบียน	
08.45-09.15 น.	พิธีเปิดการประชุมวิชาการ	อธิการบดี มหาวิทยาลัยอุบลราชธานี / นายกสมาคมเภสัชวิทยาแห่งประเทศไทย / คณบดีคณะเภสัชศาสตร์ มหาวิทยาลัยอุบลราชธานี
09.15-10.15 น.	ปาฐกถาเกียรติยศ รศ.ดร.จิรวัฒน์ สดาวงศ์วิวัฒน์ เรื่อง "Genomic medicine: a decade of successes, challenges, and opportunities in Thailand"	ศ. ดร. วสันต์ จันทราทิตย์ คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล
10.15-10.30 น.	รับประทานอาหารว่าง	
10.30-12.00 น.	Pharmacogenetic markers for drug-induced severe cutaneous adverse drug reactions Routine pharmacogenetic testing in clinical practice: dream or reality? - PGx markers for drug-induced SCARs - PGx markers and TDM for drug-induced ADR-A	ศ. ดร. ภญ. วิจิตรา ทัศนียกุล คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น ผศ. ดร. ภก. ชลภัทร สุขเกษม คณะแพทยศาสตร์ โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล
12.00-13.00 น.	Lunch symposium / รับประทานอาหารกลางวัน	บริษัท เพอร์กินเอลเมอร์ จำกัด
13.00-13.45 น.	ประชุมธุรการสมาคมฯ / นำเสนอผลงานวิชาการแบบโปสเตอร์ 1	
13.45-14.30 น.	Bioinformatics tools for genomic medicine - Systematic 3D screening of amino acid mutations in pharmacogenetics study.	อ. ดร. ภญ. ธนวดี ปรีเปรม คณะเภสัชศาสตร์ มหาวิทยาลัยอุบลราชธานี
14.30-14.45 น.	พักรับประทานอาหารว่าง	
14.45-15.45 น.	Routine whole genome sequencing for rare diseases diagnosis: The role of bioinformatician	อ. ดร. เอกวัฒน์ ผสมทรัพย์ คณะแพทยศาสตร์ โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล
15.45-17.30 น.	การนำเสนอผลงานวิชาการแบบโปสเตอร์ 2	
18.00-21.00น.	งานเลี้ยงต้อนรับ	

วันที่ 29 พฤษภาคม 2558		
เวลา	กิจกรรม / หัวข้อ	วิทยากร
08.30-09.00 น.	ลงทะเบียน	
09.00-10.00 น.	กิตติคุณประกาศ ศ.นพ. อวย เกตุสิงห์ "อวย เกตุสิงห์ ปูชนียบุคคลของชาติ ปราชญ์ของแผ่นดิน"	ศ.พิเศษ นายแพทย์ สรรใจ แสงวิเชียร ที่ปรึกษา คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล
10.00-10.15 น.	รางวัลเกียรติยศ ศ.นพ.อวย เกตุสิงห์ (The 2nd Ouay Ketusingh Honorary Award)	
10.15-10.30 น.	พักรับประทานอาหารว่าง	
10.30-12.00 น.	Novel targeted cancer therapy	อ. ดร. ศิวนนท์ จิรวัฒโนทัย คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล อ. ดร.พญ. วรรณรัศมี เกตุชาติ คณะแพทย์ศาสตร์ จุพาลงกรณ์มหาวิทยาลัย รศ. ดร. ภญ. ศรีจันทร์ พรจิราศิลป์ คณะเภสัชศาสตร์ มหาวิทยาลัยมหิดล
12.00-13.00 น.	Lunch symposium /รับประทานอาหารกลางวัน	บริษัท เมทเล่อร์-โทเลโด (ประเทศไทย) จำกัด
13.00-14.30 น.	Liver fluke associated cholangiocarcinoma: KKU's research initiative	ศ. ดร. โสพิศ วงศ์คำ คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น ผศ. ดร. ลัดดาวัลย์ เส็งกันไพร คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น
14.30-14.45 น.	พักรับประทานอาหารว่าง	
14.45-16.15 น.	Novel medications for supportive care in cancer	ผศ. ดร. ภก. สุภัสร์ สุบงกช คณะเภสัชศาสตร์ มหาวิทยาลัยขอนแก่น อ. ภก. มานิตย์ แซ่เตียว คณะเภสัชศาสตร์ มหาวิทยาลัยอุบลราชธานี
16.15-17.15 น.	Role of pharmacokinetics and pharmacogenetics for busulfan conditioning therapy prior to hematopoietic stem cell transplantation	ศ. นพ. สุรเดช หงส์อิง คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล
17.15-17.30 น.	มอบรางวัลนำเสนอผลงานวิชาการโปสเตอร์ ประชาสัมพันธ์งานประชุมวิชาการ The 13th Asia Pacific Federation of Pharmacologists Meeting (APFP)	

วันที่ 30 พฤษภาคม 2558	
เวลา	กิจกรรม / หัวข้อ
09.00 - 12.00 น.	Workshop: ความร่วมมือทางวิชาการระหว่างสถาบันในด้านงานวิจัย และการเรียนการสอนระดับบัณฑิตศึกษา
13.00 - 16.00 น.	ศึกษาแลกเปลี่ยนเรียนรู้วัฒนธรรมท้องถิ่น ภายในเขตจังหวัดอุบลราชธานี

เอกสารประกอบการบรรยาย

Pharmacogenetic Markers for

Drug-induced Severe Cutaneous Adverse Drug Reactions

Prof. Wichittra Tassaneeyakul, Ph.D.

Severe cutaneous adverse drugreactions (SCAR) including Stevens–Johnson syndrome (SJS), toxic epidermal necrolysis (TEN) and hypersensitivity syndrome (HSS) are life threatening. More than 100 drugs have been reported as culprit drugs for SCAR. Several hypotheses have been proposed to explain the immunopathogenesis of SCAR, however it is still unclear as to what extent host factors such as age, gender, genetic, comorbidities or environmental factors, might be involved. Over the last decade, several studies have reported strong associations between drug-induced SCAR and genetic polymorphism of human leukocyte antigens (HLA).

Previous studies from the post-marketing phase of abacavir (ABC), an antiretroviral drug revealed that frequencies of ABC-induced HSS in black and Asian populations were lower compared with Caucasians. Subsequent studies demonstrated a strong association between *HLA-B*57:01* and ABC-induced HSS. Data for association of ABC-induced HSS in Asian populations is, however,limited.

Carbamazepine (CBZ) and phenytoin (PHT), aromatic antiepileptic drugs,are common culprit drug for SJS/TEN. The incidences of CBZ-related SJS and TEN in Thailand and Malaysia are among the highest in the World. In 2004-2005, studies in Han Chinese showed that the *HLA-B*15:02* is strongly associated with CBZ-induced SJS/TEN but not other CBZ-induced cutaneous reactions. This strong association has also been observed in Thai, Malaysian and Indian populations. These results, however, have not been reproduced in Japanese and European populations. More recently, the *HLA-A*31:01* allele has also been linked to CBZ-induced SCAR including SJS/TEN and HSS in Japanese and Caucasian populations. For PHT, a significant association between *HLA-B*15:02* and SJS/TEN induced by phenytoin has been observed in Han Chinese, however this association could not be repeated in Thai population.

The strong association between *HLA-B*58:01* with SCAR induced by allopurinol, a uric acid lowering drug was first identified in a Han Chinese population. This strong association was subsequently confirmed in the Thai population, however, only a modest association was observed in Korean, Japanese and European populations.

Co-trimoxazole, an antibiotic consisting of sulfamethoxazole and the trimethoprim, is the most common culprit drug for SJS and TEN in Thailand. Our recent study showed that 4 HLA alleles particularly *HLA-B*15:02*, *HLA-C*06:02* or *HLA-C*08:01* were significantly associated with SJS/TEN induced by this drug.

Ethnic differences in HLA genetic polymorphism are well recognized. The allele frequency of *HLA-B*57:01* is high in Europeans (5–8%), when compared with East Asians (0-2%) and Southeast Asians (Thais, Cambodians, Vietnamese; 3-4%). In contrast to *HLA-B*57:01*, the *HLA-B*15:02* allele is most prevalent in Chinese and Southeast Asian populations (8-12%) but low in Japanese, Korean and European populations (<1%).For *HLA-B*58:01*, the allele frequencies observed in Chinese, Korean and Southeast Asian populations are quite high (5-10%) compared with Japanese and European populations (0.4-1%). The allele frequency of *HLA-A*31:01* in Japanese (7–12%) is high compared with Chinese and Thais (1-3%), Koreans (5%) and European populations (2–5%). It should be noted that the degree of relationship between these HLA alleles and drug-induced SCAR varies among ethnic subpopulations in which the associations were found in ethnic groups that had high allelic frequency of those alleles.

Routine Pharmacogenetic Testing in Clinical Practice:
Dream or Reality?
PGx markers for Drug-Induced SCARs
PGx Markers and TDM for Drug-Induced ADR-A

ผศ. ดร. ภก. ชลภัทร สุขเกษม

การคิดค้นและพัฒนายาใหม่มีวัตถุประสงค์หลักเพื่อให้ได้ยาที่มีประสิทธิผลดีขึ้น และลดโอกาสการเกิดผลข้างเคียง หรืออาการไม่พึงประสงค์จากการใช้ยา (adverse drug reaction; ADR) แต่เมื่อมีการนำมาใช้ในเวชปฏิบัติ ยังพบอุบัติการณ์ การเกิดอาการไม่พึงประสงค์จากการใช้ยาในผู้ป่วยอยู่เสมอ ซึ่งความผิดแผกทางพันธุกรรมของมนุษย์เป็นปัจจัยหนึ่งที่มี ความสำคัญกับการตอบสนองต่อยา โดยส่งผลให้เกิดความแตกต่างในแง่ประสิทธิผลและการเกิดอาการไม่พึงประสงค์จากการ ใช้ยาในแต่ละบุคคล ซึ่งลักษณะทางพันธุกรรมที่แตกต่างกันในแต่ละบุคคล (genetic variations) เช่น ความหลากหลายทาง พันธุกรรมของยีนเอชแอลเอ (human leukocyte antigen gene; *HLA* gene) และความผิดแผกทางพันธุกรรมบนยีนที่สร้าง เอนไซม์สำหรับการย่อยสลายยา (drug metabolizing enzymes) จึงมีความพยายามในการศึกษาหาความสัมพันธ์ของความผิด แผกหรือความหลากหลายทางพันธุกรรม (genetic polymorphisms) กับการตอบสนองต่อยาที่แตกต่างกันเพื่อการเลือกใช้ยา และปรับขนาดยาให้เหมาะสมในแต่ละบุคคล (individualized therapy) โดยมุ่งหวังให้ได้รับประสิทธิผลของยาสูงสุด และลด โอกาสเกิดอาการไม่พึงประสงค์จากการใช้ยา ซึ่งจะทำให้ผู้ป่วยเหล่านั้นมีคุณภาพชีวิตที่ดีขึ้น อย่างไรก็ตามแม้จะมีการศึกษา วิจัยมาโดยตลอด แต่การนำไปประยุกต์ใช้ในเวชปฏิบัติ เช่น การตรวจวินิจฉัยตัวบ่งชี้ทางเภสัชพันธุศาสตร์ก่อนการให้ยาหรือ เพื่อช่วยในการปรับขนาดยายังอยู่ในวงจำกัด ในบทนี้ได้รวบรวมข้อมูลตัวบ่งชี้ทางเภสัชพันธุศาสตร์ที่มีการใช้ในเวชปฏิบัติ อย่างแพร่หลาย ดังจะเห็นได้จากห้องปฏิบัติการเภสัชพันธุศาสตร์ ภาควิชาพยาธิวิทยา คณะแพทยศาสตร์ โรงพยาบาล รามาธิบดี รวมถึงตัวบ่งชี้ทางเภสัชพันธุศาสตร์ที่มีแนวโน้มจะได้รับการยอมรับและนำไปใช้ในเวชปฏิบัติในเวลาอันใกล้ พร้อม ทั้งแสดงปัญหาและอุปสรรคที่อาจพบได้ ตลอดจนเสนอแนะแนวทางการแก้ไข เพื่อเป็นแนวทางในการพิจารณาเลือกใช้ได้อย่าง ถูกต้องและเหมาะสม

เภสัชพันธุศาสตร์และการแพทย์เฉพาะบุคคล (pharmacogenomics and personalized medicine)

เภสัชพันธุศาสตร์ เป็นศาสตร์ที่ว่าด้วยการศึกษาถึงความหลากหลายทางพันธุกรรมในแต่ละบุคคล (individual) โดย มุ่งเน้นศึกษาในส่วนที่เกี่ยวข้องกับการตอบสนองยาทั้งในแง่ที่ดี (ประสิทธิผล : efficacy) และไม่ดี (ภาวะพิษจากยา : toxicity) โดยมีเป้าหมายที่จะนำองค์ความรู้ที่ได้จากการศึกษาวิจัยมาใช้ให้เกิดประโยชน์ในการดูแลรักษาผู้ป่วยในระดับเวชปฏิบัติ เพื่อให้ผู้ป่วยแต่ละรายได้รับประสิทธิผลสูงสุด โดยไม่เกิดอาการไม่พึงประสงค์จากการใช้ยาเลย ซึ่งการวิเคราะห์ความ หลากหลายทางพันธุกรรมในแต่ละบุคคล มีวัตถุประสงค์คือ

1. เพื่อบ่งชี้และจำแนกกลุ่มผู้ป่วยที่มีความเสี่ยงต่อการเกิดอาการแพ้ยา โดยเฉพาะการแพ้ยาแบบผื่นผิวหนังชนิด รุนแรง (severe cutaneous adverse drug reactions; SCARs)

2. เพื่อบ่งชี้และจำแนกกลุ่มผู้ป่วยที่มีลักษณะการย่อยสลายยาที่แตกต่างกัน เพื่อทำการปรับขนาดยาให้เหมาะสม ในแต่ละบุคคล

3. เพื่อบ่งชี้และจำแนกกลุ่มผู้ป่วยที่จะมีการตอบสนอง (responders) และไม่ตอบสนอง (non-responders) ต่อการใช้ยา

ดังนั้นจึงกล่าวได้ว่าเภสัชพันธุศาสตร์เป็นศาสตร์แขนงหนึ่งของการแพทย์เฉพาะบุคคล (personalized medicine หรือ individualized medicine) ซึ่งคาดว่าจะเป็นรูปแบบการดูแลรักษาผู้ป่วยที่มีความสำคัญในอนาคตอันใกล้ โดยเป็นการศึกษาที่มี เป้าหมายเพื่อเพิ่มประสิทธิผลของการรักษาและลดโอกาสเกิดอาการไม่พึงประสงค์จากการใช้ยา โดยมีองค์ประกอบที่เกี่ยวข้อง 3 ส่วนดังนี้

(1) ยา โดยยาที่ควรให้ความสำคัญในทางเภสัชพันธุศาสตร์คือ ยาที่มีอุบัติการณ์การเกิดอาการไม่พึงประสงค์ที่ รุนแรงและพบได้บ่อย คาดการณ์การตอบสนองต่อยาทั้งประสิทธิผลและอาการไม่พึงประสงค์ได้ยาก และเป็นยาที่มีความสำคัญ มีการใช้อย่างแพร่หลาย และเหมาะสมต่อภาวะทางเศรษฐศาสตร์ของประเทศนั้นๆ

(2) พันธุกรรม พันธุกรรมที่ให้ความสนใจทางเภสัชพันธุศาสตร์ มักเป็นยีนที่เกี่ยวข้องกับการสร้างโปรตีนที่มี ความสำคัญต่อกระบวนการทางเภสัชจลนศาสตร์ (pharmacokinetics) และเภสัชพลศาสตร์ (pharmacodynamics)

(3) การตอบสนองต่อยา การตอบสนองต่อยาที่เกิดภายหลังการได้รับยาในผู้ป่วยจะเกิดได้ 2 ลักษณะคือ ประสิทธิผลของยา (drug efficacy) และภาวะพิษจากยา (drug toxicity) โดยประสิทธิผลของยา เป็นสิ่งที่มุ่งหวังจากการรักษาด้วยยาในผู้ป่วย แต่ภาวะพิษจากยาเป็นสิ่งที่ต้องการหลีกเลี่ยง และไม่ต้องการให้เกิดขึ้นเลย สำหรับการตรวจยีน *CYP2B6* ในผู้ติดเชื้อเอชไอวีก่อนการให้ยาต้านไวรัสเอดส์อิฟาวิเรนซ์ (efavirenz) จะมุ่งหวังทั้งสองด้านคือ ป้องกันการเกิดภาวะพิษต่อระบบประสาท (central nervous system toxicity) และยังสามารถป้องกันภาวะระดับยาต้านไวรัสต่ำกว่าช่วงของการรักษา (therapeutic window) ซึ่งเป็นสาเหตุที่เกิดความล้มเหลวในการรักษา (treatment failure) ได้อีกด้วย

อาการไม่พึงประสงค์จากการใช้ยา : ปัญหาของการดูแลรักษาผู้ป่วย

แม้ว่าการรักษาด้วยยาจะเป็นแนวทางหลักที่ใช้ในการบำบัดรักษาโรคและความเจ็บป่วย แต่ผลการรักษาด้วยยาอาจก่อให้เกิดอาการไม่พึงประสงค์จากการใช้ยา ซึ่งหมายถึง ปฏิกิริยาที่เกิดขึ้นโดยมิได้ตั้งใจเมื่อใช้ยาในขนาดปกติ เพื่อการป้องกัน วินิจฉัย บำบัดรักษาโรค และเพื่อเปลี่ยนแปลงกระบวนการทำงานของกลไกต่างๆ ในร่างกายมนุษย์ ซึ่งมักเรียกว่า อาการข้างเคียงจากการใช้ยาหรือการแพ้ยา โดยการเกิดอาการไม่พึงประสงค์จากการใช้ยาจะมีผลให้ผู้ป่วยต้องหยุดยา เปลี่ยนชนิดยา หรือต้องมีการปรับขนาดยาและอาจส่งผลให้ผู้ป่วยบางรายต้องเข้ารับการรักษาในโรงพยาบาล และอาจต้องอยู่รักษาตัวในโรงพยาบาลนานขึ้น โดยอาจทำให้ผู้ป่วยบางรายเกิดความพิการแบบชั่วคราวหรือถาวร และอาจมีความรุนแรงจนถึงขั้นเสียชีวิตได้

อาการไม่พึงประสงค์จากการใช้ยาสามารถแบ่งได้เป็น 2 แบบ ได้แก่ ประเภทที่สามารถคาดการณ์ได้ และที่ไม่สามารถคาดการณ์ได้ ซึ่งมีแนวคิดพื้นฐานดังต่อไปนี้

1. **อาการไม่พึงประสงค์จากการใช้ยาที่สามารถคาดการณ์ได้ (Type A ADR หรือ augmented reaction)**

เป็นอาการไม่พึงประสงค์ที่เกิดจากฤทธิ์ทางเภสัชวิทยาของยา (pharmacologic effect) สามารถทำนายได้ (predictable) โดยความรุนแรงของอาการที่เกิดขึ้นจะขึ้นกับขนาดยาและการตอบสนองของแต่ละบุคคล โดยมีอุบัติการณ์การเกิดสูง (มากกว่าร้อยละ 80) แต่มีอัตราการตายต่ำ เนื่องจากอาการไม่พึงประสงค์ประเภทนี้ จะสามารถคาดการณ์ได้จากฤทธิ์ทางเภสัชวิทยาของยา ดังนั้นการป้องกันและแก้ไขจึงทำได้โดยการปรับขนาดยาลง หรืออาจเปลี่ยนไปใช้ยาอื่น ซึ่งจะช่วยลดหรือแก้ไขอาการไม่พึงประสงค์ประเภทนี้ได้ เช่น การเกิดภาวะเลือดออกจากการใช้ยาวาฟาริน ซึ่งเป็นยากลุ่มต้านการแข็งตัวของเกร็ดเลือด เป็นต้น

การตรวจวินิจฉัยทางเภสัชพันธุศาสตร์ โดยตรวจยีนที่มีความสัมพันธ์กับการย่อยสลายยา เช่น ยีนไซโตโครม พี 450 (CYP450) เพื่อประเมินความสามารถในการย่อยสลายยาในผู้ป่วยแต่ละราย ซึ่งส่งผลโดยตรงต่อระดับของยาและตัวเมแทบอไลต์ (metabolite) และมีผลกับการตอบสนองต่อยาทางคลินิก ทำให้สามารถปรับขนาดยาแต่ละชนิดให้เหมาะสมกับผู้ป่วยแต่ละรายได้

2. **อาการไม่พึงประสงค์จากการใช้ยาที่ไม่สามารถคาดการณ์ได้ (Type B ADR หรือ bizarre reaction)**

เป็นอาการไม่พึงประสงค์เมื่อให้ยาในขนาดปกติ อาการไม่พึงประสงค์ประเภทนี้เป็นอาการที่เกิดขึ้นได้ โดยที่ไม่สามารถคาดคะเนจากฤทธิ์ทางเภสัชวิทยาของยา ไม่เคยพบในระหว่างทำการศึกษาวิจัยทางคลินิกของยา แต่มักพบภายหลังจากยาได้รับการขึ้นทะเบียนและมีการใช้อย่างแพร่หลาย มีอุบัติการณ์การเกิดต่ำ (น้อยกว่าร้อยละ 20) แต่มีอัตราการตายสูง ความหลากหลายทางพันธุกรรมเป็นปัจจัยหลักในการเกิดอาการไม่พึงประสงค์ประเภทนี้ โดยส่งผลให้การตอบสนองต่อยาซึ่งถือเป็นสิ่งแปลกปลอมที่ถูกนำเข้าสู่ร่างกายแตกต่างกันไปในผู้ป่วยแต่ละราย ที่มีการศึกษาอย่างแพร่หลายคือ ความหลากหลายของยีนเอชแอลเอ (*HLA* gene) ซึ่งส่งผลให้เกิดภาวะภูมิคุ้มกันไวเกินต่อยา (drug hypersensitivity) โดยไม่เกี่ยวข้องกับปริมาณยาที่ร่างกายได้รับ

วิธีแก้ไขเพียงประการเดียวคือ ต้องเปลี่ยนชนิดยา สำหรับการป้องกันจะสามารถทำได้โดยการตรวจหาตัวบ่งชี้ทางเภสัชพันธุศาสตร์ก่อนเริ่มการรักษาหรือเลือกใช้ยา เช่น การเกิดการแพ้ยาแบบผื่นผิวหนังชนิดรุนแรง (Stevens-Johnson syndrome; SJS และ toxic epidermal necrolysis; TEN) จากการใช้ยาอัลโลพูรินอล (allopurinol) จะทำให้สามารถลดปัญหาทางสาธารณสุขต่างๆ ไปได้มาก เช่น ลดการสูญเสียงบประมาณในการดูแลรักษาป้องกันปัญหาเรื่องการฟ้องร้องต่อบุคลากรทางการแพทย์ เป็นต้น ดังนั้นนักวิจัยจึงได้มีความพยายามทำการศึกษาวิจัย เพื่อให้ทราบถึงตัวบ่งชี้ทางเภสัชพันธุศาสตร์ (pharmacogenetics marker) ที่สามารถใช้ทำนายการเกิดอาการไม่พึงประสงค์จากการใช้ยาชนิดต่างๆ ตลอดจนหาวิธีการตรวจวินิจฉัยที่สะดวกและแม่นยำ สามารถนำไปประยุกต์ใช้ในเวชปฏิบัติ เพื่อแก้ปัญหาการเกิดอาการไม่พึงประสงค์จากการใช้ยาที่ไม่สามารถคาดการณ์ได้

ซึ่งการตรวจเพื่อการปรับขนาดยาจะมีความยุ่งยาก ซับซ้อน และการนำไปใช้ในเวชปฏิบัติเป็นเรื่องที่ทำได้ยาก กว่า จึงทำให้การตรวจหาตัวบ่งชี้ทางเภสัชพันธุศาสตร์เพื่อการเปลี่ยนหรือเลือกชนิดของยา มีการใช้อย่างแพร่หลายและให้ผล การตอบสนองที่ดีและเห็นผลชัดเจนเมื่อเทียบกับการเลือกใช้ยาโดยไม่ทราบลักษณะทางพันธุกรรมของผู้ป่วยก่อนการจ่ายยา

ตัวบ่งชี้ทางเภสัชพันธุศาสตร์กับการประยุกต์ใช้ในเวชปฏิบัติ

ตัวบ่งชี้ทางเภสัชพันธุศาสตร์ตามการประยุกต์ใช้ในระดับเวชปฏิบัติ

1. การตรวจวินิจฉัยเพื่อบ่งชี้และจำแนกกลุ่มผู้ป่วยที่มีความเสี่ยงต่อการเกิดอาการแพ้ยา โดยเฉพาะการแพ้ยาแบบผื่นผิวหนังชนิดรุนแรง (SCARs)

ความหลากหลายของยีนเอชแอลเอ มีความสัมพันธ์กับการเกิดภาวะภูมิไวเกินต่อยา เนื่องจากเอชแอลเอบาง ชนิดสามารถนำเสนอยาให้กับทีลิมโฟไซต์ ซึ่งจะเปลี่ยนเป็น activated T-lymphocyte ได้มากกว่าคนทั่วไป ซึ่งเป็นจุดเริ่มต้น ของการก่อให้เกิดภาวะภูมิไวเกินต่อยาได้ ดังนั้นการตรวจหาตัวบ่งชี้ทางเภสัชพันธุศาสตร์เพื่อการเปลี่ยนหรือเลือกชนิดของยา จึงเป็นการตรวจหาความหลากหลายทางพันธุกรรมบนยีนเอชแอลเอที่มีความสัมพันธ์กับการเกิดอาการไม่พึงประสงค์จากการ ใช้ยาที่ไม่สามารถคาดการณ์ได้ ซึ่งมักเป็นการแสดงอาการทางผิวหนัง โดยอาจเป็นเพียงผื่นแพ้แบบธรรมดาถึงระดับรุนแรงถึง ขั้นเสียชีวิตได้ เช่น TEN และ SJS ซึ่งจะมีอาการผิวหนังลอกทั้งตัว มีแผลที่เยื่อบุตา ปาก และอวัยวะสืบพันธุ์ ซึ่งอาจทำให้ ผู้ป่วยถึงแก่ชีวิตได้ ซึ่งพยาธิกำเนิดไม่เกี่ยวข้องกับกระบวนการทางเภสัชจลนศาสตร์ของยาเลย ดังนั้นจึงไม่สามารถคาดคะเน จากฤทธิ์ทางเภสัชวิทยาของยาได้ วิธีการจัดการเพียงประการเดียวคือ ต้องเปลี่ยนชนิดของยาที่ใช้ในการรักษา สำหรับการ ป้องกันจะสามารถทำได้โดยการตรวจหาตัวบ่งชี้ทางเภสัชพันธุศาสตร์ก่อนเริ่มการรักษาด้วยยาหรือเลือกใช้ยา ปัจจุบันมีตัวบ่งชี้ ทางเภสัชพันธุศาสตร์ที่สามารถนำมาใช้ทางคลินิกเพื่อทำนายอุบัติการณ์การแพ้ยาในผู้ป่วยแต่ละรายได้แล้วหลายชนิด ได้แก่ ยาคาร์บามาเซปิน (carbamazepine) เนวิราปีน (nevirapine) อบาคาเวียร์ (abacavir) อัลโลพูรินอล (allopurinol)

2. เพื่อบ่งชี้และจำแนกกลุ่มผู้ป่วยที่มีลักษณะการย่อยสลายยาที่แตกต่างกัน เพื่อปรับขนาดยาให้เหมาะสมในแต่ละบุคคล

การปรับขนาดยา (dosage adjustment) ให้เหมาะสมกับผู้ป่วยแต่ละราย มีปัจจัยที่เกี่ยวข้องหลายด้าน เช่น ขนาดของยา (dose) ความแตกต่างของยีนในแต่ละบุคคล ยาที่ใช้ร่วมด้วย อาหารที่ผู้ป่วยรับประทาน อายุ เพศ น้ำหนัก พยาธิ สภาพของตับและไต และโรคที่ผู้ป่วยเป็นอยู่ เป็นต้น ทั้งนี้เมื่อยาเข้าสู่ร่างกายจะมีกระบวนการทางเภสัชจลนศาสตร์เกิดขึ้นใน ร่างกายประกอบด้วย การดูดซึมยา (absorption) การกระจายตัวของยา (distribution) การย่อยสลายยา (metabolism) และการ ขับยาออกจากร่างกาย (excretion) ซึ่งในขั้นตอนการย่อยสลายยามักเกิดขึ้นที่ตับ เนื่องจากเป็นอวัยวะที่มีเอนไซม์ทำหน้าที่ใน การย่อยสลายยา (drug metabolizing enzymes) จำนวนมาก โดยเอนไซม์หลักที่เกี่ยวข้องกับการย่อยสลายยาในร่างกายคือ ไซโตโครม พี 450 (CYP450) ซึ่งสามารถแบ่งย่อยได้อีกหลายชนิด

ปัจจุบันมีการศึกษาอย่างกว้างขวางถึงผลของความผิดแผกทางพันธุกรรมของยีนกับการตอบสนองต่อยาใน ผู้ป่วยแต่ละราย และพบว่าความผิดแผกทางพันธุกรรมของยีนต่างๆ เช่น ยีนไซโตโครม พี 450 (CYP450) ที่ส่งผลให้เอนไซม์มี ความผิดปกติทั้งในเชิงปริมาณ (quantity) และ/หรือเชิงคุณภาพ (quality) เช่น อาจเกิดความบกพร่องของเอนไซม์ เอนไซม์มี ระดับการทำงานเปลี่ยนไป ระดับของเอนไซม์เพิ่มขึ้น/ลดลง จึงสามารถจำแนกประชากรที่มีความผิดแผกทางพันธุกรรมตาม ความสามารถในการเปลี่ยนแปลงยาได้ 4 กลุ่มประชากร คือ

(1) กลุ่มประชากรซึ่งมีการย่อยสลายยาได้น้อยมากหรือไม่ได้เลย (poor metabolizers; PM)

(2) กลุ่มประชากรซึ่งมีการย่อยสลายยาได้ในน้อยกว่าคนทั่วไป (intermediate metabolizers; IM)

(3) กลุ่มประชากรซึ่งมีการย่อยสลายยาได้รวดเร็ว (extensive metabolizers; EM)

(4) กลุ่มประชากรซึ่งมีการย่อยสลายยาได้รวดเร็วมากที่สุด (ultra-rapid metabolizers; UM)

แม้ว่าการติดตามระดับยาในกระแสเลือด (therapeutic drug monitoring; TDM) โดยการวัดระดับยาในกระแส เลือดเพื่อการปรับขนาดยาให้เหมาะสมกับผู้ป่วยแต่ละราย โดยเฉพาะกับกลุ่มยาที่มีช่วงการรักษาที่แคบคือ มีระดับของยาใน กระแสเลือดที่ผู้ป่วยจะได้รับประสิทธิผลจากยา (therapeutic level) ใกล้เคียงกับระดับที่อาจก่อให้เกิดพิษ (toxic level) ซึ่งกลุ่ม ยาดังกล่าว สามารถปรับขนาดยาและลดโอกาสการเกิดอาการไม่พึงประสงค์จากการใช้ยาแบบที่สามารถคาดการณ์ได้เพียง บางส่วนเท่านั้น เพราะการปรับขนาดยาด้วยวิธีนี้จะเป็นการคำนวณขนาดยาจากปัจจัยพื้นฐาน เช่น อายุ เพศ น้ำหนัก หลังจาก นั้นทำการวัดระดับยาในกระแสเลือด แล้วจึงปรับระดับยาเพิ่มขึ้นหรือลดลงให้เหมาะสมกับผู้ป่วยแต่ละราย โดยอาจต้องทำการ วัดระดับยาในกระแสเลือดของผู้ป่วยหลายครั้ง ซึ่งผู้ป่วยจะต้องเสียค่าใช้จ่ายในการตรวจวัด และในระหว่างการปรับขนาดยา ผู้ป่วยจะมีความเสี่ยงต่อการเกิดอาการไม่พึงประสงค์จากการใช้ยาได้ ดังนั้นการตรวจวินิจฉัยทางเภสัชพันธุศาสตร์ก่อนการให้

ยา เพื่อช่วยในการปรับขนาดยาให้เหมาะสมในผู้ป่วยแต่ละราย โดยการตรวจยีนที่มีความเกี่ยวข้องกับกระบวนการเปลี่ยนแปลง
ยาในร่างกาย ซึ่งมักเกี่ยวข้องกับการเกิดอาการไม่พึงประสงค์จากการใช้ยาที่สามารถคาดการณ์ได้ (Type A ADR) เนื่องจาก
อาการไม่พึงประสงค์ลักษณะนี้จะมีความสัมพันธ์กับฤทธิ์ทางเภสัชวิทยาของยา

        อย่างไรก็ตาม การตรวจหาตัวบ่งชี้ทางเภสัชพันธุศาสตร์เพียงอย่างเดียว อาจไม่สามารถลดหรือป้องกันการเกิด
อาการไม่พึงประสงค์จากการใช้ยาได้ทั้งหมด ดังนั้นหากมีการตรวจวินิจฉัยทางเภสัชพันธุศาสตร์ก่อนการจ่ายยา ร่วมกับการ
ตรวจวัดระดับยาหรือการตรวจลักษณะทางฟีโนทัยป์ (pharmacogenetics drug monitoring; PDM) จะทำให้สามารถวาง
แผนการบริหารและการปรับขนาดยาได้อย่างมีประสิทธิภาพมากยิ่งขึ้น ช่วยลดระยะเวลาและจำนวนครั้งของการตรวจติดตาม
ระดับยาให้น้อยลง ช่วยให้แพทย์และเภสัชกรสามารถคาดการณ์ถึงประสิทธิผลของยา และอาการไม่พึงประสงค์จากการใช้ยาที่
ใช้ในการรักษาผู้ป่วย และสามารถลดความเสี่ยงต่อการเกิดอาการไม่พึงประสงค์จากการใช้ยา หรือการขาดประสิทธิภาพในการ
รักษาจากยา ซึ่งการพิจารณาปรับขนาดยาจากผลการตรวจยีนร่วมกับค่าทางคลินิกอื่นๆ ของผู้ป่วย จะช่วยเพิ่มประสิทธิภาพใน
การรักษา และลดอาการไม่พึงประสงค์จากการใช้ยา ซึ่งเป็นเป้าหมายสูงสุดในการรักษาผู้ป่วย

ห้องปฏิบัติการเภสัชพันธุศาสตร์ในระดับเวชปฏิบัติ

        โครงการเภสัชพันธุศาสตร์ภายใต้ความร่วมมือระหว่างคณะแพทยศาสตร์ โรงพยาบาลรามาธิบดี
มหาวิทยาลัยมหิดล และศูนย์ความเป็นเลิศด้านชีววิทยาศาสตร์ของประเทศไทย เริ่มดำเนินการตั้งแต่ พ.ศ. 2547 จนถึงปัจจุบัน
เพื่อสนับสนุนการวิจัยทางพันธุศาสตร์ โดยมีการจัดตั้งโครงสร้างพื้นฐานสำคัญที่จำเป็นต่อการศึกษาทางเภสัชพันธุศาสตร์
รวมทั้งได้ดำเนินการศึกษาวิจัยทางเภสัชพันธุศาสตร์ เพื่อศึกษาความสัมพันธ์ระหว่างลักษณะทางพันธุกรรมและการตอบสนอง
ต่อยาของคนไทยภายใต้ความร่วมมือกับองค์กรต่างๆ ทั้งภายในและภายนอกประเทศ ผลการดำเนินงานที่ผ่านมานำไปสู่
โอกาสในการพัฒนาองค์ความรู้ในหลายรูปแบบ ได้แก่ การสร้างฐานข้อมูลพันธุกรรมระดับจีโนมในรายบุคคล การสร้างองค์
ความรู้ในการวิเคราะห์ข้อมูลทางชีวสารสนเทศระดับจีโนม การพัฒนาชุดตรวจยีนแพ้ยาในประชากรไทย การศึกษาทางคลินิก
เพื่อยืนยันประสิทธิภาพของชุดตรวจยีนแพ้ยา และการแปลผลการตรวจยีนแพ้ยาเพื่อใช้ในการรักษาเฉพาะบุคคล รวมถึงการ
จัดตั้งห้องปฏิบัติการเภสัชพันธุศาสตร์ (Laboratory for Pharmacogenomics) ที่ได้รับการรับรองมาตรฐาน อาทิเช่น Good
Laboratory Practices (GLP) และ ISO15189 เพื่อการยอมรับในระดับสากล สำหรับใช้เป็นสถานที่เปิดให้บริการตรวจหายีนที่
มีความสำคัญต่อการตอบสนองยา และการเกิดอาการไม่พึงประสงค์จากการใช้ เป็นหน่วยงานที่เปิดให้บริการตรวจวินิจฉัย
เภสัชพันธุศาสตร์จากสิ่งส่งตรวจทั่วประเทศ โดยเน้นที่การตรวจดีเอ็นเอ และอาร์เอ็นเอ เป็นหลัก

        ในปี พ.ศ.2554 ภาควิชายาธิวิทยา คณะแพทยศาสตร์ โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล จึงมีการจัดตั้ง
ห้องปฏิบัติการเภสัชพันธุศาสตร์ (Laboratory for Pharmacogenomics) ขึ้นภายใต้อนุสาขาวิชาเภสัชพันธุศาสตร์และ
การแพทย์เฉพาะบุคคล (Division of Pharmacogenomics and Personalized Medicine) โดยสามารถตรวจวินิจฉัยตัวบ่งชี้ทาง
เภสัชพันธุศาสตร์เพื่อการเลือกหรือเปลี่ยนชนิดยา การตรวจตัวบ่งชี้ทางเภสัชพันธุศาสตร์เพื่อการปรับขนาดยา และเป็น
ห้องปฏิบัติการเพื่อสนับสนุนงานตรวจวินิจฉัยในระดับจีโนม ซึ่งการตรวจวินิจฉัยทางเภสัชพันธุศาสตร์นั้นจะสามารถนำไป
ประยุกต์ใช้ทางคลินิก ทำให้ผู้ป่วยแต่ละรายได้รับยาที่จำเพาะ มีความปลอดภัย และได้ประสิทธิผลสูงสุดในการรักษา เช่น การ
ตรวจเภสัชพันธุศาสตร์สำหรับยารักษาโรคหัวใจและหลอดเลือด ยากันชัก ยารักษามะเร็ง ยารักษาโรคเกาท์ ยาจิตเวช ยาต้าน
ไวรัสเอดส์ เป็นต้น นอกจากนี้ยังมีการวิจัยต่างๆ ทางเภสัชพันธุศาสตร์อีกมากมาย มีการรับสิ่งส่งตรวจ และให้บริการการตรวจ
วินิจฉัยตัวบ่งชี้ทางเภสัชพันธุศาสตร์แก่ผู้ป่วยก่อนได้รับการรักษาด้วยยา เพื่อให้เกิดการรักษาที่เหมาะสม มีประสิทธิภาพสูงสุด
และลดโอกาสการเกิดอาการไม่พึงประสงค์จากการใช้ยา โดยการปฏิบัติการจะครอบคลุมกระบวนการดังนี้ การสกัดสาร
พันธุกรรม (nucleic acid extraction) การตรวจหาการเข้าคู่กันของยีน (genotyping) การตรวจหาสนิปส์ (SNPs identification)
การหาลำดับพันธุกรรม (DNA sequencing) ไมโครอาเรย์ (microarray) และการแสดงออกของยีน (gene expression
analysis) การตรวจหาตัวบ่งชี้ทางพันธุศาสตร์พิษวิทยา (toxicogenomics) การจัดเก็บและรักษาตัวอย่างพันธุกรรม
(biostorage services) ชีวสารสนเทศและการวิเคราะห์ข้อมูล (bioinformatics and data analysis) ปรึกษาและออกแบบการ
ศึกษาวิจัย (consulting and custom assay design and validation) การบริหารจัดการโครงการ (project management)

        ปัจจุบันห้องปฏิบัติการเภสัชพันธุศาสตร์ ของโรงพยาบาลรามาธิบดี มีการเปิดบริการตรวจวินิจฉัยทางเภสัช
พันธุศาสตร์แบบครบวงจร ซึ่งมีส่วนงานที่สำคัญ 3 ส่วนคือ

        1. ห้องปฏิบัติการตรวจวิเคราะห์พันธุกรรม (Genetics and Genomics Laboratory) เป็นส่วนงานที่ทำหน้าที่
ตรวจวินิจฉัยความผิดแผกทางพันธุกรรมของผู้ป่วย รวมทั้งมีการวิจัย การประเมิน และพัฒนาการตรวจวิเคราะห์ทางเภสัช
พันธุศาสตร์ที่สำคัญต่างๆ โดยใช้เทคโนโลยีระดับโมเลกุล (molecular techniques) ที่ทันสมัย และเป็นแห่งเดียวที่สามารถทำ

การตรวจวินิจฉัยด้วยเครื่อง AmpliChip CYP450 ได้ นอกจากนั้นยังมีเครื่องมือสำหรับการตรวจวินิจฉัยหาตัวบ่งชี้ทาง เภสัชพันธุศาสตร์ที่ทันสมัยเทียบเท่าห้องปฏิบัติการระดับโลกได้ เช่น เครื่องวิเคราะห์ลำดับสารพันธุกรรมระดับจีโนม (whole genome sequencer; Solid 5500XL) พร้อมอุปกรณ์เครื่องเพิ่มปริมาณและวิเคราะห์สารพันธุกรรมในสภาพจริง และเครื่อง วิเคราะห์สารพันธุกรรมเชิงปริมาณแบบอัตโนมัติ โดยใช้เวลาในการวิเคราะห์และแปลผลประมาณ 1-3 วันทำการ หลังจากนั้น ทางห้องปฏิบัติการเภสัชพันธุศาสตร์จะรายงานผลการตรวจ พร้อมทั้งมีเอกสารข้อมูลเฉพาะตัวผู้ป่วยเพื่อแสดงให้แพทย์หรือ เภสัชกรทราบในกรณีที่ต้องเลือกยาหรือปรับขนาดยาด้วย

2. ห้องปฏิบัติการตรวจสอบหน้าที่ของเอนไซม์ และการตรวจติดตามระดับยา (Functional genomics, Enzyme activity and Therapeutic drug monitoring Laboratory) เป็นส่วนงานที่ทำหน้าที่ตรวจวิเคราะห์ระดับการทำงาน ของเอนไซม์ที่สำคัญต่อกระบวนการย่อยสลายและเปลี่ยนแปลงยา รวมทั้งการตรวจติดตามระดับยาในกระแสเลือดของผู้ป่วย โดยใช้เครื่องแยกวิเคราะห์ปริมาณยาในกระแสเลือด (High performance liquid chromatography/Mass spectro/Mass spectro; HPLC/MS/MS) เพื่อใช้ประกอบการพิจารณาปรับขนาดยาในเวชปฏิบัติตามหลักการ "การตรวจวัดระดับยาหรือการ ตรวจลักษณะทางฟีโนทัยป์" ซึ่งจะทำให้แพทย์และเภสัชกรมีความมั่นใจ และสามารถปรับขนาดยาได้อย่างแม่นยำและเกิด ประสิทธิภาพสูงสุดในการดูแลรักษาผู้ป่วยบางกลุ่ม เช่น ผู้ติดเชื้อเอชไอวี ผู้ป่วยมะเร็ง ผู้ป่วยที่มีการเปลี่ยนถ่ายอวัยวะ และ ผู้ป่วยที่มีการติดเชื้อรุนแรง เป็นต้น

3. คลินิกเภสัชพันธุศาสตร์ (Pharmacogenomics Clinic) เป็นส่วนงานที่เกี่ยวข้องกับกระบวนการขอรับการ ตรวจวิเคราะห์ จนกระทั่งการออกผลการตรวจวิเคราะห์ โดยทำหน้าที่ให้คำปรึกษาสำหรับแพทย์ และเภสัชกรที่ต้องการส่ง ตรวจถึงชนิด รูปแบบ วิธีการเก็บ ระยะเวลาในการเก็บ และการส่งตัวอย่างส่งตรวจ รวมถึงชนิดของยีนและรายการตรวจ วิเคราะห์ที่เหมาะสม การแปลผลและการนำผลที่ได้จากการตรวจวิเคราะห์ไปใช้ในเวชปฏิบัติ เช่น การเลือกชนิดของยาที่ เหมาะสม หรือรูปแบบการบริหารยาที่จะเกิดประสิทธิผลและความปลอดภัยต่อผู้ป่วย ภายหลังจากที่ทราบผลการตรวจ วิเคราะห์แล้ว นอกจากนั้นยังเป็นสถานที่ในการประเมินและตรวจสอบความจำเป็นของการขอรับบริการสำหรับผู้ป่วยที่มีความ ประสงค์ขอรับการตรวจเอง รวมทั้งผู้ป่วยที่ขอรับคำปรึกษาเรื่องการแพ้ยาเพื่อประสานงานกับหน่วยงานที่เกี่ยวข้องต่อไปด้วย

ห้องปฏิบัติการเภสัชพันธุศาสตร์ ตั้งอยู่ที่ชั้น 4 ศูนย์การแพทย์สมเด็จพระเทพรัตน์ คณะแพทยศาสตร์ โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล ในปี พ.ศ.2555 ได้เริ่มดำเนินการเพื่อให้ได้รับการรับรองเป็นห้องปฏิบัติการตาม มาตรฐานสากล เช่น มาตรฐาน GLP และ ISO15189 และ ISO15190 ซึ่งถือว่าเป็นห้องปฏิบัติการทางเภสัชพันธุศาสตร์เพียง แห่งเดียวในประเทศไทยที่ขอรับรองมาตรฐานสากลเหล่านี้ โดยมีการเปิดให้บริการตรวจวินิจฉัยทางเภสัชพันธุศาสตร์สำหรับ ผู้ป่วยและแพทย์ในโรงพยาบาลรามาธิบดี หน่วยงานภายนอกทั้งภาครัฐและเอกชน และพร้อมรองรับการบริการศึกษาวิจัยทาง คลินิกสำหรับบริษัทยาที่สนใจทำการศึกษาวิจัยทางคลินิกของยาใหม่ที่ต้องการตรวจยีนทางเภสัชพันธุศาสตร์ในประเทศไทย และยังสามารถสนับสนุนงานวิจัยด้านเภสัชพันธุศาสตร์และจีโนมแก่นักวิจัยภายในประเทศ และมีการตั้งเป้าหมายให้เป็น ศูนย์กลางการเรียนรู้ด้านเภสัชพันธุศาสตร์และการรักษาเฉพาะบุคคล สามารถถ่ายทอดความรู้ทางเภสัชพันธุศาสตร์ในระดับ ภูมิภาคและนานาชาติอีกด้วย สนใจรับข้อมูลเพิ่มเติมติดต่อที่ โทร 02-200-4331-2 และ 02-201-1380, 02-201-1390

Systematic 3D Screening of Amino Acid Mutations in Pharmacogenetics Study

Thanawadee Preeprem, Ph.D.

Pharmacogenomic studies examine the effects of genomic variations on diverse pharmacokinetic and/or pharmacodynamic parameters. Because variants of pharmacogenes tend to have smaller effects than disease-causing variants, the sequence conservation-based tools do not consistently classify mutations of pharmacogenes as functional. In this study, we selected 48 genes identified as "Very Important Pharmacogenes (VIPs)" by the PharmGKB database, and developed a novel tool for investigating the roles of amino acid mutations in drug responses, using 3-dimensional (3D) analysis. The 3D screening of amino acid mutations focuses on the changes in inter-residue bonding, protein stability, protein flexibility, drug binding capability, protein-protein interactions, and amino acid dissimilarity, in addition to the localization of the variants and the amino acid secondary structure preference. Distinguishable characteristic profiles for VIP variants, from functional and neutral mutations, are incorporated into the construction of a five-feature score "SDS Pharmacogenes". The score can be used to predict the impacts of unknown variants of pharmacogenes towards pharmacokinetic and/or pharmacodynamic changes.The database for SDS Pharmacogenes provides a quick access to pre-computed scores of all variants in the current structural dataset (45/48 VIPs). While expertise in 3D-protein analysis is beneficial, our implementation does not require that an individual with experience in protein structures be engaged in personalized genome evaluations. In addition, the analysis pipeline is systematic and scalable, thus expected to keep pace with the rapid accumulation of pharmacogenomic data.

Routine Whole Genome Sequencing for Rare Diseases Diagnosis:
the Role of Bioinformatician

ดร.เอกวัฒน์ ผสมทรัพย์

การทำ High throughput sequencing เป็นวิธีที่มีประสิทธิภาพสูงในการค้นหา variants ชนิด non-specific germ line, somatic mutations และ structural variants ในปัจจุบันมีการใช้ sequencing เพื่อการศึกษา whole genome sequencing, Exome sequencing และ target panel sequencing ซึ่งการทำ sequencing เหล่านี้จะทำให้ได้ข้อมูล sequence ออกมาจำนวนมาก ดังนั้นการจัดการทางด้าน bioinformatics จึงเป็นสิ่งที่จำเป็น เริ่มตั้งแต่การเก็บข้อมูล การวิเคราะห์ข้อมูล และความถูกต้องในการจัดการวิเคราะห์ข้อมูล การจัดการข้อมูล sequencing ที่ได้จากเครื่อง Next-generation sequencing เป็นสิ่งที่สำคัญมากเนื่องจากโดยปกติแล้วผลที่ออกมาจะมีความผิดปกติของ sequence จำนวนมาก ดังนั้นก่อนการใช้งานข้อมูลจึงต้องมีกระบวนการในการจัดการคุณภาพของข้อมูลตั้งแต่ต้นจนจบ การจัดการควบคุมคุณภาพของข้อมูล sequencing ที่ได้จากเครื่อง Next-generation sequencing นั้นประกอบด้วย 3 ขั้นตอนหลัก คือ การจัดการข้อมูลดิบ (raw data), การทำ alignment และการ call variant เพื่อให้ได้ความแปรผันทางพันธุกรรมจริงก่อนนำไปใช้งาน การทำการควบคุมคุณภาพนี้ต้องทำครบทั้ง 3 จะขาดขั้นตอนใดไปไม่ได้ การจัดการข้อมูลดิบเป็นส่วนแรกที่จะทำให้สามารถกรองข้อมูล sequence ที่มีคุณภาพน้อยมากๆออก การจัดการข้อมูล alignment เป็นส่วนที่สำคัญก่อนการทำ variant calling และ ในส่วนของ variant calling ก็เป็นส่วนที่สำคัญมากในการจัดการกับตัวอย่างที่ไม่ได้คุณภาพ และไม่สามารถคัดกรองได้จากการควบคุมคุณภาพก่อนหน้านี้ ซึ่งเป็นผลทำให้เกิด false-positive variants

ในปัจจุบันมีโรคพันธุกรรมจำนวนมากที่เกิดจากการกลายพันธุ์หรือผ่าเหล่าในระดับยีนแต่ไม่สามารถสืบค้นหรือตรวจพบด้วยการตรวจวินิจฉัยทางห้องปฏิบัติการเนื่องด้วยข้อจำกัดทางด้านเทคโนโลยีและบุคลากร โรคที่เกิดจากยีนหรือกลุ่มยีนผิดปกติไม่จำเป็นต้องมีการแสดงออกของโรคมาตั้งแต่กำเนิด แต่สามารถแสดงออกที่ช่วงเวลาใดของชีวิตก็ได้ ตั้งแต่เด็ก วัยรุ่น ผู้ใหญ่หรือวัยชรา โรคเหล่านี้สามารถมีผู้ป่วยเกิดขึ้นซ้ำในครอบครัว ทำให้กระทบต่อการดำรงชีวิตของผู้อื่นในครอบครัวทั้งด้านร่างกาย จิตใจ และสังคม หลายคนเกรงที่จะมีบุตรเพราะกลัวว่าโรคดังกล่าวจะถ่ายทอดสู่บุตรหลานของตน ผู้ป่วยหลายคนประสบภาวะซึมเศร้าเนื่องจากหมดหวังในชีวิตเนื่องจากไปพบแพทย์แล้วแพทย์ไม่สามารถวินิจฉัยโรคและให้คำแนะนำในการดูแลรักษาได้ โรคหลายโรคมีอาการใกล้เคียงกับโรคที่เกิดจากปัจจัยแวดล้อมและอาการไม่แตกต่างกันชัดเจน ทำให้ต้องสูญเสียเวลาในการวินิจฉัยจากแพทย์และจากทางห้องปฏิบัติการ สูญเสียทรัพยากรทางด้านการเงินในการทดลองรักษา ดังนั้นการวินิจฉัยการกลายพันธุ์หรือการผ่าเหล่าที่เกิดขึ้นบนจีโนมจึงมีความจำเป็นอย่างมาก เนื่องจากจะเป็นเครื่องมือพิเศษช่วยชี้ทางให้แพทย์ทำการดูแลรักษาผู้ป่วยได้ง่ายขึ้น โรคที่ไม่สามารถรักษาได้ในอดีตก็เริ่มมีโอกาสในการรักษาให้ทุเลา หรือบางรายสามารถดำเนินการรักษาให้หายขาดได้เนื่องจากค้นพบต้นตอหรือตำแหน่งของการกลายพันธุ์หรือผ่าเหล่าที่สามารถแก้ไขได้ หลังจากผู้ป่วยต้องทรมานด้วยอาการดังกล่าวมาเป็นเวลานานหรือตั้งแต่เกิด จากปัญหาดังกล่าวเป็นจุดกำเนิดของศาสตร์ใหม่ที่เรียกว่าเวชศาสตร์เฉพาะบุคคล (Personalized Medicine) ซึ่งหลายประเทศทั่วโลกมีการตื่นตัวที่จะพัฒนางานในด้านนี้ เนื่องจากลักษณะการกลายพันธุ์หรือผ่าเหล่าของยีนในระดับจีโนมในแต่ละเชื้อชาติมีความแตกต่างกัน สำหรับประเทศไทยแล้วแพทย์และบุคลากรทางการแพทย์ด้านนี้ยังไม่เกิดขึ้นชัดเจน จำเป็นต้องเร่งสร้างบุคลากรที่ทำงานด้านเวชศาสตร์เฉพาะบุคคลแบบบูรณาการพร้อมไปกับการพัฒนาฐานข้อมูลของโรคพันธุกรรมต่างๆที่เกิดกับประชากรไทยเพื่อใช้พัฒนาศักยภาพทางสาธารณสุขของประเทศ การตรวจวินิจฉัยกลุ่มยีนบนจีโนมยังมีผลต่อการกำหนดทิศทางการรักษา การค้นคว้าหายาหรือแนวทางการรักษาใหม่ๆ หรือป้องกันการเกิดโรคซ้ำซ้อนในครอบครัวโดยใช้เทคโนโลยีทางด้านการเจริญพันธุ์เข้าช่วย

การค้นหา variant ที่มีความสัมพันธ์กับการเกิดโรค ในปัจจุบันจะใช้ เครื่อง Next-generation sequencing ซึ่งข้อมูลที่ได้มาจะมีปริมาณมาก และบางส่วนจะเป็น sequence หรือ variant ที่มีความผิดปกติ (error) จึงจำเป็นอย่างมากที่ต้องมีการควบคุมคุณภาพข้อมูลที่ได้มา การทำการควบคุมคุณภาพประกอบด้วยหลายขั้นตอนและมีความจำเป็นที่ต้องทำให้ครบทุกขั้นตอน ขาดขั้นตอนใดขั้นตอนหนึ่งไม่ได้ และเมื่อข้อมูลทั้งหมดผ่านการควบคุมคุณภาพทุกขั้นตอนแล้วจะทำให้ได้ข้อมูล variant ที่น่าเชื่อถือ และสามารถนำไปใช้ในการคัดกรอง variant ที่ทำให้เกิดโรค จากการศึกษาต่างๆได้

การศึกษา whole genome และ Exome sequencing ในประชากรไทยนั้น มีความจำเป็นที่จะต้องมีฐานข้อมูลของประชากรไทย เนื่องจาก variant ที่มีประชากรไทยมีความแตกต่างจาก variant ที่มีในประชากรอื่นๆ และฐานข้อมูล variant นี้ก็มีส่วนสำคัญอย่างมากและเป็นหนึ่งในขั้นตอนที่ใช้ในการคัดกรอง variant ที่มีผลทำให้เกิดโรค ซึ่งหวังเป็นอย่างยิ่งว่าฐานข้อมูล Exome ที่สร้างขึ้นมาจากงานโครงการนี้จะส่งผลต่อการค้นหา variant ที่มีผลต่อการเกิดโรครวมถึงเป็นต้นแบบของการเริ่มต้นในการรวบรวมข้อมูล Exome การควบคุมคุณภาพ จนถึงการค้นหา variant ในกลุ่มประชากรไทย และใช้เป็นข้อมูลในการคัดกรอง Exome variant ที่ส่งผลต่อการเกิดโรค เพื่อเป็นยกระดับมาตรฐานการสาธารณสุขในประเทศต่อไปในอนาคต

Cancer Targeted Therapy

Siwanon Jirawatnotai, Ph.D.

Cancer targeted therapy is a novel paradigm, and a promising hope for cancer treatment. Accumulated research on the steps that contribute to the tumorigenesis, e.g. immortalization, cellular transformation, angiogenesis, and invasion and metastasis, has resulted in specific sets of gene that apparently can be proposed as suitable targets for cancer treatment. Currently, more than 100 cancer genes are labeled as "actionable" for treatment. With advance in cancer research, a lot more genes will be identified and used as a drug target, in a near future. However, drug resistance and short-term effectiveness are seriously undermining this new paradigm. The shortcomings are supposedly a result of the lack of the "larger picture" of how cancer is operating. We will discuss the usefulness, and the problem of the targeted therapy, hoping to better understand the so-called "magic bullet".

HEXIM1 is a Novel Therapeutic Target in Tamoxifen Resistant and Metastatic Breast cancer

Wannarasmi Ketchart , Ph.D.

Breast cancer is the major leading cause of death in women worldwide. Even though the development of breast cancer treatment has been a major research focus for decades, drug resistance and cancer metastasis—the main cause of patient mortality—are still difficult to overcome. Tamoxifen is the standard treatment for Estrogen receptor alpha (ERα)-positive breast cancer which is the most common type of breast cancer. After 5 years of tamoxifen therapy, 45% of patients with advanced and metastatic breast cancer develop resistance. In previous reports, Hexamethylene bisacetamide-inducible protein 1 (HEXIM1) was identified as an ERα-coregulator that can also inhibit ERα transcriptional activity and breast cancer growth. Since the major mechanism of tamoxifen is the inhibition of ERα activity, we hypothesized that HEXIM1 played a role in assisting tamoxifen in the inhibition of ER activity. Our studies demonstrated that tamoxifen increased recruitment of HEXIM1 to the promoter regions of ER target genes and the inhibitory effect of tamoxifen is attenuated when HEXIM1 is downregulated. In addition, we found that low HEXIM1 expression was associated with tumor recurrence in patients who received tamoxifen. These findings support the critical role of HEXIM1 in the response to tamoxifen.

A role for HEXIM1 in the inhibition of angiogenesis was also previously reported. Angiogenesis plays a pivotal role for tumor growth and metastasis. Our study observed that HEXIM1 expression is decreased in patients' metastatic lymph nodes when compared to their primary breast tumor tissues and re-expression of HEXIM1 in a mammary tumor of a breast cancer mouse model resulted in the reduction of tumor volume, angiogenesis, and metastasis to the lung. Moreover, we also used polymer-mediated delivery of hexamethylene bisacetamide (HMBA) to induce HEXIM1 expression in tumors and this method was able to inhibit lung metastasis in mice without inducing thrombocytopenia, the dose limiting toxicity associated with systemic HMBA treatment. 67 kDa laminin receptor (67 LR) promotes the degradation of the extracellular matrix by interacting with laminin that facilitates cell migration and invasion, both important processes during metastasis. HEXIM1 re-expression in the mammary gland modified the membrane localization of 67 LR which resulted in decreased cell migration and invasion and eventual metastasis.

Cholangiocarcinoma: One of the Thailand Grand Challenges

Prof. Sopit Wongkham, Ph D.

Cholangiocarcinoma (CCA) is rare in western countries but is considered to be one of the major public health problems in the northeast of Thailand because of its high incidence, severity and high mortality rate. The late detection and poor survival after diagnosis has led a need of more powerful markers for early diagnosis and prognostic marker of CCA. Although complete resection provides the best hope for long-term survival, it can be offered only a limited cases with early stage. At present, there is no effective therapy and specific tumor marker for CCA. In this presentation, the epidemiology, natural history, risk factors and tumor markers of CCA will be updated. The prevention, early detection and reduction of patient loss are the issues of Thailand grand challenges at present.

Liver Fluke-Associated Cholangiocarcinoma: KKU's Research Initiative "Potential Targets for Liver Fluke-Associated Cholangiocarcinoma Therapy"

Asst. Prof. Laddawan Senggunprai, Ph.D.

Cholangiocarcinoma (CCA) is one of the aggressive cancers with very poor prognosis. The high incidence of CCA in Northeastern Thailand is associated with background conditions particularly liver fluke *Opisthorchis viverrini* infection that causes long-standing inflammation. The poor prognosis of CCA is mainly caused by delayed diagnosis and resistance to cytotoxic drugs. The identification of novel potential targets for CCA therapy should be strongly encouraged in order to improve the clinical management of CCA. Several distinct findings have been highlighted the over-expression and constitutive activation of JAK/STAT3 and NF-κB signaling pathways in CCA as compared to adjacent normal tissues. This perhaps arise as the consequence of an *in vivo* tumor microenvironment which is enriched with cytokines and other growth factors. The JAK/STAT3 and NF-κB cascades have been demonstrated to play crucial roles in cholangiocarcinogenesis. Several phytochemicals including quercetin, EGCG, luteolin, curcumin and cepharanthine that target STAT3 and/or NF-κB pathways might therefore have great therapeutic potential in CCA treatment. In addition, molecular profiling studies have identified the activation of tyrosine kinase receptors and protein kinases in liver fluke-associated CCA patient tissues and cell lines. Predominately, the kinases activated downstream are those in the Ras/MAPK, PI3K/Akt, JAK/STAT and Wnt/β-catenin signaling pathways. The inhibition of multi-targeted kinase activation using kinase inhibitors, sorafenib and sunitinib, leads to CCA cell growth inhibition and apoptosis induction. This indicates that certain protein kinases are promising drug targets for CCA treatment. Moreover, abnormal increases of the antioxidant enzymes, heme oxygenase-1 (HO-1) and NADPH-quinone oxidoreductase-1 (NQO1), are also observed in opisthorchiasis-associated CCA patient tissues. The high expression of these two enzymes is significantly associated with poor prognosis. Inhibition of HO-1 and NQO1 can enhance the susceptibility of CCA to an array of chemotherapeutic agents. Hence, these two enzymes appear to be the potential targets for exploitation in CCA therapy. The potential of these candidate targets for CCA treatment need to be investigated in the prospective setting in future clinical trials.

EGFR Inhibitor–Induced Dermatologic Toxicities: MASCC Guideline Application in Prevention and Treatment

ผศ. สุภัสร์ สุบงกช

ผู้ป่วยโรคมะเร็งที่ได้รับการรักษาด้วยยาเคมีบำบัดแบบดั้งเดิม มักเกิดอาการไม่พึงประสงค์จากการใช้ยาเคมีบำบัด เช่น คลื่นไส้ อาเจียน ผมร่วง ไขกระดูกบกพร่องซึ่งเป็นอาการที่หลีกเลี่ยงได้ยาก ปัจจุบันจึงมีการพัฒนายากลุ่มใหม่ที่ออกฤทธิ์จำเพาะต่อเซลล์มะเร็งมากยิ่งขึ้น เพื่อเพิ่มประสิทธิภาพในการรักษาและเพื่อลดอาการไม่พึงประสงค์ต่าง ๆที่เกิดกับผู้ป่วยอย่างไรก็ตามพบว่ายากลุ่มใหม่ดังกล่าวยังสามารถก่อให้เกิดอาการไม่พึงประสงค์แก่ผู้ป่วยได้ซึ่งส่งผลต่อคุณภาพชีวิตของผู้ป่วยโดยตรง เนื้อหาการบรรยายส่วนนี้จะกล่าวถึงการพิษต่อระบบผิวหนัง จากการใช้ยากลุ่ม EGFR Inhibitors (EGFRIs) ตลอดจนวิธีป้องกันและรักษา โดยอ้างอิงแนวทางการรักษาของ Multinational Association of Supportive Care in Cancer (MASCC)

Medication for Supportive Care in Cancer : Focused on Chemotherapy Induced Nausea and Vomiting (CINV)

อ.ภก.มานิตย์ แซ่เตียว

อาการคลื่นไส้อาเจียน เป็นอาการไม่พึงประสงค์ที่พบได้บ่อยจากการได้รับยาเคมีบำบัด ซึ่งส่งผลต่อคุณภาพชีวิต และทำให้เกิดผลแทรกซ้อนจนถึงภาวะคุกคามชีวิตได้ ข้อมูลการศึกษาถึงกลไกในการเกิดอาการคลื่นไส้อาเจียนทางพยาธิสรีรวิทยาพบว่า vomiting center ในสมองส่วน medulla เป็นตำแหน่งสำคัญที่ทำให้เกิดอาการคลื่นไส้อาเจียนโดยรับสัญญาณประสาทจาก cerebral cortex, chemoreceptor trigger zone และ vagus nerve ในระบบทางเดินอาหาร ตัวรับสารสื่อประสาทที่สำคัญในการกระตุ้นอาการคลื่นไส้อาเจียน ได้แก่ dopamine, serotonin และ neurokinin-1

อาการคลื่นไส้อาเจียนจากยาเคมีบำบัดสามารถแบ่งออกได้เป็น

1. Acute emesis เป็นอาการอาเจียนที่เกิดขึ้นภายใน 24 ชั่วโมงหลังได้รับยาเคมีบำบัด
2. Delayed emesis เป็นอาการอาเจียนที่เกิดขึ้นตั้งแต่ 24 ชั่วโมงหลังได้รับยาเคมีบำบัดเป็นต้นไป
3. Anticipatory emesis เป็นอาการอาเจียนที่เกิดตั้งแต่ก่อนได้รับยาเคมีบำบัด ซึ่งมักมีสาเหตุจากการที่ผู้ป่วยไม่ได้รับการป้องกันอาการคลื่นไส้อาเจียนอย่างเหมาะสมในการให้ยาเคมีบำบัดในครั้งก่อน
4. Breakthrough emesis เป็นอาการอาเจียนภายหลังจากการให้การป้องกันอาการคลื่นไส้อาเจียนอย่างเหมาะสม
5. Refractory emesis เป็นอาการอาเจียนหลังได้รับยาเคมีบำบัดที่ไม่ตอบสนองจากยาในการป้องกันและรักษา

การพัฒนายาเพื่อป้องกันและรักษาอาการคลื่นไส้อาเจียนจึงมีเป้าหมายเพื่อยับยั้งการทำงานของตัวรับดังกล่าว ดังนี้

ยากลุ่ม serotonin (5-HT$_3$ receptor antagonist) เป็นยาที่มีประสิทธิภาพดีในการควบคุมอาการอาเจียน ในกลุ่ม acute emesis ยาในรุ่นแรกพบรายงานการเกิดพิษต่อหัวใจ (QT prolongation) palonosetron เป็นยาในรุ่นที่ 2 ที่สามารถจับกับตัวรับได้ดีขึ้นและมีค่าครึ่งชีวิตยาวนานขึ้น ผลการศึกษาพบว่ามีประสิทธิภาพครอบคลุมอาการอาเจียนทั้ง acute และ delayed emesis นอกจากนี้ยังไม่พบรายงานการเกิดพิษต่อหัวใจ

ยากลุ่ม dopamine receptor antagonist เป็นยาที่มีประสิทธิภาพในการควบคุมอาการคลื่นไส้และอาเจียนทั้ง acute และ delayed emesis ยา olanzapine เป็นยากลุ่ม antipsychotic ที่สามารถยับยั้งตัวรับทั้ง dopamine, serotonin, histamine และ muscarinic ที่มีการศึกษายืนยันประสิทธิภาพในการป้องกันคลื่นไส้อาเจียนได้ดี และยังนำมาใช้สำหรับ breakthrough emesis ได้อีกด้วย

ยากลุ่ม neurokinin-1 (NK$_1$) receptor antagonist เป็นยาที่มีประสิทธิภาพดีในการควบคุมอาการอาเจียน ในกลุ่ม acute emesis และ delayed emesis ยาในรุ่นแรกทั้ง apprepitant และ fosapprepitant ที่นำมาใช้สำหรับสูตรยาเคมีบำบัดที่มีโอกาสเกิดการคลื่นไส้อาเจียนในระดับสูง (มากกว่าร้อยละ 90) หรือสูตรยาที่มีการใช้ doxorubicin ร่วมกับ cyclophosphamide ยาในรุ่นที่ 2 ได้แก่ netupitant, rolapitant มีการศึกษาในข้อบ่งใช้ของยาในรุ่นแรกและยังขยายการศึกษาไปยังสูตรยาเคมีบำบัดที่มีโอกาสเกิดการคลื่นไส้อาเจียนในระดับปานกลาง (ร้อยละ 30-90)

A01 — Aeroallergens Survey in Bangsaothong District, Samutprakarn Province, Thailand

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Abstract

Preparation of allergenic extracts and allergy vaccines for proper management of allergic patients in each particular residential area is the key of "direct-to-target" specific treatment. The first step of the immunopharmacology research for this purpose must be initiated by allergens survey. Aeroallergens in Bangsaothong District, Samutprakarn Province, Thailand, Latitute 13 ° 36′14.95″ North, Longitude 100 ° 49′ 5.16″ East were therefore continuously surveyed using Aerosampler instrument for 12 months, during June 2008 to May 2009. More than 12 kinds of plants pollens and mold spores could be identified in this study. The average overall amount of pollens and mold spores were 1046 and 3990 per cubic meter of air flow respectively. The five most common pollens and mold spores found in this study were wild grass, cultivated grass, sedge, fern and _Leguminosae_; _Nigrospora_, _Cladosporium_, Yeast, 1-celled spore (mainly _Aspergillus_ and _Penicillium_), _Fusarium_. The detail findings of the incidence and the amount of plant pollens as well as mold spores were quite differ from such findings in Cholburi, the nearby province, and other provinces including those in Bangkok Metropolitan which were previously reported. The close and direct influences of the climate especially the quantity of rainfall on the occurance of aeroallergens both plant pollens and mold spores were clearly observed. The results obtained from this aeroallergens survey in Bangsaothong District should be the useful and reliable data for further preparation of allergenic test solutions and allergy vaccines to manage allergic patients in the nearby communities as well as those travellers using Suvarnabhumi International Airport.

Keywords : immunopharmacology, aeroallergen, Aerosampler, pollen, mold spore

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A02 | Pattern of Aeroallergens around Naresuan University Hospital, Phitsanulok Province, Thailand

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Abstract

Establishment of allergenic extracts and allergic vaccines for appropriate management of allergic patients in each particular residential area is the key of "direct-to-target" specific treatment. There was no official report of allergen survey in Phitsanulok. Therefore, the purpose of this study is to survey outdoor aeroallergens around Naresuan University Hospital, Phitsanulok, Thailand, latitude 16°44'55.1" north longitude 100°11'20.5" east area. The outdoor aeroallergens were continuous daily surveyed using Burkard seven-day recording volumetric spore trap instrument for 12 months, during August 2013 to July 2014. The results showed that more than 12 plant pollens and mold spores were identified. The average amounts of pollens and mold spores were 1,892.15 and 14,439.43 counts/m^3 of air flow per month, respectively. Among plant pollens, the percentages of the five most common pollens including wild grass, cultivated grass, Careless weed, Acaciae and Mimosaceae family found accumulatedly in one year were 77.32% (n=17555), 9.23% (n=2095), 8.66% (n=1967), 1.55% (n=351) and 1.10% (n=250), respectively. Among mold spores, the percentages of the five most common mold spores including 1-celled spore, Cladosporium, yeast, Rust and Nigrospora family found accumulatedly in one year were 39.86% (n=69074), 31.83% (n=55152), 10.94% (n=18948), 9.00% (n=15592) and 4.37% (n=7573), respectively. The peak levels of wild grass and cultivated grass pollens were seen in December with the total counts of 2,337.21 and 487.22 grains/m^3 per month, respectively. While one-celled spore, Cladosporium, yeast and Nigrospora spores peaked in July with total counts of 10,066.11, 7,107.60 and 2,559.20 spores/m^3, respectively. Both total rain water and relative humidity peaked in September were 359.6 millimeters per month and 85%, respectively. The continuous monitoring aeroallergens will help in establishment a pattern of aeroallergens in Phitsanulok. This pattern of aeroallergens would be beneficial for allergic patients who are allergic to pollens or molds in exposure prevention and immunotherapy.

Keywords: aeroallergen, volumetric Burkard spore trap, plant pollens, mold spores

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A03 Case Report : Adverse Event of Using Fentanyl Transdermal Patch in a 2-Month-Old Baby

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Abstract

This is the case report of an unaware use of fentanyl transdermal patch in a 2-month and 9 day-old baby. The serious event occurred when the baby's grandmother got the fentanyl patch from another cancer patient who had passed away. The patch was previously applied to her husband's skin for 24 hours. As she concerned that her grandchild might have a fever and local pain symptoms after a recent DTwP vaccination, a fentanyl patch was then reused and applied to the baby with an adult dosage (25 µg/hr). Thirty minutes later, the baby developed a sign of seizure, depression of CNS and respiratory system. The baby presented to the emergency room with rigidity and syncope. He was firstly diagnosed as having seizure from DTwP vaccination. The symptom was worsening and turned to cyanosis, low BP, and pinpoint pupils. Cardiopulmonary resuscitation had been performed for about 3 times with 2 doses of adrenaline. This adverse effect was maintained for 96 consecutive hours without using naloxone or any antidotes. Liver enzymes including AST, ALT and alkaline phosphatase were increased to 92, 57, and 365 U/L, respectively. He was treated with respiratory support with intravenous antibiotics as empirical therapy. On the 3ʳᵈ day of admission, the baby's symptom was recovered. He could open his eyes with slightly move and response to the stimulations. ET-tube was removed on the 5ᵗʰ day of admission. His mother could normally breast feed him. The baby was discharged with paracetamol syrup after he had completed the course of antibiotics. According to the manufacturer, fentanyl transdermal patch does not recommend to use in children younger than 2 years because of inadequacy of safety information. Moreover, this fentanyl patch should be used only with a severe pain and dosage should be adjusted based on level of pain, health record, and physical condition for individual patient. The unawareness was the main cause of this adverse event. Pharmacist, who is the acknowledged medication experts, must provide necessary information to the patients especially when the addictive substances are used among a population who are at risk of adverse drug reaction such as young children.

Keywords: fentanyl transdermal patch, adverse event, baby, CNS depressions, respiratory depressions

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A04 Pseudoallergic Reactions from Rapid Administration of Antibiotics

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Abstract

A 52-year-old Thai male patient had developed pseudoallergic reaction after intravenous injection (IV) of 2 grams ceftazidime rapidly in 1 minute for the first time. His unusual symptoms included nausea, coughing, sweating, palpitation and swollen lips. He also had minor skin lesion like urticarial rash on his back but no tight chest. His symptoms were slightly improved within 10 minutes after chlorpheniramine 10 mg and dexamethasone 4 mg injection. The incidence of similar anaphylactiod reaction from beta-lactam antibiotics at Sisaket hospital after rapidly IV injection was about 5 % in 2014 (25 cases out of 441 of active consultations). Ceftriaxone was the most common cause of these reactions (19 cases), followed by cefoperazone, ciprofloxacin, penicillin G sodium and clindamycin. We had reviewed the patient drug history and inquired the rate of IV injection of the drug given. Therefore, next dose of ceftazidime was given by infusing slowly for 30 minutes. He showed no signs of false allergy. This is an example of how clinical pharmacist can take an important role in minimizing patient adverse drug reaction. In this case changing only the rate of administration of beta-lactam antibiotics allowed him to use the drug without pseudoallergic reaction.

Keywords: pseudoallergic reactions, rapid administration, ceftazidime, antibiotics, false allergy

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A05 Evaluation of Nail-fold Capillary Structures (Diameter and Tortuosity) in Carbamazepine-Treated Patients

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Abstract

Carbamazepine is an anti-epileptic drug and used for treatment of several diseases such as epilepsy, neuropathic pain (neuralgia) and bipolar affective disorders. Several reports have shown vascular adverse drug effects of anti-epileptic drugs, including carbamazepine. Very few reports have been involved with micro-vascular (capillary) structural changes, which may be related to potential adverse drug reactions in patients with long-term therapy. The aim of this observational study was to evaluate nail-fold capillary structural changes in carbamazepine-treated patients in relation to serum carbamazepine concentrations and its apparent adverse drugs reactions. Patients from Chiang Mai Neurological Hospital (N=60) were recruited and enrolled according to specific inclusion and exclusion criteria. All patients were treated with monotherapy of carbamazepine. Calibrated nail-microscope Dino-Lite® was employed to measure nail-fold capillary structural changes (i.e. arterial and venous diameters and tortuous index) at the proximal nail-fold. The measurement was performed in 1st and 2nd month after starting the treatment. Serum carbamazepine concentrations were also measured by fluorescene polarization immunoassay (Abbot Axsym System®). Results showed that after one and two months of treatment the arterial loop diameters of the capillary were significantly decreased (9.05±1.07 [baseline] vs 8.88±1.10 and 8.78±1.15 μm, $p<0.001$, respectively). Tortuous index was also found to be increased from baseline after treatment for one and two months; 1.64±0.32 μm vs 1.86±0.38 μm vs 1.94±0.36 μm, $p<0.001$, respectively. Correlation between % arterial loop diameter changes of the capillary and serum carbamazepine concentrations existed (r = -0.406, $p<0.05$), but not the turtuos index. Although frequency of adverse drug reactions was greater in patients whose serum carbamazepine concentrations ≥ 8 μg/mL, there was no established association of both % arterial loop diameter changes of capillary and tortuous index with apparent short-term adverse drug reactions.

Keywords: nail-fold Capillary, carbamazepine, adverse drug reaction

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A06 Non-Genetic Risk Factors and Allopurinol-Induced Severe Cutaneous Adverse Drug Reactions

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Abstract

Allopurinol is an xanthine oxidase inhibitor that commonly used for management of gout, urate stones and recurrent calcium oxalate stones associated with urinary uric acid excretion as well as for prevention of tumorlysis syndrome in cancer therapy. This drug has been reported as one of the most common culprit drug for severe cutaneous adverse drug reactions (SCARs) including Stevens-Johnson Syndrome (SJS), Toxic Epidermal Necrolysis (TEN) and Drug reaction with Eosinophilia and Systemic Symptoms (DRESS). Although, the *HLA-B*58:01* allele has been reported as the genetic risk factor of allopurinol-induced SCARs in a Thai population,other risk factors involved in allopurinol-induced SCARs in Thai populationis still unclear. In the present study, we aimed to explore the non-genetic risk factor in allopurinol-induced SCARs in a Thai population. Medical records of 85 allopurinol-induced SCARs and 165 allopurinol-tolerant control patients were reviewed. The two-tailed Student's *t-test* and Fisher's exact test analysed found that the number of female was higher in the SCARs group with an odds ratio of 4.18 (95% CI, 2.28-7.66, *p* value 3.49 x 10^{-7}). The initial dose of allopurinol per body weight in the SCARs group was significantly higher than that of the tolerant control group. Moreover, the number of patients who had allopurinol for treatment of hyperuricemia in the SCARs group was higher than the tolerant control group. The base line of blood urine nitrogen and eosinophil of the SCARs group before receiving allopurinol was significantly higher than that of the tolerant control group and serum creatinine was higher in SCARs group. In conclusion, we found that female, high initial dose, poor kidney function and off labeled use of allopurinol were potentially non-genetic risk factors of allopurinol-induced SCARs in a Thai population. (This research was financial supported by the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program)

Keywords: allopurinol, SCARs, risk factor

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A07 Clinical Efficacy and Adherence to a 14-Day Course of Primaquine When Given with a 3-Day Chloroquine in Patients with *Plasmodium vivax* in the Thai-Myanmar Border

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Abstract

Primaquine is the only antimalarial drug available for the eradication of the hypnozoite stage of *Plasmodium vivax* in order to prevent the disease from recurring. However, one limitation of its clinical use is the long treatment course of 14 days, which may result in poor patients' adherence and low treatment efficacy. The aim of the study was to assess the clinical efficacy and adherence to primaquine therapy following the standard 14-day primaquine regimen (daily dose of 15 mg base/kg body weight) when given together with a 3-day choroquine (2,000 mg over 3 days) in 85 patients with *P. vivax* malaria in a malaria endemic area along the Thai-Myanmar border. Patients' adherence to primaquine therapy was assessed based on primaquine concentrations in finger-prick dried blood spot (DBS) samples and interview questionnaires. Results suggest that the 14-day primaquine when given as an anti-relapse, together with a 3-day blood schizontocidal chloroquine, remains an effective treatment for *P. vivax* infection in this area with a cure rate of virtually 100% during the 42-day follow-up. Based on the analysis of primaquine concentrations in DBS collected from patients on days 3, 7, and 14 of treatment, adherence rates of as high as 95-98% was observed.

Keywords: primaquine, *Plasmodium vivax*, adherence, drug concentrations, finger-prick blood sample

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A08   Pharmacokinetic Interactions between Quinine and Ritonavir-Boosted Lopinavir in Healthy Thai Subjects

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Abstract

This study was an open, three-way, sequential cross-over design in 19 healthy Thai subjects (8 males and 11 females), to investigate the potential of pharmacokinetic/pharmacodynamic interactions between quinine and ritonavir-boosted lopinavir (LPV/r) in healthy Thai subjects. The pharmacokinetic investigation was performed sequentially on three separated occasions. During period I, subjects received a single oral dose of 600 mg quinine sulfate. During period II (2 weeks washout), oral doses of LPV/r was given (400 mg/100 mg every 12 h for 14 days). During period III, 600 mg quinine sulfate in combination with LPV/r (400 mg/100 mg every 12 h for 3 days). Plasma concentrations of quinine, its active plasma metabolite 3-hydroxyquinine, lopinavir, and ritonavir were measured by HPLC. Results showed that all treatment regimens were generally well tolerated. In the presence of steady-state LPV/r, significant differences in six pharmacokinetic parameters of quinine were observed. C_{max}, AUC_{0-48h}, and $AUC_{0-\infty}$ were significantly decreased, while Vz/F and CL/F were significantly increased. Free quinine concentration at 2 hours after dosing was significantly increased. C_{max}, AUC_{0-48h}, and $AUC_{0-\infty}$ of 3-hydroxyquinine were also significantly decreased and the metabolic ratio was significantly reduced. In addition, $t_{1/2z}$ was significantly shortened. Quinine caused modest but statistically significant increase in the C_{max} of both lopinavir and ritanovir. The marked changes in the pharmacokinetics of quinine and 3-hydroxyquinine particularly systemic exposure suggest the complex pharmacokinetic interaction between LPV/r and quinine at different levels including hepatic drug metabolizing enzymes (CYP3A4 and UDP-glucuronosyl transferase), drug transporters (P-glycoprotein), and plasma protein binding (α_1-acid glycoprotein). The high magnitude of reduction in systemic exposure of quinine and 3-hydroxyquinine, raises concern regarding the risk of increase in treatment failure rate when quinine is prescribed in patients with malaria and HIV co-infection. Further investigation in patients with malaria before decision on dose optimization can be made.

Keywords: pharmacokinetic interactions, malaria, HIV, quinine, ritonavir-boosted lopinavir

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| A09 | **Population Pharmacokinetics of a Three-Day Chloroquine Treatment in Patients with *Plasmodium vivax* Infection on the Thai-Myanmar Border** |

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Abstract

The objective of the study was to investigate the pharmacokinetics of chloroquine and its active metabolite desethylchloroquine following treatment with a three-day standard course in patients with *Plasmodium vivax* infection along the Thai-Myanmar border. All patients showed satisfactory response to standard treatment with a three-day course of chloroquine with 100% cure rate within the follow-up period of 42 days. Neither recurrence of *P. vivax* parasitaemia nor appearance of *P. falciparum* occurred. Blood samples were collected from 130 (17 Thais and 113 Burmeses; 64 males and 66 females; aged 15-55 years) patients with mono-infection with *P. vivax* malaria [median (95% CI) admission parasitaemia 4,898 (1,206-29,480)/µL] following treatment with a three-day course of chloroquine (2,000 mg chloroquine phosphate over three days). Whole blood concentrations of chloroquine and its active metabolite desethylchloroquine were measured using high performance liquid chromatography. Concentration-time profiles of chloroquine and desethylchloroquine were analyzed using a population-based pharmacokinetic approach. A total of 1,045 observations from 75 participants were included in the analysis. Chloroquine disposition was most adequately described by the two-compartment model with one transit compartment absorption model into the central compartment and a first-order transformation of chloroquine into desethylchloroquine with an additional peripheral compartment added to desethylchloroquine. First order elimination from the central compartment of chloroquine and desethylchloroquine was assumed. The model exhibited a strong predictive ability and the pharmacokinetic parameters were estimated with adequate precision. Fever clearance time had a significant impact on the pharmacokinetics of chloroquine, indicating a disease effect. The developed population-based pharmacokinetic model could be applied for future prediction of optimal dosage regimen of chloroquine.

Keywords: *Plasmodium vivax*, chloroquine, desethylchloroquine, pharmacokinetics, drug resistance

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A10 Pharmacokinetic Interactions between a Three-Day Artesunate Mefloquine Combination and Lopinavir/ritonavir in Healthy Thai Adults

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Abstract

Concomitant use of antimalarial and antiretroviral drugs is becoming increasingly frequent in areas where malaria and HIV coexist. The magnitude and clinical significance of these potential interactions need elucidation particularly the interaction between artesunate-mefloquine combination and ritonavir-boosted lopinavir (LPV/r) which share the same substrate specificity on cytochrome P450 3A4. To investigate the potential of pharmacokinetic interactions between artesunate-mefloquine and LPV/r in healthy Thai adults. This study was an open, three-way, sequential cross-over design in 16 healthy Thai subjects (8 males and 8 females). The pharmacokinetic investigation was performed sequentially on three separate occasions. During Period-1, subjects received a three-day artesunate-mefloquine combination. During Period-2 (2 weeks washout), oral doses of lopinavir boosted ritonavir (LPV/r) was given (400 mg/100 mg every 12 h for 14 days). During Period-3, a three-day artesunate-mefloquine was given in combination with LPV/r (400 mg/100 mg every 12 h for 3 days). Concentrations of artesunate, dihydroartemisinin (active plasma metabolite of artesunate), lopinavir, ritonavir and mefloquine in plasma or whole blood were measured by HPLC. All treatment regimens were generally well tolerated. Artesunate AUC_{0-24h} was significantly increased. Dihydroartemisinin C_{max} and AUC_{0-24h}, and $AUC_{0-\infty}$ were significantly decreased, while $t_{1/2z}$ was significantly prolonged. The metabolic ratio (AUC_{0-24h} ratio) of dihydroartemisinin to artesunate was significantly reduced. For mefloquine, C_{max}, AUC_{0-48h}, AUC_{0-168h}, and $AUC_{0-\infty}$ were significantly decreased, while V_z/F and CL/F were significantly increased. Finally, C_{max} of lopinavir was significantly decreased and CL/F was significantly increased. C_{max}, AUC_{0-12h}, and $AUC_{0-\infty}$ of ritonavir were significantly decreased, while CL/F and Vz/F were significantly increased. The marked changes in the pharmacokinetics particularly systemic exposure of all investigated drugs suggest the complex pharmacokinetic interaction between artesunate-mefloquine and LPV/r and at different levels including hepatic drug metabolizing enzymes, drug transporters, and plasma protein binding. The high magnitude of reduction in systemic exposure of all drugs raises concern regarding the risk of increase in treatment failure rate when artesunate-mefloquine is to be prescribed in patients with malaria and HIV co-infection. Further investigation in patients with malaria before decision on dose optimization can be made.

Keywords: pharmacokinetics, drug interaction, malaria, HIV, mefloquine, artesunate, dihydroartemisinin, lopinavir, ritonavir

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A11 Application of SPECT/CT Imaging System and Radiochemical Analysis for Investigation of Blood Kinetics and Tissue Distribution of Radiolabeled Plumbagin in Healthy and *Plasmodium berghei*-Infected Mice

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Abstract

Plumbagin is a derivative of napthoquinone which is isolated from the roots of plants in several families. These compound exhibits a wide range of biological and pharmacological activities including antimalarial, antibacterial, antifungal, and anticancer activities. The aim of the study was to investigate blood kinetics and tissue distribution of plumbagin in healthy and *P. berghei*-infected mice using Single-Photon Emission Computed Tomography/Computed Tomography (SPECT/CT) and radiochemical analysis by gamma counter. Plumbagin was labelled with ^{99m}technetium (^{99m}Tc) and the reducing agent stannous chloride dihydrate (50 μg/ml) at pH 6.5. Blood kinetics and tissue distribution of the radiolabeled plumbagin were investigated in healthy and *P. berghei*-infected mice (2 males and 2 females for each experimental group). *In vitro* and *in vivo* stability of plumbagin complex suggested satisfactory stability profiles of ^{99m}Tc-plumbagin complex in plasma and normal saline (92.21 to 95.47%) within 24 h. Significant difference in blood kinetics parameters (C_{max}, AUC, $t_{1/2}$, MRT, Vd, and CL) were observed between *P. berghei*-infected and healthy mice. The labelled complex distributed to all organs of both healthy and infected mice but with high intensity in liver, followed by lung, stomach, large intestine and kidney. Accumulation in spleen was markedly noticeable in the infected mice. Plumbagin-labelled complex was rapidly cleared from blood and major routes of excretion were hepatobiliary and pulmonary routes. In *P. berghei*-infected mice, $t_{1/2}$ was significantly decreased, while V_d and CL were increased compared with healthy mice. Result suggests that malaria disease state influenced the pharmacokinetics and disposition of plumbagin. SPECT/CT imaging with radiolabeled ^{99m}Tc is a viable non-invasive technique that can be applied for investigation of kinetics and biodistribution of plumbagin in animal models.

Keywords: plumbagin, technetium-99m, biodistribution, SPECT/CT imaging system

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A12 | The Pharmacokinetic Parameters of Melatonin in Healthy Thai Male Volunteers

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Abstract

Melatonin is a hormone secreted by the pineal gland regulated by dark-light conditions. It controls the circadian rhythm in human. This substance is commonly used to treated insomnia. Moreover, several pharmacological effects of melatonin such as antioxidant, immunomodulator and chemotherapy adjuvant have been reported. However, the information about the pharmacokinetic parameters in various populations including Thai population is very limited. This study aimed to determine the pharmacokinetic parameters of melatonin in healthy Thai male volunteers. Twenty-five Thai male volunteers were enrolled in the study and 20 mg melatonin was given orally after an overnight fasting. Blood samples were collected from indwelling cannula insert in the forearm before (0) and at 0.25, 0.50, 1.0 1.25, 1.5, 2, 3, 4, 5 and 6 hours after drug administration. Plasma melatonin concentrations were determined by the validated high performance liquid chromatography coupled with fluorescence detector method. The pharmacokinetic parameters were calculated using Kinetic 2.0. The mean of the maximal concentration (C_{max}) was 55.49 ± 48.83 ng/mL (range from 10.33 – 188.19 ng/mL) and the area under the plasma concentration-time curve (AUC_{0-t}) was 66.36 ± 52.55 ng·hr/mL (range from 12.98 – 203.67 ng·hr/mL) while the mean of the time at maximal concentration (T_{max}) was 0.62 ± 0.23 hrs. The elimination rate constant (K_e) was 0.89 ± 0.16 hr^{-1} and the half-life ($T_{1/2}$) was 0.80 ± 0.14 hr. The mean clearance and Vz were 7.58 ± 5.25 L/hr·kg and 550.82 ± 399.82 L, respectively. Our results reveals the high variations in the C_{max} and AUC_{0-t} of melatonin in the study population and high variations of these pharmacokinetic parameters may affect the dose of melatonin require to archive therapeutic range for individuals.

Keywords: melatonin, pharmacokinetic parameters, Thai male

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A13   Effects of Thai Herbal Tea on P-glycoprotein Transport Function

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Abstract

Intestinal P-glycoprotein is the major efflux protein that may play a crucial role in transporter-based drug interactions with orally administered drugs. Recently, a number of herbs have been reported as a factor that can cause the interactions by alteration of drug-metabolizing enzymes and/or uptake/efflux transporters. Some herbal teas that are usually consumed as beverages have pharmacological effects, and may be used as the alternative medicine. However, consuming herbal tea with some medicines may lead to drug interactions. This study aimed to investigate the effects of Thai herbal teas on the P-glycoprotein function. To determine the influences of Thai herbal teas including safflower (*Carthamus tinctorius*), baelfruit (*Aegle marmelos*), jewel vine (*Derris scandens*), candelabra bush (*Senna alata*), and blue trumpet vine (*Thunbergia laurifolia*) on P-glycoprotein transport function, the Caco-2 cells were incubated with 0.5 µM calcein-AM, a P-glycoprotein substrate, in the absence and presence of the aqueous extract of the teas (1 mg/ml).The results showed that coincubation of safflower, candelabra bush, and blue trumpet vine with calcein-AM significantly increased calcein-AM accumulation (mean ± S.E.M) to 175.87 ±18.62%, 194.44 ± 26.36%, and 190.25 ± 22.27%, respectively, compared to that of calcein-AM alone ($p<0.01$). These results indicate that safflower, candelabra bush, and blue trumpet vine potentially inhibit the P-glycoprotein function. However, the clinical study must be performed to confirm these effects *in vivo*.

Keywords: Thai herbal tea, P-glycoprotein inhibitor, calcein-AM

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A14 *In Silico* Metabolic Modeling of Active Compounds from *Atractylodes lancea* (Thunb.) DC.

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Abstract

Atractylodes lancea (Thunb) DC. has been reported to exhibit promising cytotoxic activity against cholangiocarcinoma. The metabolic profiles of its active compounds remain to be elucidated. The aim of the present study was to apply *in silico* modeling for prediction of cytochrome P450 (CYP)-mediated metabolism of the three active constituents of *Atractylodes lancea* (Thunb) DC., *i.e.,* beta-eudesmaol, atractylodin and atractylenolide III using ADMET predictor software (Simulations Plus, Inc.). CYP2C9, CYP2C19, and CYP3A4-mediated hydroxylation were possibly involved in metabolism of beta-eudesmaol and atractylenolide III. All of these CYPs, and in addition, CYP1A2, were possibly involved in atractylodin oxidation. *In vitro* and *in vivo* studies should be performed to identify the CYP isoforms including other drug metabolizing enzymes involved in the biotransformation of these three compounds.

Keywords: *Atractylodes lancea* (Thunb.) DC., beta-eudesmaol, atractylodin, atractylenolide III, *in silico* modeling

A15 No Evidence of a Role for Renal Eicosanoid-Producing Cytochrome P450 Enzymes in Cadmium-Induced Hypertension

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Abstract

Cadmium (Cd) is an environmental pollutant which is associated with significant kidney dysfunction in exposed populations. This is due to the preferential accumulation of this metal in the kidney proximal tubules where, over-time, it causes toxicity. Animal studies have clearly shown a link between Cd exposure and hypertension where it is proposed that Cd induces renal eicosanoid-producing cytochrome P450 (CYP) expression which results in higher levels of vaso-constrictive molecules. To determine Cd toxicity and its effect on expression of CYP (4A11, 4F2, 3A4, 2B6, and 2E1), metallothionein (MT), and heme oxygenase-1 (HO-1) in kidney, we monitored CYP, MT and HO-1 protein levels in primary cultures of human proximal tubular cells (PTCs) exposed to varying concentrations of Cd (0.1−50 µM) over 48 h, compared with protein levels in the hepatoblastoma cell line HepG2 and the proximal tubular cell line HK-2. Cell toxicity was determined using the WST-1 cytotoxicity assay, lactate dehydrogenase release, and [³H]-thymidine incorporation. Expression of the five CYP and HO-1, as well as MT was determined by immunoblotting. Expression of the CYPs was also assessed in human kidney tissue (*n*=6) using immunohistochemistry. In cultured cells only CYP4F2, CYP3A4 and CYP2B6 were detected. Cd concentrations above 10 µM caused significant cell death in the three cell types. Exposure of the cells to Cd (5 µM) for 48 h had no effect on the levels of CYP4F2, CYP3A4 and CYP2B6. Expression of MT and HO-1 was, however, induced by Cd in a concentration-dependent manner. All tissue sections expressed CYP4A11, CYP4F2, CYP3A4, CYP2B6, and CYP2E1 with strong staining in proximal tubule cells. The *in vitro* study does not support a role for these CYPs in Cd-induced hypertension.

Keywords: cadmium, cytochrome P450, proximal tubular cells, hypertension

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A16   The Effect of *Garcinia mangostana* Linn. Extract on Modulation of *Plasmodium falciparum* Prostaglandins

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Abstract

Development of new promising antimalarial drugs is urgently needed due to the emergence of resistance of malaria parasite to almost all of the available drugs. *Garcinia mangostana* Linn. (pericarp) is one of the most promising candidates for further development as antimalarial drugs. It has been assumed that activity of this plant associated with the anti-inflammatory response, especially the prostaglandin production. Therefore, the aim of the present study was to investigate the effect of *Garcinia mangostana* Linn. (pericarp) crude extract on the production of prostaglandin in 3D7 clone *P. falciparum.* The parasite clone was exposed to *Garcinia mangostana* Linn. (pericarp) crude extract for 12 hours and levels of the three prostaglandins, *i.e.*, PGE2, PGD2, and PGF2α produced were determined using HPLC. The levels of all prostaglandins were significantly decreased in parasite exposed to the extract compared with control. The crude extract of the *Garcinia mangostana* Linn. possesses several pharmacological activities including anti-inflammatory activity. The current study demonstrated its inhibitory activity on prostaglandin production in malaria parasite.

Keywords: antimalarial drug, *Garcinia mangostana* Linn., prostaglandin, malaria

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A17 | *In Vitro* Antimalarial Activity of Piperine

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Abstract

Malaria remains a serious public health problem in several tropical areas and emerging artemisinin resistant of *Plasmodium falciparum* in Southeast Asia is a great concern for disease control. Development of new potential antimalarial drugs is urgently required. In the previous study, the fruits of *Piper chaba* Hunt. was demonstrated to exhibit promising antimalarial activity against asexual stage of both 3D7 and K1 *P. falciparum* clones. The aim of the present study was to investigate antimalarial activity of piperine; the major bioactive compound from *Piper chaba* Hunt. against 3D7 and K1 *P. falciparum* clones. The antimalarial activity was determined by using SYBR green-I-based assay and morphological change was observed under light microscope with giemsa staining. The median IC_{50} (concentration that inhibited parasite growth by 50%) values of piperine against 3D7 and K1 were 31.8 µg/ml and 16.8 µg/ml, respectively. The marked change in parasite morphology was observed within 48 hours post exposure to piperine. These result indicated that piperine is the promising antimalarial drug. The study is ongoing to elucidate mechanism of action of piperine at molecular and cellular levels.

Keyword: *Plasmodium falciparum*, piperine, antimalarial activity

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A18 Phenotypic and Genotypic Characters of *Plasmodium falciparum* in Artesunate-Mefloquine Treatment Patients

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Abstract

Artesunate (AS)-Mefloquine (MQ) combination is the first-line treatment for acute uncomplicated *Plasmodium falciparum* in Thailand. The increase in recrudescence cases after treatment and a decline in efficacy suggest the possibility of *P. falciparum* resistance to this combination. The study aimed to investigate the phenotypes and genotypes of *P. falciparum* collected from malaria patients who received AS-MQ combination. Twenty *P. falciparum* isolates were collected from patients prior to treatment and the treatment responses were evaluated after completed 42 days follow up. Phenotypic assessment of the parasite isolates was performed by *in vitro* sensitivity test to AS and MQ using SYBR green I assay. Genotypic character was investigated by determination of *P. falciparum multidrug resistance 1* (*pfmdr1*) copy number using SYBR green real-time PCR method. The IC_{50} (concentrations that inhibit parasite growth by 50%) values of AS and MQ in isolates collected from patients who presented adequate clinical response (n=10) were significantly lower than those from treatment failure [median: 1.0 *vs* 2.8 nM, and 18.0 *vs* 54.4 nM, for AS and MQ, respectively; $p = 0.02$ and 0.03]. *Pfmdr1* copy number was significantly higher in isolates obtained from patients with treatment failure (median: 1.0 *vs* 4.0 copy; $p = 0.01$). This preliminary study suggests that *in vitro* sensitivity and *pfmdr1* copy number may be used as markers to predict resistance of *P. falciparum* to AS-MQ combination.

Keywords: *Plasmodium falciparum*, artesunate, mefloquine, resistance, *pfmdr1* copy number

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A19 *Pvmdr1* Polymorphisms of *Plasmodium vivax* Isolates from Malaria Endemic Areas in Southern Provinces of Thailand

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Abstract

Since chloroquine is the mainstay for treatment of *Plasmodium vivax* infection, monitoring of chloroquine drug resistance marker provide useful information for effective malaria control program. The aim of the study was to investigate the genetic polymorphisms of *Pvmdr1* in *Plasmodium vivax* isolates collected from Southern Thailand. A total of 70 *P. vivax* isolates were collected by finger-prick from patients, DNA from dried blood spot samples were extracted and analyzed for *Pvmdr1* polymorphisms. Seven non-synonymous mutations and two synonymous were identified in *Pvmdr1* gene. Five haplotypes of *Pvmdr1* were observed with different frequency. However, the majority of the isolates (21/70) carried 4 mutations of 515R+698S+908L+958M. Amino acid Y976F and F1076L mutations were detected in 17 (24.3%) and 38 (54.3%) isolates, respectively. The prevalence and pattern of mutations of *Pvmdr1* obtained from this study suggest the spreading of chloroquine resistance alleles in *P. vivax* isolates from Southern part of Thailand and the monitoring of chloroquine drug resistance marker in *P. vivax* is essential for earlier warning system and expedites the appropriate drug policy.

Keywords: *Plasmodium vivax*, *Pvmdr1*, chloroquine resistance

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A20 Prevalence of Co-Morbidity of Malaria and Glucose-6-Phosphate Dehydrogenase Deficiency in Malaria Endemic Area along Thai-Myanmar Border

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Abstract

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is relatively common in populations exposed to malaria. The prevalence of G6PD deficiency variants differs widely among each country and ethnic groups. G6PD Viangchan are the most common variant in Laotians, Malaysian Malays, Cambodians and Vietnamese, G6PD Viangchan and Mahidol are most prevalent in Thailand and G6PD-Mahidol are the dominant mutation in Burmese population. This deficiency can cause hemolysis and impact on malarial treatment by primaquine, anti-malarial drug that used to prevents relapse in vivax and ovale malaria, and gametocytocide in falciparum malaria. The objective of this study was to detect the prevalence of G6PD variants in malaria patients who living in malaria endemic regions. Total of 109 dried blood spot samples (24 Thais and 85 Burmeses) were collected from malaria clinic in Mae Hong Son, Tak and Ranong provinces during 2011 to 2013. Three variants of G6PD mutation that most found in Thai-Myanmar borders including Mahidol, Viangchan and Chinese 4 variant were investigated by polymerase chain reaction (PCR). Only Mahidol variant was found with prevalence of 9.17% (10/109). Among these, one sample were found in Thai malaria patients (4.17%) and 9 samples were found in Burmese malaria patients (10.58%). The results might be indicated the ethnic effect of patients that have different common variant of G6PD or the protective effect of other G6PD variants (Viangchan and Chinese 4) against malaria infection in this population. Therefore the prevalence of G6PD variants in other malaria endemic regions should be performed to investigate the prevalence and correlation between G6PD variants and malaria disease. The information should be useful for malaria treatment by optimizing dosage regimen in endemic areas with reporting data on G6PD variants.

Keywords: glucose-6-phosphate dehydrogenase deficiency, Mahidol variant, malaria, Thailand

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A21 Prevalence of CYP3A5 Polymorphism in Thai Chronic Kidney Disease Patients

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Abstract

Cytochrome P450 3A5 (CYP3A5) is a microsomal enzyme that responsible to metabolic pathway of many drugs such as tacrolimus, vincristine etc. CYP3A5 activity is encoding by *CYP3A5* gene. Patients who carried *CYP3A5*3/*3* and *CYP*1/*3* had lower CYP3A5 enzyme activity than *CYP3A5*1/*1*. Tacrolimus is the most common immunosuppressive used for post kidney transplantation. Recent data showed *CYP3A5*1/*1* patients need lower tacrolimus dosage regimen than mutant alleles. Moreover, prevalence of *CYP3A5* genotypes is different between Asia and Caucasian population. Therefore, *CYP3A5* genotype investigated before kidney transplantation may need to improve efficacy of tacrolimus treatment. The aim of this study was to determine prevalence of *CYP3A5* genetic polymorphisms in Thai chronic kidney disease patients who are waiting list for kidney transplantation. This study was approved by ethic committee of Khon Kaen University. The patients were informed consent. Eight hundred and four patients were enrolled in this study. Blood was collected from each patient to analyze *CYP3A5* genotypes by real time polymerase chain reaction (PCR) with *Taqman*® probe. The results of *CYP3A5* genotype analysis revealed that genotype frequencies of *CYP3A5*1/*1*, *CYP3A5*1/*3* and *CYP3A5*3/*3* were 125 (15.55%), 402 (50.00%) and 277 (34.45%), respectively. Allele frequencies of **1* and **3* were 652 (40.55%) and 956 (59.45%), respectively. Conclusion, the allele frequencies of CYP3A5 from this study were similar to Asian populations, but were significantly different from Middle Eastern, Europe, American, and African populations.

Keywords: CYP3A5 polymorphisms, prevalence, Thai

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A22 Reported of 5th Year *HLA-B*58:01* Test Service at Pharmacogenetics Analysis Unit, Srinagarind Hospital

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Abstract

Allopurinol is a uric acid lowering drug, primarily used for chronic gout, kidney stones and hyperuricemia treatment. However, the most serious adverse reaction of allopurinol is Stevens–Johnson syndrome and toxic epidermal necrolysis (SJS/TEN). The correlation between *HLA-B*58:01* and SJS/TEN was found in Hun Chinese and Thai populations. Therefore, *HLA-B*58:01* testing has important role in allopurinol treatment. *HLA-B*58:01* analysis has been service at Pharmacogenetics Analysis Unit, Department of Pharmacology, Faculty of Medicine, Khon Kaen University since 2010. *HLA-B*58:01* was determined based on specific primers polymerase chain reaction. This study aimed to investigate 1) the demographic data of the patients who requested for *HLA-B*58:01* test 2) to determine the association between *HLA-B*58:01* negative test and allopurinol adverse drug reactions. The study was performed retrospectively in 69 patients with mean age 65.88 years, 52 males and 17 female. Medical records were reviewed. Indications of allopurinol treatment were gouty arthritis (79.71%), hyperuricemia (4.35%), and others. Pre-allopurinol treatment was 55 cases (79.71%). Forty five cases of pre-allopurinol treatment were *HLA-B*58:01* genotyping negative. Seven teen of them were no adverse drug reaction after started allopurinol treatment (37.78%). Twenty eight of them were no allopurinol treatment. Therefore, *HLA-B*58:01* test is useful information for avoidance of serious cutaneous adverse reaction.

Keywords: allopurinol, *HLA-B*58:01*, SJS/TEN, pharmacogenetics

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A23 — Screening for NAD(P)H: Quinone Oxidoreductase 1 (NQO1) Inhibitors in Human Cell Lysates

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Abstract

NAD(P)H:quinone oxidoreductase 1 (NQO1, DT-diaphorase, E.C. 1.6.99.2) is a cytosolic flavoprotein which regarded as a chemo-protective enzyme. Latterly, NQO1 has been recognized as a crucial source to contribute chemoresistance in many human cancers. Numerous studies have been shown that inhibition of NQO1 by pharmacological inhibitor, dicoumarol, suppressed a number of human malignant cell growths and also potentiated cytotoxicity of chemotherapeutic drug; however, dicoumarol has diverse side effects as well. The goal of this work was to determine inhibitory effects of coumarin and three of its derivatives, 7-hydroxycoumarin, esculetin, and scopoletin, on human NQO1 enzyme activity in cell lysates. Using NQO1 activity assay, the result shows that coumarin and the three derivatives, 7-hydroxycoumarin, esculetin, and scopoletin, at the concentration of 4.55 µM inhibited the NQO1 enzyme activity in liver hepatocellular carcinoma HepG2 cell lysates up to 41.81 ± 3.79, 63.45 ± 0.25, 34.88 ± 5.68, and 77.38 ± 0.70 % (mean±SE), respectively. Moreover, we also demonstrated that 7-hydroxycoumarin and scopoletin exhibited the potent inhibitory effects on human NQO1 enzyme activity with K_i values in the low micromolar range, 1.51 ± 0.53 µM and 0.55 ± 0.15 µM (mean±SE), in cholangiocarcinoma KKU-100 cell lysates. This indicates that 7-hydroxycoumarin and scopoletin could be a great alternative NQO1 inhibitors and these prompt us for the further investigation of their synergistic efficacy in combination with the chemotherapeutic drug.

Keywords: NQO1, coumarin, 7-hydroxycoumarin, esculetin, scopoletin

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A24 Anti-cancer Activities of an Isolated Steroidal Alkaloid from the Seeds of *Combretum quadrangulare* Kurz (Combretaceae)

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Abstract

The purpose of this study was to isolate new compound from the seeds of *Combretum quadrangulare* Kurz, that showed anti-cancer activities. The seeds were macerated with 95% ethanol. Purified by column chromatography using Avicel as the adsorbent and using 50% methanol in water as the eluting solvent and further purified by Avicel preparative layer chromatogram 2 times by using 80% methanol in water as developing solvent. Crude extracts showed cytotoxicity to NCI-H187 small lung cancer, human hepatocarcinoma ATCC HB-8065 and human colon adenocarcinoma and ATCC HTB-37 at 300 µg/ml. The isolated pure compound named combretin, showed significant cytotoxic at the concentration greater than 300 µg/ml. In addition the crude extract showed significant toxicity at IC_{50} 120 µg/ml in normal cells but IC_{50} in liver cancer cells was 260 µg/ml. In the normal cell test Vero cells crude extract showed cytotoxic effect at the concentration lower than in cancer cells tested. Crude extract was very toxic to the normal cell so it was not suitable to develop this pure compound to be anti-cancer drug.

Keywords: *Combretum quadrangulare*, anti-cancer activities, steroidal alkaloid

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A25 | Effects of Leukotriene Receptor Antagonists on Cell Viability and Matrix Metalloproteinase 2 Expression in Human Astrocytoma Cells

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Abstract

Expressions of matrix metalloproteinases (MMPs) including MMP-2 have been found to be up-regulated and correlated with metastasis and angiogenesis of various cancers including malignant gliomas. Antileukotriene drugs are effective treatment for asthma with limited side effects. Recent studies showed that leukotriene D4 could enhance angiogenesis and leukotriene receptor antagonists (LTRAs) and 5-lipoxygenase inhibitors inhibited tumor metastasis of liver, pancreatic and colon cancers. This study aimed to investigate the effect of two LTRAs, montelukast and zafirlukast, and a 5-lipoxygenase inhibitor, zileuton, on cell viability and MMP-2 expression in human astrocytes U373 cells. MTT assays were used to examine the effects of antileukotriene drugs on cell viability. The expression and activity of MMP-2 were measured using the gelatin zymography and quantitated using the densitometric analysis. At 24-hour post-exposure, the median toxic concentrations of montelukast, zafirlukast, and zileuton were 9.03, 18.58, and 237.3 μM, respectively. Montelukast (5 μM) and zafirlukast (5 μM), not zileuton (25 μM), were significantly decreased MMP-2 expressions at 24-hour post-exposure. These results indicated that LTRAs could inhibit tumor growth and metastasis and these drugs could be a novel therapeutic option in glioma treatment.

Keywords: matrix metalloproteinase 2 (MMP-2), montelukast, zafirlukast, astrocytoma

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A26 Salinomycin Inhibitory Effects on Cell Viability, Migration, and Its Tamoxifen Sensitization Effect in the Anti-Estrogen Resistant Breast Cancer: MCF-7/LCC2 and MCF-7/LCC9 Cells

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Abstract

Hormonal therapy is the standard adjuvant treatment of ER-positive breast cancer. However, resistance to hormonal therapy leads to more invasive cancer phenotype, tumor recurrence, and distant metastasis. Thus, the development of novel agents against anti-estrogen resistance is urgently required due to limitation of drug choices. Salinomycin is a monocarboxylic polyether ionophore which isolated from the culture supernatant of the *Streptomyces albus*. Recently, salinomycin was shown to inhibit multidrug resistant cancer cells and also functioned as P-glycoprotein inhibitor in cancer stem cells. However, the effect of salinomycin on anti-estrogen resistant breast cancer cells has not been elucidated. The objective of this study is to investigate anti-cancer activity of salinomycin on cell viability, migration, and increasing tamoxifen sensitivity in the anti-estrogen resistant breast cancer cell : MCF-7/LCC2 and MCF-7/LCC9 cells. Anti-cancer activity of salinomycin was assessed by MTT cell viability assay. The result showed salinomycin inhibited cell viability in concentration dependent manner with IC_{50} value of salinomycin in MCF-7/LCC2 and MCF-7/LCC9 cells are 24.17 and 19.74 μM at 24 hours,respectively. The anti-migration effect was evaluated by scratch assay. Salinomycin is able to inhibit cell migration in MCF-7/LCC2 ($p<0.05$) and MCF-7/LCC9 ($p<0.05$) cells after 30 hours of wound scratch. We also evaluated synergistic effect on the cell growth inhibition with tamoxifen using tamoxifen response assay. The result showed combination treatment of salinomycin and tamoxifen resulted in significantly greater inhibition on cell viability in MCF-7/LCC2 and MCF-7/LCC9 cells than tamoxifen treatment alone. Salinomycin may be a novel therapeutic agent for increasing the efficacy of treatment in patients. This study will serve as the preliminary data for further study both in animal tumor model and clinical study in anti-estrogen resistant breast cancer patients in the future.

Keywords: salinomycin, breast cancer, anti-estrogen resistant breast cancer, tamoxifen, migration

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A27 Anticancer Activity of *Atractylodes lancea* (Thunb.) DC. in Hamster Model and Application of Positron Emission Tomography for Early Detection and Monitoring the Progression of Cholangiocarcinoma

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Abstract

Opisthorchis viverrini (OV)-induced cholangiocarcinoma (CCA) is an important cancer in the Great Mekong region, particularly in Thailand. Limitation of treatment option and the lack of effective diagnostic tool for early detection of CCA are of major concerns for the control of this type of cancer. The aim of the study was to investigate anti-CCA activity of the ethanolic extract of *Atractylodes lancea* (Thunb.) DC. (AL), and to investigate the applicability of Positron Emission Tomography-Computed Tomography (PET-CT) imaging system as a tool for detection and monitoring the progression of CCA in *Opisthorchis viverrini* (OV)/dimethylnitrosamine (DMN)-induced CCA hamsters. Male Syrian hamsters were used for toxicity tests and anti-CCA activity evaluation. Development of CCA in hamsters was induced by initial feeding of 50 metacercariae of OV, followed by drinking water containing 12.5 ppm of DMN. AL was administered orally for 30 days. PET-CT was performed every 4 weeks after initiation of CCA using ^{18}F-fluorodeoxyglucose (^{18}F-FDG). Results from the present study demonstrated promising anti-CCA activity of AL to prolong survival time and safety profile compared to the control group in hamster model. In conclusion, AL exhibited anti-CCA activity in hamster model and modification approach of radiolabeling to successfully apply PET-CT as a tool for early detection of tumor development and progression is required to improve its specificity to CCA cells.

Keywords: cholangiocarcinoma, anticancer, PET-CT, *Atractylodes lancea* (Thunb.) DC.

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A28 Cytotoxic and Anti-invasion Activities of Plumbagin against Cholangiocarcinoma Cell Line

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Abstract

Cholangiocarcinoma (CCA) is a cancer that arises from the epithelial cell of the bile duct both inside and outside the liver. Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), the major constituent isolated from the root of *Plumbago indica* Linn., has been shown to exhibit a wide spectrum of pharmacological activities. The aim of this study was to investigate cytotoxic and cellular invasion activities of plumbagin in a CCA cell line (CL-6). Cytotoxic activity was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and cellular invasion activity was investigated using QCM 96-well cell invasion chambers kit. Plumbagin showed significantly potent cytotoxic activity against CL-6 compared with the standard drug 5-FU (mean ± SD IC_{50} = 24.18 ± 3.33 µM *vs* 1036 ± 137.77 µM, respectively, p = 0.05). In addition, plumbagin exhibited 49% inhibitory activity on CL-6 cell invasion at 25 µM compared with control. Results suggest that plumbagin could be a promising candidate for further development as chemotherapeutic drug against CCA.

Keywords: cholangiocarcinoma, plumbagin, cytotoxicity

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A29 Cytotoxic Activity of Ethanolic Extract of *Atractylodes lancea* (Thunb.) DC. against Cholangiocarcinoma Cell Line

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Abstract

Atractylodes lancea (Thunb.) DC. has been ethno-traditionally used as crude extracts/decoctions or as a major component in several herbal formulations for treatment of several diseases or conditions. The aim of the study was to investigate cytotoxic activity against cholangiocarcinoma (CCA) cell line of the ethanolic extract of *A. lancea* (ALE) obtained from two traditional herbal shops in Nakhon-Pathom (NP) and Bangkok (CP). Three different extraction procedures, namely, maceration, sonication, and heat-reflux were used to prepare ALE. Cytotoxic activity of the extracts and 5-fluorouracil (standard control) on CCA cell line --CL-6 and normal human embryonic fibroblast cells --OUMS-36T was evaluated using standard MTT assay at the concentration range of 0-250 µg/mL. ALE from both sources exhibited selective cytotoxic activity towards CL-6 with selectivity index (SI) of 2.5-3.0. Specifically, the ALE obtained from both NP and CP prepared by sonication exhibited the highest cytotoxic activity against CL-6 cells with IC_{50} (concentration that inhibits cell growth by 50 percent) values (mean $\pm$ SD) of 26.15 $\pm$ 0.48 and 28.29 $\pm$ 0.13 µg/mL, respectively. The ALE obtained by maceration and heat-reflux from both sources showed relatively lower activity (IC_{50} 33.03 - 39.23 µg/mL). The potency of activity of all ALE preparation was about 2-fold of 5-flurouracil (IC_{50} 72.86 µg/mL).

Keywords: *Atractylodes lancea* (Thunb.) DC., cholangiocarcinoma, cytotoxicity

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A30 Antiproliferative Activity and Apoptosis-Inducing Mechanism on Human Hepatocellular and Colorectal Carcinoma Cells of Extracts and Alkaloids from *Coscinium fenestratum* Stems

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Abstract

Stems and roots of turmeric tree (*Coscinium fenestratum*) have been used in traditional medicine as bitter tonic and to treat a variety of diseases *e.g.* jaundice, liver diseases and cancers including liver and colon. Our previous study demonstrated that its chloroform and methanolic extracts exhibited potent antiproliferative activity on a panel cancer cells; LLC, A549, MCF-7 and MDA-MB-231 cells. Moreover, berberine, a main isoquinoline alkaloid isolated from the active extracts, inhibited the proliferation of lung cancer cells (LLC) by inducing apoptosis and blocking the NF-κB signaling pathway. In the present work, we evaluated the antiproliferative activity of its extracts and three bioactive alkaloids; berberine, palmatine and jatrorrhizine on human hepatocellular (HepG2), colorectal (HT-29 and HCT-116) carcinoma cells using MTT assay. Additionally, HPLC profiling of stem extracts were determined. Cellular mechanisms of apoptosis induction on treated cancer cells were then detected as follows; morphological changes by Hoechst 33342 staining, cell cycle arrest and Annexin-V/PI apoptosis induction by flow cytometry as well as caspase-3 activation by caspase-3 enzyme assay, respectively. Treatment of HepG2, HT-29 and HCT-116 cells with various concentrations of *n*-hexane, chloroform and methanolic extracts at 24, 48 and 72 hr resulted in a dose-and time-dependent manner. Chloroform extract exhibited strongest antiproliferative effect on cancer cells with IC_{50} values of 12.25, 22.66 and 17.79 µg/mL after 72 hr incubation, respectively. HPLC profiling indicated the presence of berberine, palmatine and jatrorrhizine in the active extracts. Berberine displayed the most potent antiproliferative efficacy than that of palmatine and jatrorrhizine. Interestingly, berberine and palmatine induced apoptosis in treated cells as shown by loss of viability, chromatin condensation, sub-G1 accumulation as well as caspase-3 activation, whereas jatrorrhizine had lowest effect. Our findings suggest that *C. fenestratum* extracts and its bioactive alkaloids have antiproliferative effects on hepatocellular and colorectal cancers and these effects are mediated through apoptosis.

Keywords: antiproliferative activity, apoptosis induction, *Coscinium fenestratum,* isoquinoline alkaloids, hepatocellular and colorectal carcinoma cells

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A31 Dihydrocapsaicin Induces Tumor Necrosis Factor-α-Mediated G1 Cell Cycle Arrest and Apoptosis in Human Cervical Cancer Cells

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Abstract

Capsaicinoids, as the spicy pungent taste, are found in red peppers of the Capsicum genus, used as spice, food additive and drug. Capsaicin and dihydrocapsaicin which are the two most abundant capsaicinoids in peppers have been known to induce selectively apoptosis in malignant cells but not in the normal cells. This study investigated the effect of dihydrocapsaicin on the cell cycle and apoptosis in tumor necrosis factor-α (TNF-α)-treated human cervical cancer HeLa cells. Dihydrocapsaicin showed cytostatic activity on HeLa cells at 12 hr, especially in the presence of TNF-α by inducing cell cycle arrest in the G1 phase correlated with the downregulation of cyclin D1 expression and RB protein phosphorylation. At 24 and 48 hr after the treatment, dihydrocapsaicin significantly enhanced TNF-α-induced cancer cells death in a concentration-dependent manner. Regarding mode of cell death, we found that dihydrocapsaicin enhanced TNF-α-induced cell death via apoptosis pathway indicated by the presence of poly-(ADP-ribose) polymerase (PARP-1) cleavage. Furthermore, the results obtained from immunoblotting assay showed that dihydrocapsaicin in combination with TNF-α significantly reduced the activated forms of survival proteins including NF-κB, TAK-1, IKK-α, P65, p38, Akt, JNK, and Erk. Taken together, this study indicated that dihydrocapsaicin sensitized TNF-α-induced G1 cell cycle arrest and apoptosis in HeLa cervical cancer cells by suppressing survival signaling pathways.

Keywords: dihydrocapsaicin, G1 cell cycle, apoptosis, survival proteins

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A32 Effect of Combination of Gefitinib and Bufotalin on Non-Small Lung Cancer Cells Apoptosis

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Abstract

This study investigated the effects of bufotalin, a bufodienolide compound isolated from toad venom, in combination with gefitinib on apoptosis of three non-small lung cancer cell lines, including A549, RPC-9 and PC-9 cells. The cells were treated with bufotalin in the presence or absence of gefitinib. The cells viability was observed using WST-1(4-[3-(4-iodophenyl)-2-(4-nitrphenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate) assay at 24 hr. The results showed that bufotalin alone caused cytotoxic to A549, RPC-9 and PC-9 lung cancer cells with IC_{50} values of 11.03, 6.27 and 4.31 µM, respectively, while 10 µg/mL gefitinib showed weak cytotoxicity on all of cells. In combination experiment, bufotalin enhanced gefitinib-induced apoptosis of A549, RPC-9 and PC-9 cells from the lowest to highest respectively. After 1 hr pretreatment with 0.1 µM bufotalin, the cells were treated with or without 10 µg/mL gefitinib for 4 hrs, the apoptotic proteins were then examined by immunoblotting assays. The results showed that bufotalin and gefitinib alone induced the activation of caspase-3 and PARP-1, markers of physiological apoptosis in PC-9 and RPC-9. The combination of bufotalin and gefitinib markedly promoted a cleavage of PARP-1 and caspase-3 in PC-9 and RPC-9. The results indicated that bufotalin enhanced gefitinib-induced apoptosis of PC-9 and RPC-9 non-small lung cancer cells.

Keywords: gefitinib, bufotalin, apoptosis, non-small lung cancer cells

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A33 Effects of *Polygonum odoratum* Lour Extract on Improvement of Memory Deficit Induced by Scopolamine in Mice

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Abstract

In searching for a promising candidate for treatment of neurodegenerative diseases such as Alzheimer/dementia, the ethanol extract of *Polygonum odoratum* Lour was investigated for neuroprotective effects by both *in vitro* and *in vivo* models. The results showed that the ethanol extract of *Polygonum odoratum* Lour at the concentration of 100 µg/mL exhibited ABTS radical scavenging activity and inhibitory activity on acetylcholinesterase with % inhibition of 99.18 and 48.41, respectively. The neuroprotection study in cell culture revealed that the *Polygonum odoratum* Lour extract can reduce neuronal death induced by oxidative stress and β-amyloid toxicity. For the Morris water maze test which reflects in long-term memory, pretreatment mice with the ethanol extract of *Polygonum odoratum* Lour at the concentration of 250 mg/kg/day for 7 days significantly decreased escape latency time when compared with the only scopolamine treated mice. In addition it significantly increased % alternation and discrimination ratio measured by the Y-maze and object recognition test which were used as a measure of short-term memory. All results indicated that the extracts of *Polygonum odoratum* Lour could improve both short term and long term memory. Thus, the *Polygonum odoratum* Lour extract might be a potential candidate for further developing as the drug for Alzheimer's disease.

Keywords: *Polygonum odoratum* Lour, Alzheimer, antioxidant, acetylcholinesterase inhibitor, neuroprotection

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A34 The Effects of *Streblus asper* Lour. on Motor Functions in MPTP-Treated C57BL/6 Mice: a Model of Parkinson's Disease

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Abstract

Parkinson's disease (PD) is one of the common neurodegenerative disorders caused by degeneration of dopaminergic neurons in the substantia nigra leading to disruption of neuronal structures and/or functions. Among all the etiologies suggested to be the causes of PD, the important common mechanisms of causing neurodegeneration are oxidative damage and inflammation. *Streblus asper* Lour. is a medicinal plant that widely used in Thailand which has an anti-oxidative and anti-inflammatory properties. This study aimed to investigate effects of *Streblus asper* Lour. (SA) leaf extract on motor functions of C57BL/6 mice treated with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). Animals were divided into 3 experimental groups including control, MPTP, and MPTP+SA200 groups. C57BL/6 mice were injected with 80 mg/kg MPTP (i.p.) in a 7-day period, after that the animals were tested with catalepsy (on day 3, 5, 7, and 9), beam balance (on day 5, 7, and 9) and spontaneous locomotor activity (on day 18) tests. The results showed that MPTP-treated mice had significant positive score in the catalepsy test, increase number of foot faults in beam balance, and decrease locomotor activities in activity chamber when compared to the control group. Interestingly, SA leaf extract at dose of 200 mg/kg/day (MPTP+SA200 group) could reverse MPTP-induced motor dysfunctions in all tests. Conclusion, SA leaf extract can ameliorate MPTP-induced motor dysfunctions in C57BL/6 mice. SA leaf might be useful as an additional or alternative treatment in PD.

Keywords: *Streblus asper* Lour., MPTP, Parkinson's disease

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A35 Neuroprotective Effects of Nicergoline and Its Toxicity on Human Neuronal and Astrocyte Cells

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Abstract

Nicergoline, a selective α_1 adrenergic receptor antagonist, is currently used for treatment of cognitive impairment. Previous studies showed neuroprotective activity of nicergoline in murine models. Herein, we studied the effects of nicergoline and its protective effect against amyloid-β peptides. High concentration of nicergoline decreased viability of human cortical neuronal (HCN-2) cells with a half maximal toxic concentration(TC_{50}) at 92.2 µM at 24 hours post-exposure. Nicergoline at 30 µM increased cell survival and reduced the loss of mitochondrial membrane potential ($\Delta\Psi m$) in HCN-2 exposed to amyloid-β fragment 25-35 ($A\beta_{25-35}$). Nicergoline was also toxic to human astrocytoma U-87 MG cells with TC_{50} at 112.40 µM at 24 hours post-exposure. Nicergoline and $A\beta_{25-35}$ did not enhance reactive oxygen species levels orelevate lipid peroxidation production in human neurons and astrocytes as detected by dichlorofluorescein (DCF) and thiobarbituric acid reactive substances (TBARs) assays. Additionally, U-87 MG cells treated with nicergoline at 10 µM showed increase in mRNA levels of glial cell-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF) and catalase at 24 hours post-exposure. This study indicated that the non-toxic concentration of nicergoline showed limited direct neuroprotection against amyloid-β peptides and could elevated neuronal survival by increasing the expression of neurotrophic factors and antioxidant enzymes.

Keywords: nicergoline, neuron, astrocyte, beta-amyloid, neurotrophic factors

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A36 — Comparing the Platelet Inhibitory Effect of Ticagrelor 180 mg and Clopidogrel 600 mg Loading Dose in Thai Patients Who Planned Undergoing Coronary Angiography

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Abstract

Pretreatment with clopidogrel, an antiplatelet is beneficial for patient undergoing coronary angiography (CAG) as it reduces rate of morbidity and mortality due to cardiovascular events after the procedure. Ticagrelor, a newer antiplatelet, has been shown to be superior than clopidogrel in terms of effects on platelet activity and improvement of clinical outcomes. However, there is no evidence comparing these two antiplatelets in Thai patients. This encourages us to do this study, since variation of pharmacokinetics, pharmacodynamics properties, and efficacy of clopidogrel among populations has been reported. This is a randomization-controlled study, 26 patients undergoing CAG from King Chulalongkorn Memorial Hospital. Patients were randomized (1:1) to receive either clopidogrel 600 mg loading dose or ticagrelor 180 mg loading dose prior to the procedure. Blood specimens were obtained before and at 6 hours after antiplatelet administration. Platelet function was evaluated using light transmission aggregometry (LTA) technique after an induction with adenosine diphosphate (ADP). Both clopidogrel and ticagrelor significantly decreased platelet activity. Patients receiving ticagrelor had significantly higher inhition of 20 mM ADP-induced platelet aggregation. But with lower concentration of ADP (5 mM), non-significant difference of platelet activity was obtained. However, a tendency towards higher efficacy in ticagrelor group is observed. Therefore, antiplatelet activity of ticagrelor is likely to be more potent than clopidogrel in Thai population.

Keywords: platelet aggregation, ticagrelor, clopidogrel, coronary angiography

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A37   *Momordica cochinchinensis* Aril Extract Reduces Blood Pressure and Oxidative Stress in L-NAME-Induced Hypertensive Rats

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Abstract

Momordica cochinchinensis (*M. cochinchinensis*) is a plant of Cucurbitaceae family commonly known as Gac fruit or Fak-Khaoin Thai. *M. cochinchinensis* possesses strong antioxidant and pharmacological activities. N$^\omega$-nitro-L-arginine methyl ester (L-NAME) is a nitric oxide synthase (NOS) inhibitor causes reducing nitric oxide (NO) bioavailability, and enhances oxidative stress that induces hypertension. L-NAME-induced hypertension is a well established experimental model of hypertension. This study aimed to investigate the effect of *M. cochinchinensis* aril extract (MCE) on prevention of high blood pressure progression and preservation of antioxidant status in L-NAME-induced hypertensive rats. Male Sprague-Dawley rats received L-NAME (50 mg/kg/day) via drinking water for 3 weeks, together with intragastric administration of deionized water (DI) or MCE at dose of 100 or 500 mg/kg/day. Rats received tap water and intragastrically administered with DI were served as normotensive controls. It is found that blood pressure, vascular superoxide production and plasma malondialdehyde levels were markedly increased in L-NAME treated-rats. Moreover, blood glutathione level was also decreased after L-NAME administration. MEC significantly alleviated these deleterious effects by reducing blood pressure (approximately 20% from L-NAME-treated group), decreasing oxidative stress and enhancing antioxidant glutathione ($p<0.05$). The results of this study suggest the beneficial effect of *M. cochinchinensis* or Fak-Khao for prevention of hypertension.

Keywords: hypertension, L-NAME, *Momordica cochinchinensis*, nitric oxide, antioxidant

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A38 Subchronic Toxicological Evaluation of Protein Hydrolysates from Sung-Yod Rice Bran in Sprague-Dawley Rats

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Abstract

Sung-Yod rice (*Oryza sativa* L.), a red-violet pigmented rice, is grown generally in the southern part of Thailand. Hydrolysates-derived from Sung-Yod rice bran (RBH) contains contains amounts of nutrients and biological active compounds, such as tocopherols, tocotrienols, oryzonols, andphenolic substances. The objective of this study was to evaluate the sub-chronic toxicity of RBH from the Southern part of Thailand in Sprague-Dawley rats. According to the Organization of Economic Cooperation and Development guideline (OECD-408) for sub-chronic toxicity testing, male and female rats (8 weeks old) were randomly divided into 2 groups (N=10/group). Rats in the control group received a normal rat chow diet whereas rats in the RBH-treated group received a normal rat chow diet mixed with 1.5% of RBH (or equivalent to 1 g/kg/day) for 90 consecutive days. After 90 days, all animals were sacrificed with overdose of pentobarbital sodium (120 mg/kg, i.p.). Blood samples and organs were collected for evaluation of hematological and biochemical parameters, gross anatomy and histopathology. There was no death of animals in both groups. The body weights of all rats are normally increased. There were no obvious changes in general appearance and histopathology of the organs of rats treated with RBH compared to the control. The hematological and biochemical levels were within normal ranges. Interestingly, it is found that the serum level of triglyceride was decreased (90.1±4.5 vs 62.1±3.9, $p<0.05$), whereas the level of HDL-cholesterol was increased in RBH treated rats compared to the control (64.7±1.4 vs 71.2 ± 1.7, $p<0.05$). Results of this study indicate that RBH (1 g/kg/day) is generally safe with no adverse effects in rats. These findings suggest that RBH is non-toxic and can be used as a food supplement for prevention of the dyslipidemia.

Key words: rice bran hydrolysates, Sung-Yod rice (*Oryza sativa* L), Sprague-Dawley rats, sub-chronic toxicity

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A39 The Toxicity of *Spilanthes acmella* (L.) Murray from Methanol Crude Extract for Fruit Fly Life Cycle

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Abstract

Spilanthes acmella is a species of flowering herb in the family Asteraceae and natural source of bioactivities such as antioxidant, anti-inflammatory and treatment for toothache. The most important taste-active molecules in *Spilanthes acmella* present are fatty acid amides such as spilanthol [(2E,6Z,8E)-deca-2,6,8-trienoic acid isobutyl amide], (2E,7Z,9E)-Undeca-2,7,9-trienoic acid isobutyl amide and (2E)-Undeca-2-en-8,10-diynoic acid isobutyl amide. This study reported the toxicity of *Spilanthes acmella* methanol crude extract at various concentrations of crude extract from 0.1 – 1000 ppm by adding in fly food (the recipe of the genetic experiment fly food in Maejo-Phrae University), and studied life cycle of fruit fly for 15 days. It was found that methanol crude extract of *Spilanthes acmella* was non-toxic for life cycle of fruit fly. Furthermore, the concentrations of crude extract best growth supported to more adult drosophila than other concentrations when compared with control (dimethyl sulfoxide, DMSO) were 1 and 10 ppm.

Keywords: *Spilanthes acmella*, Asteraceae, spilanthol, extraction, fruit fly

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A40 Effects of *Nelumbo nucifera* Seed Extract on Testicular Histology of Normal Male Rats

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Abstract

Lotus (*Nelumbo nucifera,* Nelumbonaceae) seeds have long been conducted to use as an anti-fertility agent in human. However, few scientific data are available to explain these effects and the underlying mechanisms of action are unclear. Therefore, the aim of this research was to evaluate the effects of NNSE on testicular histology of normal male rats. To determine the effects of *N. nucifera*, dried seeds were extracted using 70% ethanol. Animals were divided into 4 groups (5 animals per group). Group 1 received the distilled water orally and served as the control group. Group 2-4, animals received the suspension the extract at the dose of 100, 500 and 1000 mg/kg body weight, respectively for a period of 21 days. Testes were collected and processed for histological preparation. A number of spermatogonia, spermatocytes, spermatids and Leydig cells were counted. Furthermore, the seminiferous tubule diameter, seminiferous tubule lumen and germinal thick cell in the testis were measured. The results revealed that NNSE significantly decreased in testicular weight and a number of spermatogonia, spermatocytes, spermatids and Leydig cells in a dose-dependent manner ($p \leq 0.05$). In addition, NNSE at the dose of 500 and 1000 mg/kg body weight significantly reduced the seminiferous tubule diameter, seminiferous tubule lumen and germinal thick cell in the testis of the tested groups when compared to the control group ($p \leq 0.05$). There is evidence that *N. nucifera* seeds contain alkaloids, flavonoids and triterpenoids. It was found that alkaloids produced anti-androgenic and anti-spermatic properties in male rats. We hypothesized that alkaloids contents might be responsible for anti-fertility in this present study. The action of NNSE could be due to the interruptions of spermatogenesis which may partially be associated with the inhibition of testosterone production by Leydig cells. We conclude that NNSE may be useful as a novel anti-fertility agent in males.

Keywords: lotus, *Nelumbo nucifera*, testis, male rat, anti-fertility

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A41 Aphrodisiac Potential of Watermelon (*Citrullus lanatus*) Rind Juice cv. Kinnaree in Male Rats

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Abstract

Watermelon (*Citrullus lanatus*, Cucurbitaceae) Rind is rich in citrulline and arginine; the contents that could be useful for the treatment of erectile dysfunction (ED). Thus, the aim of this study was to investigate aphrodisiac potential of watermelon cv. Kinnaree in male rats. Watermelon rind juice (WRJ) was prepared. The animals were divided into 5 groups (5 animals per group). Group 1 received distilled water at the dose of 1 mL/day and served as a control group. Groups 2 to 4 received WRJ at the doses of 0.5, 0.75 and 1 mL/day, respectively, daily for 35 days. Group 5 received Sildenafil citrate at the dose of 60 mg/kg body weight, one hour prior to the experiment and served as a positive control group. We found that WRJ significantly increased mounting frequency and intromission frequency ($p \leq 0.05$) and significantly decreased mounting latency and intromission latency ($p \leq 0.05$) in a dose-dependent manner compared to the control group. The relative organ weights of penis, testes and prostate gland of treated animals were significantly higher than those in control group ($p \leq 0.05$). No significant differences in epididymis and seminal vesicle were observed between the groups ($p > 0.05$). WRJ produced a significant increase in the seminiferous tubule diameter, germinal thick cell, spermatogonia, spermatocytes and spermatids and caused a significant decrease in seminiferous tubule lumen in the testis of the experimental groups compared to the control group ($p \leq 0.05$). Previous reports found citrulline and arginine to be the main constituents of watermelon. We hypothesized that the aphrodisiac potential of WRJ may be due tocitrulline and arginine.The actions of WRJ might be associated with the activation of nitric oxide-cGMP pathway in penile smooth muscle, leading to smooth muscle relaxation and penile erection. We suggest that WRJ could be used as a natural aphrodisiac agent for the treatment of ED in man.

Keywords: aphrodisiac, watermelon, *Citrullus lanatus*, reproductive system, male rat

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A42 Antiviral and Anti-inflammatory Effects of Atractylodin from *Atractylodes lancea* (Thunb.) DC. Rhizome in Dengue Virus Infection

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Abstract

Dengue virus is one of the most important mosquito-borne human pathogen that causes a serious public health problem to the people who live in tropical and subtropical regions of the world including Thailand. A great deal of evidence suggests that an increased risk of severe disease is mediated by immunopathological mechanisms which create excessive immune activation. Currently, neither a vaccine to prevent nor an effective therapeutic agent to treat dengue infection is available. This study aimed to investigate antiviral and anti-inflammatory activities of atractylodin from *Atractylodes lancea* (*A. lancea*) (Thunb.) DC. rhizome against dengue virus infection. Antiviral and anti-inflammatory activities against dengue virus infection of atractylodin were evaluated by determining its effect on cellular viral load, viral replication, viral production, and cytokine expression and secretion profile after the cells were infected with dengue virus. These results suggest that atractylodin from *A. lancea* rhizome significantly reduced dengue virus induced cytokine expression and secretion. Atractylodin is a promising candidate for using as an anti-inflammatory agent against dengue virus infection.

Keywords: dengue virus, *Atractylodes lancea*, antiviral activity, anti-inflammatory activity

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A43 Antibacterial Activity of Ethanolic Extract from *Alstonia scholaris* Leaves against *Enterococcus faecalis* and the Cytotoxicity Effect on Human Gingival Fibroblasts

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Abstract

Alstonia scholaris has been used as traditional medicine to treat some diseases such as malaria, diabetes and skin infection. The aim of this study was to determine the antibacterial activity of ethanolic extract from *Alstonia scholaris* leaves against *Enterococcus faecalis* (*E. faecalis*), the major oral bacteria associated with re-colonization in failure endodontic treated teeth. The cytotoxicity on human gingival fibroblast cells was also basically investigated. The *in vitro* antimicrobial activity was examined in comparison with 2.5% sodium hypochlorite (the most effective endodontic irrigant), and the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined by the microdilution method. The cytotoxicity of *Alstonia scholaris* was also investigated by exposure of primary human gingival fibroblasts to various concentrations of the ethanolic leaf extract from 0.01-1 mg/ml for 24 hours, and the cell viability was measured by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Results showed the antimicrobial activity of *Alstonia scholaris* leaf extract against *Enterococcus faecalis* with the MIC and MBC were 75and 125 µg/ml, respectively. There was no significant cytotoxic effect of the extract on gingival fibroblasts as observed by MTT assay or inverted microscopy at the 24 hour-exposure time. This study suggests that *Alstonia scholaris*is potential to be a natural source in the development of anti-infective agent used in the tooth root canal treatment. However, further investigations and clinical trials are required to be considered prior clinical usage.

Keywords: *Alstonia scholaris*, *Enterococcus faecalis,* antibacterial activity, cytotoxicity, human gingival fibroblasts

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F01
Clinical Effectiveness, Quality of Life and Costs of Treatment in Transfusion-Dependent Thalassemia Patients Undergoing Iron-Chelating Therapy; Deferasirox

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Abstract

Deferasirox (DFX) is an oral iron chelator was approved for the treatment of transfusional iron overload. Iron chelation treatment aims to remove excess iron after blood transfusion to minimize complications such as cardiac and endocrine dysfunction. The objectives of this study were to determine clinical effectiveness, quality of life and treatment costs of DFX therapy in transfusion-dependent thalassemia patients, at Maharaj Nakorn Chiang Mai Hospital. Serum ferritin levels which represented clinical effectiveness and treatment costs were obtained from medical record and hospital database. The quality of life was determined by using self-report questionnaire. There were 32 patients enrolled in this study. Frequent blood transfusion of at least once a month and high baseline ferritin levels of > 1,000 µg/L were observed in 27 and 29 patients, respectively. Iron chelation with DFX monotherapy and DFX combination with deferoxamine were prescribed in 26 and 6 patients, respectively. The mean serum ferritin levels (µg/L) after receiving DFX were slightly decreasing from baseline; 3,032±2,249 to 2,957±2,190 and 7,471±5,349 to 5,556±4,335 for DFX monotherapy and DFX combination therapy, respectively. However, reduction in ferritin levels from baseline was not statistically significant due to a continued iron load from multiple blood transfusions. The average health utility score EQ-5D-3L was 0.83±0.15 correlated with EQ VAS was 0.71±0.16. The total annual treatment costs were estimated to be 374,898 THB and 84% of the expenditure was due to DFX costs. In conclusion, this study showed that DFX appears to stabilize ferritin levels after frequent blood transfusions in thalassemia patients. The health related quality of life was favorable as patients rated their health approximately more than 70% of perfect health. Nevertheless, the high costs of treatment with DFX should be concerned.

Keywords: deferasirox, thalassemia, clinical effectiveness, quality of life, costs of treatment

ประสิทธิผลทางคลินิกคุณภาพชีวิตและต้นทุนการรักษาในผู้ป่วยธาลัสซีเมียที่พึ่งพาการรับเลือดและได้รับยาขับเหล็กดีเฟอราซิรอกซ์

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บทคัดย่อ

ดีเฟอราซิรอกซ์เป็นยาขับเหล็กชนิดรับประทานใช้ในผู้ป่วยที่มีภาวะเหล็กเกินจากการรับเลือดเป็นประจำได้ วัตถุประสงค์การศึกษา เพื่อประเมินประสิทธิผลทางคลินิก คุณภาพชีวิต และต้นทุนการรักษาในผู้ป่วยธาลัสซีเมียที่พึ่งพาการรับเลือดและได้รับยาดีเฟอราซิรอกซ์ซึ่งรักษาและตรวจติดตามที่โรงพยาบาลมหาราชนครเชียงใหม่ การประเมินประสิทธิผลทาง

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คลินิก และต้นทุนการรักษาเก็บข้อมูลจากเวชระเบียนและฐานข้อมูลของโรงพยาบาล การประเมินคุณภาพชีวิตเก็บข้อมูลจาก
แบบสอบถามที่ผู้ป่วยกรอกเอง มีผู้ป่วยที่เข้าร่วมการศึกษาจำนวน 32 คน ผลการศึกษา มีกลุ่มที่ได้รับการรักษาด้วยยาดีเฟอรา
ซิรอกซ์ชนิดเดียว 26 คน และกลุ่มที่ได้รับการรักษาด้วยยาดีเฟอราซิรอกซ์ร่วมกับยาดีเฟอร์รอกซามีน 6 คน การประเมิน
ประสิทธิผลทางคลินิกพบว่าค่าเฉลี่ยของระดับซีรัมเฟอริทีนที่จุดสุดท้ายลดลงจากจุดเริ่มต้นทั้ง 2 กลุ่ม แต่ไม่พบความแตกต่าง
อย่างมีนัยสำคัญในทั้ง 2 กลุ่ม การประเมินคุณภาพชีวิตพบว่าค่าอรรถประโยชน์ด้านสุขภาพจากคะแนน EQ-5D-3L และ EQ
VAS มีค่าใกล้เคียงกันเท่ากับ 0.83±0.15 และ 0.71±0.16 ตามลำดับ ส่วนค่าเฉลี่ยค่าใช้จ่ายต่อปีของผู้ป่วยทั้งหมดที่ได้รับยาดี
เฟอราซิรอกซ์เท่ากับ 374,898 บาท โดย 84% เป็นค่ายาดีเฟอราซิรอกซ์ การศึกษานี้กล่าวได้ว่า ยาดีเฟอราซิรอกซ์สามารถ
ช่วยรักษาระดับซีรัมเฟอริทีนให้คงที่จากจุดเริ่มต้นในผู้ป่วยธาลัสซีเมียที่พึ่งพาการรับเลือด จากค่าอรรถประโยชน์ด้านสุขภาพ
กล่าวได้ว่าผู้ป่วยให้คะแนนสุขภาพตัวเองโดยประมาณมากกว่า 70 เปอร์เซ็นต์ของคะแนนสุขภาพที่ดีที่สุด และในส่วนค่าเฉลี่ย
ค่าใช้จ่ายต่อปีของผู้ป่วยทั้งหมดพบว่ามีค่าค่อนข้างสูง

คำสำคัญ: ดีเฟอราซิรอกซ์, การรักษาภาวะเหล็กเกินในผู้ป่วยธาลัสซีเมีย, ประสิทธิผลทางคลินิก, คุณภาพชีวิต, ต้นทุนการรักษา

Introduction

Thalassemia is an inherited autosomal recessive blood disorders resulting from a decrease in the production of globin chain, the protein component of hemoglobin. A decrease in the synthesis of globin chain results in an ineffective erythropoiesis and hemolysis of the red blood cell. The severe forms of thalassemia are β-thalassemia major and β-thalassemia with hemoglobin E disease. These patients have severe anemia that require multiple blood transfusion. The consequent of each transfusion is an excess in iron load which gradually accumulates in various organs causing tissue damage and organ failure.[1-2]

The primary goal of iron chelating therapy is to remove excess iron from the body. The treatment is important and necessary for transfusion-dependent thalassemia patients to reduce morbidity and increase survival since long-term blood transfusions lead to various complications. Iron level in the body is indirectly represented by serum ferritin level. Serum ferritin level of >2,500 µg/L are associated with a high risk of cardiac disease. Effective iron chelating therapy keeps iron balance between transfusion iron input and chelated iron output. In addition, the excretion of excess iron by iron chelating therapy lead to decrease serum ferritin to nontoxic levels of < 1,000 µg/L.[1,3]

Deferasirox (DFX) is an oral iron chelator was approved by the US Food and Drug Administration in November 2005. Currently, DFX is approved in many countries worldwide for the treatment of chronic iron overload due to blood transfusions in patients aged ≥ 2 years. DFX is given once daily with generally good safety and efficacy profiles. DFX possesses a high iron binding potency and selectivity. It is a tridentate iron chelators that can chelated iron from intracellular, extracellular and blood circulation.[1,4]

The objective of this study was to determine clinical effectiveness, quality of life and costs of treatment of deferasirox in transfusion-dependent thalassemia patients at MaharajNakon Chiang Mai Hospital.

Materials and Methods

Study design

The cross-sectional study was conducted in all thalassemia patients who were treated with DFX at Maharaj Nakorn Chiang Mai Hospital. Inclusion criteria were transfusion-dependent thalassemia patients aged ≥ 10 years who could understand, read and write Thai language. The informed consents were performed prior to the study in patient ≥ 18 years as well as parental informed consent and child's assent in patients <18 years. The study was approved by Research Ethics Committee, Faculty of Medicine, Chiang Mai University.

Data collection

The data of serum ferritin level, laboratory monitoring results and the treatment costs were obtained from medical records and hospital database. The quality of life were obtained from self-report questionnaire which included patients' demographic, clinical data related to any adverse events over the previous 30 days and patients' adherence to medication over the previous 7 days.

Clinical effectiveness

Serum ferritin level which indirectly indicated the total amount of iron stored in the body was used as clinical marker for DFX effectiveness.The baseline level was defined as the level obtained in the past 12 months or the level before initiation of DFX while the final level was the recent available serum ferritin level.

Quality of life

The Health Related Quality of Life (HRQoL) was measured by using self-report questionnaire; EuroQoL. The first part, EQ-5D-3L descriptive system comprises of 5 dimensions: mobility, self-care, usual activities, pain or discomfort and anxiety or depression. Each dimension has 3 levels: no problems, some problems, and extreme problems. The second part, visual analogue scale (EQ VAS) records patients self-rated health on a vertical line ranging from 0 to 100, with 100 score indicating 'Best imaginable health state' and 0 score indicating 'Worst imaginable health state'.[5] The score of EQ-5D-3L and EQ VAS were calculated based on Thai value set.[6] In addition, the laboratory result that monitored DFX adverse events including liver function test (aspartate aminotransferase: AST and alanine aminotransferase: ALT) and serum creatinine were obtained from medical record. Any adverse events that occurred during previous 30 days were recorded in self-assessment questionnaire.

Costs of treatment

The total costs of treatment paid to the hospital were estimated as annual cost per patient (costs/ patient /year). The total costs consisted of direct medical costs and direct non-medical costs. The direct medical cost comprised of DFX cost and other treatment-related costs (e.g. medical visit, concomitant medications, blood transfusion, laboratory and instrumental etc.) were estimated from hospital database. While the direct non-medical costs (i.e. transportation, meals, accommodation and other expense) were estimated from self-assessment questionnaire. All costs were reported as mean Thai Baht (THB) (year 2013 values).

Statistical analysis

Demographic data, clinical data, laboratory results, total HRQoL (EQ-5D-3L, EQ VAS) score and treatment costs were reported as descriptive analysis; mean and/or median, standard deviations (SD), minimum (min) and maximum (max) values. Wilcoxon Signed Ranks Test was used to compare means different between baseline serum ferritin level and final serum ferritin level. Spearman's correlation was used to examine relationship between HRQoL (EQ-5D-3L, EQ VAS) score with some patients'

demographic and clinical characteristics. The data were analyzed by program Microsoft Excel 2010 and SPSS (Statistical Package for the Social Sciences) version 17.0. The p-values less than 0.05 were considered significant.

Results

Demographic and clinical data

A total of 32 patients were enrolled in this study. Their demographic and clinical characteristics data were showed in Table 1. Their mean age was 24.7 years (range: 12-62 years). There were 7 patients' ages less than 18 years. Male and female were in equal number. Homozygous β-thalassemia and β-thalassemia with hemoglobin E disease were diagnosed in 23 and 9 patients, respectively. Monotherapywith DFX and DFXcombination with DFO were given to 26 patients (81.3%) and 6 patients (18.7%), respectively. The duration of treatment ≥ 12 months was reported in 11 patients (34.4%). Splenectomy was carried out in 21 patients (65.6%). Fifty-six percentage of patient had good compliance assumed from no missing dose in previous 7 days.

Clinical Effectiveness

The values of baseline versus final serum ferritin level (μg/L) of the 3 groups; All patients with DFX, DFX monotherapy and DFX combination with DFO were 3,864±3,434 VS 3,444±2,821, 3,032±2,249 VS 2,957±2,190 and 7,471±5,349 VS 5,556±4,335, respectively (Figure 1). There were no statistically significant differences between baseline and final serum ferritin level in all groups ($p > 0.05$). The mean serum ferritin changes from baseline (μg/L) of patients with DFX monotherapy and DFX combination with DFO were -74 (median:-51.5, range: (-3,984 to 4,549)) and -1,916 (median:-600, range: (-10,239 to 1,276)), respectively.Subgroup analysis in 7 pediatrics (<18 years) and 25 adult (≥18 years), the mean serum ferritin changes from baseline (μg/L) were 216 (median: 67, range: (-335 to 1,276)) and -598 (median:-350, range: (-10,239 to 4,595)), respectively.
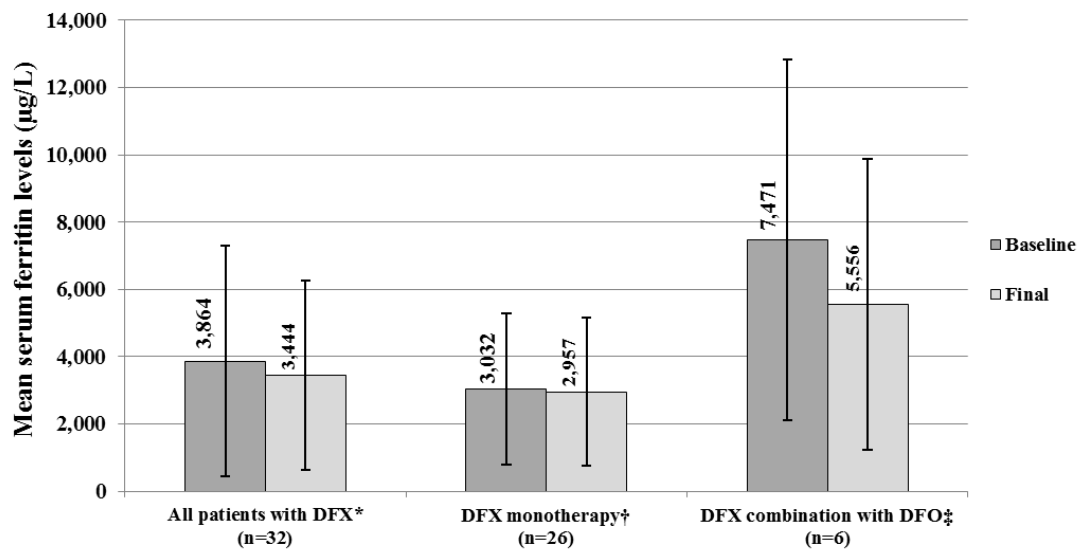
Figure 1. Mean baseline and final serum ferritin levels (μg/L)
*The *p*-value of this group was 0.22 (*p* > 0.05).
†The *p*-value of this group was 0.59 (*p* > 0.05).
‡The *p*-value of this group was 0.25 (*p* > 0.05).

Table 1. Demographic and clinical characteristics of patients (n=32)

Characteristics	Values (n, %)
10-18	7 (21.9)
≥18	25 (78.1)
Mean (SD)	24.7 (11.0)
Median (range)	22 (12-62)
Gender	
Male	16 (50.0)
Female	16 (50.0)
Diagnosis	
Homozygous β-thalassemia	23 (71.9)
β-thalassemia/Hb E	9 (28.1)
Iron chelation treatment	
Deferasirox	26 (81.3)
Deferasirox+Deferoxamine	6 (18.7)
Duration of treatment with DFX	
< 12 months	21 (65.6)
≥ 12 months	11 (34.4)
Frequency of blood tranfusions (times/month)(n=27)	
1	20 (74.1)
1-2	5 (18.5)
2	2 (7.4)
Average of blood tranfusions (Units/year) (n=27)	
< 10	1 (3.7)
10-20	12 (44.4)
> 20	14 (51.9)
Mean serum ferritin change from baseline (mean change, %)	
DFX monotherapy (n=26)	-74.3 (-2.5)
Median (μg/L) (range)	-51.5 (-3,984 to 4,549)
DFX combination with DFO (n=6)	-1,915.5 (-34.5)
Median (μg/L) (range)	-600.0 (-10,239 to 1,276)
Splenectomy	
Yes	21 (65.6)
No	11 (34.4)
Compliance (previous 7 days)	
No missed dose	18 (56.3)
Missed dose 1-2 times	12 (37.5)
Missed dose 3-4 times	2 (6.3)

Health Related Quality of life

The results of health related quality of life (HRQoL) were summarized in Table 2. The EQ-5D-3L self-assessment reported no extreme problems in all domains. Moreover, in self-care domain 100 % of patients had no problems. Only 2 (6.3%) and 1 (3.1%) patients reported some problems in mobility and usual activities domains, respectively. Some problems in pain/discomfort and anxiety/depression domain were reported as 47% and 28%, respectively. The values of EQ-5D-3L were 0.83±0.15 (median=0.75; range=0.605-1.000) while the values of EQ VAS were 0.71±0.16 (median=0.71; range=0.5-1.0).

Relationship between ED-5D-3L score and EQ VAS score with patients' demographic and clinical characteristics included age, gender, blood transfusion (Units/year), Hb and final serum ferritin level showed no significant correlation to predict HRQoL values (relationship between EQ-5D-3L; r= (-0.143), 0.025, 0.143, (-0.048), 0.053, respectively and relationship between EQ VAS; r= 0.326, 0.034, 0.040, (-0.221), (-0.066), respectively) ($p > 0.05$).

The adverse events (AE) occurred in the previous 30 days obtained from self-assessment questionnaire in patients treated with DFX monotherapy were summarized in Table 3. The liver function test and serum creatinine levels were determined in 17 and 11 patients, respectively. The results were within the acceptable range (AST, ALT; not more than 5 times upper limit of normal and/or not more than 2 times from baseline[7], serum creatinine level; 0.6-1.3 mg/dL).

Costs of treatment

The total annual costs were showed in Figure 2. The treatment costs expense in patients who received DFX less than 1 year were adjusted to annual costs. The mean total annual cost was estimated to be 374,898 THB per patient. The direct medical costs were 96% which 84% of this expense was the DFX costs (315,676 THB). The other medical costs were 45,443 THB (12%) spent to medical visit, concomitant medications, blood transfusion, laboratory and instrumental etc. The direct-non medical costs were 13,799 THB (4%) spent for transportation, meals, accommodation and other expense

.

Table 2. The health related quality of life results

Domain and levels	Values (n, %)
Mobility	
No problem	30 (93.8)
Some problems	2 (6.3)
Extreme problems	0
Self-care	
No problem	32 (100.0)
Some problems	0
Extreme problems	0
Usual activities	
No problem	31 (96.9)
Some problems	1 (3.1)
Extreme problems	0
Pain/Discomfort	
No problem	17 (53.1)
Some problems	15 (46.9)
Extreme problems	0
Anxiety/Depression	
No problem	23 (71.9)
Some problems	9 (28.1)
Extreme problems	0
EQ-5D-3L scores	
Mean±SD	0.83±0.15
Median (range)	0.75 (0.605-1.000)
EQ VAS scores	
Mean±SD	0.71±0.16
Median (range)	0.71 (0.50-1.00)

Table 3. Adverse events (AE) in previous 30 days of DFX monotherapy (n=26)

Adverse events (AE) in previous 30 days	n	(%)
Diarrhea	3	(11.54)
Headache	3	(11.54)
Fever	3	(11.54)
Abdominal pain	2	(7.69)
Loss of appetite	2	(7.69)
Decrease hearing	1	(3.85)
Back pain	1	(3.85)
Nausea/Vomiting	1	(3.85)
Arthralgia	1	(3.85)
>1 AE*	4	(15.38)

* Some patients had more than one AE.
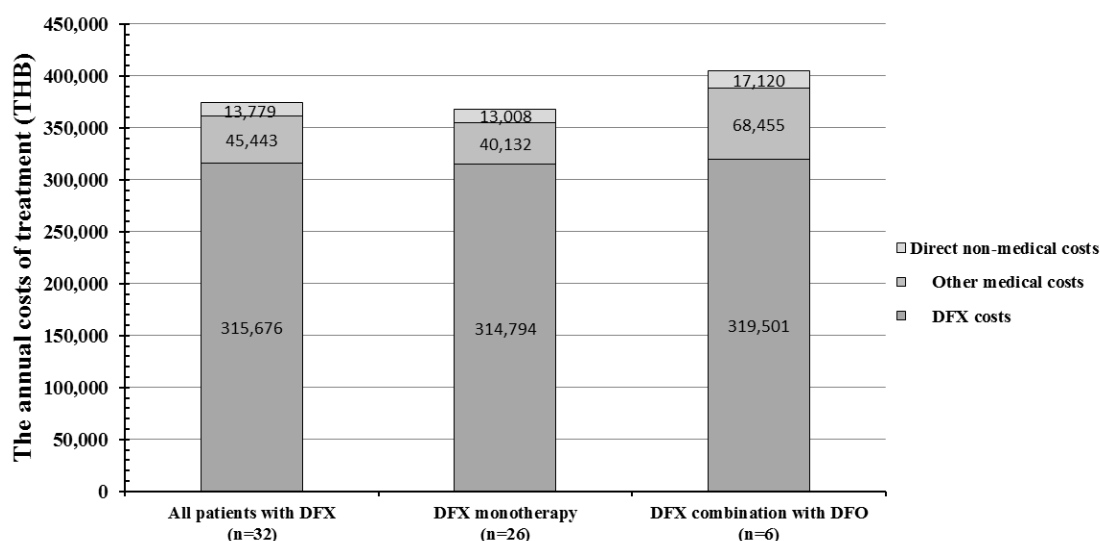
Figure 2. The annual costs of treatment in 3 groups (All patients with DFX, DFX monotherapy and DFX combination with DFO).

Discussion

This study was conducted in 32 transfusion-dependent thalassemia patients who received iron chelating therapy with DFX in Maharaj Nakon Chiang Mai Hospital. The baseline serum ferritin level of > 1,000 µg/L was observed in 29 patients. The efficacy of DFX was represented as a decreasing in final serum ferritin level from baseline. However, if the patients required multiple transfusions, the iron load would exceed the drug chelating effect. Therefore, in these cases the baseline and final serum ferritin level might not be statistically significant difference in DFX monotherapy and DFX combination with DFO ($p > 0.05$) due tocontinued iron load from multiple blood transfusions. The duration of treatment may be another factor since more than 60% of our patients received DFX < 1 year. However, the efficacy of DFX in adult (≥18 years) in reducing mean serum ferritin level was higher than in children (<18 years) and corresponded to previous study. [8]

The health related quality of life showed the mean health utility score EQ-5D-3L and EQ VAS of 0.83 and 0.71, respectively. The calculation was based on the Thai value set, supported that the patients assessed their health of approximately > 70% of perfect health. Moreover, in self-care domain 100 % of patients had no problems. The finding of health utility score in this study was in agreement with previous studies of Thai patients with chronic disease such as peritoneal dialysis and HIV/AIDS showing that EQ-5D-3L and EQ VAS scores are 0.65 and 0.65, 0.80 and 0.79, respectively.[9,10] The adverse events reported in this study were minimal and consistent with previous study.[4,8]

Base on the mean annual costs of treatment, this study found that more than 80% of the expense belonged to DFX costs. The drug is the most expensive iron chelator[11] and difficult to access. However, Thailand has the program called "EXPAP"[12] that kept DFX at lower prices and 26 patients (81%) in this program were enrolled to this study. The annual expense of DFX combination therapy was slightly higher than DFX monotherapy since the cost of other iron chelator such as DFO was much less than DFX. Luangasanatip et al. 2009 used the costs of iron chelators as cost data inputs to evaluate cost-effectiveness compared between DFX versus DFO and

DFP in Thai transfusion-dependent thalassemia patients.[13]

This study had small sample size,larger of sample size that should be considered. Moreover, the medical information was retrospectively collected. The data of EQ-5D-3L scores and EQ VAS scores in Thai's healthy population have not been study yet, so the comparison between healthy and the results obtained in this study could not be done. Therefore, the results of this study are more beneficial for pharmacoeconomics study in the future of thalassemia patients with DFX. The total costs of treatment can be used for financial planning in these patients.

Conclusion

This study showed deferasirox could maintain final serum ferritin level from baseline level despite frequent blood transfusions in thalassemia patients. The health related quality of life assessed by the patients was approximately > 70% of perfect health. However, the total annual costs of this treatment regimen were higher than other regimen available in Thailand. In further study, the severity of thalassemia and duration of treatment in deferasirox treated Thai thalassemia patients should be studied by using situation analysis and cost-effectiveness model for determine budget impact.

Acknowledgements

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F02

Clinical Effectiveness, Quality of Life and Costs of Treatment in Transfusion-Dependent Thalassemia Patients Undergoing Iron-Chelating Therapy; Deferoxamine

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Abstract

Severe anemia in thalassemia patient requiresblood transfusion therapy topromote normal growth and suppress ineffective erythropoiesis. However, the consequence of this treatmentis iron overload and accumulation of iron in the body. Since the body has no mechanism to excrete excess iron, iron chelating therapy is mandated to remove iron burden and minimize the iron overload complications. Deferoxamine (DFO) is an iron chelating agent indicated in transfusional patients. The drug has long been used in thalassemia patients at MaharajNakorn Chiang Mai Hospital. However, a high drug cost and parenteral routes of administration limit its accessibility. Therefore the clinical effectiveness, quality of life and treatment costs of DFO were determined. There were 48 thalassemia patients received DFO. DFO monotherapy and combination with deferiprone (DFO + DFP) were prescribed in 19 and 29 patients, respectively. Serum ferritin levels which represented clinical effectiveness and the direct cost of treatment were obtained from medical record. The quality of life was obtained from the self-reported questionnaire.The mean baseline versus final ferritin level (μg/L) were 2,909 $\pm$ 3,319 VS 2,786 $\pm$ 3,495 and 2,818 $\pm$ 1,733 VS 2,482 $\pm$ 1,571 in DFO and DFO+DFP, respectively. Statistical analyses showed no different between the two ferritin levels ($p > 0.05$). The mean EQ-5D-3L and VAS scores were 0.88 $\pm$ 0.16 and 0.75 $\pm$ 0.2, respectively. The total annual direct costs of DFO and DFO+DFP were 81,282.40 and 69,073.81 THB, respectively. Notably, DFO accounted for 80% of direct medical expenses. In conclusion, DFO was effective in maintaining serum ferritin levels in transfusion–dependent thalassemia patients with favorable quality of life. A high cost of treatment spent to DFO required further pharmacoeconomic evaluation.

Keywords: deferoxamine, transfusion-dependent thalassemia, iron overload, iron-chelating therapy

ประสิทธิผลทางคลินิก คุณภาพชีวิต และต้นทุนการรักษาในผู้ป่วยธาลัสซีเมียที่พึ่งพาการรับเลือดและได้รับยาขับเหล็กดีเฟอรอกเซมีน

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บทคัดย่อ

ผู้ป่วยโรคธาลัสซีเมียที่มีโลหิตจางรุนแรงต้องเติมเลือด เพื่อลดภาวะแทรกซ้อน ทำให้เกิดภาวะเหล็กเกินเนื่องจากร่างกายขับเหล็กไม่ได้ จึงต้องได้รับยาขับเหล็กดีเฟอรอกเซมีน ซึ่งยานี้ใช้ในผู้ป่วยที่ได้รับการเติมเลือดในโรงพยาบาลมหาราช

นครเชียงใหม่ แต่ยามีราคาแพงและต้องให้ด้วยวิธีฉีด ทำให้มีข้อจำกัดของการใช้ยา จึงต้องมีการศึกษาเพื่อประเมินประสิทธิผล
ทางคลินิกของยา คุณภาพชีวิต และต้นทุนการรักษา จากการศึกษาในผู้ป่วยธาลัสซีเมียจำนวน 48 ราย พบ19 ราย ได้รับ
ดีเฟอรอกซามีนอย่างเดียว และ 29 ราย ได้รับดีเฟอร์รอกซามีนร่วมกับดีเฟอริโพรน ระดับซีรั่มเฟอริทีนเฉลี่ยพื้นฐานเทียบกับ
ล่าสุดคือ 2,909 ± 3,319 กับ 2,786 ± 3,495 และ 2,818 ± 1,733 กับ 2,482 ± 1,571 ไมโครกรัม/ลิตร ของกลุ่มที่ได้รับ
ดีเฟอรอกซามีน อย่างเดียวและกลุ่มที่ได้รับดีเฟอรอกซามีนร่วมกับดีเฟอริโพรนตามลำดับและความแตกต่างระหว่างปริมาณ
ซีรั่มเฟอริทีนพื้นฐานและล่าสุดเฉลี่ยของทั้งสองกลุ่มไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ (p>0.05) ค่าเฉลี่ย EQ-5D-3L
คือ 0.88 ± 0.16 และ VAS คือ 0.75 ± 0.2 ผลรวมของต้นทุนการรักษาทางตรงของผู้ป่วยที่ได้รับดีเฟอรอกซามีนอย่างเดียว
และที่ได้รับร่วมกับดีเฟอริโพรนโดยประมาณเฉลี่ยคือ 81,282.40 และ 69,073.81 บาท ของทั้งสองกลุ่มตามลำดับ คิดเป็นค่ายา
80% สรุปดีเฟอรอกซามีนช่วยรักษาระดับซีรั่มเฟอริทีนในร่างกายซึ่งส่งผลให้ผู้ป่วยมีคุณภาพชีวิตที่เหมาะสม แต่ถูกจำกัดการ
เข้าถึงเนื่องจากต้นทุนสูงจึงต้องการเภสัชเศรษฐศาสตร์ช่วยจัดการ

คำสำคัญ: ดีเฟอรอกซามีน, ยาขับเหล็ก, ผู้ป่วยธาลัสซีเมียที่พึ่งพาการรับเลือด

Introduction

Thalassemia is the most prevalent genetic blood disorder in Thailand. Approximately 40% of Thai population carries thalassemia gene traits and about 1% manifests severe anemia which requires blood transfusion.[1] These patients are beta thalassemia patients with hemoglobin < 7 mg/dL. Blood transfusion therapy is aimed toprevent growth retardation, bony change, hypersplenism and suppress endogenous erythropoiesis.[2] A consequence of blood transfusion is iron overload since a single unit of transfused blood has 200-250 mg of iron and the body has no mechanism to excrete excess iron. Excess iron will accumulate in the organs such as myocardium and endocrine glands leads to cardiomyopathy or eventually heart failure, diabetes mellitus, growth failure and delayed onset of puberty in juvenile patients.[3]

Iron overload can be prevented by deferoxamine (DFO), aniron chelating agent. DFO has been demonstrated to decrease hepatic iron accumulation, ameliorate cardiac, pancreatic and other organ dysfunctions, as well as improve growth, sexual maturation and a reduction in morbidity and mortality.[4] DFO treatment is generally initiated between the ages of 3-5 years. Due to its relatively large molecular size, it is poorly absorbed by the gut. Moreover, it has a short half-life of 0.3 hour thus parenteral administration (subcutaneous or intravenous) was required, with a dosing regimen of 8-12 hours, 5-7 times per week. These inconvenient factors lead to poor compliance.[5] The adverse reactions of DFO include hypersensitivity reactions, sensory neural loss, growth retardation, acute renal failure and hepatic dysfunction.[6] Because of parenteral administration, time-intensive dosing regimen and adverse effects, DFO may impair patients' quality of life.[7] In addition to the high cost of drug, parenteral administration that requires more equipment may increase the cost of treatment.[8] Because of these reasons, the objectives of this study were to evaluate clinical efficacy, quality of life and cost of treatment of DFO in transfusion-dependent thalassemia patients.

Materials and Methods

Study design

A cross sectional study was conducted in thalassemia patients at Maharaj Nakorn Chiang Mai hospital. Approval for the study was granted by Research Ethics Committee, Faculty of Medicine, Chiang Mai University and all patients signed informed consent forms before entered the study.

Clinical efficacy and safety

Hospital medical records were retrospectively reviewed to obtain serum ferritin levels which represented iron storage in the body, serum creatinine level and liver function test. Baseline ferritin levels and final levels were defined as the level obtained in the past 12-month or the level before initiation of DFO and a recent available serum ferritin level, respectively. Adherence to DFO treatment in the past 7-day and adverse events which occurred within the prior 30-day were obtained from self-reported questionnaire.

Health related quality of life (HRQoL)

The quality of life using self-report questionnaire consisted of 2 parts; the EuroQoL EQ-5D-3L descriptive system Thai version (EQ-5D-3L) and the EQ visual analogue scale (VAS). Each patient reported their health status in the preceding 24 hours then assessedusing a Thai value set. The EQ-5D-3L consists of 5 dimensions: mobility, self-care, usual activities, pain or discomfort and anxiety or depression; each dimension has 3 levels: no problem, some problems and extreme problems. The second part, EQ-VAS evaluated patients' self-rated overall health status on a vertical line ranging from 0

to 100; 0 indicatesworst imaginable health stateand 100 indicates best imaginable health state.[9]

Costs

The total annual cost consisted of direct medical costs and non-medical costs. The direct medical cost included the drug cost and equipment costswhich were obtained from the hospital database. While the direct non-medical cost included transportation, meal and facility costs. All costs were approximated per annum, in Thai currency (THB), for the financial year 2013.

Statistical Analyses

All descriptive data were presented asmean ± SD and frequency.Wilcoxon signed-rank test was used for comparison between baseline and post serum ferritin levels. Spearman correlation test was used to assess correlation between the EQ-5D score and the VAS score with laboratory values and demographic data. P values of less than 0.05 were considered significant. All analyses were performed using SPSS version 17.0 software and Microsoft excel version 2013.

Results

A total of 48 thalassemia patients were enrolled in this study (Table 1). Beta thalassemia and beta thalassemia/Hb E disease were diagnosed in 26 and 22 patients, respectively. Their average age was 23.8 ± 11.9 years and 22 (45.8%) patients were less than 18 years. Twenty eight patients were splenectomized patients. DFO and DFO + DFP were prescribed in 19 (39.6%) and 29 (60.4%) patients, respectively. Thirty-nine patients had no underlying disease while heart, liver, kidney and endocrine disease that might associated with iron overload were observed in 5, 3, 2 and 2 patients, respectively. Blood transfusion of 11- 35 unit/year was recorded in 30 patients, and 3 patients had < 11 transfusion/year, while 15 patients had no record of blood transfusion in our hospital

Clinical efficacy

DFO therapy resulted in a decline in final serum ferritin level (2602 ± 2484 µg/L) from baseline level (2854 ± 2451µg/L), albeit the change was not reached statistically significant difference ($p>0.05$). A decline in a mean final ferritin levelsfrom baseline (µg/L)in DFO+DFPwas higher (2818 ± 1733 versus 2482 ± 1571) than a mean final ferritin levels in DFO monotherapy (2909 ± 3319 versus 2786 ±3495), as shown in Figure 1. However statistical analysis showed no significant differences between the baselines

Safety data

No adverse effects were founded in 19 patients (31.6% and 44.8% in DFO and DFO+DFP, respectively). Eighteen patients reported gastrointestinal symptoms including stomachache, nausea, vomiting and diarrhea (47.4% and 31% of DFO and DFO+DFP, respectively). Fourteen patients had joint and backache (21.1% and 34.5% of DFO and DFO+DFP,respectively). Redness and irritation around the injection sitewere found in 8 patients. Nine patients had respiratory symptoms, blurred vision and temporary hearing loss which occurred more often in DFO+DFP.Thirty-onepatients had normal serum creatinine level (0.58±0.15mg/dl). Liver function test was determined in 40 patients. Abnormal elevated alanine aminotransferate (ALT) level of >105 U/L (3-foldof the upper limit)was reported in 6 patients, 13.75% and 12.5% in DFO and DFO+ DFP, respectively, Six patients (31.6%) and 20 patients(69%) in DFO and DFO+DFP, respectively, reported missing at least 1 dose in the prior 7-day.

Health related quality of life (HRQoL)

Twenty nine patients; 13 patients (68.4%) and 16 patients (55.1%) in the DFO and DFO+DFP groups, respectively, graded their own health as full health (maximum QOL score) (Table 2). Moderate pain or discomfort was reported in 15patients (31.3%) declared that they had some problems in drug administration. All patients reported no problem in washing or dressing themselves as well as no report of extreme disability across all five EQ-5D-3L dimensions. Correlation coefficient (r^2) between EQ-5D-3L assessment VS age was -0.45. The older patients assessed their *HRQoL* less than younger patients which reached statistical significant different ($p = 0.014$). Majority of the patients (60.4%) graded their high EQ-VAS score of 80 from 100.A maximum score of 100 or "the best imaginable state as possible" was reported in 6 patients (12.5%) in both groups. There were no correlation between EQ-VAS with demographic data, DFO, DFO+DFP and laboratory data (Hb and final serum ferritin level).

Cost

The total annual cost of DFO treatment could be retrospectively estimated only in 34 patients as shown in Table 3. The total annual direct costs (THB/patient)were estimated to be 81,282.40 and 69,073.81in patients treated with DFO and DFO+DFP, respectively. The corresponded expenses for DFO cost were 89% and 72%, respectively. Conversely the mean annual direct non-medical cost of DFO (5,900.77 THB/patient) was less than those of DFO+DFP (11,134.33 THB/patient).

Table 1. Socio-demographic and clinical characteristics of enrolled patients (n=48).

Description of variables	DFO: n (%)		DFO+DFP: n (%)	
No. of patients	19	(100.0)	29	(100.0)
Gender				
Female	9	(47.4)	17	(58.6)
Male	10	(52.6)	12	(41.4)
Age in years				
10-18	6	(31.6)	16	(55.2)
18-30	6	(31.6)	7	(24.1)
>30	7	(36.8)	6	(20.7)
Occupation				
Student	7	(36.8)	17	(58.6)
Business owner	4	(21.1)	2	(6.9)
Freelance	1	(5.3)	2	(6.9)
Unemployed	3	(15.8)	3	(10.3)
Government officer	1	(5.3)	2	(6.9)
Others*	3	(15.8)	3	(10.3)
Educational level				
Primary school	0	0.0	11	(37.9)
Secondary school	8	(42.1)	10	(34.5)
Diploma	1	(5.3)	4	(13.8)
Bachelor's degree	7	(36.8)	4	(13.8)
Others†	3	(15.8)	-	
Missed dose in 7 days				
No	13	(68.4)	9	(31.0)
Yes	6	(31.6)	20	(69.0)
Type of Thalassemia				
β-thalassemia	12	(63.2)	14	(48.3)
β-thalassemia/Hb E	7	(36.8)	15	(51.7)
Splenectomy				
Yes	15	(78.9)	13	(44.8)
No	4	(21.1)	16	(55.2)
Underlying disease				
None	13	(68.4)	26	(89.7)
Heart disease	4	(21.1)	1	(3.4)
Kidney disease	1	(5.3)	1	(3.4)
Liver disease	1	(5.3)	2	(6.9)
Endocrinopathy	2	(10.5)	-	
Others‡	5	(26.3)	-	
Transfusion (unit/year)				
unknown	9	(47.4)	6	(20.7)
1-10	3	(15.8)	-	
11-35	7	(36.8)	23	(79.3)

*Others occupation were consisted of state enterprise employee, monk, agriculture and trader.

†Others educational level were consisted of unknown and master's degree.

‡Others were consisted of Systemic Lupus Erythematosus, Hearing loss, Osteoporosis, Rheumatoid and Cataract.
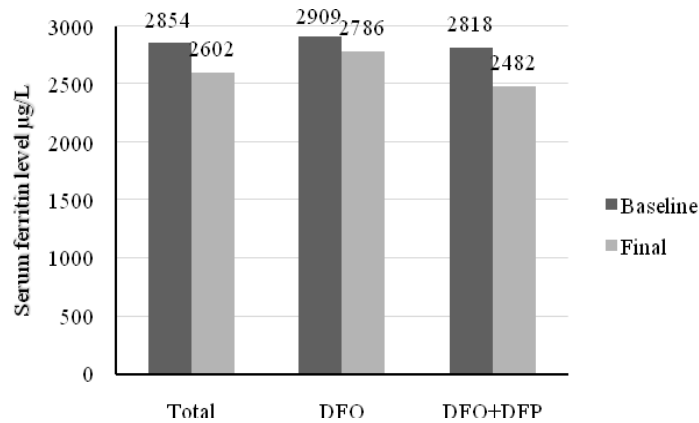
Figure 1. Mean serum ferritin level between baseline and Final in Total, DFO and DFO+DFP groups and the final values in both groups.

Table 2. Descriptive statistics of EQ-5D domains, the EQ-5D score and the EQ-VAS scorein DFO and DFO+DFP groups (n=48)

Description	Total n=48		DFO (n=19)		DFO+DFP (n=29)	
EQ-5D Domain	n	(%)	n	(%)	n	(%)
Mobility						
No problem	44	(91.7)	16	(84.2)	28	(96.6)
Some problem	4	(8.3)	3	(15.8)	1	(3.4)
confined to bed	-		-		-	
Self-care						
No problem	48	(100.0)	19	(100.0)	29	(100.0)
Some problems	-		-		-	
Unable to wash of dress self	-		-		-	
Usual activities						
No problem	42	(87.5)	16	(84.2)	26	(89.7)
Some problem	6	(12.5)	3	(15.8)	3	(10.3)
Unable to perform	-		-		-	
Pain/discomfort						
No pain or discomfort	33	(68.8)	15	(78.9)	18	(62.1)
Moderate	15	(31.3)	4	(21.1)	11	(37.9)
Extreme	-		-		-	
Anxiety/depression						
Not anxious or depressed	41	(85.4)	15	(78.9)	26	(89.7)
Moderate	7	(14.6)	4	(21.1)	3	(10.3)
Extreme	-		-		-	
EQ-5D scores						
Mean ± SD	00.88 ± 0.16		0.89 ± 0.18		0.87 ± 0.15	
Median (range)	1	(0.513-1)	1	(0.51-1)	1	(0.57-1)
VAS scores						
Mean ± SD	0.75 ± 0.20		0.73 ± 0.24		0.75 ± 0.17	
Median (range)	0.80	(0-1)	0.80	(0-1)	0.80	(0.40 -1)

Table 3. The total annualized direct costs (THB) based on the treatment groups

Description of costs	Mean	% with in regimen
Treatment with deferoxamine monotherapy (n=13)		
Total	81,282.40	100.00
DFO	72,756.55	89.51
Equipment	2,625.07	3.23
Direct non-medical	5,900.77	7.26
Treatment with combination DFO+DFP (n=21)		
Total	69,073.81	100.00
DFO	50,169.00	72.63
DFP	5,623.88	8.14
Equipment	2,146.60	3.11
Direct non-medical	11,134.33	16.12

Discussion

Despite receiving multiple blood transfusions, DFO as monotherapy or in combination with DFP was able to decrease final serum ferritin levels from baseline in transfusional dependent thalassemia patients. The combination regimen appeared declined ferritin levels more than monotherapy however the reversal effect was not statistically significant ($p>$ 0.05) in addition a larger sample size can enhance a statistical power.[10, 11]

Mental health was also an integral aspect of healthcare and holistic medical treatment. Accordingly, this study evaluated the health related quality of life (HRQoL) in patients using the EQ-VAS and EQ-5D-3L psychometric instruments. The result showed a significant percentage (60.4%) of patients which reported high score of 80 (with a maximum score of 100) consistent to previous studies that found the mean HRQoL scores of > 70 in Thai thalassemia patients.[12] An association of younger age with better EQ-5D score was also found in this study which correlates with previous studies.[13,14] Our findings reinforce the premise that older patients tended to be adverse events when they have used DFO therapy more than younger patients. This suggests that DFO may not be a suitable drug of choice for older patients but an alternative iron chelating agents should be considered in this subset of patients.

Known adverse events of DFO included gastrointestinal symptoms, joint pain, backache, redness and irritation around the injection site were reported in this study. Blurred vision and temporary hearing loss were still reported in receiving patients as average dose 15 mg/kg/day. The risk factors for DFO adverse effects correlate to a high dose administration (more than 100 mg/kg/day).[6] Although laboratory monitoring found normal serum creatinine level and the liver function test found abnormal elevated ALT level in 6 out of 40 patients. Therefore, when DFO was prescribed, the renal and liver function should be monitored. Adherence of compliance in DFO was better than DFO+DFP since found only 31.6% in DFO who reported missing at least 1 dose in the prior 7-day compared with 69% in DFO+DFP.

The total annual cost of DFO monotherapy was higher than those of DFO+DFP. Nevertheless, the effectiveness of combination regimen in chelating ironwas higher which indicated for the treatment in patients with high serum ferritin levels. The selection of DFO monotherapy or combination therapy may be more economical if ferritin level is monitored.

Conclusion

DFO was an effective iron chelating agent with respected to reduce iron load however the annual costof DFO might affect patients' access. The health-related quality of life in youngerwas better thanolder patients therefore the drug of choice in older patients should be reassessment. The larger prospective clinical studies could provide further clarification in this respect.

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F03 — Method Validation for Determination of Clopidogrel Active Metabolite in Human Plasma by LC-MS/MS

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Abstract

Clopidogrel is an antiplatelet drug and widely used to prevent ischemic vascular events. However, the data on the pharmacokinetics of clopidogrel active metabolite is still limited. The study aimed to develop and validate the method for determining theclopidogrel active metabolite in human plasma by liquid chromatography tandem mass spectrometry (LC-MS/MS). Plasma clopidogrelactive metabolite was extracted by organic solvent withtert-Butyl ethyl ether. LC-MS/MS analysis was performed using a reverse phase BDS Hypersil C18 column and the mobile phase used for gradient elution consisted of 0.1% (v/v) formic acid in water (A) and acetonitrile(B). The calibration at the range of 0.5-40 ng/ml concentration was obtained with the correlation coefficient of 0.99. The intra-day and inter-day as well as precision were within the range of acceptance criteria. The lower limit of quantitation (LLOQ) was 0.5 ng/ml. The intra-day and inter-day accuracy of LLOQ were 96.16±0.028 and 95.87±0.037, respectively and the intra-day and inter-day precision (%CV) were 3.56% and 7.62%, respectively. The developed method was suitably applied to a pharmacokinetic study of clopidogrel active metabolite in human receiving a tablet of clopidogrel.

Keywords: clopidogrel active metabolite, method development, pharmacokinetics

วิธีการตรวจวัดสารออกฤทธิ์ของโคลพิโดเกรลในพลาสมาคนโดยใช้เครื่อง **LC-MS/MS**

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บทคัดย่อ

โคลพิโดเกรลเป็นยาต้านการเกาะกลุ่มของเกล็ดเลือดใช้แพร่หลายเพื่อป้องกันการอุดตันของหลอดเลือด อย่างไรก็ตาม ข้อมูลเกี่ยวข้องกับเภสัชจลนศาสตร์ของสารออกฤทธิ์โคลพิโดเกรล (clopidogrel active metabolite)มีค่อนข้างจำกัด การศึกษานี้มีวัตถุประสงค์เพื่อพัฒนาวิธีวัดสารออกฤทธิ์ของโคลพิโดเกรล (clopidogrel active metabolite) ในพลาสมาคน โดยใช้เครื่อง LC-MS/MS โดยนำพลาสมาที่มีสารออกฤทธิ์ของโคลพิโดเกรล (clopidogrel active metabolite) สกัดด้วยเอทิลอีเทอร์ชนิด tert-Butyl ethyl ether วิเคราะห์โดยใช้เครื่อง LC-MS/MS แยกชนิดสารโดยใช้คอลัมน์ C18 เป็นเฟสคงที่ ใช้เฟสเคลื่อนที่เป็น 0.1% กรดฟอร์มิกในน้ำ และอะซิโตไนไตรล์กราฟมาตรฐานในช่วงความเข้มข้นระหว่าง 0.5-40 ng/ml มีค่าสัมประสิทธิ์สหสัมพันธ์ 0.99 สำหรับค่าความถูกต้องและความแม่นยำในวันเดียวกันและระหว่างวันซึ่งอยู่ในเกณฑ์การยอมรับค่าต่ำสุดที่

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สามารถตรวจวัดได้เท่ากับ 0.5ng/ml ค่าความถูกต้องในวันเดียวกันและระหว่างวันของค่าต่ำสุดที่เครื่องสามารถตรวจวัดได้
เท่ากับ 96.16±0.028 และ 95.87±0.037 ตามลำดับและมีค่าความแม่นยำในวันเดียวกันและระหว่างวันของค่าต่ำสุดที่เครื่อง
สามารถตรวจวัดได้ เท่ากับ 3.56% และ 7.62% ตามลำดับ วิธีการตรวจนี้เหมาะสมที่จะนำไปใช้ศึกษาเภสัชจลนศาสตร์ของสาร
ออกฤทธิ์โคลพิโดเกรลในคนที่ได้รับยาเม็ดโคลพิโดเกรล

คำสำคัญ: สารออกฤทธิ์ของโคลพิโดเกรล, การพัฒนาวิธี, เภสัชจลนศาสตร์

Introduction

Clopidogrel was a thienopyridine groups. The drug inhibits platelet aggregation and it is used in the treatment of ischemic cardiovascular disease.[1] Clopidogrel was a prodrug that rapidly absorption after administration and undergoes biotransformation via hepatic pathway by cytochrome P450, including CYP2C19, CYP3A4, CYP1A2, CYP2C9 and CYP2B6 to form its active metabolite.[2]The drug was firstly oxidized to 2-oxo-clopidogrel and subsequently hydrolysed to active metabolite.[1] The clopidogrel active metabolite has a thiol group. The main inactive metabolize found in human plasma was a carboxylic acid derivative taken part about 85% and a thiol active metabolite about 15% of total metabolite. Clopidogrel active thiol metabolite has presented in biological body as four diastereoisomers: H1 and H2 which are E compounds, while H3 and H4 are Z compound.[3] After clopidogrel was absorbed, about 15% of clopidogrel dosage was transformed to be clopidogrel active thiol metabolite, which H3 and H4 isomer were presented in plasma and only H4 isomer can inhibit platelet aggregation.[4] The metabolite selectively and irreversibly inhibits platelet function by the binding of adenosine diphosphate (ADP) at platelet P2Y12 receptor.[5] Direct determination of the active metabolite should be the essential tool to specifically study the efficacy of clopidogrel. However, the data on the pharmacokinetics of clopidogrel active metabolite is still limited. Therefore, the aimof this study was to develop and validate method for determining the clopidogrel active metabolite determination in human plasma by liquid chromatography tandem mass spectrometry (LC-MS/MS).

Materials and Methods

Chemical and reagent

(E)-2-bromo-3-methoxyacetophenone (MPB) derivatized clopidogrel active metabolite hydrochloride (CAMD) was purchased from Alsachim (Illkirch, France). Ticlopidine hydrochloride (Internal standard), tert-butyl ethyl ether, formic acid and ammonium acetate were purchased from Sigma Aldrich (St. Louis, MO). Acetonitrile HPLC grade was purchased from Merck (Darmstadt, Germany).

Standard stock solutions of (E)-2-bromo-3-methoxyacetophenone (MPB)-derivatized clopidogrel active metabolite (CAMD) 1mg/mland the ticlopidine

hydrochloride (internal standard; IS) stock solutions 1 mg/ml were prepared in acetonitrile.

LC- MS/MS equipment and condition

LC-MS/MS analysis was performed on the Shimadzu LC-MS-8040, which a triple quadrupole tandem mass spectrometry. The mobile phase containing of 0.1% (v/v) formic acid in water (A) and acetonitrile (B) and a reverse phase BDS Hypersil C18 column were used via gradient elution.

Sample preparation

Spike 10 μL of standard solution into 250μL plasma and add 10 μL of 60 ng/ml of ticlopidine hydrochloride(internal standard; IS) and then mix. 50 mM of 50μL ammonium acetate was added and mixed.The sample was extracted by 4 ml of tert-Butyl ethyl ether and mix 3 minutes at room temperature. The upper organic layer was collected into a new glass tube, and evaporated under vacuum at 25 °C. The yield residue was reconstituted by 250 μL of the mobile phase, vortexed and mixed, then centrifuged at 13,000 g for 5 minutes at 15 °C. The samples were filtered using nylon filters and transferred into vial. A 10 μL was injected into the LC-MS/MS system for analysis.

Bioanalytical method validation

The bioanalytical method was validated to verifythe determination of clopidogrel active metabolite in human plasma following by US FDA Guidance for Industry on Bioanalytical Method Validation.[6] Blank plasma sample has been donated from the blood bank atThe Thai Red Cross Society.

Specificity and selectivity

For assessment of peak interference, six different plasmadonors were tested according to the method of sample preparation and analysis as described above.

Lower limit of quantification

The lower limit of quantification (LLOQ) was defined as the lowest concentration of clopidogrel active metabolite as the method is able to detect. That was determined by an assay precision with %CV≤20% and accuracy within of 80-120% of measured value.

Linearity

A calibration curve was constructed from a plasma sample with 0.5, 1, 2, 5, 10, 20, and 40 ng/ml ofclopidogrel active metabolite.

Accuracy and precision

Five set of quality control (QC) sample at low (1.5 ng/ml), medium (12 ng/ml) and high (30 ng/ml) concentrations of clopidogrel active metabolite were set to be QC samples. The QC sample was evaluated using five replicates the intra-day and inter-day assay for precision and accuracy of each QC set for three day. The intra-day and inter-day should be within 100±15% whiles the intra-day and inter-day precision (%CV) were less than 15%.

Recovery of extraction

Low (1.5ng/ml), medium (12 ng/ml), high (30 ng/ml) concentration of clopidogrel active metabolite and ISticlopidine (60 ng/ml) were tested for percent recovery of extraction. Five replication of each standard solution were spiked into mobile phase and plasma. Plasma sample was extracted by the sample preparation as described above. The percentage recovery of clopidogrel active metabolite and IS was determined by mean of peak area of active metabolite clopidogrel and IS of plasma sample compared with those of solution.

Stability

The stability of clopidogrel active metabolite was measured in the autosampler. Samples were analyzed in 24 h after the initial analysis at low concentration (1.5 ng/ml) and high concentration (30 ng/ml).

Results

LC MS/MS analysis

The transition m/z 504.10→155.10, m/z 504.1→534.10 for clopidogrel active metabolite and m/z 264.00→125.05, m/z 264.00→154.00 for ticlopidine (IS), respectively.

Chromatogram of clopidogrel active metabolite and IS in human plasma. The result was shown in Figure 1.

Specificity and selectivity

Blank human plasma was no found any endogenous compound in six different plasma donors as the condition shown no peak interference of clopidogrel active metabolite and ticlopidine hydrochloride (internal standard; IS) chromatogram. The result was shown in Figure 2.

Linearity

The calibration curve was linear at the range 0.5-40 ng/ml of clopidogrel active metabolite concentration. The linear was obtained by using 1/C weighting factor with the correlation coefficient of 0.99.

LLOQ, Accuracy and Precision

The lower limit of quantitation (LLOQ) was measured at 0.5 ng/ml for clopidogrel active metabolite.The intra-day and inter-day accuracy of LLOQ were 96.16±0.028 and 95.87±0.037, respectively and intra-day and inter-day precision (%CV) were 3.56% and 7.62%, respectively.The intra-day and inter-day precision were less than 20%.

Five replication of 3 set QC sample (1.5, 12, and 30 ng/ml) were determined during 3 days of validation. The measured concentration of clopidogrel active metabolite at low, medium and high concentration was 100.01±0.07, 109.46±0.29 and 102.61±1.26, respectively for intra-day accuracy and 98.11±0.12, 102.38±1.43 and 101.28±2.04 for inter-day accuracy, respectively. The precision was 4.78%, 2.20%, 4.09%, respectively,for intra-day and 8.29%, 11.60 and 6.72%, respectively, for inter-day. The result presented that intra and inter-day accuracy and precision were less than 15%. (Table 1.)
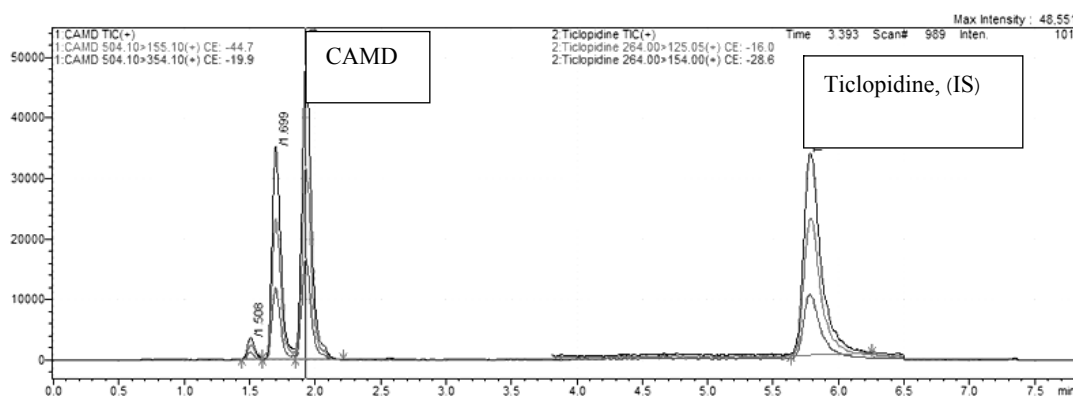
Figue 1. Chromatograms of extracted medium (12 ng/ml) concentration clopidogrel active metabolite and IS in human plasma were separated and retention time were 1.929 and 5.789 min, respectively.
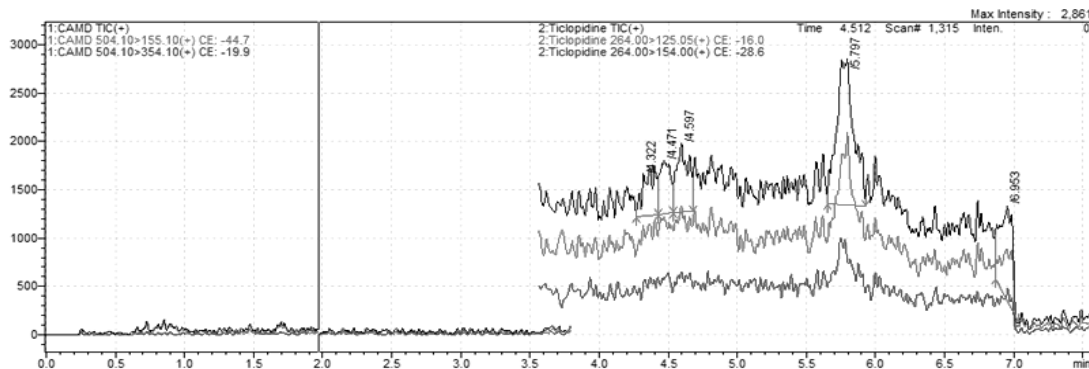
Figure 2. Chromatogram of an extracted blank plasma sample.

Table1. Intra-day and inter-dayaccuracy and precision at three concentrations of quality control samples.

Nominal concentration (ng/ml)	Intra-day (n=5)				Inter-day(n=3)			
	Mean assay value (ng/ml)	SD	CV (%)	Accuracy* (%)	Mean assay value (ng/ml)	SD	CV (%)	Accuracy* (%)
1.5	1.50	0.07	4.78	100.01±0.07	1.47	0.12	8.29	98.11±0.12
12	12.89	0.29	2.20	109.46±0.29	12.29	1.43	11.60	102.39±1.43
30	30.76	1.26	4.09	102.61±1.26	30.38	2.04	6.72	101.28±2.04

*Mean±SD

Recovery of extraction

The percentage recovery of clopidogrel active metabolite and IS were determined by comparing peak area of extracted plasma samples of clopidogrel active metabolite at low (1.5 ng/ml), medium (12 ng/ml), high (30 ng/ml) and IS (60 ng/ml) to those of them in mobile phase. Mean percentage recovery of clopidogrel active metabolite was 90.08% of low (1.5 ng/ml), 86.16% of medium (12 ng/ml), 88.27% of high (30 ng/ml) and 88.04% of IS (60 ng/ml).

Stability

Clopidogrel active metabolite had high stability in the autosampler at 15°C for 24 h of both low and high concentrations. After the initial analysis at low concentration (1.5 ng/ml) was 104.7±0.03 and high concentration (30 ng/ml) was 95.97±1.49, respectively

Discussion

The LC-MS/MS method with triple quadrupole tandem mass spectrometer was developed and validated to determine the concentration of clopidogrel active metabolite in plasma.The method was modified from the method mentioned in Cody J et al. 2012.[2]

The instrument conditions were optimized for obtaining a signal and sensitivity for clopidogrel active metabolite and ticlopidine hydrochloride (IS) in plasma sample. The transition m/z

504.10→155.10, m/z 504.1→534.10 for clopidogrel active metabolite and m/z 264.00→125.05, m/z 264.00→154.00 for ticlopidine (IS), respectively. The setting condition can measure clopidogrel active metabolite with no endogenous interference in each six different plasma donors. The assay method showed the lower limit of quantitation was 0.5 ng/ml for clopidogrel active metabolite. The intra-day and inter-day accuracy of LLOQ were 96.16±0.028 and 95.87±0.037, respectively and the intra-day and inter-day precision (%CV) were 3.56% and 7.62%, respectively. The accuracy and precision of the LLOQ sample, which were less than 20%. The measured concentration of clopidogrel active metabolite at low, medium and high concentration was 100.01±0.07, 109.46±0.29 and 102.61±1.26, respectively for intra-day accuracy and 98.11±0.12, 102.38±1.43 and 101.28±2.04 for inter-day accuracy, respectively. The precision was 4.78%, 2.20%, 4.09%, respectively, for intra-day and 8.29%, 11.60 and 6.72%, respectively, for inter-day. The intra-day and inter-day should be within 100±15% whiles the intra-day and inter-day precision (%CV) were less than 15%.The percentage recovery at low, medium and high concentration of clopidogrel active metabolite was 90.08%, 86.16% and 88.27%, respectively. Whereas the percentage recovery of ticlopidine (IS) was 88.04%. Clopidogrel active metabolite had high stability in the autosampler at 15°C for 24 h of both low and high concentrations.The procedure of clopidogrel

active metabolite and IS extraction had high efficiency with almost 90 percent of recovery and it had longtimest able in the assay condition that was suitable for determination.

Karazniewicz-Ladaet,al. 2012 studied pharmacokinetic parameters of clopidogrel active metabolite in Polish subjects receiving 75 mg clopidogrel tablet that presented C_{max}= 13.3±9.8ng/ml, t_{max}= 1.33±0.58 h and $h_{1/2}$= 0.57±0.25 h.[4] According to the reference C_{max}, this present method was adequately sensitive to detect clopidogrel active metabolite in human receiving clopidogrel 75 mg tablet.

Conclusion

The method for quantitation assay of clopidogrel active metabolite in human plasma was high valid and sensitive to determine the level in human plasma. The procedure was suitably applied to pharmacokinetic study of clopidogrel active metabolite in human receiving clopidogrel tablet.

Acknowledgements

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F04 CYP2A6 Allele Frequencies in Thai Smokers Attending the Medical Check-up Clinic at King Chulalongkorn Memorial Hospital

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Abstract

Enzymes of the cytochrome P450 2A6 (CYP2A6) are responsible for the metabolism of >90% of nicotine. Genetic polymorphism of CYP2A6 plays a major role in the large inter-individual and inter-ethnic difference in nicotine metabolism. We assessed genetic variations of CYP2A6 alleles in 125 Thai smokers attending the medical check-up clinic at King Chulalongkorn Memorial Hospital. The allele frequency of the CYP2A6*9 was determined using real-time PCR with Step one software V2.2 program. The allele frequency of the CYP2A6*1A, CYP2A6*1B and CYP2A6*4 were determined using restriction fragment length polymorphism method (PCR-RFLP). The frequencies of the variant alleles CYP2A6*9, CYP2A6*1A, CYP2A6*1B and CYP2A6*4 in the study population were 17.2, 69.6, 17.4 and 13.0%, respectively. These results showed that the distributions of CYP2A6 alleles in Thai smokers attending the medical check-up clinic at King Chulalongkorn Memorial Hospital were different from previously reported in other ethnic groups. This warrants further studies investigating the effects of genetic variations of CYP2A6 on pharmacokinetics and pharmacodynamics of nicotine in Thai smokers.

Keywords: CYP2A6, genetic polymorphism, nicotine, smoking

การกระจายตัวของอัลลีล CYP2A6 ในผู้สูบบุหรี่ชาวไทยที่มารับบริการตรวจสุขภาพ ณ โรงพยาบาลจุฬาลงกรณ์

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บทคัดย่อ

เอนไซม์ไซโตโครมพี 450 (CYP2A6) เป็นเอนไซม์ที่เกี่ยวข้องกับกระบวนการเมแทบอลิซึมของนิโคติน โดยร้อยละ 90% ของนิโคตินที่เข้าสู่ร่างกายถูกเมแทบอลิซึมผ่านเอนไซม์ CYP2A6 อย่างไรก็ตามพบว่า ประชากรในแต่ละคนแต่ละเชื้อชาติมีกระบวนการเมแทบอลิซึมที่แตกต่างกัน ดังนั้นงานวิจัยนี้จึงได้ทำการตรวจสอบการกระจายตัวของอัลลีล CYP2A6 ในผู้สูบบุหรี่ชาวไทยที่มารับบริการตรวจสุขภาพ ณ โรงพยาบาลจุฬาลงกรณ์ จำนวน 125 คน โดยทำการตรวจสอบอัลลีล ของ CYP2A6*9 โดยใช้ real-time PCR จากนั้นวิเคราะห์ผลโดยใช้โปรแกรม Step one software V2.2 และตรวจสอบอัลลีลของ CYP2A6*1A, CYP2A6*1B และ CYP2A6*4 โดยใช้เทคนิค PCR-RFLP พบว่าการกระจายตัวของอัลลีล CYP2A6*9,

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CYP2A6*1A, CYP2A6*1B และ CYP2A6*4 ในกลุ่มประชากรที่ศึกษาเท่ากับ 17.2, 69.6, 17.4 และ 13.0% ตามลำดับ ผลการศึกษานี้แสดงให้เห็นว่าการกระจายตัวของอัลลีล CYP2A6 ในผู้สูบบุหรี่ชาวไทยที่มารับบริการตรวจสุขภาพ ณ โรงพยาบาลจุฬาลงกรณ์มีความแตกต่างจากเชื้อชาติอื่นๆ ที่มีการรายงานก่อนหน้านี้ ซึ่งผลของการศึกษานี้สามารถนำไปใช้เป็นแนวทางในการตรวจสอบผลของความแตกต่างทางพันธุกรรมของ CYP2A6 ต่อเภสัชจลนศาสตร์และเภสัชพลศาสตร์ของนิโคตินในผู้สูบบุหรี่ชาวไทยต่อไปในอนาคต

คำสำคัญ: ไซโตโครมพี 450 (CYP2A6), ความหลากหลายทางพันธุกรรม, นิโคติน, สูบบุหรี่

Introduction

Cigarette smoking is a major problem in many countries around the world including Thailand. Cigarette smoking is a risk factor of respiratory diseases such as emphysema including chronic obstructive pulmonary disease (COPD) and chronic cough. Diseases are caused by nicotine compounds in tobacco which is also the substance that causes cigarette addiction.[1] Cytochrome P450 (CYP) is the main enzyme responsible for the metabolism of clinically used pharmaceuticals such as hormones or drugs. CYP2A6 is an important metabolic pathway of nicotine. Nicotine is mainly metabolized by CYP2A6 to cotinine which is further metabolized to *trans*-3'-hydroxycotinine.[2] The rate of nicotine metabolism by CYP2A6 affects cigarette smoking behavior and highly influences on nicotine dependence.[3]

Genetic polymorphisms of CYP2A6 (over 30 alleles) have been reported; yet many of the polymorphisms are at low frequency or have not been characterized for functional impact.[3,4] In humans, large inter-individual differences in nicotine metabolism were resulted from the genetic polymorphism of CYP2A6 gene.[5] The wild type of CYP2A6 gene was known as CYP2A6*1 which is relatively prevalent among Caucasians and Black Americans (up to 90%) but is at lower frequency in Mongolians.[3] CYP2A6*2 is a rare frequency genetic variants (approximate frequency of 2%) among Caucasians and not has been found in Asian populations. CYP2A6*4 allele where the mutation is the deletion of the entire CYP2A6 gene resulting in complete loss or lack of the enzyme activity is the most common variant seen in Asian populations. Some variant alleles possess a single nucleotide polymorphism (SNP) for example CYP2A6*7 allele which is with a point mutation in exon 9 leading to a single amino acid substitution (I471T) and CYP2A6*9 allele which is with a point mutation in the TATA box (-48T>G) of the promoter region, which decreases the transcriptional activity and *in vivo* metabolic activity of CYP2A6, was detected in 16–22% of Asians.[1]

As previously mentioned, genetic variations of CYP2A6 differ in different population. Genetic polymorphism of CYP2A6 gene or SNPs identification of disease candidate genes for each population is necessary. In the present study genetic variations of CYP2A6 alleles in Thai smokers attending the medical check-up clinic at King Chulalongkorn Memorial Hospital were investigated.

Materials and Methods

Chemicals and reagents

PureLink® Genomic DNA mini kit was from Invitrogen (Invitrogen, USA). Takara LA Taq DNA polymerases and restriction enzymes were from Takara (Kyoto, Japan). Agarose, 100 bp DNA ladder plus and 6X loading dye were from Vivantis (Vivantis, Malaysia). AmpliTaq DNA polymerase was from Applied Biosystem (Invitrogen, USA). All other chemicals and solvents were of molecular biology grade in quality.

Subjects

Whole blood samples used in this study were obtained from the study entitled: Nicotine dependence, depression, anxiety and type of metabolizer alleles with CYP2A6 genotype among Thai smokers who visited the medical check-up clinic at King Chulalongkorn Memorial Hospital. The inclusion criteria of the subject of the above study were Thai male or female, aged 18 to 60 years old. All of them are smokers who consume at least 100 cigarettes within the past 5 months. Subjects who had used any medication or consume food that may induce or inhibit activity of CYP2A6 enzyme within 14 days prior to the initiation of the study were excluded. The study did not include pregnant or breast feeding women and subjects with kidney or liver diseases. The study protocol was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University. Each subject provided written informed consents prior to the start of the study.

DNA extraction

Genomic DNA was extracted from peripheral lymphocytes using a PureLink® Genomic DNA mini kit (Invitrogen, USA). The concentrations of DNA were determined by NanoDrop-2000c Spectrophotometer at 260 nm. DNA samples were then dissolved in distilled pyrogenic free water.

Genotyping of CYP2A6*9

The genotyping of CYP2A6*9 alleles was assessed using real-time PCR with Stepone software V2.2 program and Applied Biosystems 7500 Real-Time PCR System. The assay was designed for the detection of SNP genotypes of specific gene sequence of CYP2A6*9. The PCR was performed with TaqMan MGB probe in this protocol as follow: Reporter 1 (VIC-TGG TTT GCC TTT **A**TA CTG CCT-NFQ) and Reporter 2 (FAM- TGG TTT GCC TTT **C**TA CTG CCT-NFQ). Final reactions were assembled so that they contained 1X TaqMan genotyping master mix, 1X TaqMan assay (rs28399433) and 3 µL of genomic DNA (stock 10 ng/µl), the volume was increased to a final volume of 20 µL of pyrogenic free water. Reactions were carried out in an Applied Biosystems 7500 Real-Time PCR System. The cycling parameters for the detection of CYP2A6*9 included an initial step at 95 ºC for 10 minutes, the amplification was performed by denaturation at 95 ºC for 15 second, annealing and extension at 60 ºC for 1 minute for 40 cycles. The data were analyzed using Stepone software V2.2 program.

Genotyping of the CYP2A6*1A, CYP2A6*1B and CYP2A6*4

The genotyping of CYP2A6*1A, CYP2A6*1B and CYP2A6*4 alleles were determined using the restriction fragment length polymorphism method (PCR-RFLP).[6] The PCR was performed with primers of 2A6-B4 (5'-CAC CGA AGT GTT CCC TAT GCT G-3') and 2A6-UTRAS (5'-TGT AAA ATG GGC ATG AAC GCC C-3'). The 2A6-B4 primer anneals with the corresponding region of CYP2A6 as well as CYP2A7 whereas the 2A6-UTRAS anneals only with CYP2A6. Genomic DNA samples (approximately 50 ng) were added to the polymerase chain reaction mixtures 25 µL that consisted of 2.5 mM $MgCl_2$, 1X PCR buffer, 0.2 µM of each primer, 0.4 mM dNTPs and 1U LA Taq polymerase. After an initial denaturation at 94 ºC for 3 minutes, the amplification was performed by denaturation at 94 ºC for 1 minute, annealing at 56 ºC for 1 minute and extension at 72 ºC for 2 minutes for 30 cycles. The PCR product was digested using two restriction enzymes, *Acc*II and *Eco*81I. After an overnight digestion at 37 ºC, samples were analyzed by electrophoresis with a 1.5% agarose gel.

Results

125 whole blood samples of Thai smokers attending the medical check-up clinic at King Chulalongkorn Memorial Hospital was entered into study. Male subjects (n=120) had an average age of 36.9 years and female subjects (n=5) had an average age of 46.4 years. The average of the number of cigarettes consumption per day of each participants was 11.7. Genotypes (wild type, heterozygous or homozygous for the variant alleles) and allele frequencies is depicted in Table 1. The CYP2A6*9 allele was found in 31 individuals. 94 persons had wild type for the CYP2A6*9 allele.

Table 1. CYP2A6 genotypes and allele frequencies in 125 Thai smokers attending the medical check-up clinic at King Chulalongkorn Memorial Hospital

Genotype	Number of subjects (n = 125)	Frequency (%)
CYP2A6*1/CYP2A6*1	94	75.2
CYP2A6*1/CYP2A6*9	19	15.2
CYP2A6*9/CYP2A6*9	12	9.6

Allele	Number of alleles (n = 250)	Frequency (%)
CYP2A6*1	207	82.8
CYP2A6*9	43	17.2

CYP2A6*1A, CYP2A6*1B and CYP2A6*4 alleles in human genomic DNA were assessed in 23 subjects. At the beginning of the test, the original forward primer 2A6-B4 and reverse primer 2A6-UTRAS were applied. After amplification of a PCR product (approximate 1.3 Kb products), a restriction digestion with two enzymes, *Acc*II and *Eco*81I were conducted. The CYP2A6*1A yielded 1322, 789, 429 and 280 base pair fragments (Figure 1). The CYP2A6*1B yielded 1322, 1041, 789, 429 and 280 base pair fragments. The CYP2A6*4 yielded 1040, 728, 428 and 280 base pair fragments. In our study, the allele frequencies of CYP2A6*1A, CYP2A6*1B and CYP2A6*4 were 69.6%, 17.4% and 13.0%, respectively (Table 2).

Table 2. Prevalence of CYP2A6*1A, CYP2A6*1B and CYP2A6*4 alleles in 23 Thai smokers attending the medical check-up clinic at King Chulalongkorn Memorial Hospital

Allele	Number of alleles (n = 46)	Frequency (%)
CYP2A6*1A	32	69.6
CYP2A6*1B	8	17.4
CYP2A6*4	6	13.0
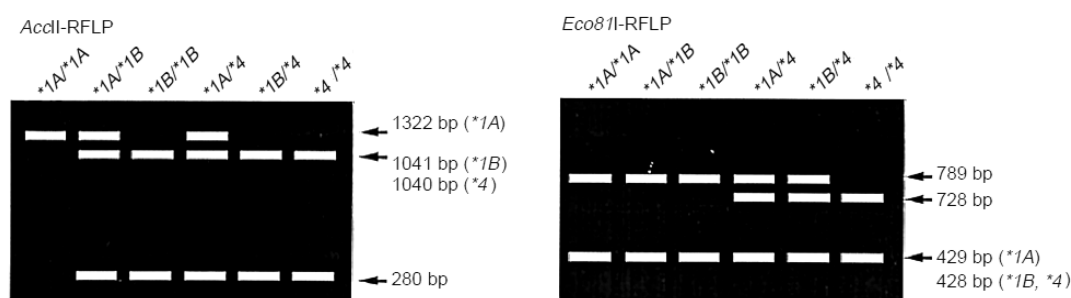
Figure 1. Typical RFLP pattern of CYP2A6*1A, CYP2A6*1B and CYP2A6*4 alleles

Discussion

CYP2A6 enzymes are the predominant cytochrome P450 proteins in the human liver and play an important role in the metabolism of nicotine. This study determined the allele frequencies of CYP2A6 in Thai smokers attending the medical check-up clinic at King Chulalongkorn Memorial Hospital. The alleles frequencies of CYP2A6*9 was higher than that previously reported in 120 healthy Thai (17.2% vs. 12.1%),[6] and Caucasians (7.9%).[3] and lower than those of the Japanese (21.3%) and Korean (22.3%).[2] Here, the finding suggests that the frequency of the CYP2A6*9 alleles is higher in Asian populations compared to White populations.

Additionally, CYP2A6*1A, CYP2A6*1B and CYP2A6*4 alleles seen here were 69.6%, 17.4% and 13.0%, respectively where the previous report in 209 Korean subjects were 54.7%, 42.8% and 11.0%, respectively.[7] Ariyoshi, *et al* showed that in French populations (n =242) the data were 65.7%, 30.3% and 4.0%, respectively and for Japanese (n=444) the frequencies were 40.4%, 41.2% and 18.4%, respectively.[8]

Conclusion

Genetic polymorphism of CYP2A6 was seen in Thai smokers attending the medical check-up clinic at King Chulalongkorn Memorial Hospital which may affect their response to nicotine and other related substances. Studies on the relationships of pharmacokinetics and pharmacodynamics of nicotine and CYP2A6 gene will provide more detail on this and warrant further research.

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F05 The Effects of Ayurved Siriraj Ha-Rak Recipe and Its Herbal Components on Cytochrome P450 mRNA Expression (CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4) in HepaRG Cell Culture

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Abstract

AyurvedSiriraj Ha-Rak Recipe (AVS022), a Thai traditional polyherbal formula has been widely used as an antipyretic drug in Thailand. Previous scientific studies have suggested that AVS022 had an antioxidant and anti-inflammatory effects. However, we still lack scientific data that investigate the risk of herb-drug interaction. The objective of this study was to assess the effects of AVS022 and its herbal components on CYP (CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4) mRNA expression. TheCYP expression has been assessed using Human hepatoma (HepaRG) cell culture. To study the induction and inhibition effects of AVS022 and its components on CYP mRNA expression, cells were treated with positive control inducer (omeprazole and rifampicin), positive control inhibitor (furafylline, sulfaphenazole, disulfiram, quinidine and ketoconazole), AVS022 and its five components. The overall CYP mRNA expression were systematically analyzed using the principal component analysis. The results suggested that AVS022 and its five components did not strongly affect CYP mRNA expression when we compared with positive control inducer and positive control inhibitor. Moreover, we also observed biphasic dose-dependent effects of AVS022 and its five components on CYP mRNA expression. Thus, our *in vitro* results support that AVS022 does not significantly affect the expression of the CYP isoenzymes.

Keywords: Ayurved Siriraj Ha-Rak, CYP, herb-drug interaction

การศึกษาผลของยาสมุนไพรตำรับอายุรเวทศิริราชห้ารากและสมุนไพรที่เป็นองค์ประกอบของยา ตำรับต่อการทำงานของยีนเอนไซม์ไซโตโครม P450 (CYP1A2, CYP2C9, CYP2D6, CYP2E1 และ CYP3A4) ในเซลล์เพาะเลี้ยง HepaRG

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บทคัดย่อ

ยาสมุนไพรตำรับอายุรเวทศิริราชห้าราก (AVS022) เป็นสมุนไพรตำรับที่มีการใช้อย่างแพร่หลายในประเทศไทยมีสรรพคุณบรรเทาอาการไข้มีงานวิจัยหลายฉบับแสดงให้เห็นว่ายาสมุนไพรตำรับนี้มีฤทธิ์ทางเภสัชวิทยาทั้งต้านอนุมูลอิสระและลดการอักเสบ แต่ยังไม่เคยมีการศึกษาปฏิกิริยาระหว่างกันของยาจากสมุนไพรและยาแผนปัจจุบันมาก่อนดังนั้นการศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาปฏิกิริยาระหว่างกันของยาจากสมุนไพรและยาแผนปัจจุบันในหลอดทดลองผ่านการแสดงออกของยีนเอนไซม์

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ไซโตโครม P450 (CYP1A2, CYP2C9, CYP2D6, CYP2E1 และ CYP3A4) ในเซลล์เพาะเลี้ยง Human hepatoma (HepaRG) โดยศึกษาผลของ AVS022 และสมุนไพรที่เป็นองค์ประกอบต่อการเพิ่มขึ้นหรือการยับยั้งการแสดงออกของยีน เอนไซม์ไซโตโครม P450 เปรียบเทียบกับกลุ่มควบคุม โดยใช้ยาโอมิพราโซลและยาไรแฟมพิซินเป็นชุดควบคุมการเพิ่มขึ้นของการ แสดงออกของยีนเอนไซม์ไซโตโครม P450 ยาฟูราฟิลลีน ยาซัลฟาเฟนาโซล ยาไดซัลฟิแรม ยาควินิดีน และยาคีโตโคนาโซลเป็น ชุดควบคุมการยับยั้งของการแสดงออกของยีนเอนไซม์ไซโตโครม P450 ผลการศึกษาเชิงระบบต่อการแสดงออกของยีนเอนไซม์ ไซโตโครม P450 โดยการแสดงในรูปแบบกราฟทางคณิตศาสตร์ที่เรียกว่า Score plot และ Heat map พบว่า AVS022 และ สมุนไพรที่เป็นองค์ประกอบมิได้มีผลในการกระตุ้นหรือยับยั้งอย่างรุนแรงต่อการแสดงออกของยีนเอนไซม์ไซโตโครม P450 เมื่อ เทียบกับกลุ่มควบคุม นอกจากนี้จากกราฟ Heat map ยังแสดงให้เห็นผล biphasic dose-dependent ของ AVS022 และสมุนไพร ที่เป็นองค์ประกอบต่อการแสดงออกของยีนเอนไซม์ไซโตโครมอีกด้วย การทดลองในหลอดทดลองนี้เป็นข้อมูลเบื้องต้นเพื่อ สนับสนุนว่า AVS022 และสมุนไพรที่เป็นองค์ประกอบมิได้มีผลอย่างมีนัยสำคัญทางสถิติต่อการแสดงออกของยีนเอนไซม์ ไซโตโครม P450

คำสำคัญ: ยาห้าราก, เอนไซม์ไซโตโครม P450, อันตรกิริยาระหว่างยาสมุนไพรกับยาแผนปัจจุบัน

Introduction

Ayurved Siriraj Ha-Rak Recipe (AVS022), a Thai traditional polyherbal formula has been widely used in Thailand as an antipyretic drug. The formula has been included in the list of herbal medicinal products since 2006 by National Drug Committee of Thailand.[1] AVS022 is prepared by the combination of five dried roots powder in the equal parts by weight; dried root of *Capparis micracantha* DC. (*C. micracantha* or Ching-Chi), *Clerodendrum petasites* (Lour.) S. Moore. (*C. petasites* or Thao-Yai-Mom), *Harrisonia perforata* (Blanco) Merr. (*H. perforata* or Khontha), *Ficus racemosa* Linn. (*F. racemosa* or Madur-Chumporn) and *Tiliacora triandra* (Colebr) Diels. (*T. triandra* or Yanang). Pharmacological activities of AVS022 have been previously studied such as antioxidant, anti-inflammatory, antipyretic, and antimycobacterial.[2-5] Many scientific studies[6-18] showed that the main chemical constituents of each AVS022's herbal components, excluding *C. micracantha*, were phenolic acid and flavonoids which are known as pharmacological substances of antioxidant, anti-inflammatory and anticancer actions. The use of Ha-Rak Recipe as an alternative or complementary therapy with modern therapeutic drugs has increased. Although their exact mechanisms of action are generally unknown, there are enough clinical evidences to support their efficacy. The AVS022's components consisted of many chemical compositions[19], so it is possible that herb-drug interactions will occur. Currently, there has been no report about the metabolism of Ha-Rak Recipe and its herbal components via CYP isoenzymes. In this study, we thus investigated how the mRNA expressionof major CYP isoenzymes (CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4) might be affected by Ha-Rak Recipe or more specifically by each ofits herbal components.

Materials and Methods

Chemicals

Ethanol (analytical grade) were purchased from Scharlau (Spain). Omeprazole, rifampicin, furafylline, sulfaphenazole, disulfiram, quinidine and ketoconazole were purchased from Sigma (USA). Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (1:1) or D-MEM/F-12 medium were purchased from Gibco (USA). Fetal bovine serum or FBS were purchased from Biochrom AG (Germany). Penicillin G sodium and streptomycin were purchased from general drug house of Thailand.

Herbal materialsand Preparation of AVS022 and its components

AVS022 and its five herbal components (root of *Capparis micracantha* DC., *Clerodendrum petasites* (Lour.) S. Moore, *Harrisonia perforata* (Blanco) Merr., *Ficus racemosa* Linn. and *Tiliacora triandra* (Colebr.) Diels) were prepared by the GMP certified Manufacturing Unit of Herbal Medicines and Products, Center of Applied Thai Traditional Medicine (CATTM), Faculty of Medicine, Siriraj Hospital, Mahidol University, Thailand. All of them were authenticated by experienced Thai traditional practitioners. Then, the powders were stored and preserved at room temperature in dry condition.The AVS022 and its herbal components powders were dissolved in 80% ethanol at final concentration 100 mg/mL, mixed for 24 hr. The extract solution was filtered through Whatman® glass microfiber filter, Grade GF/A(Whatman International Ltd, England) with a Buchner funnel. The residual was evaporated ethanol using rotary evaporator (Rotavapor R110, Buchi, Switzerland) and dried by lyophilizer

(Freezone, Labconco, USA). The herbal extracts were store at 4 ºC until testing.

Human hepatoma (HepaRG) cell treatment and culture conditions

HepaRG cells (Lot Number: 933777) were obtained from Invitrogen Corporation. Cells were cultured in cell culture flask 75 cm^3 with D-MEM/F-12 medium in the present of 10% (V/V) FBS 100 units/mL penicillin G and 100 µg/mL streptomycin. They were maintained in a humidified atmosphere of 5% (V/V) CO_2 at 37 ºC. The culture medium was changed every 3-4 days and grown to confluence until used. Upon 100% confluence, HepaRG cells were trypsinized and passaged for the next expansion. HepaRG cells were differentiated by 2% DMSO. The CYP mRNA expression assay was started after 3 days of cells differentiation, representing day 0 of the study. The medium was changed into the medium in the absence of 2% DMSO plus treatments and took into the incubator at 37 °C in 5% (V/V) CO_2 atmosphere for 3 days. For CYP induction and inhibition experiments, the treatments were 10 µM of positive control inducers (omeprazole and rifampicin), 10 µM positive control inhibitors (furafylline, sulfaphenazole, disulfiram, quinidine and ketoconazole) and the extractions of AVS022 and its herbal components at 0, 10, and 100 µg/mL.The incubation mediums were changed every 24 hours for 72 hours. The CYP mRNA expression studies were performed at 72 hours of triplicate experiments.

RNA extraction and quantitative RT-PCR

After 72 hours cell treating in 24 well plate, the medium was removed and washed with PBS 2 times. Total RNA was isolated using illustraRNAspin Mini Isolation Kit following the manufacturer's instructions. The quality and quantity of the total mRNA were determined by using a NanoVue Spectrophotometer. cDNA were synthesized from the total isolated mRNA by using Improm-II reverse transcription system according to the manufacturer's protocol.The CYP mRNA expression included CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4. All primers were designed by Vector NTI software version 10 and ordered from 1st BASE[20] (Table 1). The expressions were amplified using KAPA SYBR® FAST qPCR kit Master Mix (2X) ABI Prism™ and quantified by StepOnePlus™ Real-time PCR System. qPCR assay was performed following amplification conditions: Enzyme activation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 3 sec, annealing/extending at 60°C for 20 sec and followed by data acquisition at 72°C for 40 sec. The fluorescent-PCR products were measured at the last step of each cycle.The relative fold changewere subtracted with house-keeping gene (GAPDH) of the same cells.

Results

The effects of AVS022 on CYP mRNA expression in HepaRG cells were shown in figure 1. AVS022 100 µg/mL significantly upregulated the CYP1A2 mRNA expression (Figure 1A) when compared with the control (non-treated). However, AVS022 had no effects on CYP2C9, CYP2D6 and CYP3A4 mRNA expression in comparison with the control (Figure 1B, 1C and 1E). Figure 1D showed the mRNA expression levels of CYP2E1 induced or inhibited by AVS022. AVS022 (100 µg/mL) had inhibition effects when compared with disulfiram 10 µM. They significantly decreased CYP2E1 mRNA expression in HepaRG cells as compared with the control treatment.

Table 1. Primer sets and conditions used in quantitative real-time PCR

| Gene	Genbank Accession	Sense primer 5'----> 3'(Tm°C)	Antisense primer 3'----> 5'(Tm°C)	Size amplicon (bp)	Annealing Temp. (°C)	Putative function
CYP1A2	AF182274	ACCCCAGCTGCCCTACTTG (64.5)	GCGTTGTGTCCCTTGTTGT (62.4)	101	60	Cytochrome P450 1A2
CYP2C9	NM_000771	CCTCTGGGGCATTATCCATC (62.4)	ATATTTGCACAGTGAAACATAGGA (57.7)	137	60	Cytochrome P450 2C9
CYP2D6	NM_000106	CTAAGGGAACGACACTCATCAC (62.7)	GTCACCAGGAAAGCAAAGACAC (62.7)	289	60	Cytochrome P450 2D6
CYP2E1	NM_000773	ACCTGCCCCATGAAGCAACC (64.5)	GAAACAACTCCATGCGAGCC (62.4)	246	60	Cytochrome P450 2E1
CYP3A4	AK298451	GCCTGGTGCTCCTCTATCTA (62.4)	GGCTGTTGACCATCATAAAAGC (60.8)	187	60	Cytochrome P450 3A4
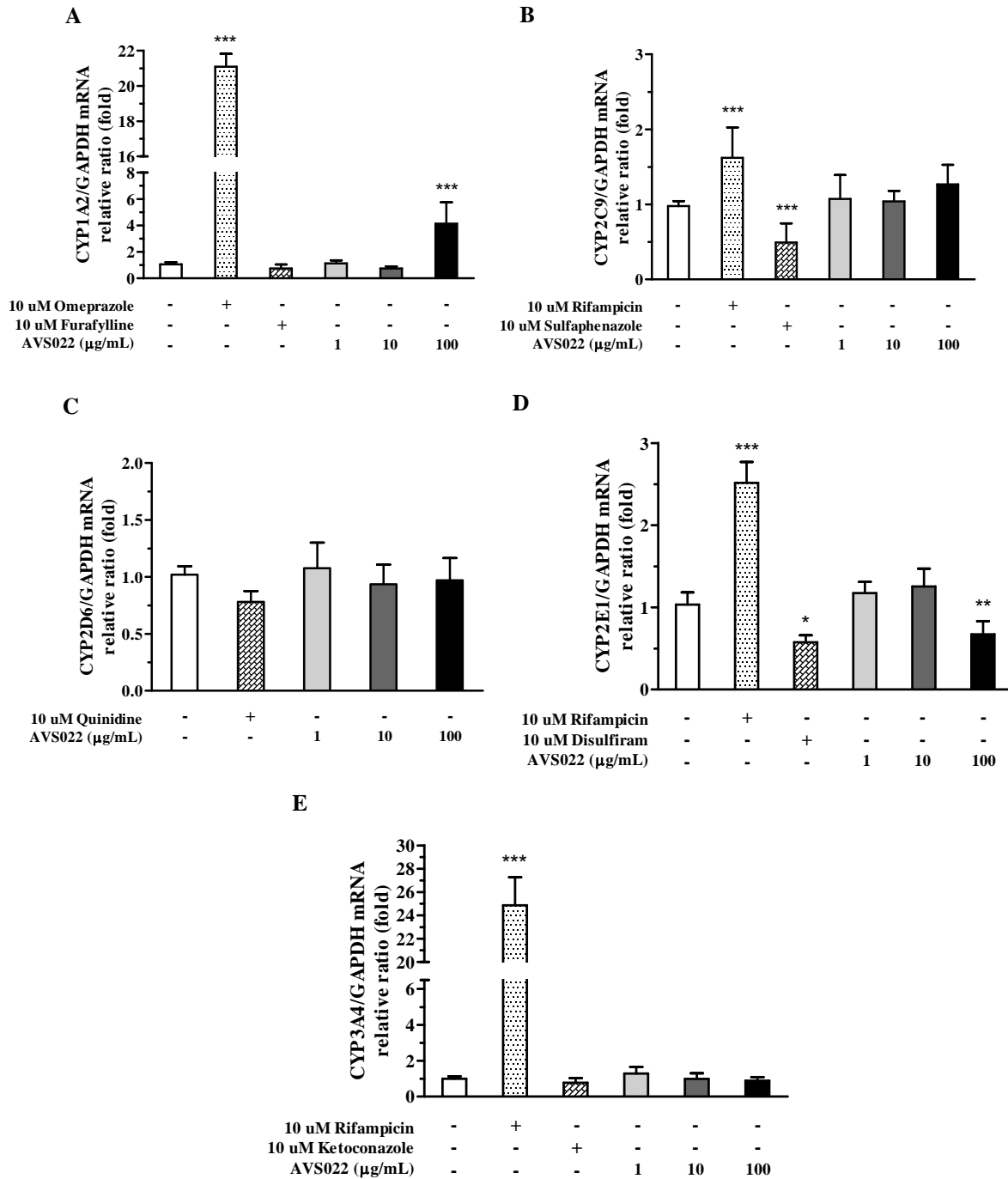
Figure 1. CYP1A2 (A), CYP2C9 (B), CYP2D6 (C), CYP2E1 (D) and CYP3A4 (E) mRNA expression induced or inhibited by 10 µM positive control inducer, 10 µM positive control inhibitor, 1, 10 or 100 µg/mL AVS022 in HepaRG cells treatment. The results are expressed as means ± SD of triplicate experiments. One-way ANOVA followed by Dunnett's *post hoc* test was applied for statistical study compared with control: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Table 2. The induction or inhibition effects of AVS022's components on CYP mRNA expressionin HepaRG cells treatment at 72 hr

Treatment	Concentration	CYP1A2	CYP2C9	CYP2D6	CYP2E1	CYP3A4
F. racemosa	1 µg/mL	↑*	-	-	↓*	-
	10 µg/mL	-	-	-	-	-
	100 µg/mL	↑***	↓**	-	-	↓**
C. micracantha	1 µg/mL	-	-	-	-	↑*
	10 µg/mL	-	-	-	-	-
	100 µg/mL	↑*	-	-	-	-
H. perforata	1 µg/mL	-	-	-	-	-
	10 µg/mL	↑***	-	-	-	-
	100 µg/mL	↑***	-	-	↑***	-
T. triandra	1 µg/mL	↑*	-	-	-	-
	10 µg/mL	-	-	-	-	-
	100 µg/mL	-	-	-	↓***	-
C. petasites	1 µg/mL	↑**	-	-	-	-
	10 µg/mL	↑***	-	↓***	↓***	↑**
	100 µg/mL	↑***	-	↓***	↓***	↑***

Note: ↑ = induction, ↓ = inhibition, - = no effect

One-way ANOVA was applied for statistical study compared with control: * $p < 0.05$,
** $p < 0.01$ and *** $p < 0.001$.

Table 2 summarized the induction or inhibition effects of AVS022's components on CYP mRNA expression in HepaRG cells treatment at 72 hr. *F. racemosa* (1 and 100 µg/mL), *C. micracantha* (100 µg/mL), *H. perforata* (10 and 100 µg/mL), *T. triandra* (1 µg/mL) *and C. petasites* (1, 10 and 100 µg/mL) significantly increased CYP1A2 mRNA expression in HepaRG cells compared with the control (non-treated). All treatments excluding *F. racemosa* (100 µg/mL) gave no effect on the CYP2C9 mRNA expression. Interestingly, the CYP2C9 mRNA expression was significantly decreased by *F. racemosa* (100 µg/mL). All treatments except *C. petasites* (10 and 100 µg/mL) showed no effect on CYP2D6 mRNA expression. *H. perforate* (100 µg/mL) showed induction effect by significantly increasing the CYP2E1 mRNA expression in HepaRG cells whereas *F. racemosa* (1 µg/mL), *T. triandra* (100 µg/mL) and *C. petasites* (10 and 100 µg/mL) exerted the inhibitory effects by significantly decreasing the CYP2E1 mRNA expression. *C. micracantha* (1 µg/mL)*, H. perforate* (100 µg/mL) and *C. petasites*(10 and 100 µg/mL) showed induction effect by significantly increased CYP3A4 mRNA expression in HepaRG cells compared with control whereas *F. racemosa* (100 µg/mL) gavean inhibitory effect on the CYP3A4 mRNA expression in HepaRG cells.

The systematic analysis of the tested compounds on CYP mRNA expression were generated using principal component analysis. The score plot (shown in Figure 2) illustrates the overall difference in the CYP mRNA expression change for the positive control inducer (omeprazole and rifampicin), the positive control inhibitor (furafylline, sulfaphenazole, disulfiram, quinidine and ketoconazole), AVS022 and its components (*F. racemosa*. (FR), *C. petasites* (CM)*, H. perforata* (HP), *T. triandra* (TT) and *C. micracantha* (CP)). The results suggested that AVS022 and its five components did not strongly affect the CYP mRNA expression ascompared with the positive control inducer and the positive control inhibitor. The heat map shown in Figure 3, gradient color 0.00-1.00 and 3.00-0.00 represent the induction and inhibition of CYP mRNA expression. The Figure 3, exhibited a biphasic dose-dependent effects of AVS022 and its five components on CYP mRNA expression. The result indicates non-monotonic effects with dose variation, showing the highest effect at the middle dose, and the least effect at either the low and high concentrations.
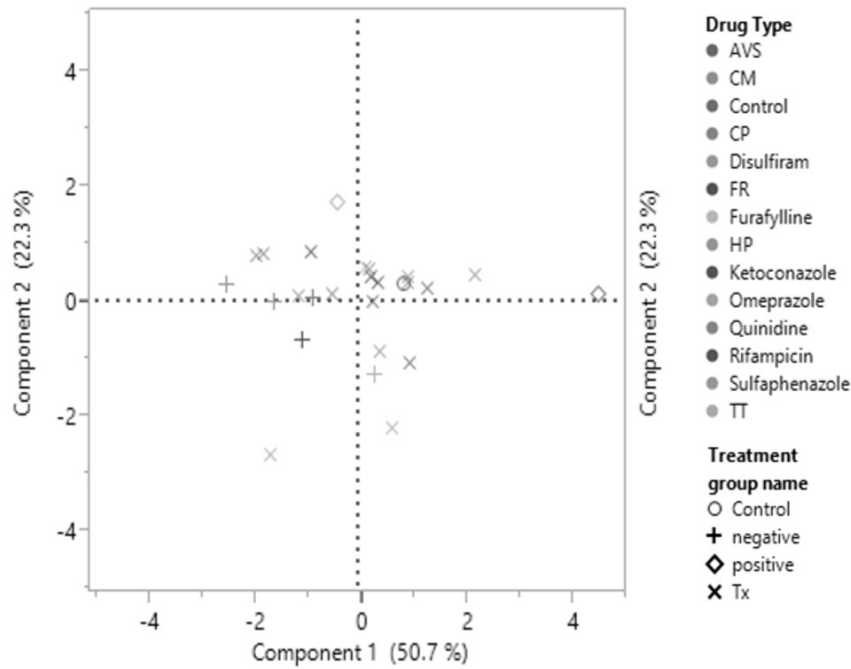
Figure 2. Example of principal component analysis, showing the score plot from the effects of 1) positive control inducer (omeprazole and rifampicin), 2) positive control inhibitor (furafylline, sulfaphenazole, disulfiram, quinidine and ketoconazole), 3) AVS022 and 4) each of its individualcomponents (*F. racemosa.* (FR), *C. petasites* (CM), *H. perforata* (HP), *T. triandra* (TT) and *C. micracantha* (CP)) on the CYP mRNA expression in HepaRG cells.
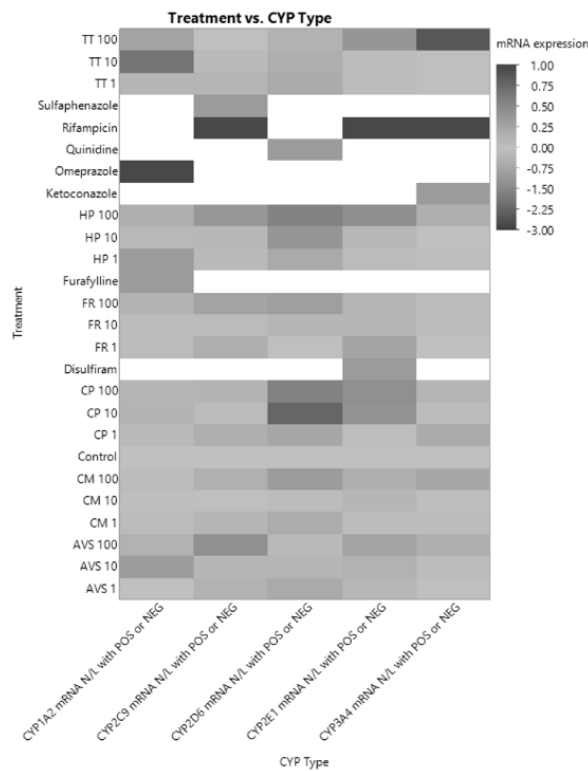

Figure 3. Example of systematic analysis, Heat map of positive control inducer (omeprazole and rifampicin), positive control inhibitor (furafylline, sulfaphenazole, disulfiram, quinidine and ketoconazole), AVS022 and its components (*F. racemosa.* (FR), *C. petasites* (CM), *H. perforata* (HP), *T. triandra* (TT) and *C. micracantha* (CP)) effects on CYP mRNA expression in HepaRG cells.

Discussion and Conclusion

The AVS022 and its components consisted of many chemical compositions, so it is possible that herb-drug interactions via CYP enzymes will occur. The interactions are discovered in order to avoid clinical implications, as shown for example between St. John's wort and oral contraceptives[21], St. John's wort and cyclosporine[22], or Ginkgo and warfarin[23] Cytochrome P450 plays a key role in the metabolism of numerous important drugs in clinical use. Drug interactions involving the CYP isoforms generally are of two types, enzyme induction or enzyme inhibition. Enzyme inhibition reduces metabolism, whereas induction can increase it. Then, understanding these mechanisms of enzyme inhibition or induction is extremely important in order to give appropriate multiple-drug therapies. This study, demonstrated the effect of AVS022 and its herbal components on CYP isoenzymes (CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4) mRNA expression. HepaRG cells were used as an *in vitro* model for study drug metabolism. It is a human hepatoma cell line derived from a hepatocellular carcinoma established by Griponand the other.[24] HepaRG cells were shown to maintain hepatic functions and to express genes for various liver-specific proteins, including CYP and transporters of the Phase II metabolism.[25, 26] Gene expression stability over up to 30 days was reported.[27] Furthermore, They can express high functional levels of most of the major xenobiotic metabolizing CYP such as CYP1A1/2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 excluded CYP2A6 and CYP2E1.[28] The addition of 2% DMSO to the culture medium induced HepaRG cells into more granular cells with one or two nuclei and abundant vacuole which closely to adult primary hepatocytes. Yu ZW and Quinn PJ, 1994[29] reported that DMSO may exert an inducer of cell differentiation via changes in the physical properties of the membrane lipid bilayer. The optimal concentration of all treatments were chose from cell viability assay and studied for dose-response relationship. All treatments at 1, 10 and 100 µg/mL gave the percent cell viability more than 80% so they were chose for this experiment. The results exhibited an appropriate incubation time for all CYP mRNA expression were 72 hr. Omeprazole and rifampicin were used as positive control inducers for CYP1A2 and CYP2C9, CYP2D6, CYP2E1 and CYP3A4 respectively. For positive control inhibitors of CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 were furafylline, sulfaphenazole, disulfiram, quinidine and ketoconazole respectively.

As shown in the results, AVS022 1 and 10 µg/mL had no effects on CYP isoenzymes. However, at the highest concentration 100 µg/mL, AVS022 had the induction effect on CYP1A2 mRNA expression. Moreover, it had the inhibition effect on CYP2E1 mRNA expression. Thus, low concentration AVS022 had no effects on CYPisoenzymes but co-administration of high concentration AVS022 with the drug, that was the substrate of CYP1A2, CYP2E1 and CYP3A4 such as caffeine, phenacetin, chlorzoxazone, midazolam and lovastatin, should be avoided due to the risk of herb-drug interaction. *F. Racemosa* showed the induction effect on CYP1A2 and also showed the inhibition effect on CYP2C9, CYP2E1 and CYP3A4 mRNA expression. The induction effects of CYP1A2 and CYP3A4 mRNA expression were observed after *C. Micracantha* treatment. *H. Perforata*had the induction effects on CYP1A2 and CYP2E1. *T. Triandra* exhibited the induction effects on CYP1A2, while it exhibited the inhibition effect on CYP2E1 mRNA expression. The induction effects of CYP1A2 and CYP3A4 were investigated by *C. Petasites*treatment. Moreover, *C. Petasites* had the inhibition effects on CYP2D6 and CYP2E1. Therefore, the use of these herbs should be concerned herb-drug interaction with western drug that was substrate of CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 such as phenacetin, warfarin, dextromethorphan, acetaminophen and cyclosporine, respectively. In addition, the effects of AVS022 on CYP1A2 and CYP2E1 mRNA expression as same as shown in the effects from AVS022's component.

According to the example of overall CYP mRNA expression systematic analysis, AVS022 and its five components effects on CYP mRNA expression are not strong as the effect of positive control inducer and inhibitors. Moreover, Heat map exhibited a biphasic dose-dependent effects of AVS022 and its five components on CYP mRNA expression. It showed different effects at different concentrations of AVS022 with the highest effects at the middle concentration. The biphasic dose-dependent effects of AVS022 and its five components were possible from the complexity of chemical components. In addition, the 100 µg/mL highest concentration may trigger cellular stress response in HepaRG cells.[30] When the cellular stress response occurred, cells lost their functions. For the further study, the highest concentration should be adjust to lower than 100 µg/mL. The clinical study will be established to enhance the reliability of scientific data for the effect of AVS022 on CYP isoenzymes.

Acknowledgements

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F06 Effect of Chronic Kidney Disease on Hepatic CYP3A in a Mouse Model

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Abstract

Chronic kidney disease (CKD) is a common and an important problem of public health in Thailand. Patients with CKD have been reported to be at risk of adverse drug reactions and drug-drug interaction since CKD is associated with alteration of cytochrome P450 (CYP) enzyme in the liver. The aim of this study was to investigate the effect of an induction of CKD in mice on the function of hepatic CYP3A, the most abundant CYP enzyme involved in phase I metabolism of numerous drugs using a new mouse model. ICR mice were randomized to the control group (n=4) or to the CKD group (n=4). CKD was induced by a new ischemic reperfusion (I/R) of left kidney by ligating renal artery for 50 minutes then right nephrectomy was performed after 7 days afterwards. Fourteen days later, mice were sacrificed. Serum and liver tissue were harvested to measure serum creatinine levels and CYP3A activity using QuantichromTM Creatinine assay kit and high-performance liquid chromatography (HPLC), respectively. Serum creatinine levels were significantly higher in the CKD group compared with control group (0.816 ± 0.21 compared to 0.359 ± 0.10 mg/dL, respectively, $p < 0.01$). Hepatic CYP3A activity of the CKD group was less than the control group (0.21 ± 0.05 compared to 0.43 ± 0.04 pmol/mg of microsome protein/min, respectively, $p < 0.01$). This data demonstrated that the activity of CYP3A enzyme in CKD-induced miceby new procedure is decreased compared to the controls. This result suggests that further studies on the pharmacokinetics properties of drugs that are substrates of CYP3A are of interest in CKD-induced mice.

Keywords: chronic kidney disease, mouse, CYP3A, liver, metabolism

ผลของโรคไตวายเรื้อรังในหนูเมาซ์ต่อเอนไซม์ *CYP3A* ในตับ

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บทคัดย่อ

โรคไตวายเรื้อรังเป็นโรคที่พบบ่อย และเป็นปัญหาสำคัญด้านสาธารณสุขของประเทศไทย จากรายงานพบว่าผู้ป่วยกลุ่มนี้มักประสบปัญหาด้านการใช้ยาเนื่องจากโรคไตวายเรื้อรังไปรบกวนการทำงานของเอนไซม์ไซโตโครมพี 450 โดยเฉพาะเอนไซม์ CYP3A การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของโรคไตวายเรื้อรังในหนูเมาซ์ที่ถูกเหนี่ยวนำให้เกิดไตวายเรื้อรังต่อการทำงานของ CYP3A ซึ่งเป็นเอนไซม์สำคัญในกระบวนการเมแทบอลิซึมของยาหลายชนิดด้วยโมเดลที่พัฒนาขึ้นใหม่โดยแบ่งหนูเมาซ์ออกเป็น 2 กลุ่ม ๆ ละ 4 ตัว ได้แก่ กลุ่มควบคุมและกลุ่มที่เกิดโรคไตวายเรื้อรังเป็นระยะเวลา 14 วันด้วยการผูกไต

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ข้างซ้ายเป็นเวลา 50 นาทีและ 7 วันต่อมาตัดไตข้างขวาออกจากนั้นทำการุณยฆาตเพื่อเก็บเลือดมาตรวจวัดระดับ serum creatinine และเก็บตับเพื่อศึกษาการทำงานของเอนไซม์ CYP3A ด้วยเทคนิค HPLC พบว่าระดับ serum creatinine ของหนู เมาซ์ที่เกิดไตวายเรื้อรังเพิ่มขึ้นอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มควบคุม (0.816 ± 0.10 mg/dL ต่อ 0.359 ± 0.05 mg/dL ที่ $p < 0.01$) และการทำงานของเอนไซม์ CYP3A ในตับหนูเมาซ์ที่เกิดไตวายเรื้อรังลดลงอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มควบคุม (0.43 ± 0.02 pmol/mg of microsome protein/min ต่อ 0.21 ± 0.03 pmol/mg of microsome protein/min ที่ $p < 0.01$) การศึกษานี้ บ่งชี้ว่าโรคไตวายเรื้อรังที่ถูกเหนี่ยวนำด้วยวิธีใหม่ดังกล่าว ส่งผลกระทบต่อการทำงานของเอนไซม์ CYP3A ในตับหนูเมาซ์ ผลที่ได้จะเป็นแนวทางในการศึกษาเกี่ยวกับเภสัชจลนศาสตร์ของยาที่เป็นสับสเตรทของเอนไซม์ CYP3A ในหนูเมาซ์โรค ไตวายเรื้อรังต่อไป

คำสำคัญ: โรคไตวายเรื้อรัง, หนูเมาซ์, เอนไซม์ไซโตโครมพี 450 3A, ตับ, เมแทบอลิซึม

Introduction

Chronic kidney disease (CKD is the presence of structural or functional abnormalities of the kidneywith or without a decreased glomerular filtration rate (GFR) for three months or longer or GFR less than 60 ml/min/1.73m^2 with/without kidney damage for at least three months.[1] CKD emerges as a worldwide public health problem. The incidence of CKD in Thailand has increased steadily over the past decade.[2] CKD has abundant comorbidity such as diabetes and hypertension, etc. Patients with CKD therefore require numerous medicines to manage both their kidney diseases and associate complications. The disease itself is an important risk factor for systemic accumulation of drug and may cause adverse effects or ineffective therapy. CKD affects pharmacokinetics process particularly, drug metabolism and elimination.[3] Furthermore, studies have demonstrated that CKD also affects drug metabolism via a decrease in hepatic cytochrome P450 (CYP) in the liver. The CYP3A subfamily, is an important CYP family involved in drug metabolism. It plays an important role in the oxidative metabolism of up to 50% of all drugs used in the clinic. As a result, drug-drug interactions associated with modulation of CYP3A-mediated metabolism can be of substantial clinical importance.[4] The CYP3A subfamily has very broad substrate specificity. Large interindividual differences were reported in CYP3A-mediated metabolism.In mouse liver, CYP3A11 is the isoform most similar to human CYP3A4, the most abundant CYP isoform of the CYP3A family, with 76% amino acid homology.[5] Several studies using erythromycin, testosterone and midazolam as CYP3A probes reported that CYP3A activity is decreased in CKD rats. To our best knowledge, very few CKD studies using a mouse model have been published. One study revealed that, CYP3A is also down-regulated in CKD mouse using erythromycin as a CYP3A probe.[11] In addition, there is no CKD mouse model induced by ischemic reperfusion (I/R) of one kidney followed by nephrectomy of the other side of the kidneys. This study aimed to use this new mouse model to induce

CKD in mice and see whether CKD causes any changes in the hepatic CYP3A-mediated pathway using testosterone as a CYP3A probe.

Materialsand Methods

Chemicals and reagents

6β-hydroxytestosterone was purchased from Cayman chemical (Ann Arbor, MI, USA). Testosterone and 11α-hydroxyprogesterone were purchased from Tokyo chemical industry Co., Ltd. (Chuo-ku, Tokyo, Japan). Phosphate buffered saline (PBS), ethylenediaminetetraacetic acid (EDTA), glucose-6-phosphte, glucose-6-phosphate dehydrogenase and β-nicotinamide adenine dinucleotide phosphate sodium salt hydrate (NADP) were purchased form Sigma-Aldrich (St. Louis, MO, USA). Methanol HPLC grade was purchased from Merch KGaA (Darmstadt, Germany). A Milli-Q Synthesis (Millipore, SA, Molsheim, France) water purification system was used for purify deionized water.

Experimental Model

All experiments were performed in male ICR mice, aged 6 to 8 weeks, weighing 20 to 30 g. All were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakorn Pathom, Thailand. All animals were housed as 5 animals / cage in the room 12:12 light-dark cycle at 25 ± 2 ℃ at the Faculty of Medicine, Chulalongkorn University's animal laboratory facilities. Food and water were provided *ad libitum*. The animals were allowed acclimatization for 3-5 days before the experiments were conducted. The experimental protocol was approved by the Ethics Committee for animal study of the Faculty of medicine, Chulalongkorn University.

Experimental Protocol

Studies were performed in two groups of mice: mice with normal kidney function (n=4), and mice with CKD group (n=4). CKD was induced by ischemic reperfusion (I/R) of left kidney. The left

renal artery was ligated for 50 minutes then right nephrectomy was performed 7 days afterwards. In brief, mice were anesthetized by inhaled isoflurane in anesthetic chamber. The clinical signs, including movement and respiration were observed for the deep sleep. Then, the left kidney was exposed via left costal margin approach. The left renal artery was then identified with temporary clamping with a surgical clip for 50 minutes. After removal of the clamp, clinical signs were observed at 2, 4 and 12 hours after operation. Seven days later, the right nephrectomy was done via right costal margin approach. The skin was then sutured layer by layer and then the clinical signs were observed at 2, 4 and 12 hours post-nephrectomy. All mice were weighed daily. Fourteen days after right nephrectomy operation, CKD mice were sacrificed. Blood and liver tissue samples were harvested. The liver tissue samples were flash frozen in liquid nitrogen, and stored at -80 ℃ until further analysis. Serum creatinine was determined by QuantichromTM Creatinine assay kit (Hayward, CA, USA).

Preparation of mice hepatic microsomes

Mice liver samples were immediately excised after death and hepatic microsomes were prepared by differential centrifugation as described previously.[12] The pellets containing microsomes were resuspended in 0.1 M of Tris base (pH 7.4) containing 20% glycerol and stored at -80 ℃ until further analysis. Total protein concentrations of the liver microsomes were determined using the Bio-Rad DC assay kit (Bio-Rad, CA, USA), with bovine serum albumin as a standard.

Testosterone hydroxylation assay

Hepatic CYP3A activity was determined as described previously.[12] In brief, 34.67 mM of testosterone was incubated with 200 µg of liver microsomal protein at 37 ℃ for 15 minutes with gentle agitation in the presence of an NADPH regenerating system consisting of 5 mM NADP, 100 mM of glucose-6-phosphate, and 100 mM of magnesium chloride, and 5 units of glucose-6-phosphate dehydrogenase in a total volume of 1 mL. At the end of the incubation, the reactions were quenched with dichloromethane and 11α-hydroxyprogesterone was added as an internal standard. The organic phase was evaporated and samples were dissolved in 200 µL of methanol. All samples were transferred to an autosampling vial for high performance liquid chromatography (HPLC) analysis.

Determination of metabolite by HPLC

Testosterone metabolite was quantified by HPLC technique according to Wonganan P, *et al* with modifications.[12] HPLC apparatus was Shimadzu, Kyoto, Japan with a UV-VIS detector (wavelength of 280 nm). Chromatographic separation was performed by a reversed-phase HPLC, using a symmetry C18 column (3.5-µm, 4.6 x 100 mm; Waters, Tokyo, Japan). The temperature of the column oven was maintained at 40 °C. Analysis was allotted under a gradient condition using a mobile phase consisting of methanol and water. The cycle time was 30 minutes. The linearity of this method was assessed with seven-point calibration curve (5 to 100 µM). The inter-day and intra-day precisions were assessed from quality control samples at the concentrations of 15, 50 and 90 µM and expressed as coefficient of variation (%CV). The inter-day and intra-day accuracies were assessed from the same samples and expressed as relative difference between the measured and true value. The lower limit of quantification (LLOQ) was determined at 5 µM. The precision and accuracy were < 20%. The extraction recovery of 6β-hydroxytestosterone standard (3, 15, and 90 µM) and 11α-hydroxyprogesterone as internal standard (100 µg/mL) prepared in methanol or samples were determined by comparisons between peak areas of standard or internal standard in methanol versus peak areas of standard or internal standard in methanol in samples within the same run.

Data analysis

Data are presented as mean ± standard deviation (SD). Differences between normal groups versus CKD groups were assessed by Mann-Whitney U test. A *p*-value of less than 0.05 was considered significant. All analyses were performed by SPSS version 20 for Windows (SPSS Inc. IL, Chicago).

Results

Biochemical parameters and body weight in control and CKD mouse

As shown in Table 1, CKD mice had higher levels of serum creatinine compared with the control group (0.816 ± 0.21 mg/dL versus 0.359 ± 0.10 mg/dL, *p* = 0.035). Body weight in CKD mice were significantly reduced with respect to the control group (30.1 ± 0.08 g versus 27.8 ± 1.27 g, *p* = 0.007).

Table 1. Effect of CKD on body weight and serum creatinine.

	Control group	CKD group	*p*-value
Number of mice	4	4	-
Body weight, g	30.1 ± 0.04	27.8 ± 0.63*	< 0.035
Serum creatinine, mg/dL	0.359 ± 0.05	0.816 ± 0.10**	< 0.007

CKD, chronic kidney disease
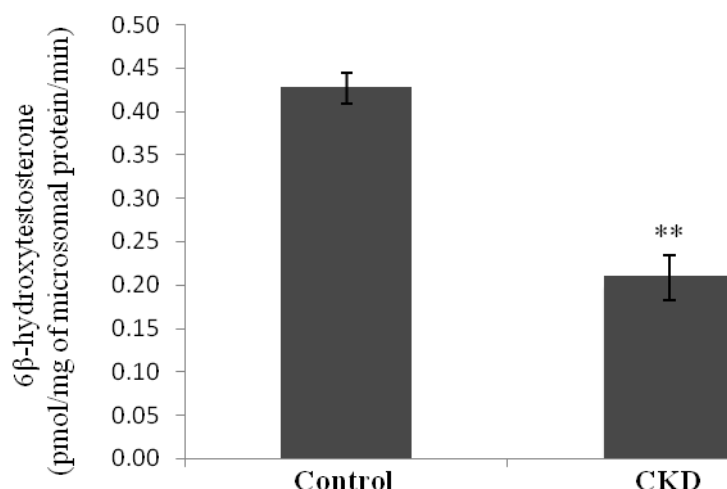*p < 0.05* compared with the controls
**p < 0.01* compared with the controls

Figure 1.   Effect of CKD on hepatic CYP3A activity. *In vivo* catalytic activity of CYP3A microsomal protein was measured by the production of testosterone metabolite, 6β-hydroxytestosterone. **, *p*< 0.01 compared with control.

Hepatic CYP3A-mediated drug metabolism

A primary CYP3A-specific metabolite of testosterone, 6β-hydroxytestosterone was assessed in mouse liver microsomes to determine the effects of CKD on hepatic CYP3A-activity. As shown in Figure 1, the amount of 6β-hydroxytestosterone generated in samples from CKD mice (0.21 ± 0.05 pmol/mg of microsome protein/min) was significantly lower than that of the control group (0.43 ± 0.04 pmol/mg of microsome protein/min), indicating that hepatic CYP3A activity was dramatically decreased in our CKD mouse model.

Discussion

In the present study, we developed a new CKD mouse model to be used in assessing the effect of CKD on hepatic CYP function using testosterone hydroxylation assay, a well-established substrate as a probe. Our results demonstrated that, there was a rapid increased in serum creatinine in CKD mice (0.359 ± 0.10 mg/dL in control group compared to 0.816 ± 0.21 mg/dL in CKD group, *p* < 0.01), indicating that CKD has been established in mice. Compared to a previous reported of CKD model, [11] our model takes a shorter

time for CKD induction, and shows a higher survival rate of mouse, suggesting that ischemic reperfusion of one kidney followed by nephrectomy of the other can be a new technique to develop CKD *in vivo*.

Using our CKD mouse model, the current result showed that hepatic CYP3A in the CKD group (0.21 ± 0.05 pmol/mg of microsome protein/min) was reduced compared to the control group (0.43 ± 0.04 pmol/mg of microsome protein/min), *p* < 0.01). Several studies have reported that the activity of hepatic CYP3A enzyme is reduced in CKD, however, most studies were performed in rats. [7, 8, 9, 10] Although one study was done in mice, they used erythromycin as a CYP probe. Interestingly, Velenosi *et al* have found that midazolam and testosterone can be used as a probe for evaluating hepatic CYP3A function as both compounds are more selective to CYP3A than erythromycin. Additional studies to determine hepatic CYP3A activity in CKD using other CYP3A substrates are currently under active investigation in our laboratories.

Conclusion

In the present study, we developed a new technique to establish a CKD mouse model. Using testosterone as a probe, we found that hepatic CYP3A-mediated metabolism is decreased in mice with CKD. This result suggests that further research on the pharmacokinetics properties of drugs that are substrates of CYP3A is essential in CKD-induced mice.

Acknowledgements

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F07 Effects of a Single Dose Administration of Fresh- and Cooked Betong Watercress on Caffeine Metabolism via *N*-demethylation and C-8 Hydroxylation in Rats

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Abstract

Fresh Betong watercress contains phenethyl isothiocyanate (PEITC) which has an influence on hepatic enzymes. This compound derived from a hydrolytic reaction of glucosinolates via myrosinase in plant cell. Since cooking cause enzyme inactivation, the present study was aimed to investigate effects of fresh- and cooked Betong watercress juice in form of dry powder on caffeine metabolism via *N*-demethylation and C-8 hydroxylation reactions in rats. Three groups of male Wistar rats (n=6) were included. They were pretreated with a single oral dose of PEITC (2 mg/kg), dry powder of fresh Betong watercress (1.3676 g/kg), and cooked Betong watercress (0.3594 g/kg) followed by caffeine (25 mg/kg). Plasma concentrations of caffeine (CF), theobromine (TB), paraxanthine (PX), theophylline (TP), and 1,3,7-trimethyluric acid (137-U) after 3h of caffeine dosing were measured. The caffeine metabolic ratios (TB/CF, TP/CF, PX/CF, and 137-U/CF) were determined and compared with those after receiving caffeine alone. PEITC slightly decreased all caffeine metabolic ratios. Fresh Betong watercress significantly decreased all caffeine metabolic ratios by 19-37%, while cooked Betong watercress decrease only TB/CF and TP/CF by 3-13%. Inversely, it significantly increased PX/CF and 137-U/CF, compared with fresh Betong watercress. The results indicated that fresh Betong watercress inhibited caffeine metabolism via *N*-demethylation and C-8 hydroxylation in rats while cooked Betong watercress slightly inhibited 1-*N* and 7-*N* demethylation and enhanced 3-*N* demethylation and C-8 hydroxylation.

Keywords: Betong watercress, phenethyl isothiocyanate, caffeine, metabolism

ผลของการได้รับผักน้ำเบตงสดและผักน้ำเบตงปรุงสุกต่อเมแทบอลิซึมของคาเฟอีนผ่านการเกิดปฏิกิริยาเอ็น-ดีเมทิลเลชัน และ ซี-8 ไฮดรอกซิเลชันในหนูขาว

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บทคัดย่อ

ผักน้ำเบตงสดมีสารเฟนเอทิลไอโซไธโอไซยาเนท (PEITC) ซึ่งมีอิทธิพลต่อการทำงานของเอนไซม์ในตับ สารชนิดนี้เกิดจากปฏิกิริยาไฮโดรไลซิสของสารกลุ่มกลูโคซิโนเลตโดยอาศัยเอนไซม์ไมโรซิเนสที่มีอยู่ในเซลล์พืช เนื่องจากการปรุงอาหารด้วยความร้อนทำให้เอนไซม์ทำงานไม่ได้ การศึกษาในครั้งนี้จึงมีวัตถุประสงค์เพื่อศึกษาผลของน้ำคั้นน้ำเบตงสดและผักน้ำเบตงปรุงสุกต่อเมแทบอลิซึมของคาเฟอีนผ่านการเกิดปฏิกิริยาเอ็น-ดีเมทิลเลชัน และ ซี-8 ไฮดรอกซิเลชันในหนูขาว ใช้หนูขาวเพศผู้

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สายพันธุ์วิสตาร์ 3 กลุ่ม (กลุ่มละ 6 ตัว) หนูขาวได้รับ PEITC (2 มิลลิกรัม/กิโลกรัม), ผงแห้งผักน้ำเบตงสด (1.3676 กรัม/กิโลกรัม), และ ผงแห้งผักน้ำเบตงปรุงสุก (0.3594 กรัม/กิโลกรัม) ล่วงหน้าครั้งเดียวตามด้วยคาเฟอีนขนาด (25 มิลลิกรัม/กิโลกรัม) วัดความเข้มข้นของคาเฟอีน (CF), ธีโอโบรมีน (TB), พาราแซนทีน (PX), ธีโอฟิลลิน (TP) และ 1,3,7-ไตรเมทิลยูริกแอซิด (137-U) ในพลาสมาที่เวลา 3 ชั่วโมงหลังจากได้รับคาเฟอีน วัดและเปรียบเทียบค่าอัตราส่วนเมทาบอลิคของคาเฟอีน (TB/CF, TP/CF, PX/CF และ 137-U/CF) กับเมื่อได้รับคาเฟอีนอย่างเดียว PEITC ลดอัตราส่วนเมทาบอลิคของคาเฟอีนทุกค่าเพียงเล็กน้อย ผักน้ำเบตงสดลดอัตราส่วนเมทาบอลิคของคาเฟอีนทุกค่าอย่างมีนัยสำคัญ 19 ถึง 37% ในขณะที่ผักน้ำเบตงปรุงสุกลดเฉพาะค่า TB/CF และ TP/CF 3-13% ในทางกลับกันผักน้ำเบตงปรุงสุกเพิ่มค่า PX/CF และ 137-U/CF อย่างมีนัยสำคัญ เมื่อเปรียบเทียบกับผักน้ำเบตงสด ผลการศึกษาชี้ให้เห็นว่าผักน้ำเบตงสดยับยั้งเมทาบอลิซึมของคาเฟอีนผ่านปฏิกิริยาเอ็น-ดีเมทิลเลชัน และ ซี-8 ไฮดรอกซิเลชันในหนูขาว ในขณะที่ผักน้ำเบตงปรุงสุกยับยั้งปฏิกิริยา 1-เอ็น และ 7-เอ็น-ดีเมทิลเลชัน เพียงเล็กน้อย และเพิ่มการเกิดปฏิกิริยา 3-เอ็น-ดีเมทิลเลชัน และ ซี-8 ไฮดรอกซิเลชัน

คำสำคัญ: ผักน้ำเบตง, เฟนเอทิลไอโซไธโอไซยาเนท, คาเฟอีน, เมทาบอลิซึม

Introduction

Betong watercress (Phak Num Betong; *Nasturtium officinale* R.Br.) is a variety of watercress cultivated in Southern Thailand. It is a member of the *Cruciferae* (*Brassicaceae*) which contains glucosinolates that can be hydrolyzed to isothiocyanates (ITCs) by the action of myrosinase, an enzyme naturally existing in plant cells. The hydrolysis of glucosinolates can occur when plant cells are damaged such as from cutting or chewing. Glucosinolates can also be hydrolyzed by intestinal microflora.[1] Among cruciferae members, watercress contains the largest amounts of ITCs, mostly phenethyl isothiocyanate (PEITC) which has preventive effect against chemical carcinogenesis possibly by influencing on hepatic enzymes used in activation or inactivation of chemical procarcinogens.[2-4]

Many studies showed that watercress and PEITC interfered with cytochrome P450 isoforms such as CYP2E1 and CYP1A2. A previous study investigated effect of Betong watercress and PEITC on caffeine metabolism via *N*-demethylation in rats by determining primary metabolites (theobromine, paraxanthine, theophylline) and caffeine metabolic ratios.[4] The decrease in caffeine metabolic ratios indicated that fresh Betong watercress and PEITC inhibited *N*-demethylation of caffeine and they have inhibitory activity on CYP1A2 and CYP2C.

Since myrosinase in plant cells can be inactivated by cooking, it is hypothesized that cooking may alter effect of Betong watercress on caffeine metabolism. In addition, in rats, C-8-hydroxylation is a major route and mainly regulated by CYP1A2 while *N*-demethylation, is a minor route and regulated by CYP1A2 and CYP2C. The present study is therefore aimed to investigate effects of fresh- and cooked Betong watercress juice on caffeine metabolism via *N*-demethylation and C-8 hydroxylation reactions in rats.

Materials and Methods

Chemicals and reagents

PEITC, caffeine, theobromine, theophylline, paraxanthine, 1,3,7-trimethyluric acid, and 7-(β-hydroxyethyl)theophylline (an internal standard) were purchased form Sigma-Aldrich (St. Louis, MO, USA.). Sodium acetate was purchased from Thermo Fisher Scientific Inc. (Waltham, MA USA). Acetonitrile and methanol (HPLC grade) were purchased from Avantor Performance Materials, Inc. (Center Valley, PA, USA.). Acetic acid, sodium hydroxide, and isopropanol (AR grade) were obtained from Merck (Darmstadt, Frankfurter, Germany).

Animals

The study included seven-week-old male Wistar rats (190-220 g) provided by Southern Laboratory Animal Facility, Prince of Songkla University. The experiment was approved by the Animal Ethic Committee, Prince of Songkla University (ref.17/51). The animals were housed under the controlled environment (temperature 25±2ºC, light/dark cycle 12/12 h) with food and water *ad libitum.* Before treatment, they were fasted overnight (12 h) with free access to water

Study design

Three groups (n=6 each) of animals were included. An experiment consisted of two phases. Phase I, the animals were administered a single oral dose of caffeine (25 mg/kg). Phase II, the same animal was pretreated with PEITC (2 mg/kg), a dry powder of fresh Betong watercress juice (1.3676 g/kg), or a dry powder of cooked Betong Watercress juice (0.3594 g/kg) before receiving the same dose of caffeine. All treatments were administered by gastric gavage.

Blood samples were collected by tail clipping at 0 and 3 h after caffeine administration in phase I and phase II. Plasma was obtained by centrifugation at 1600 x g for 10 min and immediately stored at -80º C until analysis.

Preparation of dry powder of fresh and cooked Betong watercress juice

Betong watercress (3 kg) was purchased from a local farm at Betong district, Yala province, Thailand. The vegetable was cleaned thoroughly and divided into portions for preparing fresh and cooked preparations. Each portion (100 g) was chopped and added with deionized water (80 mL). For cooked preparation, the vegetable was heated using a microwave (800 W, 3 min). The mixture was blended into a fine paste using homogenizer before filtration. The filtrate was centrifuged 12,000 rpm at 4°C for 10 min). The supernatant was then lyophilized. The dry powder was immediately stored at -80°C until analysis.

Determination of PEITC in dry powder of Betong watercress juice

The amount of PEITC in dry powder of fresh Betong watercress and cooked Betong watercress was determined based on the previous method.[5] Briefly, the dry powder samples (25 mg) were hydrolyzed by myrosinase before derivatization with 1,2-benzenedithiol prior to HPLC injection.

The HPLC system consisted of a Waters 2695 Separations Module and a Waters 5487 dual λ absorbance detector (Milford, MA, USA). Data were processed using the Empower TM Software System (Milford, MA, USA). Samples were separated using a Novapak® C18 column (3.9 ×150mm, 4 μm) at 25ºC. The mobile phase was a methanol-water mixture (90:10, v/v) flowed at 1.1 mL/min. The aloquot (20 μL) was detected at 365 nm.[5]

Quantification of caffeine and its metabolites in plasma

The HPLC system as stated above was used. Separation was modified based on the previous method.[6] A Symmetry® C18 column (250×4.6 mm i.d., 5 μm) connected to a Symmetry TM guard column (20×3.9 mm i.d., 5 μm) was used and controlled at 40ºC. The mobile phase was a mixture of 20 mM sodium acetate buffer (pH 4.8): acetonitrile: methanol (900:20:80 v/v/v). The flow rate was 1.1 mL/min, and the detection wavelength was 275 nm

Sample preparation

Plasma (80 μL) was combined with 20 μL of the internal standard. Then acetonitrile was added and vortex mixed before centrifugation at 14,000 rpm for 10 min. An aliquot of 200 μL of the supernatant was transferred to a new tube and evaporated under a stream of nitrogen at room temperature. The residue was reconstituted in 100 μL of mobile phase and a 10 μL was injected into the HPLC system.

Data analysis

All data were expressed as mean ± SE. Metabolic ratios (MRs) were calculated as the ratios of the plasma concentration of each metabolite to that of CF (TB/CF, PX/CF, TP/CF and 137-U/CF). MRs in phases I (without pretreatment) and II (after pretreatment) experiments were compared using nonparametric test. The percentage of changes in MRs among different groups was analyzed by using Kolmogorov-Smirnov test. A statistically significant difference was accepted at $p<0.05$.

Results

Quantification of PEITC in dry powder of fresh and cooked Betong watercress juice

To analyze PEITC, the derivative product of PEITC was identified. The derivative product was eluted at 1.986 min with no interference (data not shown). The calculated amounts of PEITC in dry powder of fresh Betong watercress juice and cooked Betong watercress juice were 36.56 μg/25 mg of dry powder and 139.12 μg/25 mg of dry powder, respectively.

Quantification of caffeine and its metabolites

Caffeine, paraxanthine, theobromine, theophylline, 1,3,7 trimethyluric acid, and internal standard were clearly separated within 32 min with no interference (Figure 1).

Caffeine metabolic ratios in rats after a single pretreatment with PEITC (2 mg/kg, p.o.), fresh Betong watercress (1.3676 g of dry powder/kg, p.o.) and cooked Betong watercress (0.3594 g of dry powder/kg, p.o.) are shown in Figure 2. The results showed that pretreatment with PEITC decreased all of metabolic ratios but this reduction was not significant when compared with without pretreatment. Significant reduction of metabolic ratios (TB/CF, PX/CF, TP/CF, and 137-U/CF) were observed after pretreatment with fresh Betong watercress when compared with phase I ($p<0.05$), whereas metabolic ratios after pretreatment with cooked Betong watercress were not significantly different

The percent changes in metabolic ratios are shown in Figure 3. After single pretreatment, PEITC changed metabolic ratios by -10 to -24%. Dry powder of fresh Betong watercress changed metabolic ratios by -19 to -37 %.

The changes in metabolic ratios caused by cooked Betong watercress were -2 to +24%. This effect was significantly different when compared with fresh Betong watercress pre-treatment ($p<0.05$).
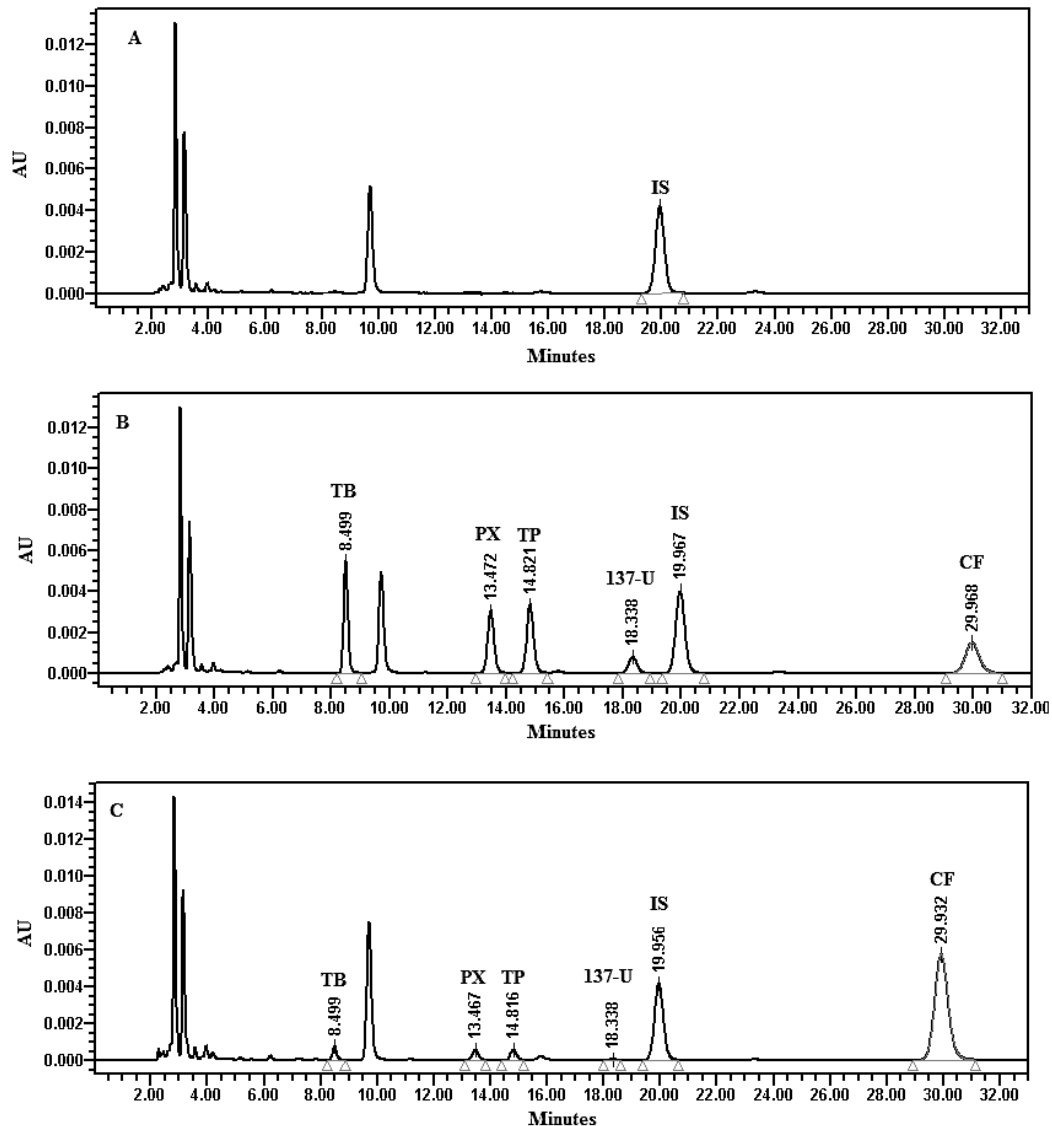

Figure 1. Representative chromatograms of caffeine (CF), paraxanthine (PX), theobromine (TB), theophylline (TP), and 1,3,7 trimethyluric acid (137-U); (A) blank rat plasma spiked with internal standard (IS; 10 µg/mL); (B) Blank rat plasma spiked with internal standard (IS; 10 µg/mL) and standard TB, PX, TP, 137-U and CF (5 µg/mL); (C) rat plasma at 3 h after an oral administration of CF (25 mg/kg).
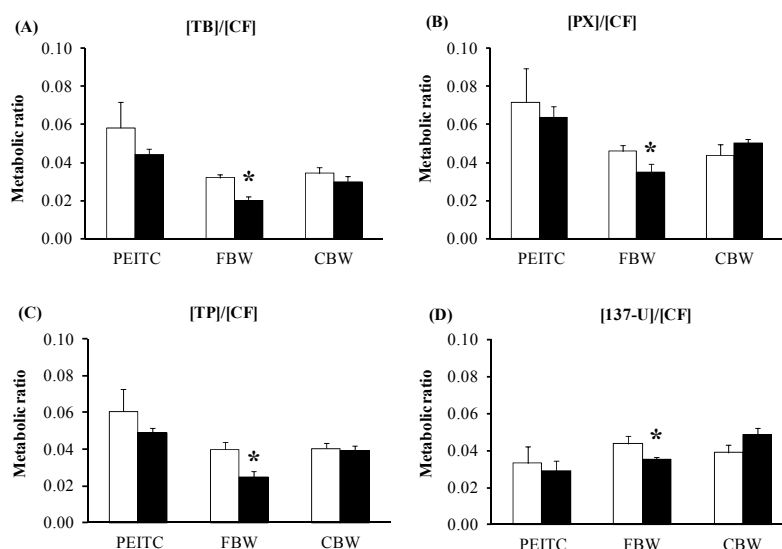
Figure 2. Metabolic ratios of caffeine (mean ± SE) (A-D) in rats (n=6) receiving caffeine (CF) alone (phase I; □) and caffeine after pretreatment with PEITC (2 mg/kg), dry powder of fresh Betong watercress (FBW) (1.3676 g of dry powder/kg), and dry powder of cooked Betong watercress (CBW) (0.3594 g of dry powder/kg) (phase II; ■). * $p < 0.05$, compared with phase I.
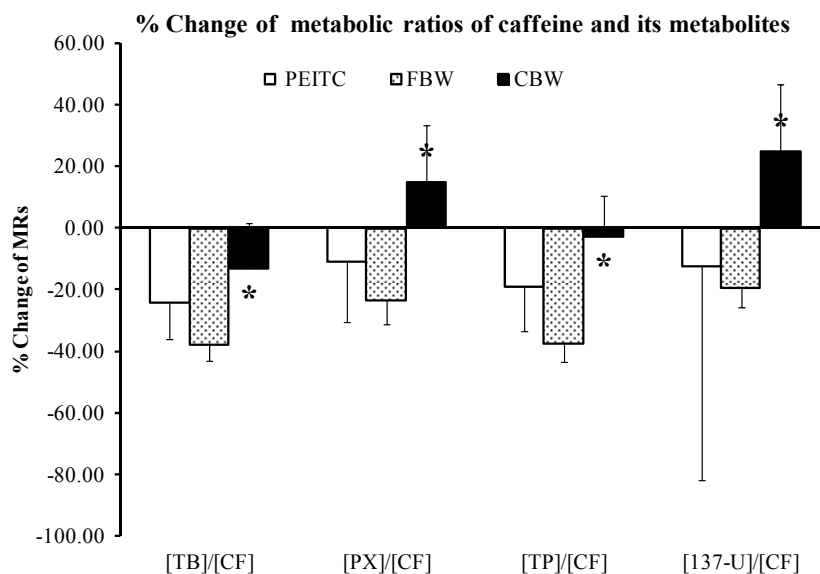
Figure 3. Percentage of the change in metabolic ratios of caffeine (MRs) (Mean ± SE) of caffeine and its metabolites in rats receiving a single dose of PEITC (2 mg/kg), dry powder of fresh Betong watercress (FBW) (1.3676 g of dry powder/kg), and dry powder of cooked Betong watercress (CBW) (0.3594 g of dry powder/kg). * $p<0.05$, compared with FBW pre-treatment.

Discussion

Doses of fresh and cooked Betong watercress were 1.3676 g of dry powder/kg and 0.3594 g of dry powder/kg, respectively. Those were calculated from the amounts determined from the dry powder of fresh and cooked Betong watercress juice. This study was designed to compare the effect of fresh and cooked Betong watercress containing the same amounts of PEITC. The result showed that dry powder of cooked Betong watercress juice, after hydrolysis with myrosinase *in vitro*, contained a higher amount of PEITC compared with that of fresh Betong watercress juice, i.e. 139.12 µg/25 mg of dry powder vs. 36.56 µg/25 mg of dry powder. The amount of dry powder of cooked Betong watercress

administered to the rats was therefore lower than that of dry powder of fresh Betong watercress.

The doses of Betong watercress used in this study were equivalent to 2 mg PEITC/kg. That was based on the previous study[7] that showed a significant reduction of caffeine metabolic ratios (TB/CF, PX/CF, and TP/CF) in rats pretreated with 2-20 mg/kg PEITC. This study also used PEITC at the dose of 2 mg/kg as a reference. However, the reduction of all caffeine metabolic ratios (TB/CF, PX/CF, TP/CF, and 137-U/CF) was not statistically significant.

Fresh Betong watercress caused a significant reduction of all caffeine metabolic ratios, both those represent *N*-demethylation (TB/CF, PX/CF, and TP/CF) and C-8 hydroxylation (137-U/CF). The percent changes of caffeine metabolic ratios via *N*-demethylation ranged from -23% to -37%, while that via C-8 hydroxylation was -19%. This effect indicated an inhibition of caffeine metabolism via *N*-demethylation and C-8 hydroxylation by fresh Betong watercress. In rats, C-8 hydroxylation is a major route and mainly regulated by CYP 1A2, while *N*-demethylation is regulated by CYP 1A2, CYP 2C6, and CYP 2C11.[8] Therefore it is likely that dry powder of fresh Betong watercress juice inhibited CYP 1A2 and CYP 2C.

In contrast to fresh Betong watercress, dry powder of cooked Betong watercress juice caused a small reduction in caffeine metabolic ratios, i.e. 13% and 3% for TB/CF and TP/CF, respectively. It also caused an increase in metabolic ratios, i.e. 15% and 25% for PX/CF and 137-U/CF, respectively. Different result observed from fresh and cooked Betong watercress may be a result from different bioactive constituents in both preparations. Fresh Betong watercress juice contains mostly PEITC derived from hydrolysis of glucosinolates by myrosinase in plant cell during blending of fresh plant. In contrast, during cooking process, myrosinase in plant cells was inactivated.[9,10] Homogenate and juice of cooked Betong watercress therefore contained mostly glucosinolates. After an oral administration, these precursors were naturally converted to PEITC and other metabolites by colonic microflora.[10, 11]

Conclusion

In conclusion, heat from cooking Betong watercress altered its activity on caffeine metabolism via *N*-demethylation and C-8 hydroxylation. Fresh Betong watercress inhibited both *N*-demethylation and C-8 hydroxylation, while cooked plant slightly inhibited 1-*N* and 7-*N* demethylation, and accelerated 3-*N* demethylation and C-8 hydroxylation.

Both metabolic pathways are regulated mainly by CYP 1A2 and CYP 2C. It was suggested that cooked and fresh Betong watercress affected the activity of CYP 1A2 and CYP 2C in an opposite direction. In humans, ingestion of cooked Betong watercress may exhibit different outcome from ingestion of fresh plant.

Acknowledgements

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F08

Prevalence and Enzyme Activity of Cytidinedeaminase Variations in Northeast Thai Healthy Volunteers

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Abstract

Gemcitabine (dFdC) is a high efficacy chemotherapy drug for patients with pancreatic cancer, non-small cell lung cancer, bladder cancer, ovarian cancer and cholangiocarcinoma (bile duct cancer). However, its clinical outcome and adverse effects vary among patients, depending on the variations of gemcitabine pharmacokinetics among individuals. Gemcitabine is metabolized by cytidinedeaminase (CDA) to an inactive form. Recent study reports $CDA*2$ 79A>C (K27Q, rs2072671) mutant allele is correlated with CDA enzyme activity. Patients who carry $CDA*2$ genotype have higher CDA activity and lower response rate than patients with wild type CDA. Patients who carry $CDA*3$ 208G>A (A70T, rs60369023) genotype have lower CDA activity than patients with wild type CDA, and experience a higher incidence of neutropenia. The prevalence of CDA gene variations and their enzymatic activities have not been reported in Thai populations. This study aims to determine the frequency of the two major CDA variants in sixty one Northeast Thai healthy volunteers, and to examine the correlation between CDA genotype and the enzyme activity.The $CDA*2$ and $*3$ genotypes were determined using real-time PCR technique, and the plasma CDA activity was evaluated by spectrophotometric technique. The prevalence of $CDA*1/*2$ was observed at 22.95%. Neither the homozygous mutant allele of $CDA*2$, nor any forms of $CDA*3$ were detected in this study. This study concludes that the allele frequency of $CDA*2$ in Thai population significantly differs from American ($p = 0.002$) and European populations ($p = 0.001$),but it is not differentfrom Chinese ($p = 0.828$), Japanese ($p = 0.114$), Korean ($p = 0.490$)and African ($p = 0.061$) populations. In addition, the mean of CDA activity in $CDA*1/*1$ volunteers was not significantly different from $CDA*1/*2$ group ($p = 0.08$). The majority of insignificance statistics for CDA allele frequencies among populations could result from the small sample size effects.

__Keywords__: cytidinedeaminase, CDA gene, prevalence, enzyme activity, allele frequency

การศึกษาความชุกของความหลากหลายทางพันธุกรรมของยีน *CDA* กับการทำงานของเอนไซม์ cytidinedeaminaseในอาสาสมัครสุขภาพดีจากภาคตะวันออกเฉียงเหนือของประเทศไทย

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บทคัดย่อ

Gemcitabine (dFdC) เป็นยาเคมีบำบัดที่พบว่าให้ผลการรักษาดีกว่าสูตรยาอื่นในการรักษาผู้ป่วยโรคมะเร็งตับอ่อน มะเร็งปอดชนิดเซลล์เล็ก มะเร็งกระเพาะปัสสาวะ มะเร็งรังไข่และมะเร็งท่อน้ำดี อย่างไรก็ตามผลการรักษาและอาการไม่ พึงประสงค์ในผู้ป่วยที่ได้รับยา gemcitabine แตกต่างกันเนื่องจากเภสัชจลนศาสตร์ของผู้ป่วยแต่ละรายต่างกัน เอนไซม์ cytidinedeaminase (CDA) ทำหน้าที่เปลี่ยนยา gemcitabine เป็นสารที่ไม่ออกฤทธิ์ รายงานการศึกษาที่ผ่านมาพบว่าความ หลากหลายของยีน $CDA*2$ 79A>C (K27Q, rs2072671) สัมพันธ์กับการทำงานของเอนไซม์ CDA ผู้ป่วยที่มียีนชนิด $CDA*2$

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มีการทำงานของเอนไซม์ CDA สูงกว่าผู้ป่วยที่เป็น wild type ขณะที่ผู้ป่วยที่มียีนชนิด CDA*3 208G>A(A70T, rs60369023) มีการ ทำงานของเอนไซม์ CDA ลดลงและยังเพิ่มอุบัติการณ์การเกิด neutropenia สูงขึ้นซึ่งยังไม่มีข้อมูลความหลากหลายทางพันธุกรรม ของยีน CDA และการทำงานของเอนไซม์ CDA ในประชากรไทย คณะผู้วิจัยจึงได้ศึกษาความชุกของยีน CDA รวมทั้งความสัมพันธ์ ระหว่างการทำงานของเอนไซม์ CDA และความหลากหลายทางพันธุกรรมของยีนในอาสาสมัครสุขภาพดีที่มีภูมิลำเนาเป็นคน ภาคตะวันออกเฉียงเหนือจำนวน 61 ราย การศึกษาความหลากหลายทางพันธุกรรมของยีน CDA*2 และ CDA*3 ใช้เทคนิค Real-time PCR ส่วนการศึกษาการทำงานของเอนไซม์ CDA ในพลาสมาใช้เทคนิค spectrophotometric พบว่าความหลากหลายทาง พันธุกรรมของยีน CDA*1/*2 เท่ากับ 22.95% ของประชากรทั้งหมด โดยยังไม่พบกลุ่ม homozygous mutant และ allele mutant ของยีน CDA*3 ในประชากรชาวไทยอีสานเมื่อเปรียบเทียบการเปลี่ยนแปลงความถี่ของแอลลีลกับประชากรกลุ่มอื่นพบว่ามีความ แตกต่างกันอย่างมีนัยสำคัญกับประชากรอเมริกัน (p = 0.002) และในทวีปยุโรป (p = 0.001) แต่ไม่มีความแตกต่างกันอย่างมี นัยสำคัญกับประชากรชาวเอเชีย เช่น จีน (p = 0.828) ญี่ปุ่น (p= 0.114), เกาหลี (p = 0.490) และชาวแอฟริกัน (p= 0.061)นอกจากนี้ การศึกษานี้ไม่พบความสัมพันธ์ระหว่าง CDA activity และความหลากหลายทางพันธุกรรมของยีน CDA(p = 0.08) ซึ่งอาจเป็นผลจาก มีจำนวนอาสาสมัครเข้าร่วมโครงการน้อยเกินไป

คำสำคัญ: Cytidinedeaminase (CDA),ยีนCDA, ความชุก, เอนไซม์,การเปลี่ยนแปลงความถี่ของแอลลีล

Introduction

Gemcitabine (2',2'-difluorodeoxycytidine, dFdC) is a high efficacy chemotherapy dug for patients with pancreatic cancer, non-small cell lung cancer, bladder cancer, breast cancer, ovarian cancer and cholangiocarcinoma (bile duct cancer). However, the therapeutic response and adverse effectsof this drug differ among patients due tothe variations of gemcitabine pharmacokinetics. Human cytidinedeaminase (CDA) is a cytoplasmic enzyme (EC 3.5.4.5) which involves in pyrimidine nucleotide synthesis; therefore, CDA enzyme plays a crucial role in the degradation of gemcitabine (dFdC) to 2',2'-difluorodeoxyuridine(dFdU), an inactive form. Recent data showed patients with low CDA activity have better clinical outcomes than those with high CDA activity. Low CDA activity patients have a significant higher response rate (37.7% vs.13.8%; p = 0.006), higher clinical benefit (91.8% vs.51.7%; p< 0.001), longer time to disease progression (8.0 vs.3.0 months; p < 0.001) and longer overall survival (19.0 vs.6.0 months; p = < 0.001) than the high CDA activity group.[1]

More than ten single nucleotide polymorphisms of CDA gene have been reported. Previous study showed CDA*2 (79A>C, K27Q) (rs2072671) mutant allelecorrelates with CDA enzyme activity. Patients who carry CDA*2 genotype have higher CDA activity than those with wild type enzyme.[2] Non-small cell lung cancer patients who carry CDA*1/*1 genotype have lower mean of CDA activity than those with CDA*1/*2 and CDA*2/*2 (8963.9 ± 6614.9 vs.11686.5 ± 9339.9 vs.15450.6 ± 10155.6 pmol/h/mg proteins, respectively). CDA*2 patients have lower response rate than wild type (37.7% vs. 13.8%; P = 0.006).[1] Patients who carry CDA*3 208G>A, A70T) (rs60369023) genotype have

lower CDA activity than patients with wild type enzyme. CDA*3 patients have a higher incidence of neutropenia than wild type (60% and 40% respectively, p = 0.205).[3]

The prevalence of CDA genotype and the correlation between CDA activity and CDA genotype has not been reported in Thai healthy volunteers. The aims of this study were to determine the frequency of the two major CDA variants in Northeast Thai healthy volunteers, and to examine the correlation between CDA genotype and the enzyme activity.

Materials and Methods

Subjects

Sixty one healthy volunteers (21 women and 40 men) with a family history ofliving for three generations in Northeast Thailand were enrolled from the blood donation center of Srinagarind Hospital, Faculty of Medicine, Khon Kaen University. The mean age among volunteers is 34±10.4 years.

The research protocol was approved by the Khon Kaen University Ethic Committee for Human Research, Khon Kaen University, Thailand (HE571402). Written informed consents were obtained from all subjects before participating in this study.

Sample collection

Three milliliters of blood from each subject were collected in potassium ethylene iaminetetra-acetic acid (EDTA)-containing vacutainer tubes. Then, blood samples were centrifuged at 2,500 rpm for 10. Plasma and buffy coat were collected. Plasma samples were used for CDA activity analysis. Buffy coat was stored at -20°C until used.

CDA Polymorphism Screening

Genomic DNAs extraction was isolated from buffy coat, using the QIAamp® DNA blood extraction kit (Qiagen, Hilden, Germany). The CDA*2 and CDA*3 polymorphism were verified by real-time PCR technique with TaqMan® probes. Analyses were performed by using the Light-Cycler 480 technology (Roche Diagnostics, Meylan, France). Primer sequences and PCR condition were used as previous reported.[4]

CDA enzyme activity assay

CDA enzyme activity was evaluated by measuring of residual enzyme activity in plasma. The principle of the assay is based on the conversion of cytidine to uridine which releases ammonium (NH_3).[5] Each plasma sample was mixed with 2 mMcytidine as the substrate and incubated for 16 hours at 37°C. NH_3 released was then measured by spectrophotometry incubation (625 nm). CDA activity was expressed as mU/mg protein (1 U equals to 10^{-4} µmol of ammonium released per minute per milliliter of plasma). Total proteins in plasma were assayed using the standard Bradford method.[6]

Statistical analysis

The distribution test of CDA activity was assessed by Kolmogorov-Smirnov test. The distribution was displayed as a histogram using SPSS statistic software version 17.0 (SPSS Inc., Chicago, USA). The mean of CDA activity in male and female volunteers were compared by Mann-Whitney Rank Sum Test. The allele frequency of CDA*2 was calculated following the Hardy-Weinberg equilibrium (Hardy-Weinberg equilibrium: $p^2 + 2pq + q^2 = 1$; when p is wild type allele or allele "A" and q is the variant allele frequency or allele "C"). The difference in allele frequencies of CDA*2 among ethnic populations was evaluated using the Pearson Chi-square test in STATA statistical software version 13.0 (Stata corporation, TX, USA). Association between CDA*2 genotype and CDA activity were assessed using Mann-Whitney Rank Sum Test. The association was displayed as a box plot using Sigmaplot software version 10.0 (Systat Software Inc. California, USA).

Results

CDA polymorphism

The prevalence of CDA*1/*1 and CDA*1/*2 genotype in the healthy volunteers were 77.05% and 22.95%, respectively. No homozygous mutant allele of CDA*2 was found. CDA*3 mutant allele was not found in this study. The calculated allele frequencies of CDA*1 and CDA*2 were 88.52% and 11.48%, respectively. The allele frequency of CDA*2in our sample was significantly different from American and European populations, as shown in Table 1. However, CDA*2 allele frequency of the Northeast Thai population was not significantly different from other Asian populations (Table 1).

Table 1. Allele frequencies of CDA*2in Northeast Thai healthy volunteers compared with other populations

Ethnic groups	N	n	Allele frequency (%)		p-value
			*1	*2	
Asian					
Thai	61	122	88.5	11.5	
Chinese[7]	102	204	87.7	12.3	0.828
Japanese[8]	206	412	79.6	20.4	0.114
Korean[8]	200	400	84.8	15.2	0.490
Malay[9]	20	40	82.5	17.5	0.380
Indian[9]	17	34	88.2	11.8	0.974
Caucasians					
American[8]	150	300	67.3	32.7	0.002
Dutch[10]	100	200	65	35	0.001
European[11]	95	190	64	36	0.001
African[11]	85	170	96	4	0.061
African-American[8]	150	300	91.3	8.7	0.518
Chinese-American[8]	200	400	84.5	15.5	0.435

N = Number of healthy subjects
n = Number of alleles

CDA activity

The mean plasma CDA activity of the healthy volunteers was 1356.9±509.2 mU/mg protein (range 609.9-3471.1 mU/mgproteins), it follows a non-normal distribution (Kolmogorov-Smirnov test, $p = 0.014$; Figure 1). The averaged CDA activity among male volunteers was 1402.2±412.6 mU/mg protein, whereas the mean activity in females was 1282.1±641.4 mU/mg protein. Males and females showed a non-significantlydifferent mean of CDA activity (Mann-Whitney Rank Sum Test, $p = 0.100$).

Correlation between CDA polymorphism and activity

The means CDA activity of wild type and *CDA*1/*2* genotype were 1422.4±545.9 and 1127.4±285.1 mU/mg protein, respectively. CDA activity in *CDA*1/*1* group was notsignificantly statistically different from *CDA*1/*2* (Mann-Whitney Rank Sum Test, $p= 0.08$; Figure 2.).
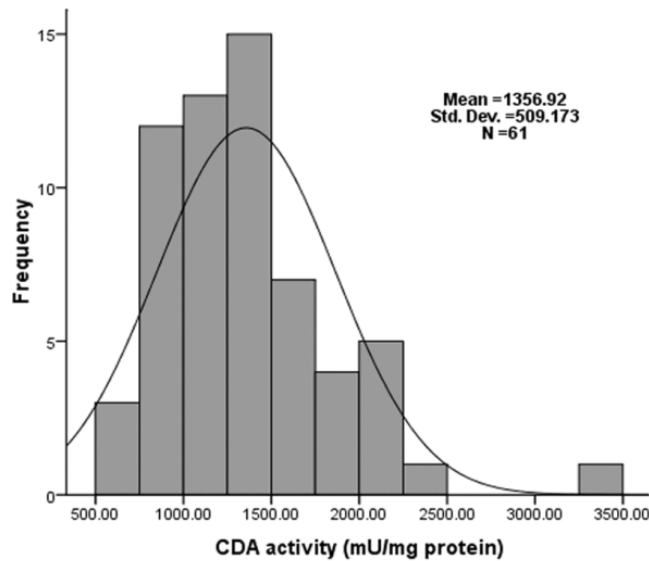
Figure 1. Distributionof CDA activity of healthy population in northeast Thailand.
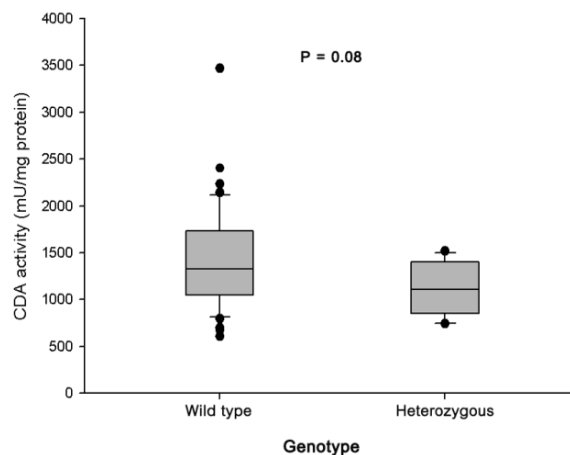
Figure 2. Correlation between *CDA* genotype and CDA activity of healthy populations in northeast Thailand

Discussion

The response rate and toxicity of gemcitabine are related to its metabolism. CDA is a cytoplasmic enzyme with an important role in gemcitabine metabolism. Variations of CDA activity affect gemcitabine response and toxicity.

Many studies reported *CDA*2* and *CDA*3* mutant allelesto be associated with CDA activity and clinical response of gemcitabine.[1,3] However the study on the association between *CDA* polymorphism and CDA activity, and the prevalence of *CDA* mutant alleleshad been limited in Thailand.

Our study is the first report on the prevalence of *CDA*2* and *CDA*3 mutant alleles in Thailand. We found the allele frequency of *CDA*2* in Thai population is significantly different from American ($p = 0.002$) and European populations ($p = 0.001$), but it isnot different from Chinese ($p = 0.828$), Japanese ($p = 0.114$), Korean ($p = 0.490$) and African ($p = 0.061$) populations. *CDA*2/*2* was not found in this study, but it was observed in 3.4% of Japanese, and 16% of Caucasian populations. Moreover, this study did not find the *CDA*3* mutant allele.

Previous studies reported patients who carried *CDA*2* have higher enzyme activity than patients with wild type CDA.[1] However, this study did not show a statistically significant difference between CDA activity and *CDA*2* genotype ($p = 0.08$). An increased sample size may be needed to further investigate the potential correlation.

Conclusion

*CDA*1/*2* genotype was observed in 22.95% of the Northeast Thai healthy volunteers, but the *CDA*2/*2* and *CDA*3* mutant alleles were not found in this study. Allele frequency of *CDA*2* in the Thai population is similar to the other Asian populations, but is different from Caucasian populations. Although a previous study showed patients who carried *CDA*2* have higher activity than those with wild type[1], our study showed no statistically significant correlation, possibly due to the small sample size effect.

Acknowledgements

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| F09 | **A Novel ROS-Suppressive Role of a Cell Cycle Protein, Cyclin D1 in Breast Cancer Cells** |

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Abstract

Cyclin D1 is an important regulator of cell cycle progression that drives cell cycle from G1 to S phase. Overexpression of cyclin D1 have been linked to the development and progression of various cancers, including breast, lung, melanoma, mantle cell lymphoma, and oral squamous cell carcinoma. However, the precise role of cyclin D1 in cancer formation is still unclear. Here, we demonstrated that cyclin D1 depletion by short hairpin RNA (shRNA) resulted in cellular senescence in a breast cancer, which associated with decreased cell proliferation, changed cell morphology; their volume increased and they lose their original shape by acquiring a flattened cytoplasm. This was confirmed by the senescence-associated β-galactosidase (SA β-gal) assay and increasing in expression of senescence markers, such as p16, p21. We found accumulated level of endogenous reactive oxygen species (ROS), and attenuated response to exogenous ROS in the cyclin D1-depleted breast cancer cells. Pretreatment of cyclin D1-depleted cancer cells with an antioxidant N-acetylcysteine (NAC) reduced the number SA β-gal positive cells significantly. Therefore, cyclin D1 maybe required to maintain low non-toxic level of ROS in the cancer cells, and to prevent the cancer senescence. In addition, we found that suppression of ROS by cyclin D1 is independent of the retinoblastoma protein (Rb) inactivating function of CDK4-cyclin D1. These findings suggested a potential function of cyclin D1 as a regulator of intracellular oxidative stress, which allows cancer cells to grow under the ROS stress, and that cyclin D1 may be a strong candidate for cancer therapy.

Keywords: cyclin D1, oxidative stress, senescence, breast cancer

การศึกษาหน้าที่ใหม่ของโปรตีนไซคลิน ดี1 ในการลดระดับสารอนุมูลอิสระในเซลล์มะเร็งเต้านม

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บทคัดย่อ

โปรตีนไซคลิน ดี1 (cyclin D1) เป็น โปรตีนที่สำคัญตัวหนึ่งในการควบคุม วงจรของเซลล์ (cell cycle regulator) มีหน้าที่ในการ ขับเคลื่อนเซลล์จากระยะ G1 ให้เข้าสู่ระยะ S นอกจากนี้ยังพบว่าในเซลล์มะเร็งบางชนิดเช่น มะเร็งเต้านม มะเร็งปอด มะเร็งผิวหนัง (melanoma) มะเร็งต่อมน้ำเหลือง (mantle cell lymphoma) และมะเร็งช่องปาก ที่ปริมาณของโปรตีน ไซคลิน ดี1 เพิ่มมากขึ้นกว่าปกติซึ่งมีความสัมพันธ์กับการเกิดและการดำเนินไปของมะเร็งดังกล่าว แต่อย่างไรก็ตามหน้าที่ที่ แท้จริง ที่ไซ-คลิน ดี1 ใช้ในการก่อมะเร็งยังไม่เป็นที่ทราบแน่ชัด จากผลการทดลองพบว่า เมื่อกำจัดไซคลิน ดี1 ออกไปจาก เซลล์มะเร็งเต้านมโดยใช้วิธี อาเอ็นเอสายสั้น (short hairpin RNA; shRNA) พบว่าสามารถลดการเจริญเติบโต ของเซลล์ได้ และที่สำคัญทั้งยังทำให้เซลล์มีรูปร่างที่เปลี่ยนไป เช่น ไซโตพลาสซึมที่แผ่ขยายใหญ่ขึ้น ต่างจากเซลล์เริ่มต้นอย่างชัดเจน บ่งชี้

ถึงภาวะเซลล์ชราภาพหรือ cellular senescence ซึ่งสามารถยืนยันได้ด้วยการทดสอบ senescence-associated β-alactosidase (SA β-gal) assay และการแสดงออกที่เพิ่มขึ้นของยีนบางชนิดได้แก่ p16, p21 เซลล์ที่ถูกกำจัดโปรตีนไซคลินดี1 มีการสะสมของสารอนุมูลอิสระ (reactive oxygen species) เพิ่มมากขึ้น และมีความสามารถ ในการต้านสารอนุมูลอิสระที่เติมจากภายนอกได้ลดลง นอกจากนี้ การเติมสารต้านอนุมูลอิสระ N-acetylcysteine (NAC) สามารถลดการเกิดเซลล์ชราภาพที่เกิดจากการกำจัดโปรตีนไซคลิน ดี1 ได้อีกด้วย ดังนั้น โปรตีนไซคลิน ดี1 อาจจะมีหน้าที่ใหม่ ในการควบคุมระดับสารอนุมูลอิสระภายในเซลล์มะเร็งเต้านมให้อยู่ในระดับที่ไม่ก่อให้เกิดอันตรายกับเซลล์ และหน้าที่นี้ไม่เกี่ยวข้องกับการทำงานของเรติโนบลาสโตมาโปรตีน (Rb) ส่งผลให้โปรตีนไซคลิน ดี1 อาจเป็นเป้าหมายที่สำคัญในการรักษาโรคมะเร็งต่อไป

คำสำคัญ: ไซคลิน ดี1, ความเครียดจากสารอนุมูลอิสระ, เซลล์ชราภาพ, มะเร็งเต้านม

Introduction

Breast cancer is the most common cancer among women worldwide, as well as Thai women. About 1 in 8 (12%) women in the US will develop invasive breast cancer during their lifetime.[1] Several targeted therapies have been proposed for treating breast cancer but mostly only acts at surface of the cells. One of the strategies for treating cancer was suggested by Weinstein and Joe, 2008.[2] "Cancer cells contain multiple genetic and epigenetic abnormalities. Despite this complexity, their growth and survival can often be impaired by the inactivation of a single oncogene. This phenomenon, called oncogene addiction". These oncogenes become the primary targets for drug development to block their effect on cancer cells, while not affecting to the normal cells.

Cyclin D1 is a critical regulator of cell cycle progression that drives cell cycle from G1 to S phase. Overexpression of cyclin D1 protein due to amplification or chromosomal rearrangement have been linked to development and progression of various types of cancer including breast, lung, melanoma, mantle cell lymphoma, and oral squamous cell carcinomas.[3] but, the precise role of cyclin D1 in cancer formation remains to be clarified. Although working as an essential cell cycle regulator protein, requirement of cyclin D1 for cell proliferation is cancer-specific, not for the survival of normal cells. Gene targeting cyclin D1 in mice resulted in normal development and life span.[4]

Several lines of evidence suggest other CDK-independent roles of cyclin D1 in DNA repair[5,6], estrogen receptor activation[7,8], inhibition of histone acetyltransferase activity[9], etc. These evidences indicate that cyclin D1 may have other roles besides cell cycle control. Moreover, recent studies have shown that depletion of cyclin D1 caused cells to become larger in size, diminish cell growth, arrest cells in G1 and, most remarkable, loss of tumorigenicity.[10,11]

Cellular senescence was first described more than fifty years ago by Leonard Hayflick and Paul Moorhead as a state of "permanent cell cycle arrest" resulting from the limited replicative capacity of normal human diploid fibroblasts (HDFs) in culture.[12] now refer to "replicative senescence". While entering to the senescence state, cells show dramatically change in their morphologies; the cells increase the volume and lose original shape, acquiring a flattened or elongated cytoplasm. This alteration follows by changes in nuclear structure, gene expression, protein processing, and metabolism.[13] Although senescent cells are not dividing, they differ from cell cycle arresting cells in many ways. The most important different is that they are no longer capable of returning to cell cycle. They also contain several unique markers, including the presence of senescence-associated β-galactosidase (SA-β-gal) activity, expression of tumor suppressors and cell cycle inhibitors, and often also of DNA damage markers, nuclear foci of constitutive heterochromatin and prominent secretion of signaling molecules.[14]

Senescence has been shown to play role in tumor suppression that restricts the progression of benign tumors to become malignant. Expression of oncogenic Ras[G12V] in normal cells induces senescent phenotypes, which are almost indistinguishable from replicative senescence. It was termed "oncogene-induced senescence; OIS".[15] Besides the oncogene RAS, agents causing DNA damage, oxidative stress, chemotherapeutic drugs, or even the process of reprogramming to induced pluripotent stem cells can also trigger premature senescence or stress-induced senescence.[16,17] Since senescence can forms a universal block to tumorigenesis which impacts on all hallmarks of cancer, making it an attractive target for drug discovery. In addition to apoptosis, nowadays therapy-mediated premature senescence has also been identified as a desirable outcome of cancer therapy.[15,18]

We found that cyclin D1 depletion uniquely resulted in breast cancer senescence. In this study we investigate the novel anti-ROS function of cyclin D1 in the cancer that may prevent cancer senescence. Understanding this pathway may bring about the proper way to block cancer growth using cyclin D1 as a therapeutic target.

Materials and Methods

Cell culture and chemical reagent

The human breast carcinoma MCF7 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). Cell were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin/streptomycin and maintained at 37°C in a humidified incubator with 10% CO_2. All shRNA expressing cells were maintained in selection medium with one of these antibiotics, Puromycin (Calbiochem, Merck millipore, USA), Geneticin; G418 (Gibco, Life technologies, USA), Hygromycin (Invitrogen, CA, USA). An antioxidant N-acetyl cysteine; NAC was from Amresco, OH, USA.

Gene silencing with shRNAs

For gene silencing, cells were transfected with short hairpin RNA (shRNA). Lentiviral plasmids containing shRNA pLKO.1 (Mission shRNA, Sigma) of cyclin D1 and Rb (Rb139) and pBabe-hygro-E7 (Addgene) were transfected into HEK293 cells using Lipofectamine® LTX and Plus reagent (Invitrogen, CA, USA). Transfected cells were cultured in DMEM containing 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin for 48 hours. To infect MCF7 cells, cells were plated in approximately 80% confluency in a 10 cm plate, transduceded with the lentivirus, supplimenteded with polybrene for overnight, and selected over 4 days with appropriate drug and changed into fresh complete medium plus selected drug every 3 days .

Cell proliferation assay

Cells were seeded in twenty four-well plates at a density of approximately 10,000 cells/well and counted wells in triplicates. The fold increase was calculated with normalization by the first day cell number.

SA-β-gal assay

Cells were seeded in six-well plates at a density of approximately 100,000 cells/well and performed a staining protocol as described by Dimri et al.[19] For N-acetylcysteine (NAC) pretreatment, NAC was added as indicated concentration during drug selection period after shRNA transduction and changed every 3 days. Plates were incubated overnight at 37 °C in a dry incubator (no CO_2). Cells were then detected for blue staining under a bright-field microscope. The percentage of SA-β-gal-positive cells was calculated by counting the cells in five random fields.

Quantitative real-time PCR (qRT–PCR)

Total RNA was extracted with the RNA extraction Kit (Ambion, life technologies, USA). Complementary DNA generated by Superscript III reverse transcriptase and oligo-dT primers (Invitrogen, CA, USA) was analysed by qRT–PCR (Applied Biosystems StepOnePlus™ Real-Time PCR). Transcript levels were normalized using internal expression of GAPDH gene. For all primers used in this experiment were synthesized by bioDesign, Thailand.

Reactive oxygen species (ROS) measurement

Carboxy-H_2DCFDA dye was used to study the intracellular ROS levels in MCF7 cells. The study was performed in 6-well plates, wherein the cells were seeded at a density of 5×10^5 cells/well. After 24 hours of cell attachment, the cells were washed twice with PBS, carboxy-DCFDA dye in serum free media was added at a concentration of 3 μM in 2ml/well, and the plates were incubated in a 37 °C, 5% CO_2 incubator for 30 minutes. Following incubation, the dye solution was removed; the cells were washed twice with PBS and were treated with or without hydrogen peroxide (H_2O_2) as indicated concentrations and incubated 37 °C, 5% CO_2 incubator for 30 minutes then removed and washed with PBS twice. Collecting cells by trypsinization following add flow buffer (pre-cold PBS with 1% fetal bovine serum). The fluorescence measurement of the oxidized DCFDA dye was measured at the respective excitation and emission wavelengths of 488 nm and 530 nm by flow cytometer. The results were calculated and presented as a mean fluorescent intensity analyzed using FlowJo® 7.6 software.
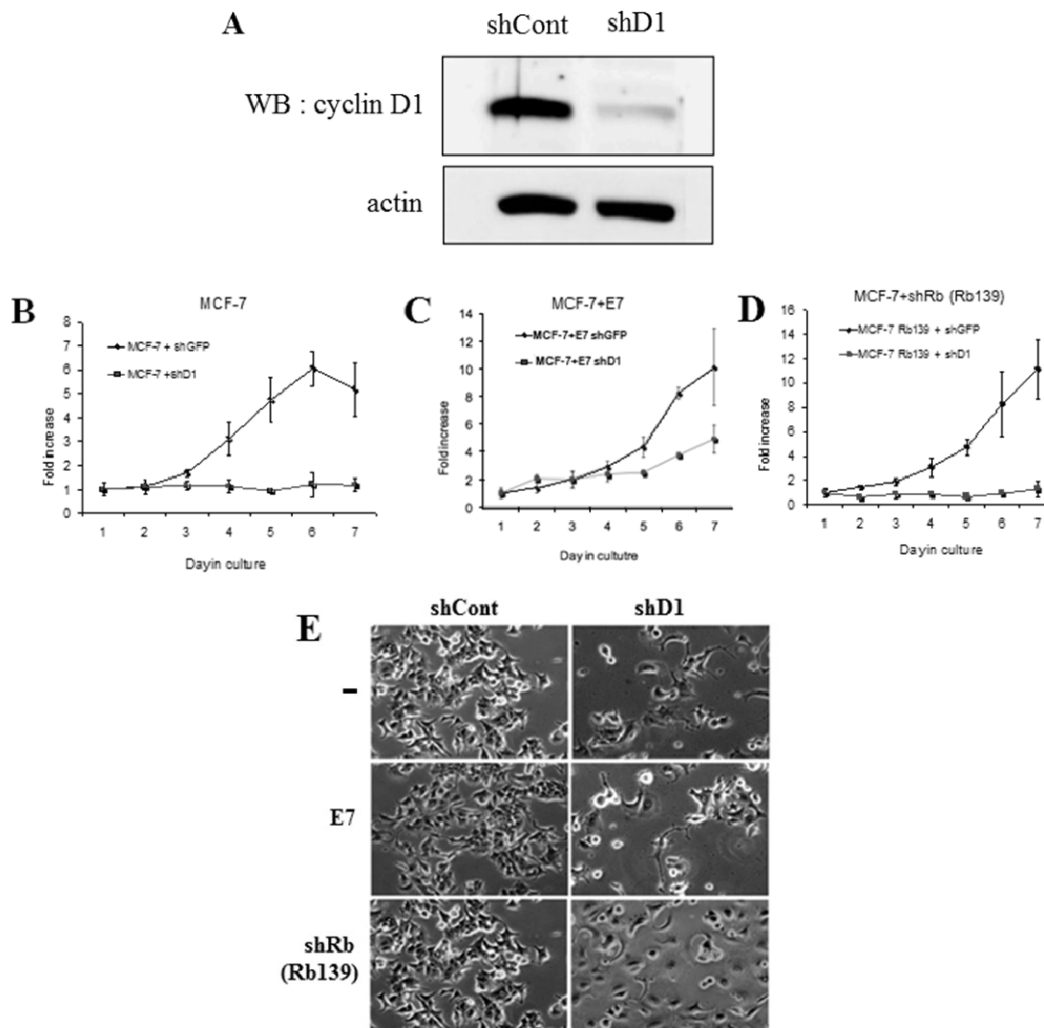
Figure 1. Knockdown of cyclin D1 decreased cell proliferation and introduced senescence-like phenotypes .

A. Western blot analysis of cyclin D1 knockdown, actin as a loading control

B. Growth curves of MCF7 cells expressing either anti-cyclin D1 shRNA (shD1) , or anti-GFP shRNA (shGFP). shGFP was used as a negative control.

C. Growth curves of MCF7 cells expressing either shD1 plus E7, or shGFP plus E7(left)

D. Growth curves of MCF7 cells expressing either shD1 plus anti-pRb shRNA (Rb139) or shGFP plus Rb139 (right) .

E. Cell morphology shown by phase contrast microscopy of MCF7 cells expressing either shD1 or shcont with or without E7, Rb139.
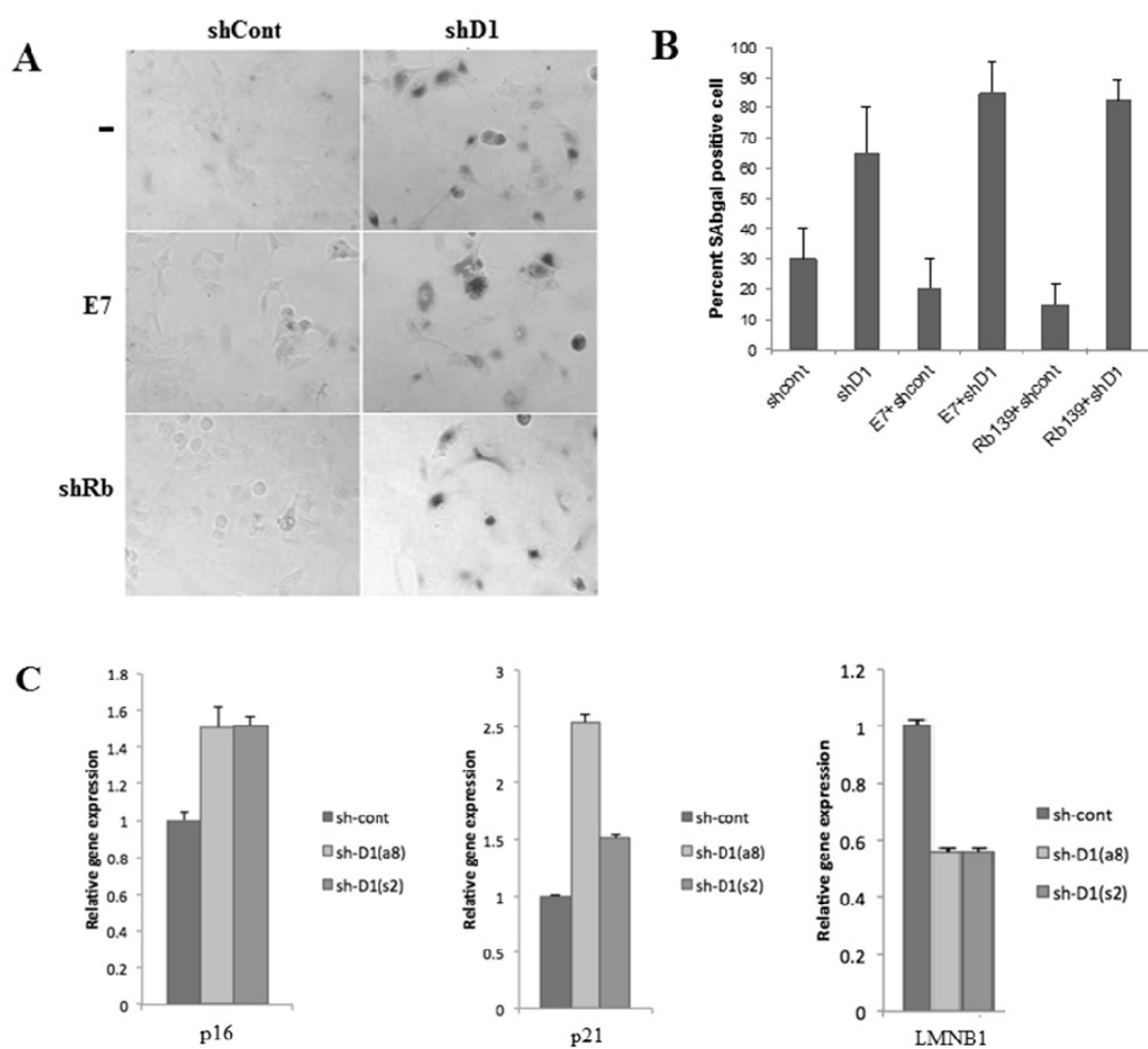
Figure 2. Depletion of cyclin D1 from a breast cancer cell line MCF7 caused cellular senescence confirmed by senescence-associated β-galactosidase (SA β-gal) assay
A. SA-β-gal staining of cyclin D1- depleted and control MCF7 cells with the expression of with or without E7, Rb139
B. The graphs show percentages of SA-β-gal-positive cells by counting the cells in five random fields at least 200 cells. Bars represent average ± S.D.
C. The graphs show relative gene expression as indicated (p16, p21 and LMNB1) measured by quantitative real-time PCR using GAPDH to normalize transcript level and compare fold change to control group. Bars represent average ± S.D. (n=3).
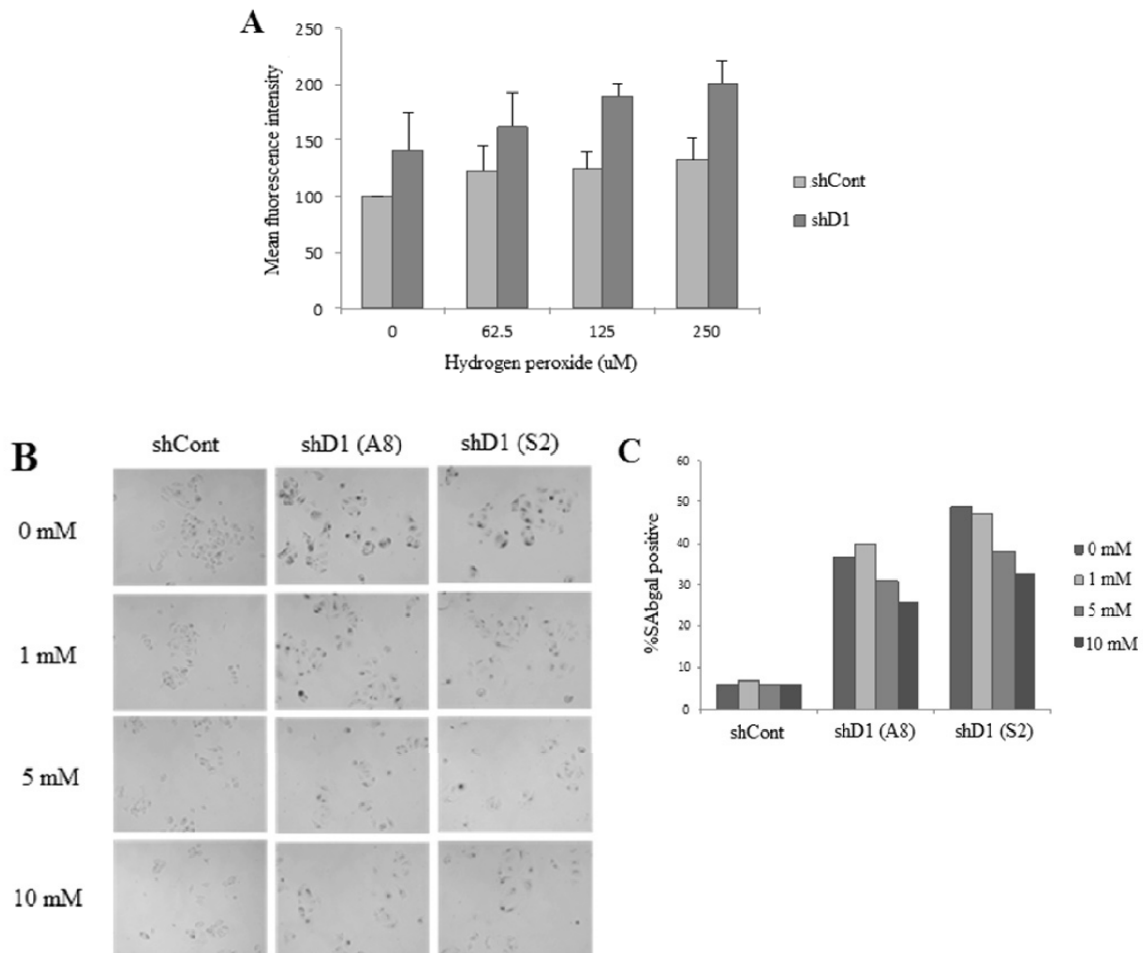
Figure 3. Cyclin D1 is required for suppression of both endogenous and exogenous reactive oxygen species (ROS).

A. ROS levels were measured by flowtometry as mean fluorescence intensity of Dichlorofluorescein diacetate (DCFDA) with or without adding hydrogen peroxide (H_2O_2) in the indicated concentrations (62.5, 125, 250 µM).

B. SA-β-gal staining of cyclin D1- depleted by two different sequences of shRNA (A8, S2) and control MCF7 cells with or without pretreatment of *N*-acetylcysteine (NAC) as an antioxidant in the indicated concentrations (1, 5, 10 mM).

C. The graphs show percentages of SA-β-gal-positive cells quantified by counting the cells in five random fields at least 200 cells.

Results

Cyclin D1 depletion decreased cell proliferation and changed cellular morphology independent from the retinoblastoma protein (pRB)

We use the stable short hairpin RNA expression to create a stable depletion of cyclin D1 in a breast cancer cell line MCF7 resulted over 80% of cyclin D1 were depleted (Figure 1A). As a critical cell cycle regulatory protein, knockdown of cyclin D1 decreased cell proliferation ability (Figure 1B). Interestingly, the depletion caused increased cell size (Figure 1E). When analyzed, cyclin D1-depleted cells

also lost their original shape by acquiring a flattened cytoplasm and changed in nuclear structure, which indicated the stage of cellular senescence (Figure 1E upper panel).

We examined if the inhibition of cell division caused by cyclin D1 knockdown mediated by the retinoblastoma protein (pRB), a cell cycle repressor and a direct-target of cyclin D1/CDK4, by inactivating pRB using either the papilloma viral protein (E7) expression or a pRB-specific short hairpin RNA which can deplete over 80% of Rb protein (data not shown). Unexpectedly, E7 or pRB-specific shRNA slightly increased cyclin D1-depleted cell division, but did not abolish the cell cycle arresting phenotype of cyclin D1 depletion (Figure 1C, 1D), and did not rescue the

cyclin D1-depleted cell morphologies (Figure 1E middle and lower panel).

Cyclin D1 depletion caused cellular senescence in MCF7 cells

We stained MCF7 cells using senescence-associated β-galactosidase (SA β-gal) assay as described by Dimri, *et al*. 1995.[19] The results showed that knockdown of cyclin D1 in MCF7, resulted approximately 60% of cells become senescent (Figure 2A upper panel, B). Interestingly, the phenomenon was also clearly detected in Rb-inactivated cells (Figure 2A middle and lower panel, B). Another important marker used to identify cellular senescence is senescence-related genes expressions. Various studies have shown upregulations of these genes in senescent cells, and generally accepted as markers for cellular senescence. These genes include p16, p21.[14] In addition to these, some genes were reported to reduce the expression in senescent cells, such as LMNB1 (lamin B1). Two sequences of cyclin D1 shRNA (S2, A8) were used to knockdown cyclin D1 from MCF7 and expressions of the senescence-related genes were analyzed. We found upregulations of p16, p21 and downregulation of LMNB1 (Figure 2C), indicating that knockdown of cyclin D1 caused cellular senescence in MCF7 cells.

Cyclin D1 depletion resulted in ROS-mediated senescence

Elevated level of ROS can cause cellular senescence.[20,21,22] We investigated whether ROS was the cause of senescence observed in cyclin D1-depleted cells by measuring intracellular ROS levels. We found that in cyclin D1-knockdown cells higher levels of endogenous ROS was detected when compared to control cells. In addition, the difference became more pronounce, when exogenous ROS (hydrogen peroxide; H_2O_2) were added in the dose dependent manner (Figure 3A). To investigate the connection between ROS upregulation and cellular senescence found in cyclin D1-depleted cancer cells, we treated cyclin D1-depleted MCF7 with an antioxidant (*N*-acetylcysteine; NAC) and measured senescence by SA-β-gal assay. We found that the senescence marker was significantly downregulated in the dose dependent manner (Figure 3B, C).

These results indicated that elevated level of ROS is causal for the cellular senescence observed in the cyclin D1-depleted MCF7, and that cyclin D1 expression is required for the suppression of intracellular senescence.

Discussion

Here, we have gather some evidences that shed the light on cyclin D1 as an oncogene that the breast cancer cells addicted to. Cyclin D1 depletion by short hairpin RNA (shRNA) decreased cell proliferation ability, and triggered cellular senescence. There are many factors that lead to irreversible proliferative arrest.[16] All of these stimuli converge on the accumulation of reactive oxygen species. We next found higher levels of endogenous ROS in cyclin D1-depleted cells. Furthermore, the cells are defective in response to the exogenous ROS. Elevated levels of ROS found in cyclin D1-depleted cells are the mediator for senescence, since treatment of cyclin D1-depleted cancer cells with an antioxidant (NAC) significantly prevent cellular senescence. Therefore, cyclin D1 may be required to maintain low ROS level, at which is not harmful for the cancer cells. The suppression of ROS by cyclin D1 did not require retinoblastoma protein (Rb), since pRB-specific shRNA did not recue the senescence caused by cyclin D1 depletion. The intracellular ROS level can be balanced by controlling both production and eliminate the excessive amount of ROS which disturbance by the absence of cyclin D1 might be involved in either ways. However, the underlying mechanism(s) of how cyclin D1 suppress ROS need to be further explored. These findings suggested a potential novel function of cyclin D1 as a regulator of intracellular oxidative stress, which allows cancer cells to grow under the ROS stress, and that cyclin D1 may be a strong candidate for cancer therapy.

Conclusion

Cyclin D1 may have a novel anti-senescence role in cancer cells by inhibiting endogenous level of ROS and this role is Rb-independent.

Acknowledgements

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F10 Synergistic Anticancer Effect of Rhinacanthin-C and Doxorubicin in Human Breast Cancer Cell Lines

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Abstract

Rhinacanthin-C (RN-C) is a major bioactive naphthoquinone ester found in *Rhinacanthus nasutus* Kurz (Acanthaceae). This compound has potential therapeutic value as an anticancer and antiviral agent. The purpose of this study was to determine the capability of RN-C to enhance cytotoxicity of doxorubicin (DOX) in a breast cancer cell line MCF-7. The cytotoxicity was assessed by an MTT assay. The growth inhibition IC_{50} values of DOX and RN-C were 1.36 ± 0.02 and 8.57 ± 0.15 µM, respectively. RN-C at the non-cytotoxic concentration (0.1 µM) was able to enhance DOX-mediated cytotoxicity. The apparent IC_{50} of DOX in the presence of RN-C decreased by 1.4-fold. The interaction between RN-C and DOX was synergism with the combination index (CI) value of 0.71. Furthermore, the interference of RN-C on the ABC drug transporters (P-gp, MRP1 and MRP2) was evaluated by a substrate accumulation assay, using fluorescence spectroscopy technique. We found that RN-C at 0.1 µM after 12-hr treatment could increase intracellular accumulation of transporter substrate [i.e., calcein by 1.45 fold (P-gp), DCDF by 1.37 fold (MRP1) and CDCF by 1.84 fold (MRP2)]. Our findings suggested that RN-C potentiated doxorubicin-mediated cytotoxicity in breast cancer cells possibly through interference on drug efflux pumps. The interaction between these two compounds was synergism.

Keywords: breast cancer cell, cytotoxicity, doxorubicin, rhinacanthin-C, drug transporters

ผลการเสริมฤทธิ์ของไรนาแคนทิน-ซี และ ด๊อกโซรูบิซินในเซลล์มะเร็งเต้านม

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บทคัดย่อ

ไรนาแคนทิน-ซี (RN-C) เป็นสารในกลุ่ม Napththoquinone ester ที่ได้จากต้นทองพันชั่ง โดยสารนี้มีฤทธิ์ทางเภสัชวิทยาหลากหลายรวมถึงฤทธิ์ต้านมะเร็งและต้านไวรัส การศึกษานี้มีวัตถุประสงค์เพื่อตรวจวัดความสามารถของไรนาแคนทิน-ซี ในการเสริมความเป็นพิษต่อเซลล์มะเร็งเต้านมของด๊อกโซรูบิซิน ทั้งนี้การศึกษาความเป็นพิษต่อเซลล์ทำด้วยวิธี MTT พบว่าด๊อกโซรูบิซินและไรนาแคนทิน-ซี สามารถยับยั้งการเจริญของเซลล์โดยมีค่า IC_{50} ที่ 1.36 ± 0.02 และ 8.57 ± 0.15 ไมโครโมลาร์ ตามลำดับ สำหรับไรนาแคนทิน-ซีในความเข้มข้นที่ไม่เป็นพิษต่อเซลล์ (0.1 ไมโครโมลาร์) สามารถเพิ่มความเป็นพิษของด๊อกโซรูบิซินได้ โดยค่า IC_{50} ของด๊อกโซรูบิซิน ลดลง 1.4 เท่าเมื่อให้ร่วมกับไรนาแคนทิน-ซี มีทั้งนี้การออกฤทธิ์ร่วมกันของสารทั้งชนิดมีลักษณะเป็นแบบ Synergism ซึ่งมีค่า Combination index อยู่ที่ 0.71 ในส่วนการศึกษาผลของไรนาแคนทิน-ซี ต่อตัวขนส่งโปรตีน (P-gp, MRP1 และ MRP2) ซึ่งประเมินได้โดยวิธี วัดการสะสมของสับสเตรทภายในเซลล์ ด้วย

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เทคนิค fluorescence spectroscopy พบว่าหลังจากให้สารไปเป็นเวลา 12 ชั่วโมง ที่ความเข้มข้น 0.1 ไมโครโมลาร์ สามารถเพิ่มการสะสมของสับเสตรทภายในเซลล์ได้ ดังนี้ Calcein เพิ่มขึ้น 1.45 เท่า (P-gp), DCDF เพิ่มขึ้น 1.37 เท่า (MRP1) และ CDCF เพิ่มขึ้น 1.84 เท่า (MRP2). ดังนั้นจึงสรุปได้ว่าไรนาแคนทิน-ซี มีผลเสริมความเป็นพิษของด๊อกโซรูบิซิน โดยอาจออกฤทธิ์ผ่านการยับยั้งการทำงานของโปรตีนที่ขับยาออกในเซลล์มะเร็งเต้านม

คำสำคัญ: เซลล์มะเร็งเต้านม, ความเป็นพิษต่อเซลล์, ด๊อกโซรูบิซิน, ไรนาแคนทิน-ซี, โปรตีนขนส่งยา

Introduction

Breast cancer is the leading cause of death in women worldwide. In 2014, more than 83% of new cancer cases were breast cancer.[1] Doxorubicin (DOX) is one of the most effective cytotoxic drugs in breast cancer chemotherapy. Its adverse effects include myelosuppression and cardiotoxicity.[2] Because the adverse effects are concentration-dependent, the less DOX concentrations produce the less severity of adverse events and accommodate the more patient's tolerability.[2] The combination between a cytotoxic anticancer drug and a non-cytotoxic agent is one approach to enhance drug efficacy without titrating up the concentration. In this regard, a non-cytotoxic agent may be able to potentiate the cytotoxic effect of anticancer drug through several mechanisms including inhibition of drug efflux pumps.[9] Moreover, it was well established that multidrug resistant (MDR) cancer cells were often associated with high level of transporter proteins such as P-glycoprotein (P-gp) and multidrug resistance associated protein (MRP).[10] Recently, we found that rhinacanthin-C (RN-C), a major naphthoquinone ester from the root of *Rhinacanthus nasutus* Kurz. (Acanthacae), could inhibit the function of P-gp transporter in the Caco-2 cells[4]. This compound has various pharmacological activities including antiviral, antimicrobial, anti-proliferative and anti-cancer.[5] It could be hypothesized that RN-C at the non-cytotoxic concentration was able to potentiate the cytotoxic effect of doxorubicin in MCF-7 breast cancer cell. The type of interaction between RN-C and doxorubicin was also determined.

Materials and Methods

Plant materials

Rhinacanthin-C (RN-C; Figure 1) was obtained from the roots of *R. nasutus* Kurz, using the purification and identification processes as previously described[4.] Doxorubicin (DOX) was purchased from Merck Calbiochem (KGaA, Darmstadt, Germany). RN-C and DOX were dissolved in 99.9% DMSO and stored at −20 °C until use. The final concentration of DMSO in each experiment was less than 0.1% (v/v).

At this concentration, DMSO had no cytotoxic effect on MCF-7 cells.

Cell cultures

The human breast cancer MCF-7 (ATCC® HTB-22™) was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin mixture at 37 °C in a humidified atmosphere of 5% CO_2.

Cell viability assay

The cytotoxic effects of RN-C and DOX on MCF-7 cells were determined by an MTT assay.[4] Briefly, the cells were seeded onto 96-well plates at the density of 5×10^3 cell per well and cultured overnight. Then, the cells were treated with either RN-C (0-10 μM) or DOX (0-2 μM) for 24 hours. At the end of the treatment peroid, the cells were washed and further incubated in serum-free medium containing MTT reagent (0.83 mg/ml) for another 4 hours at 37 °C. The intracellular formazan crystals were dissolved with DMSO and measured spectrophotometrically at 570 nm with a microplate reader (Wallac 1420 VICTOR 3 PerkinElmer Inc., USA)

The combinatorial effects of RN-C and DOX on cell viability were also assessed. The cells were treated with DOX (0-2 μM) in the presence of RN-C at non-cytotoxic concentration for 24-hr. The cell viability was determined by an MTT assay, as described above.

Combination index analysis

Half maximal inhibitory concentration (IC50) values were estimated and used for combination index (CI) analysis according to the following equation:

$$CI = \frac{C50\ (DOX)}{IC50\ (DOX)} + \frac{C50\ (RN-C)}{IC50\ (RN-C)}$$

Where C50 (DOX) and C50 (RN-C) were the concentrations in a combination regimen that generated 50% cytotoxicity. IC_{50} (DOX) and IC_{50} (RN-C) were the concentrations that produce 50% cytotoxicity when the compound was used alone. The CI values suggested as following; antagonism when CI > 1, additive interaction when CI = 1, and synergism when CI < 0.1.[6]
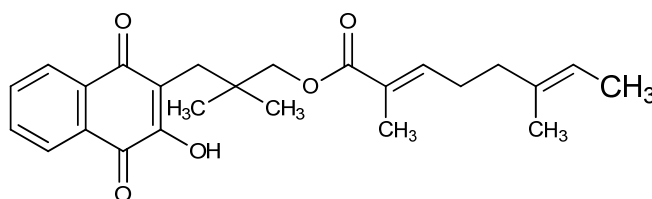
Figure 1. Chemical structure of rhinacanthin-C (RN-C).

Uptake assays for transporter function

The function of P-gp, MRP1 and MRP2 transporters were determined by substrate accumulation assay as described previously.[7] The MCF-7 cells were seeded onto 96-well plates at the density of 2.2×10^5 cell per well. On day 3 after seeding, the cells were washed and treated with inhibitor for 30 min at 37 °C. Then, a specific substrate of each transporter was added and further incubated for another 30 min. In this study, cyclosporine A (CsA; 50 µM) and indomethacin (Indo; 500 µM) were used as positive inhibitors. The selected substrates for evaluation of transporter activity included P-gp substrate calcein-AM (0.4 µM), MRP1 substrate DCDF (5.2 µM) and MRP2 substrate CDCFDA (5 µM). Furthermore, the inhibitory effect of RN-C on substrate accumulation was also determined at the 12 hr-incubation period in the presence of a specific substrate. At the end of incubation period, the cells were washed with ice-cold PBS and lysed with 0.1% Triton X-100. The fluorescence intensity of calcein, DCDF and CDCF was determined with a microplate reader (Wallac 1420 VICTOR 3, PerkinElmer Inc., USA) at excitation/emission wavelengths of 485/535 nm. The amount of proteins in each sample were determined with Bradford reagent® at 595 nm.[4]

Statistical analysis

Data were expressed as mean ± S.E.M. Statistical analysis was performed by one-way ANOVA, followed by *post-hoc* Least Significant Different (LSD) test. *P*<0.05 was considered significance.

Results

Effect of RN-C and DOX on cell viability

Both DOX and RN-C caused concentration-dependent cytotoxicity on MCF-7 cell after 24-hr treatment (Figure 2). RN-C at the concentrations up to 1.5 µM were not toxic to the cells after 24-hr exposure. RN-C at the non-cytotoxic concentration (0.1 µM) was able to reduce cell survival rate from DOX exposure (Figure 3). Upon addition of RN-C 0.1 µM, the IC50 of DOX decreased by 1.4-fold with the CI value of 0.71. The IC50 values of each treatment were shown in Table 1.

Effect of RN-C on P-gp, MRP1 and MRP2 function

In this experiment, our MCF-7 cells expressed appreciable levels of P-gp, MRP1 and MRP2 activities. The presence of cyclosporine A (50 µM) and indomethacin (500 µM) increased accumulation of calcein, DCDF, and CDCF by approximately 2.02, 2.16 and 4.01 folds, respectively (Figure 4).

RN-C at the concentration of 0.1 µM was able to increase intracellular accumulation of calcein (by 1.20 fold), DCDF (by 1.37 fold) and CDCF (by 1.84 fold) in MCF-7 cells after 12-hr treatment (Figure 5). Moreover, RN-C caused more CDCF retention within the cells than a known MRP2 inhibitor indomethacin at the equimolar concentration of 0.1 µM did. These data suggested that RN-C at the very low concentration was able to interfere P-gp, MRP1 and MRP2 activities. In addition, RN-C could inhibit MRP2 activity more than P-gp and MRP1 activities.
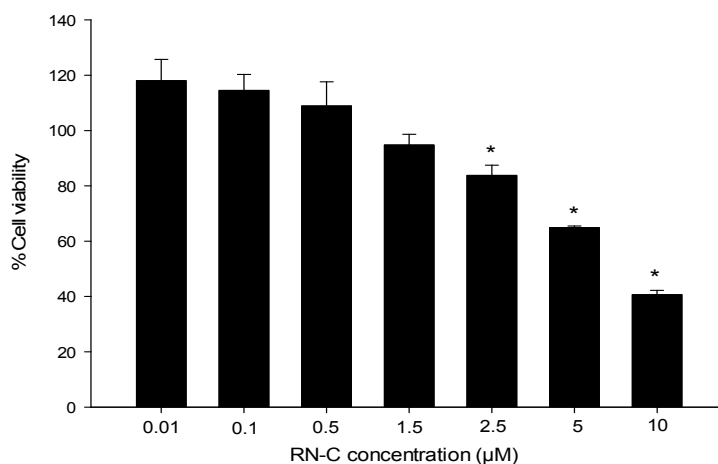
Figure 2.   Effect of RN-C on the MCF-7 viability after 24-hr treatment. Each value represented the mean ± S.E.M. (n=4) *P < 0.05 vs untreated group.
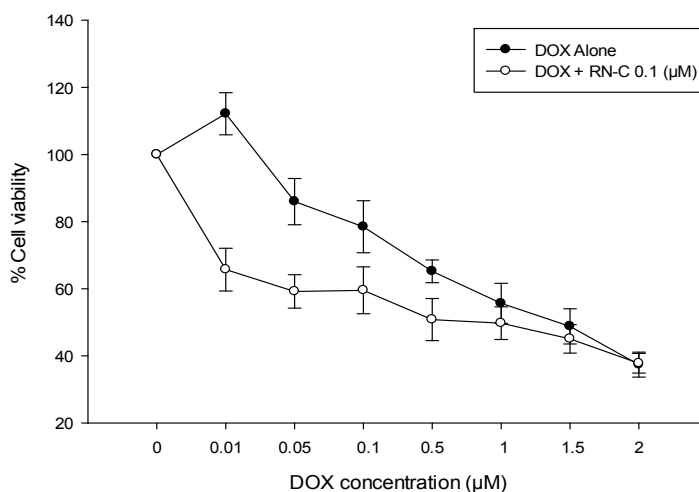
Figure 3.   Effect of RN-C on DOX-mediated cytotoxicity. The cytotoxicity at the 24 hours treatment period was determined in the presence and absence of RN-C 0.1 µM. Each value represented the mean ± S.E.M. (n=4).

Table 1. IC$_{50}$ and combination index (CI) value of doxorubicin (DOX) and rhinacanthin-C (RN-C) in the MCF-7 cells after the treatment period of 24 hours.

24-hr Treatments	IC$_{50}$ (µM)	CI
RN-C	8.57 ± 0.15	-
DOX	1.36 ± 0.02	-
DOX + 0.1 µM RN-C	0.95 ± 0.38	0.71
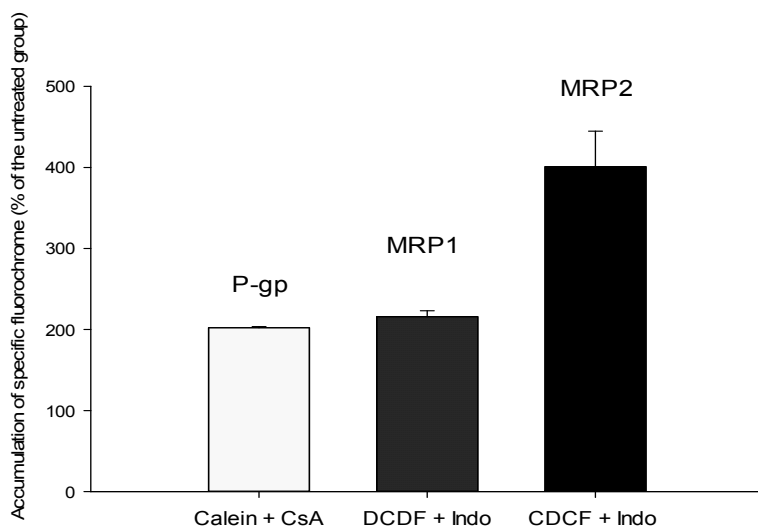
Figure 4. P-gp, MRP1 and MRP2 activities in the MCF-7 cells. Intracellular accumulation of calcein, DCDF and CDCF were determined in the presence and absence of in Cyclosporine A (CsA; 50 μM) and indomethacin (Indo; 500 μM). Data were calculated and expressed as the percentage of the untreated group.
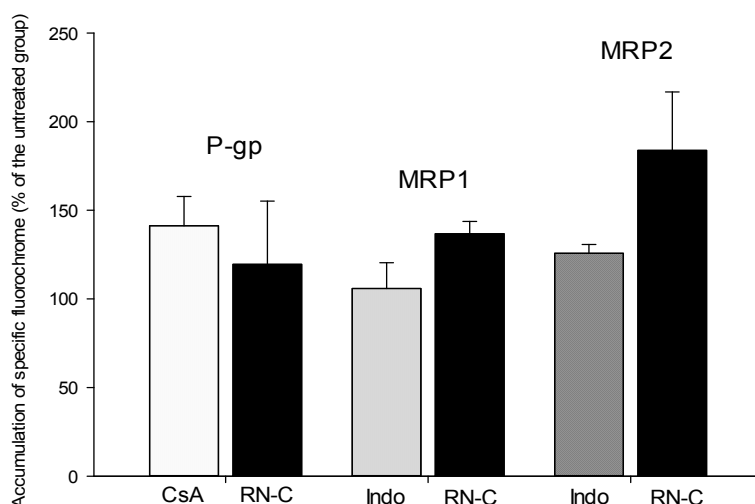
Figure 5. Effect of RN-C on P-gp, MRP1 and MRP2 activities in MCF-7 cells after 12-hr treatment. The cells were treated with test compound [i.e., RN-C (0.1 μM), cyclosporine A (CsA, 0.1 μM), Indomethacin (Indo, 0.1 μM) for 12 hr. At the end of 12-hr treatment period. Intracellular accumulation of calcein, DCDF and CDCF were determined for P-gp, MRP1 and MRP2 respectively. Data were calculated and expressed as the percentage of the untreated group.

Discussion

In this study, we demonstrated that rhinacanthin-C (RN-C), a natural naphthoquinone derivative, was cytotoxic to breast cancer cell after 1-day treatment. Apparently, the potency of RN-C was about 6.3 fold less than doxorubicin. At the non-cytotoxic concentration of 0.1 μM, RN-C could enhance doxorubicin sensitivity in breast cancer cell line. The IC$_{50}$ value of doxorubicin decreased 1.4 fold in the presence of RN-C 0.1 μM. According to combination index (CI) analysis, the interaction

between RN-C and doxorubicin was apparent synergism with the CI values of 0.71.

Inhibition of drug efflux pump is a known mechanism for improving cell sensitivity to cytotoxic drugs in multidrug resistant (MDR) cancer cells.[9] It has been demonstrated that MDR cancer cells have high expression of the ABC transporters in particular P-gp, MRP1 and MRP2.[7,8] The inhibition of these efflux transporters can increase intracellular accumulation of its cytotoxic drug substrate up to the level that can cause cell death. In this study, we demonstrated that RN-C at the low concentration (0.1

μM) was able to increase intracellular levels of transporter substrates in the MCF-7 cells. These data suggested that RN-C at low concentration could disrupt the activities of drug efflux pumps including P-gp, MRP1 and MRP2. Moreover, the inhibitory action of RN-C was more pronounced toward MRP2 function than toward MRP-1 and P-gp functions. As known, DOX is a substrate of drug efflux pumps in the ABC superfamily including P-gp, MRP1 and MRP2.[9] Hence, it was likely that RN-C enhanced doxorubicin-mediated cytotoxicity in the MCF-7 cells through inhibition of these ABC drug efflux pumps. onsequently, intracellular doxorubicin increased up to the cytotoxic level and caused cell death.

Conclusion

Rhinacanthin-C could potentiate doxorubicin-mediated cytotoxicity in breast cancer cells. The interaction between this compound and doxorubicin was synergism. The MDR reversal property of RN-C was possibly related to its inhibitory action against the ABC transporters in particular MRP2.

Acknowledgements

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Effect of Apigenin on Stem Cell Marker Expression in Hypoxic Head and Neck Squamous Cell Carcinoma.

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Abstract

Cancer stem cells play an important role in tumor recurrence. Many studies demonstrated that hypoxic condition increased number of cancer stem cells in several tumors. This study aimed to investigate whether apigenin, a potent chemopreventive agent, could inhibit stem cell marker expression under hypoxic condition in head and neck squamous cell carcinoma.Using semi quantitative RT-PCR, we demonstrated that hypoxic incubation clearly increased mRNA expression of *REX-1*, *NANOG*, *CD44*, *CD105* and *VEGF*.Interestingly, real time RT-PCR showed that apigenin at a concentration of 40 µM significantly inhibited the mRNA expression of these stem cell markers. These data suggest that apigenin may reduce the number of cancer stem cells. Therefore our study supports the future use ofapigenin as the promising anti-cancer agent.

Keywords:head and neck squamous cell carcinoma, hypoxia, apigenin, cancer stem cells

ผลของอะพิเจนินต่อการแสดงออกของยีนที่บ่งชี้ความเป็นเซลล์ต้นกำเนิดในเซลล์มะเร็งศีรษะและลำคอที่ถูกเหนี่ยวนำภายใต้สภาวะพร่องออกซิเจน

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บทคัดย่อ

เซลล์ต้นกำเนิดมะเร็งเป็นหนึ่งในสาเหตุของการกลับมาเป็นซ้ำของรอยโรคมะเร็งการศึกษาก่อนหน้านี้พบว่าสภาวะพร่องออกซิเจนมีผลต่อการเพิ่มขึ้นของเซลล์ต้นกำเนิดมะเร็งในมะเร็งหลายชนิด งานวิจัยชิ้นนี้จึงมีวัตถุประสงค์เพื่อศึกษาผลของอะพิเจนินซึ่งมีฤทธิ์ทางเภสัชวิทยาในการป้องกันการเกิดต่อการแสดงออกของเซลล์ต้นกำเนิดมะเร็งที่ถูกเหนี่ยวนำการแสดงออกภายใต้สภาวะพร่องออกซิเจนในมะเร็งศีรษะและลำคอ ผลการทดสอบโดยใช้เทคนิคพีซีอาร์พบว่าสภาวะพร่องออกซิเจนมีผลเหนี่ยวนำการแสดงออกในระดับเอ็มอาร์เอ็นเอของยีน*REX-1*, *NANOG*, *CD44*, *CD105*และ*VEGF*และจากการศึกษาโดยใช้เทคนิคเรียลไทม์พีซีอาร์พบว่าอะพิเจนินสามารถยับยั้งการแสดงออกของยีนดังกล่าวภายใต้สภาวะพร่องออกซิเจนได้ โดยอะพิเจนินความเข้มข้น 40 µM ให้ผลยับยั้งการแสดงออกได้ดีที่สุด จากผลการศึกษาครั้งนี้แสดงให้เห็นว่าอะพิเจนินน่าจะมีผลในการลดจำนวนของเซลล์ต้นกำเนิดมะเร็งและสามารถนำไปใช้ในการพัฒนาเป็นสารต้านมะเร็งได้ในอนาคต

คำสำคัญ: เซลล์มะเร็งศีรษะและลำคอ, สภาวะพร่องออกซิเจน, อะพิเจนิน, เซลล์ต้นกำเนิดมะเร็ง

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Introduction

Head and neck squamous cell carcinoma is the sixth most common cancer in worldwide. The therapeuticoptions of this tumor involves radiotherapy, surgery, chemotherapy, or concurrent chemo-/radiotherapy.[1] Although the treatments of this disease have been advanced, survival rate of patients in last 30 years remains unchanged.[2] Discovering new therapeutic strategy to improve survival rate of patients is challenging.

Cancer stem cells are the small population of cells that are responsible for tumor recurrence and metastasis.[3] Cancer stem cells have been identified in many solid tumors from various origins, including brain,[4] head and neck,[5] colon,[6] and breast.[7] The characteristics of cancer stem cells contain four key elements-: self-renewal, differentiation capacities, tumorigenicity, and specific cell surface marker expression.[8] The common markers that are used to identify cancer stem cells include*CD44*, *CD133*, *NANOG* and *OCT-4*. However these markers can be found in other cell types, thus not specific to cancer stem cells.[2]Therefore, the researchers try to find the combination of markers in order to specifythe cancer stemcell population.

Hypoxia, a condition of low oxygen levels and poorly vascularized regions, is a common characteristic of solid tumors. It plays an important role in promoting many oncogenic events such as proliferation and angiogenesis.[9] Recent evidence showed that hypoxia enhanced stemness of cancer stem cells. For example hypoxia($0.5\%O_2$) increased the level of both *OCT-4* and *NANOG* expression in prostate cancers.[10] Correspondingly, Zhizhong's study showed that hypoxia increased *OCT-4*expression in hypoxic brain cancers.[11]

Apigenin (4',5,7,-trihydroxyflavone), a phytopolyphenol, is widely studied for its anticancer activity in many types of cancer cells. For example, apigenin is shown to reduce cell proliferation and induce apoptosis in human gastric carcinoma.[12] Moreover it can suppress tumorigenesis and angiogenesis in prostate and lung carcinomas.[13,14] Apigenin inhibited cancer cell proliferation and angiogenesis through suppression of *HIF-1* and vascular endothelial growth factor (*VEGF*).[15,16]In head and neck cancer cells, it has been shown that apigenin exhibited various pharmacological properties, including antioxidant, chemopreventive, and apoptosis.[17] However, the effect of apigeninon cancer stem cell is not known yet. Therefore, in this study, we investigated whether apigenin could reduce cancer stem cell population in head and neck squamous cell carcinoma by determining stem cell marker expression.

Materials and methods

Cell culture and hypoxic treatment: HN-30, head and neck cancer cell lines fromsquamous cell carcinomas of the esophagus were utilized. Cells were culturedin DMEM supplemented with 10% fetal bovine serum (FBS), L-glutamine (100μg/ml) and antibiotics (100 μg/ml), at 37ºC in humidified atmosphere with 5% CO_2. Forhypoxic condition, cells were put into hypoxic chamber (0.5-1% O_2).Oxygen levels wereconstantly monitored by oxygen detector(AsunGadget).

Cytotoxic assay: Cells were seeded into 24well-plate at the density of 20×10^4 cells/well. After reaching 80%confluent, cells were treated with various concentrations of apigenin(Sigma, USA)for 24 and 48 hr. At the end ofthe experiment, the culture medium was replaced with MTT solution(USB, USA). The formazan productwas dissolved with DMSO. The absorbance of formazan dye was measured at 570 nm. Thepercentage of cell survival was calculated by a following formula:

Percentage of cell survival
$$= OD(sample)/OD(negative\ control)\times100\%$$

Semi-quantitative reverse transcription polymerase chain reaction(RT-PCR) and Real-time PCR:Cells were seeded into 6well-plateat the density of 20×10^4cells/well. After reaching 80% confluent,the cells were treated with various concentrations of apigeninand incubated in either normoxic or hypoxic conditions for 6 and 24 hr. At the end of the experiment,total RNA was extracted with TRIzol (Invitrogen).cDNAwas synthesized using Superscript II reverse transcriptase (Invitrogen).ThemRNA expression of cancer stem cell markers was detected by TaqPCR (Sigma). For real-timePCR, SYBR Green PCR Kit (Sigma) was used.

Statistical analysis:Data are presented as means ± SD of three-independent experiments. One wayANOVA with least significant difference (LSD) *post hoc* test was used to determine the statistical significance. The *p*-value less than 0.05 wasconsidered as statistically significance.
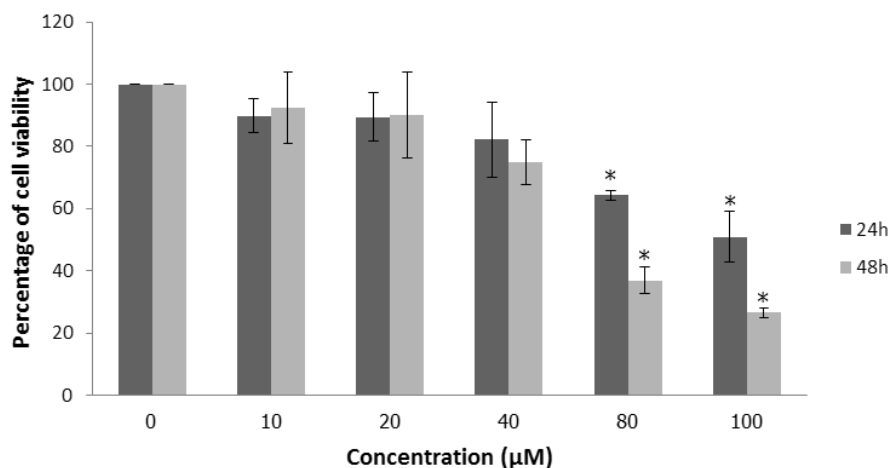
Figure 1. Cytotoxic effect of apigenin.Toxic concentration of apigenin on HN30 cell at 24 hr and 48 hr was studied by MTT assay.*$p < 0.05$ compared with control (0 µM apigenin).
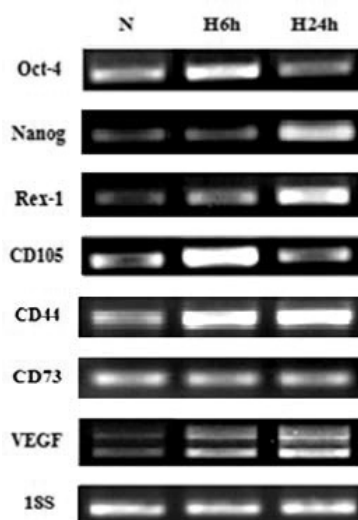

Figure 2 . Effect of hypoxic condition (H) on the level of stem cell marker expression inHN-30 cell at 6 and 24 hr by RT-PCR.

Results

Effect of apigenin on the viability of HN-30 cells

Cells were treatedwith 0, 10, 20, 40, 80 and 100 µM of apigenin for 24 and 48 hr As shown in Figure 1, apigenin significantly decreased cell viability, in a dose- and time-dependent manner. Apigenin at the concentration of 10-40 µM did not significantly affect cell viability. Significant decreased in cell viability was observed when cells were treated with 80-100 µM apigenin. The IC$_{50}$ values of apigenin at 24 hr and 48 hr were 106.95 and 67.96 µM respectively.

Hypoxia induced stem cell marker expression

Next, we determined whether hypoxic condition could induce the expression of cancer stem cell markers in HN-30 cell. After 6 and 24 hr of hypoxic incubation, the mRNA expression of *OCT-4, NANOG, REX-1, CD44, CD105* and *VEGF* was studied by semi-quantitative-PCR. As shown in Figure 2, mRNA expression of *OCT-4* and *CD105* was increased at 6 h of hypoxic condition. The mRNA expression of *NANOG* and*REX-1* was enhanced after 24 hr The mRNA expression of *CD44* and *VEGF* was increasedat both 6 and 24 hr However, *CD73* mRNA expression was not changed during the treatment.

Effect of apigenin on stem cell marker expression under hypoxic condition.

To test the effect of apigenin on the expression of stem cell markers, cells were treated with 20 and 40 µM of apigenin, the concentrations that did not affect cell viability. As the induction of stem cell markers was observed at different time point, the inhibitory effect of apigenin was tested at 6 hr for *OCT-4, CD44* and *CD105* (Figure 3A) and

at 24 h for *REX-1, NANOG* and *CD44* (Figure 3B). Using real-time PCR, we found that, at 6 hr, the mRNA expression of *CD44* and *CD105* significantly reduced when cells were treated with 40 µM apigenin (Figure 3A). At 24 hr, the mRNA expression of *NANOG* and *VEGF* significantly decreased when cells were treated with 20 and 40 µM of apigenin (Figure 3B). However, apigenin was not able to inhibit the expression of *OCT-4* and *REX-1*.

A
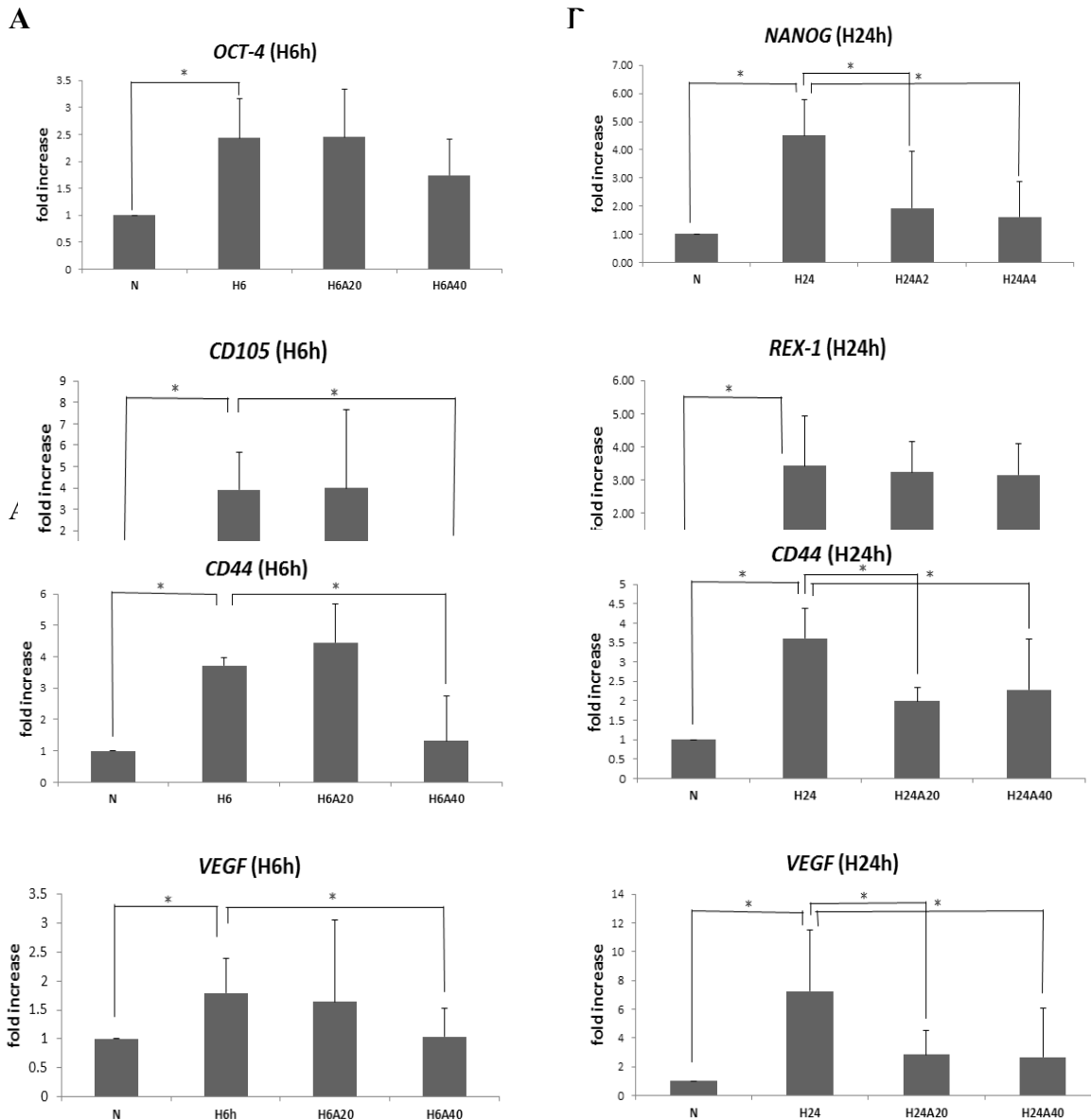
Figure 3. Effect of apigenin on the expression levels of stem cell markersunder hypoxic condition. Cell were treated with 20-40 µM apigenin and incubated at hypoxic condition for 6 (A) and 24 (B) hour. H, Hypoxia ; A, Apigenin.

Discussion and Conclusion

The traditional cancer therapies aim to eliminate as many cancer cell as possible. However, regarding cancer stem cell hypothesis, only small subpopulations of cells, known as cancer stem cells, are critical to the growth and progression of many cancers.[16] Therefore, to gain more effective cancer therapy, it is important to seek for the agent that can eliminate cancer stem cell as well. Apigeninis a phytochemical compound that possess various anticancer activities. However, its effect on cancer stem cellsis poorly understood. In the present study, we found thatapigeninreduced a variety of stem cell marker expression under hypoxic condition.Most importantly, apigenin could reduce the expression ofCD44, the main marker for head and neck cancer stem cellidentification.[18] Moreover,it can reduce the expression of NANOG,the embryonic stem cell marker.[19-21] andCD105, themarker for tumor angiogenesis.[22]These finding imply that apigenin could inhibit self-renewal capacity of cancer stem cell.In the future, the effect of apigenin on the number of cells expressing stem cell markers will be studied using Flow cytometry.

In summary, our present data demonstrated thatapigeninhad anticancer effect on cancer stem cell in HN-30cells. Therefore, apigenin could be a useful therapeutic agent for development of cancer stem cell based therapy. Though, the future experiment is certainly required to prove this hypothesis.

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| F12 | Expression and Roles of xCT and GLUT1 among Colorectal Cancer Cell Lines with Different Genetic Backgrounds |

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Abstract

Colorectal cancer (CRC) is one of the most common diagnosed cancers with high mortality rate world wide due to its resistance to chemotherapeutic drugs. In addition, the carcinogenesis of CRC is complex and associated with multiple genetic alterations. CRC upregulates several nutrient transporters such as glucose transporter 1 (GLUT1) and cystine/glutamate exchanger (xCT) to provide sufficient nutrients for their metabolic requirement and protection against the oxidative stress. However, the expression pattern and the role of nutrient transporters in CRC cell lines with different genetic backgrounds have not been documented. Herein, we examined the expression of xCT and GLUT1 in HT29, HCT116, SW480 cell lines. HT29 expressed the highest level of xCT, whereas GLUT1 was detected in SW480 >> HT29 > HCT116 cell lines. Inhibition of xCT and GLUT1 by a specific inhibitor, had no dramatic effect on CRC cell viability. However, the sensitivity of SW480 but not HT29 to 5-FU was enhanced by co-treatment with an xCT inhibitor, sulfasalazine. Interestingly, combination treatment between sulfasalazine and WZB117, a GLUT1 inhibitor, markedly reduced SW480 cell viability induced by 5-FU. These results indicate that the expression pattern of xCT and GLUT1 transporters might depended on the differences in genetic alteration in CRC cells. Inhibition of these transporters may enhance the sensitivity of specific CRC cells to chemotherapeutic drugs. The results further support the concept of personalized medicine, in which the genetic background information should be taken into account for medical decision.

Keywords: colorectal cancer, xCT, GLUT1, drug resistance, personalized medicine

การแสดงออกและบทบาทของ xCT และ GLUT1 ในเซลล์มะเร็งลำไส้ใหญ่ที่มีรหัสทางพันธุกรรมที่แตกต่างกัน

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บทคัดย่อ

โรคมะเร็งลำไส้ใหญ่ เป็นโรคมะเร็งที่มีอุบัติการณ์และมีอัตราการเสียชีวิตสูงอยู่ในอันดับต้นๆ ของโลกรวมทั้งประเทศไทย สาเหตุเนื่องจากการดื้อยา การเกิดมะเร็งลำไส้ใหญ่มีความซับซ้อนและมีความสัมพันธ์กับการเปลี่ยนแปลงของรหัสทางพันธุกรรมหลายชนิด เซลล์มะเร็งลำไส้ใหญ่มีการแสดงออกของตัวขนส่งสารอาหารหลายชนิดเพิ่มขึ้น เช่น ตัวขนส่งน้ำตาล 1 (glucose transporter 1; GLUT1) และ ตัวแลกเปลี่ยน ซิสทีน/กลูตาเมท (cysteine/glutamate exchanger; xCT) เพื่อให้

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เพียงพอต่อความต้องการพลังงานและต่อสู้กับภาวะเครียดออกซิเดชั่นของเซลล์มะเร็ง อย่างไรก็ตามในปัจจุบันยังไม่มี การศึกษารูปแบบการแสดงออก และบทบาทหน้าที่ของตัวขนส่งสารอาหารในเซลล์มะเร็งลำไส้ที่มีรหัสทางพันธุกรรมที่แตกต่าง กัน ดังนั้นในการวิจัยครั้งนี้จึงได้ทำการศึกษาการแสดงออกของตัวขนส่ง GLUT1 และ xCT ในเซลล์มะเร็งลำไส้ใหญ่ 3 ชนิดคือ HT29 HCT116 และ SW480 ซึ่งพบว่า เซลล์ HT29 มีการแสดงออกของ xCT มากที่สุด ในขณะที่ GLUT1 มีการแสดงออก มากในเซลล์ SW480 HT29 และ HCT116 ตามลำดับ การยับยั้งการทำงานของ xCT และ GLUT1 ด้วยสารยับยั้งที่มี ความจำเพาะต่อตัวขนส่งสารอาหารนี้ไม่มีผลยับยั้งการเจริญเติบโตของเซลล์มะเร็งลำไส้ อย่างไรก็ตาม เซลล์มะเร็งชนิด SW480 มีความไวต่อยาต้านมะเร็ง 5-FU เพิ่มขึ้นเมื่อให้ร่วมกับ sulfasalazine ซึ่งเป็นสารยับยั้งตัวขนส่ง xCT และที่สำคัญ เมื่อ บ่มเซลล์ในสาร sulfasalazine ร่วมกับสาร WZB117 ซึ่งเป็นตัวยับยั้งตัวขนส่ง GLUT1 สามารถลดการเจริญเติบโตของเซลล์ SW480 จากฤทธิ์ของยา 5-FU เพิ่มขึ้น การศึกษานี้แสดงให้เห็นว่ารูปแบบการแสดงออกของตัวขนส่งสารอาหาร xCT และ GLUT1 อาจเกี่ยวข้องกับความแตกต่างทางพันธุกรรมในเซลล์มะเร็งลำไส้ใหญ่ การยับยั้งการทำงานของตัวขนส่งเหล่านี้อาจ ช่วยเพิ่มความไวต่อเคมีบำบัดของเซลล์มะเร็งลำไส้ใหญ่ที่จำเพาะ การศึกษานี้สนับสนุนแนวคิดของการใช้ข้อมูลพื้นฐานทาง พันธุกรรมในการประกอบการตัดสินใจเลือกแนวทางการรักษาในผู้ป่วยแต่ละบุคคล

คำสำคัญ: มะเร็งลำไส้ใหญ่, xCT, GLUT1, การดื้อยา, การแพทย์ส่วนบุคคล

Introduction

Colorectal cancer (CRC) is the fourth most commonly diagnosed cancer and mortality worldwide.[1] The incidence rate of CRC in Thailand has rapidly been increased since 2001-2003 in both male and female with a male/female ratio of 1.4:1.[2] In 2012, National cancer institute (NCI) of Thailand reported that colorectal cancer ranked as the second most common cancer (11.59% of total) after breast cancer, and was the third leading cause of cancer-related death (8.1% of total) after liver and lung cancer. The highest incidence rate of CRC for both sexes has been reported in Bangkok.[2] The risk factors for colorectal cancer development appear to be associated with smoking, physical inactivity, obesity, and alcohol consumption.[3] Primary tumors without metastasis are usually treated by surgical resection whereas the majority of patients with advanced colon cancer require cytotoxic chemotherapy as a primary treatment. The first-line chemotherapeutic drugs for treatment of metastatic colorectal cancer worldwide is 5-fluorouracil (5-FU), which inhibits cancer cell growth and initiates apoptosis by targeting thymidylate synthase (TS).[4] Nevertheless, either intrinsic resistance to these drugs or acquired resistance during treatment remains one of the major problems.[4] The treatment of outcome for Thai patients with advanced stages of disease is relatively poor. The 5-year survival rate after treatment of Thai colorectal cancer patients for stage I, II, III and IV were 100%, 68%, 44%, and 2%, respectively.[5] Therefore, in order to improve the response rates to chemotherapy, the use of 5-FU in combination with other drugs or agents have been reported.[6] Due to the narrow therapeutic windows of the existing chemotherapeutic drugs,

these synergistic or additive interactions may improve the therapeutic results and decrease the necessary doses of current chemotherapeutic agents. However, the optimal combination treatment has not yet been identified.

Many types of nutrient transporter have been proposed to be crucial to supply the nutrients to meet metabolic demands of tumor cells for their proliferation and survival. Of these, cysteine/glutamate exchanger (xCT) and glucose transporter 1 (GLUT1) are identified to be significantly linked to the tumor pathogenesis and carcinogenesis.[7] xCT (SLC7A11), a plasma membrane cysteine/glutamate exchanger transporter enables the intracellular uptake of cystine, which is required for the synthesis of glutathione (GSH).[7,8] It requires 4F2hc/CD98 for its functional expression at plasma membrane.[8] GSH is a major antioxidant that is essential for maintaining intracellular redox balance and considered essential for protection of cells from oxidative stress. Moreover, the expression of xCT has been reported to play a crucial role in the tumor progression and drug resistance.[9,10] For example, the elevation of xCT in human pancreatic cancer cells (PANC-1) was associated with cell growth, viability and resistance to gemcitabine.[11] Furthermore, overexpression of xCT induced ovarian cancer cells resistance to cisplatin treatment.[12] Besides xCT, most of cancer cells express glucose transporter 1 (GLUT1) at high level.[7] GLUT1 or SLC2A1 is a facilitative glucose transporter.[13] Prognostic significance of GLUT1 in colorectal cancer has been reported.[14] Notably, several reports have shown that inhibition of xCT and GLUT1 suppressed tumor growth. Inhibition of the xCT transporters leading to cystine/cysteine starvation and subsequent glutathione depletion, has been proposed as a potential

therapeutic approach in a variety of cancers.[11,15-17] Down-regulation of xCT increased the sensitivity of lung adenocarcinoma (HOP62) cells to an HSP90 inhibitor, geldanamycin.[18] Likewise, inhibition of GLUT1 suppressed glycolysis pathway and inhibited cancer cell growth *in vivo*.[19] Interestingly, 5-FU resistant colorectal cancer cells showed overexpression of GLUT1, and inhibition of GLUT1 by a specific inhibitor increased sensitivity to 5-FU in resistant cells.[20] Collectively, these findings suggest that reduction of drug resistance by specific inhibition of the xCT and GLUT1 transporters could provide a new approach for treatment colorectal cancer.

Carcinogenesis of colorectal cancer is known to be a multistage process that involves several genetic alterations. Currently, many established CRC cell lines are widely used as *in vitro* model system to study drug responsiveness and biological pathways related to cancer development or progression. Each cell line presents the same mutational status or genetic alteration as in primary tumors.[21,22] and have the different in malignancy characteristics.[23] However, the comparison between the expression and its role of these nutrient transporters among CRC cell lines isolated from patients with different genetic background has not been documented. Herein, we examined the expression of two nutrient transporters, xCT and GLUT1 in three different types of CRC cell lines, namely HT29, HCT116 and SW480 which are reported to have the different genetic background, as well as the effect of their inhibitors on the sensitivity of the chemotherapeutic drug, 5-FU.

Objectives of the study
1. To compare the expression pattern of xCT and GLUT1 in three human colorectal cancer cell lines.
2. To investigate the effect of xCT and GLUT1 inhibitors on the sensitivity of human colorectal cancer cell lines to 5-FU.

Materials and Methods

Cell culture, antibodies and reagents

Human colorectal cancer cell lines (HT29, HCT116 and SW480) were obtained from American Type Culture Collection (ATCC). All three cell lines were cultured in complete DMEM high glucose medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma–Aldrich, St. Louis, MO) and containing 1% antibiotic-antimycotic (Gibco, Carlsbad, CA). Cells were incubated at 37 °C with 5% CO_2 incubator. The following antibodies were used: anti-xCT from Abcam (Cambridge, ENG, UK); anti-GLUT1 from Thermo scientific (Rockford, MD); anti-β-actin monoclonal antibodies from

Sigma-Aldrich (St. Louis, MO); HRP goat anti-rabbit IgG (H+L) and HRP goat anti-mouse IgG (H+L) antibodies from Jackson Immuno Research Laboratories, Inc. (West Grove, PA). The following reagents were used: 5-fluorouracil, WZB117 and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) were purchased from Sigma-Aldrich (St. Louis, MO); TRIzol reagent from Invitrogen (Carlsbad, CA); cDNA kit from Bio-Rad (Hercules, CA); SYBR kit from Biosystem (Woburn, MA); complete Mini EDTA-free from Roche (Mannheim, Germany); Luminata Crescendo Western HRP Substrate (Millipore, Billerica, MA).

Western blot analysis

Cells were cultured in a 60 mm dish at density 5 x 10^5 cells/dish and incubated at 37 °C with 5% CO_2 incubator for 24 h. Cell were lysed with modified RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% TritonX-100, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, protease inhibitor cocktail). After 30 min incubation on ice, cells were centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was collected and the protein concentrations were measured. Equal amount of protein samples were mixed with Laemmli's sample buffer with or without DTT as indicated before resolved on SDS-PAGE, subsequently transferred onto polyvinylidene fluoride (PVDF) membrane by electro-blotting. Membranes were incubated with anti-CD98, anti-GLUT1, anti-xCT antibody or anti β-actin overnight at 4°C before incubating with HRP-conjugated goat anti-rabbit or anti-mouse IgG for 1 h at room temperature. The signals were detected using the enhanced Luminata Crescendo Western HRP Substrate reagent.

Total RNA extraction and RT-PCR Quantification

Colorectal cancer cells were cultured in a 60 mm dish at density 5 x 10^5 cells/dish and incubated at 37 °C with 5% CO_2 incubator for 24 h. Total RNAs were extracted from cells by using Trizol reagent according to the recommendation of the manufacturer. cDNA synthesis was conducted by using iScript^{TM} cDNA synthesis kit. Quantitative real time PCR of GLUT1 and xCT were analyzed using SYBR Green I dye with ABI PRISM 7500 Sequence Detection System and analysis software (Applied Biosystem). Primers were designed using NCBI/Primer-Blast and 7 IDT Scitools Olioanalyzer 3.1. The primers used are summarized in Table 1. The mRNA level of each gene was normalized to mRNA level of a housekeeping gene, GAPDH.

Table 1. The primers for quantitative real time PCR

xCT	5'-GGTCAGAAAGCCTGTTGT-3'	5'-GATGAAGATTCCTGCTCC-3'
GLUT1	5'-TCACTGTGCTCCTGGTTCTG-3'	5'-CCTGTGCTCCTGAGAGATCC-3'
GAPDH	5'-ACCACAGTCCATGCCATCAC-3'	5'-TCCACCACCCTGTTGCTGTA-3'

Cell viability assay

Cells were seeded into 96 well plate at density of 1×10^4 cells/well and incubated at 37 °C with 5% CO_2 incubator for 24 h. Cells were treated with 5-Fluorouracil (0.1, 0.5, 1, 5, 10, 50 µM) alone or in combination with WZB117 (10 µM) and sulfasalazine (500 µM) as indicated for 72 h. The percentage of viable cells were assessed using 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. Medium was removed and replaced with the complete D-MEM medium containing 0.5 mg/ml of MTT solution and incubation at 37 ºC for 4 h. Medium was removed and formazan product was solubilized by DMSO. Cell viability was determined by measurement the absorbance at 540 nm by Multiskan Go microplate spectrophotometer (Thermo Scientific, Rockford, MD).

Statistical analysis

All data were expressed as mean and standard error of mean (mean ± S.E.M). The statistical analysis was performed by using the statistical software package, GraphPad Prism version 5.0. The statistically significant differences among groups were compared using one-way analysis of variance (ANOVA) followed by Tukey-Kramer *post hoc* test. Statistical significance was considered when $p < 0.05$.

Results

mRNA expression of xCT and GLUT1 in human colorectal cancer cell lines

The systematic study on the expression of the therapeutic targets as well as their roles among these colorectal cancer cell lines are needed to be examined. In present study we compared the expression levels of xCT and GLUT1, two nutrient transporters which have

been demonstrated to highly expressed in multiple cancers, was compared among the three different colorectal cancer cell lines (HT29, HCT116 and SW480) that differ in mutation status. The mRNA expression of xCT and GLUT1was examined by quantitative real time PCR. As shown in Fig. 1, HT29 showed approximately 15 folds higher mRNA expression of xCT than HCT116 and SW480 cell lines (Fig. 1A). The expression level of GLUT1 was highly detected in SW480 and HT29 cell lines (Fig. 1B). However, the expression of GLUT1 was low in HCT116 cell line.

The expression of these nutrient transporters was further confirmed by western blotting. For xCT, HCT116, HT29 and SW480 cell lysates were separated on SDS-PAGE in the presence [DTT(+)] and absence [DTT(−)] of DTT followed by staining with anti-xCT antibody. As shown in Fig. 2A, the ~125 kDa bands corresponding to xCT-4F2hc heterodimer complexes were detected in all colorectal cancer cells lines in the absence of DTT [DTT(-)]. However, under the reducing condition [DTT(+)], xCT was detected at ~35 kDa bands corresponding to the monomeric form of xCT as was previously reported.[24] Consistent with the mRNA expression study, HT29 showed the highest xCT protein expression compared to HCT116 and SW480 cells. Together with previous reports, this result indicates that xCT forms heterodimeric complexes with other proteins, presumingly 4F2hc (CD98) in these 3 colorectal cancer cell lines. In addition, the expression of GLUT1 protein was also examined and demonstrated the consistent result as in mRNA expression in which the highest expression was found in SW480 cells followed by HT29 and HCT116 cell lines, respectively (Fig. 2B).
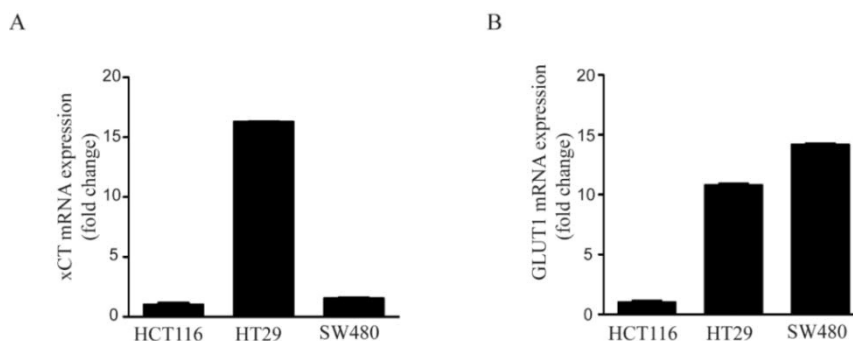
Figure 1. mRNA expression of xCT and GLUT1 in colorectal cancer cell lines. Total RNA was isolated from HCT116, HT29 and SW480 cells and used for quantitative real time PCR by specific primer of xCT(A) and GLUT1(B). The mRNA expression level of each gene was normalized to mRNA level of a housekeeping gene, GAPDH. The results are means ± S.E.M (n=3) and represented as fold change compared to the expression level in HCT116 cells.
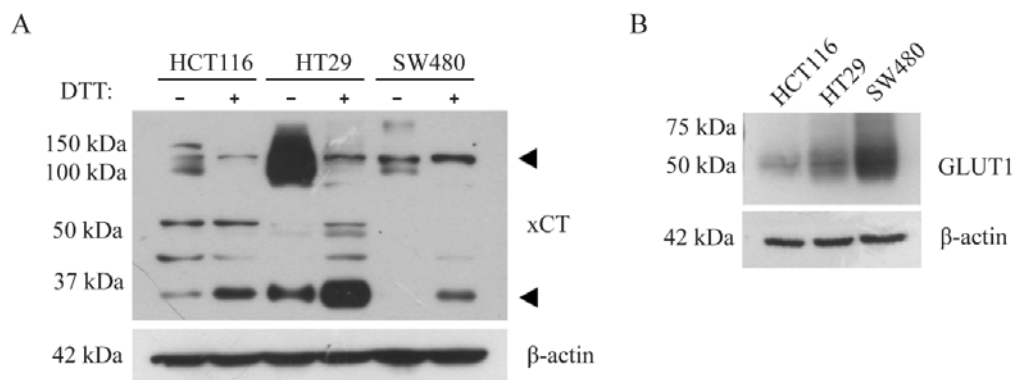
Figure 2. Protein expression of xCT and GLUT1 in three colorectal cancer cell lines. (A) Expression of xCT protein in colorectal cancer cell lines. HCT116, HT29 and SW480 cells were lysed with modified RIPA lysis buffer and subjected for western blotting in the presence [DTT(+)] or absence [DTT(-)] of DTT with anti-xCT and anti-β-actin antibodies. (B) Expression of GLUT1 protein in colorectal cancer cell lines. Colorectal cells were lysed and subjected for Western blotting with anti-GLUT1 and anti-β-actin antibodies.
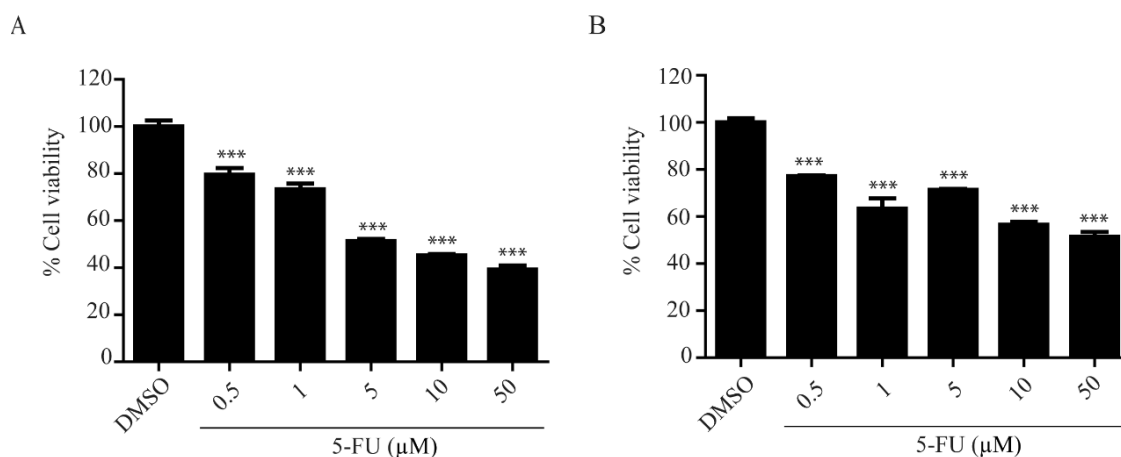
Figure 3. 5-FU treatment decreases cell viability in colorectal cancer cell lines. (A) HT29 cells and (B) SW480 cells were treated with indicated concentration of 5-FU for 72 h followed by the measurements of cell viability by using MTT assay. The results were expressed as % of control group (DMSO) and represented as mean ± S.E.M. (n=3). *** $p<0.0001$ vs control (DMSO).

The effects of sulfasalazine, WZB117 and 5-FU treatments on colorectal cancer cell viability

To determine the optimal dose of 5-FU on colorectal cancer cell viability, HT29 and SW480 cells were treated with various doses of 5-FU for 72 h following by MTT assay. As shown in Fig. 3, 5-FU decreased cell viability in dose-dependent manner in HT29 and SW480 cell lines. (Fig. 3A and B). We next determined whether inhibition of xCT and GLUT1 by specific inhibitors increase sensitivity of HT29 and SW480 to 5-FU. Cells were treated with 5-FU or inhibitors alone or in combination between 5-FU and inhibitors for 72 h before assessment cell viability by MTT assay. As shown in Fig. 4A, single treatment of xCT and GLUT1 inhibitors had no effect on HT29 cell viability.

In addition, the sensitivity of HT29 to 5-FU drug was not affected by these inhibitors. In contrast, SW480 cell viability significantly reduced after sulfasalazine but not WZB117 treatments. Consistent with this notion, the sensitivity of SW480 cells to 5-FU was also increased in the presence of an xCT inhibitor (Fig. 4B). Co-treatment of 5-FU and sulfasalazine suppressed SW480 significantly compared to 5-FU treatment alone. Most interestingly, co-treatment with WZB117 and sulfasalazine further increased the sensitivity of SW480 cells to 5-FU. Taken together, these results indicate that inhibition of xCT and GLUT1 function could be used for cancer treatment particularly in combination with other chemotherapeutic drugs.

A

B
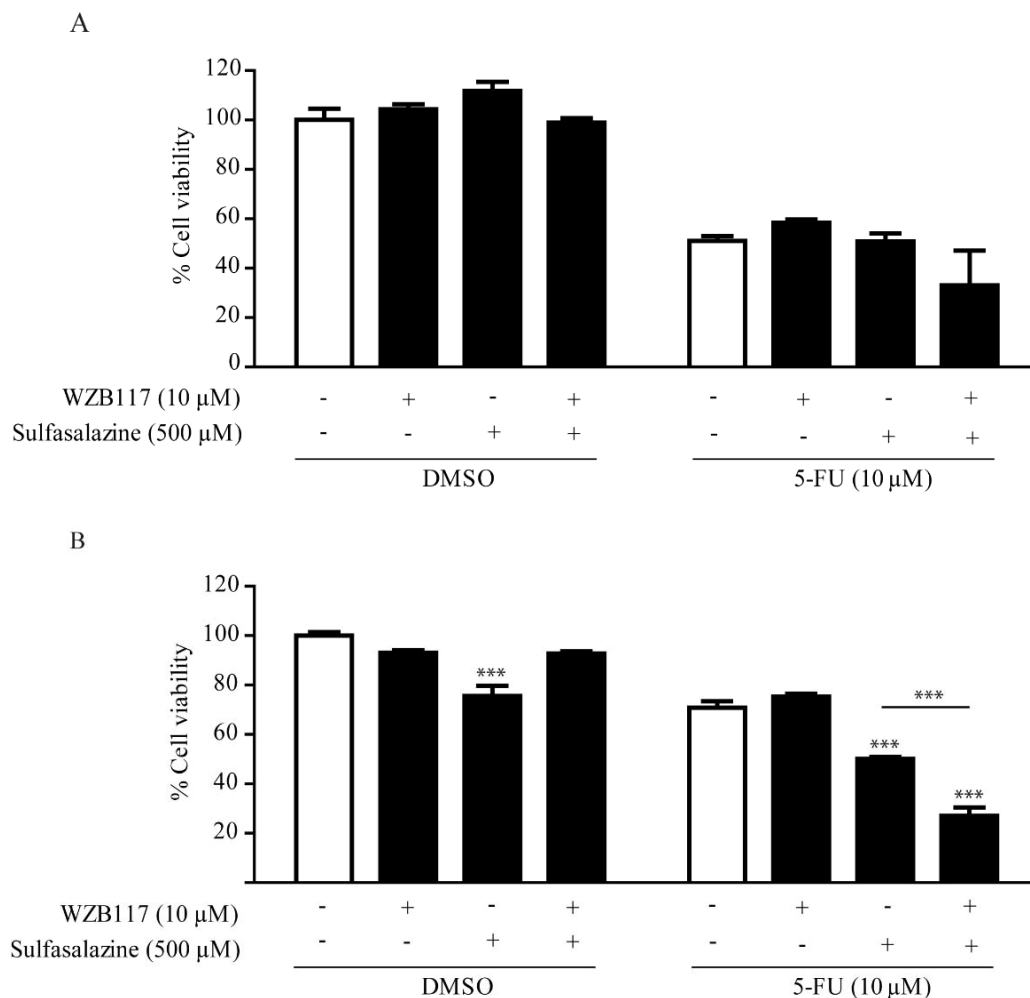
Figure 4.   Inhibition of GLUT1 and xCT increase sensitivity of SW480 colorectal cancer cell line to 5-FU.
(A) HT29 cells and (B) SW480 cells were treated with of 5-FU (10 μM), WZB117 (10 μM) and
sulfasalazine (500 μM) alone and combination treatment as indicated for 72 h followed by the
measurements of cell viability by MTT assay. The results were expressed as % of control group
(DMSO) and represented as means ± S.E.M. (n=3). *** $p<0.0001$ vs control (DMSO)

Discussion and Conclusions

We examined the expression of two nutrient
transporters, xCT and GLUT1 in three different types
of CRC cell lines, namely HT29, HCT116, SW480. We
found that the tested CRC cell lines showed different
levels of xCT and GLUT1 expression. HT29 expressed
the highest level of xCT whereas GLUT1 was detected
at high level in SW480 and HT29 cell lines. Inhibition
of xCT and GLUT1 by a specific inhibitor had no effect
on CRC cell viability. However, the sensitivity of
SW480 but not HT29 to 5-FU was enhanced by
co-treatment with an xCT inhibitor, sulfasalazine.
Interestingly, combination treatment between sulfasalazine
and WZB117, a GLUT1 inhibitor, markedly reduced
SW480 cell viability induced by 5-FU. These results
indicate that the expression pattern of xCT and GLUT1
transporters may depend on the differences in genetic

alteration in CRC cells. Inhibition of these transporters
may enhance the sensitivity of specific CRC cells to
chemotherapeutic drugs.

The genetic alteration of HT29, SW480 and
HCT116 cell lines have been extensively
characterized.[21,22] and showed distinct mutation pattern.
HCT116 contains *KRAS* (G13D), *PIK3CA* (H1047R)
and β-catenin mutations. SW480 contains *KRAS*
(G12V) and *TP53* (R273H; P309S) and APC
(adenomatous *polyposis coli*) mutations. HT29 contains
BRAF (V600E), *PIK3CA* (P449T), *TP53* (R273H) and
APC (adenomatous polyposis coli) mutations. The
malignancy characteristic of these CRC cell lines have
also been studied in an orthotopic nude mice model.[25]
The growth indices of HCT116 and SW480 cell lines
were higher than that of HT29 cells. In contrast, the
highest of lymph node metastasis percentage was

observed in HT29 cell lin.[25] Reactive oxygen species (ROS) play a role in inducing apoptosis. In order for cancer cells to survive against oxidative stress from ROS production, most of cancer cells upregulate cysteine transporter (xCT). xCT is essential for the uptake of cysteine required for synthesis of intracellular glutathione (GSH), a substance that play a role in prevention of oxidative stress induced apoptosis.[26] Our current study showed that the molecular weight of xCT protein in these three CRC cell lines were detected at ~125 kDa in the absence of reducing agent [DDT(-)]. However, under reducing condition [DDT(+)] the bands were shifted to ~35 kDa corresponding to the original size for xCT. Together with previously reported, this result indicates that xCT forms the heterodimeric complex with chaperone protein 4F2hc or CD98 in these CRC cell lines.[8] The multiple bands observed in HCT116 and HT29 cell lines could be an alternatively spliced or translationally modified of xCT. Indeed, the xCT modified 55 kDa band was detected in astrocyte culture.[24] Among three CRC cell lines, we found that HT29 expressed the highest mRNA and protein levels of xCT. At present the regulation of xCT expression in HT29 cell line is not known. Unlike other CRC cell lines, HT29 cell line contains *KRAS* wild-type. Previous studies in human endothelial cells have reported that *KRAS* has a potent anti-apoptotic activity induced by ROS.[27] Therefore, the expression of xCT in this cell line might be regulated by *KRAS* and awaiting for further investigation.

The biological significance of GLUT1 in carcinogenesis remains unclear. Some evidences suggest that GLUT1 expression in colorectal adenocarcinomas is associated with a high incidence of lymph node metastasis and distal recurrence after operation.[14,28] Therefore, the expression of GLUT1 was suggested to use as a prognostic marker for CRC.[14,29] Recent study found that GLUT1 expression was observed in SW480 >> HT29 > HCT116 cell lines. The cross-talks between Wnt/β-catenin signaling pathway and GLUT1 expression have recently been reported. The study in head and neck squamous cell carcinoma have found that the hyperactivation of Wnt/β-catenin induced by WNT2B increased GLUT1 expression.[30] Therefore, the expression of GLUT1 in CRC might be regulated by Wnt/β-catenin signaling pathway. Indeed, hyperactivation of this Wnt/β-catenin signaling pathway is well documented in colorectal cancer.[31] SW480 and HT29 contain the APC mutations that leads to aberrant activation of Wnt/β-catenin signaling pathway. However, the hyperactivation of Wnt/β-catenin signaling pathway in HCT116 is due to activated β-catenin mutation.[22] Thus, the upregulation of GLUT1 in SW480 and HT29 cell lines might be related with the signaling protein(s) upstream of β-catenin.

Previous studies have reported that HT29 cells could be induced to apoptosis by 5-FU.[32] and exposure of SW480 cells to 5-FU resulted in growth inhibition in a dose-dependent manner.[33] Consistent with these, we showed that 5-FU reduced HT29 and SW480 cell viability. Most of colorectal cancer cell lines including HT29 and SW480 contain p53 tumor suppressor gene mutation, which often leads to resistant to anticancer drugs such as 5-fluorouracil (5-FU).[22,34] 5-FU triggers their cytotoxicity by increasing the intracellular ROS level in the p53-dependent pathway.[35] Thus, we reasoned that, the suppression of GSH synthesis by inhibition xCT mediated cysteine uptake should increase the intracellular ROS and sensitize CRC to 5-FU. The growth inhibitory effects of sulfasalazine (SSZ), the specific inhibitor for xCT amino acid transporter have been seen in lymphoma and pancreatic cancer.[16,36] SSZ-induced sensitization of pancreatic cancer cells to gemcitabine treatment was observed both *in vitro* and *in vivo*.[36] Furthermore, the specific GLUT1 inhibitor WZB117, downregulates glycolysis pathway, induces endoplasmic reticulum (ER) stress leading to cell-cycle arrest, and inhibits lung cancer cell growth both *in vitro* and *in vivo*.[19] The combination of WZB117 and cisplatin or paclitaxel showed synergistic anticancer effects.[19] These information suggest the possible use of SSZ and WZB117 for the treatment of colorectal cancer. However, we found that treatment with SSZ or WZB117 alone, or in combination did not reduced HT29 cell viability. In addition, the sensitivity of HT29 to 5-FU was not improved. This might be due to the presence of *v-raf* murine sarcoma viral oncogene homolog B1 or *BRAF* proto-oncogene gene mutation (V600E) in HT29 cell line. *BRAF* (V600E) activating mutation has been associated with the resistance of metastatic colorectal cancer patients to EGFR inhibitors, panitumumab or cetuximab.[37] Consistent with this speculation, combination treatment of SSZ and WZB117 increased the sensitivity of *BRAF* wild type SW480 cell line to 5-FU. In contrast, WZB117 an increased the sensitivity of resistant colorectal cancer cell lines (HT29 cells) to 5-FU have been reported.[20] This discrepancy might be affected by the passage number and culture conditions that has been reported for the sensitivity of human prostatic cancer cell line LNCaP to androgen.[38]

In conclusion, we demonstrated the expression pattern of two nutrient transporters, xCT and GLUT1 in three CRC cell lines with difference in genetic alterations. The mutational status of oncogene in CRC may have an influence on the expression of these transporters and may determine the drug resistance phenotypes or drug resistance of cancer cells. These observations are consistent with the concept of personalized medicine. To increase the sensitivity of

CRC cells to anticancer drug treatments, the genetic background information should be taken into account for medical decision.

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Cytotoxicity of Aqueous and Methanolic Leaves Extract of *Pseuderanthemum palatiferum* (Nees) Radlk. on Human Non Small Cell Lung A549 Cells

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Abstract

Pseuderanthemum palatiferum (Nees) Radlk. leaves have been traditionally consumed to cure various kind of diseases including cancer. This study therefore, aimed to investigate the cytotoxic effects of aqueous and methanolic leaves extract of *P. palatiferum* on human non small cell lung cancer A549 cells. The *in vitro* cytotoxic potential of *P. palatiferum* was carried out by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The results demonstrated that aqueous and methanolic extract of *P. palatiferum* exhibited potent cytotoxic effect against A549 cells with an IC_{50} of 0.22 and 0.36 mg/mL respectively after 72 hr treatment. The present study suggested that aqueous leaves extract of *P. palatiferum* might contain substances for further studies to develop new anticancer drug.

Keywords: *Pseuderanthemum palatiferum* (Nees) Radlk., non small cell lung cancer, A549, cytotoxic effect

ความเป็นพิษของสารสกัดจากใบพญาวานรต่อเซลล์มะเร็งปอด

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บทคัดย่อ

ใบพญาวานรได้ถูกนำมาใช้รับประทานเพื่อใช้ในการรักษาโรคชนิดต่าง ๆ รวมทั้งมะเร็ง ดังนั้นการศึกษานี้มีวัตถุประสงค์เพื่อศึกษาความเป็นพิษของสารสกัดจากใบพญาวานรด้วยน้ำและเมทานอลต่อเซลล์มะเร็งปอดชนิดไม่ใช่เซลล์เล็ก (เซลล์ A549) การศึกษาความเป็นพิษต่อเซลล์ของสารสกัดจากใบพญาวานร โดยใช้วิธี MTT assay ผลการทดลองแสดงให้เห็นว่าสารสกัดทั้งสองมีความเป็นพิษต่อเซลล์ และยังพบว่าสารสกัดด้วยน้ำมีประสิทธิภาพมากกว่าสารสกัดด้วยเมทานอล โดยมีค่า IC_{50} เท่ากับ 0.22 และ 0.36 mg/mL ที่ 72 ชั่วโมง จากผลการทดลองชี้ให้เห็นว่าสารสกัดใบพญาวานรด้วยน้ำน่าจะมีประสิทธิภาพในการพัฒนาเป็นยารักษามะเร็ง

คำสำคัญ: พญาวานร, มะเร็งปอดชนิดไม่ใช่เซลล์เล็ก, A549, ความเป็นพิษต่อเซลล์

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Introduction

Lung cancer is one of the most common cancers which is a leading cause of death. It has been classified into small cell lung cancer (SCLC) (representing 15%-20%) and non small cell lung cancer (NSCLC) (representing 80%-85%). Unfortunately, patients underlying NSCLC are asymptomatic therefore most cases are not diagnosed in early stage until the disease progress to advance stage in which the survival rate is low.[1, 2] Thereby, the development of more effective cancer therapy remains an ongoing challenge.[3]

Pseuderanthemum palatiferum (Nees) Radlk. (*P. palatiferum*) belongs to Acanthaceae family. It is also known as Hoan-ngoc or Payawanorn.[4-5] The leaves of this plant are traditionally consumed to treat various kind of diseases in folk medicine including hypertension, diarrhea, peptic ulcer, hepatitis, inflammatory disease and cancer.[6] The phytochemical investigation of *P. palatiferum* found flavonoid, saponin, phenols, *n*-pentacosan-l-ol, β-sitosterol, stigmasterol, β-sitosterol 3-O- β-glucoside, stigmasterol 3-O-β-glucoside, kaempferol 3-methyl ether 7-O- β-glucoside and apigenin 7-O- β-glucoside.[4, 7] Previous studies reported the potential effect of *P. palatiferum* on colon cancer and breast cancer cell lines.[8, 9] However, to date, there is no scientific report on human non small cell lung cancer. Therefore, the purpose of this study was to investigate the cytotoxic effects of aqueous and methanolic leaves extract of *P. palatiferum* on human non small cell lung cancer A549 cells.

Materials and Methods

Preparation of P. palatiferum leaves extracts

P. palatiferum were obtained from Hoan-Ngoc Thailand herb garden, Bangkok, Thailand and identified by a botanist. Voucher specimens were deposited as BKF: 187517 at the Forest Herbarium, Bangkok, Thailand. Dried powder of *P. palatiferum* (1 kg) was extracted with milli-Q water and 80% methanol at 1: 10 ratio for 24 hr, followed by filtration through Whatman filter paper No. 1. The residues were then re-extracted twice. The combined fractions were subjected to remove solvent under reduced pressure with a rotary evaporator and then lyophilized with the freeze drier. The yield of crude aqueous and methanol extract was 17.37% and 9.72%, respectively.

Cell culture

Human lung adenocarcinoma epithelial cell line (A549) was purchased from the Cell Lines Service GmbH (CLS, Eppelheim, Germany). Cells were grown onto 75 cm^2 tissue culture flasks in DMEM/F12 medium supplemented with 10% FBS, 1% penicillin-streptomycin and 1% L-glutamine and maintained under 5% CO_2 in a humidified incubator at 37 °C.

In vitro assay for cytotoxicity

Cell viability was examined by the MTT assay. A549 cells were seeded in a 96-well plate ($1x10^4$ cells/well) for 24 hr. After exposure the cells with aqueous and methanolic extract of *P. palatiferum* at various concentrations of 0.10, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75 and 2.00 mg/mL for 24, 48 and 72 hr. At the end of each exposure time, the treated cells were then incubated with MTT (5 mg/mL) for 4 h at 37 °C. The blue formazan crystals of viable cells were solubilized in dimethylsulfoxide (DMSO) and then the absorbance was measured at a wavelength of 570 nm using a microplate reader (BioTek, Highland Park, USA). The half inhibitory concentration (IC_{50}) was determined from the dose-response relationship between the *P. palatiferum* concentration (mg/mL) and the percentage of cell viability. The assays were performed three times in triplicate.

Statistical analysis

The data were presented as mean ± standard error of mean (SEM). Statistical comparisons were performed by using one-way ANOVA, followed by Turkey *post-hoc* analysis. Probability values of $p<0.05$ was considered to be statistically significant.

Results

Cytotoxic effect of P. palatiferum on A549 cells

In order to evaluate the cytotoxic effect of aquoues and methanolic leaves extract of *P. palatiferum* against human non small cell lung cancer A549 cells, MTT assay has been carried out. As shown in Figure 1A, viable cells were significantly inhibited after treatment with aqueous extract of *P. palatiferum* in dose- and time-dependent manner. The IC_{50} values of 24, 48, 72 hr were 0.58, 0.41, 0.22 mg/mL, respectively. Concerning to treatment with methanolic extract of *P. palatiferum* (Figure 1B), it was found that the methanolic extract showed less potent than the aqueous extract with IC_{50} values of 24, 48, 72 hr were 0.79, 0.56, 0.36 mg/ml, respectively.
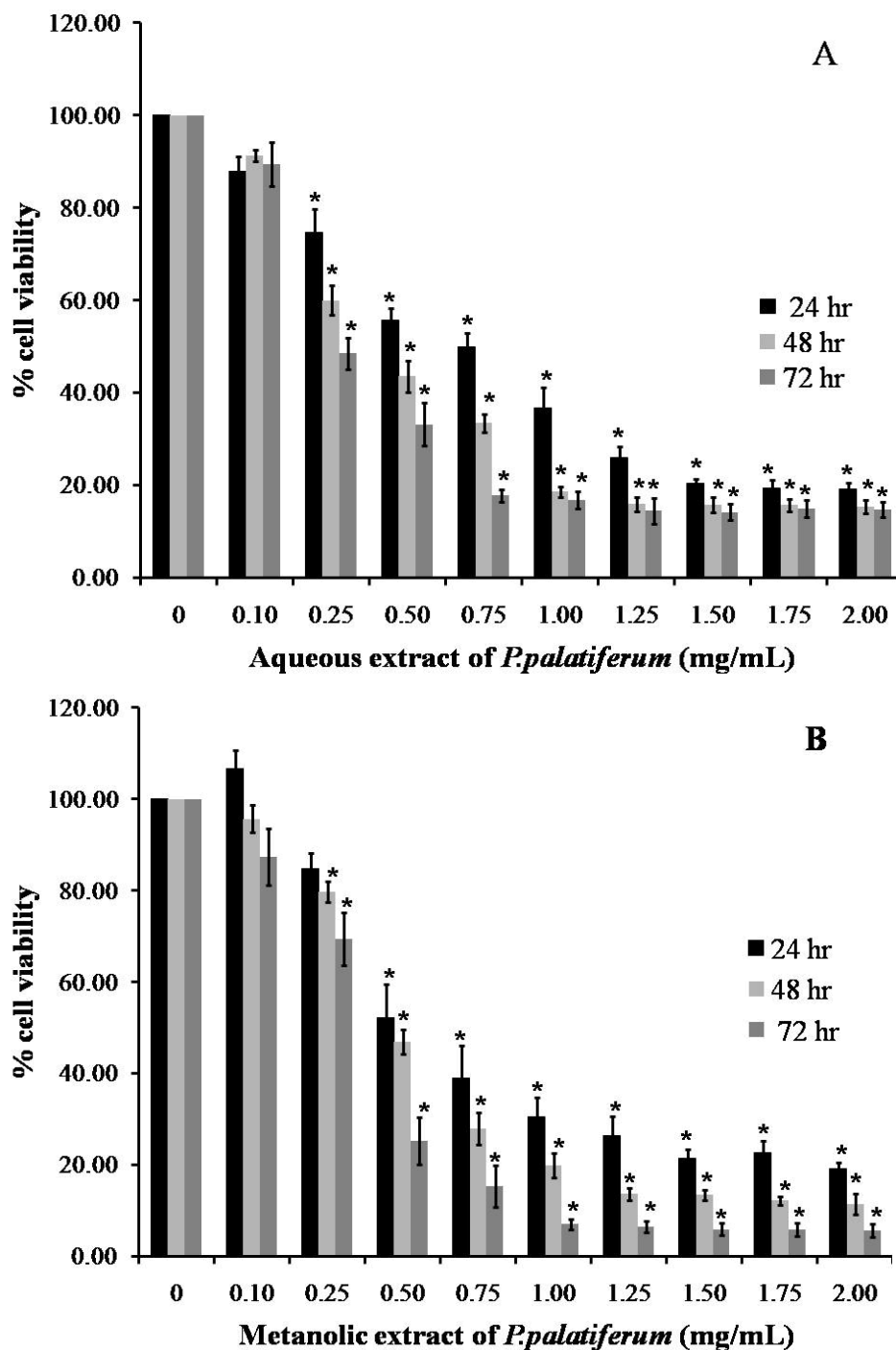
Figure 1. Cytotoxic effect of *P. palatiferum* leaves extracts on A549 cells. A549 cells were treated with various concentrations (0.1, 0.25, 0.50, 1.00, 1.25, 1.50 1.75 and 2.00 mg/mL) of aqueous (A) and methanolic extract (B) for 24, 48 and 72 hr. Data were presented as mean ± SEM of three independent experiments. *$p<0.05$ were considered to be statistically significant.

Discussion and Conclusion

Several attempts have been made on lung cancer treatment and prevention. Natural products, particularly from plants source are highlighted for effective drug discovery. In the present study, we demonstrated the cytotoxic potential of aqueous and methanolic leaves extract of *P. palatiferum* on human non small cell lung cancer A549 cells. Our results showed that aqueous extract exhibited more

potent cytotoxicity against A549 cells than that of methanolic extract, with IC_{50} values of 0.58, 0.41, 0.22 mg/mL for the treatment of aqueous extract at 24, 48 and 72 hr, respectively. Previous studies found that aqueous leaves extract of *P. palatiferum* inhibited colon cancer cell proliferation including HCT15, SW48 and SW480 with the IC_{50} value of 0.52, 0.50, 0.51 mg/mL at 72 hr, respectively.[8] Besides, ethanolic leaves extract of *P. palatiferum*

was also found to inhibit colon cancer (Caco-2) and breast cancer (MCF-7) with IC_{50} values of 0.45 and 0.59 mg/mL at 48 hr.[9] Polyphenols are a group of chemical substances found in plants and well-known for their anti-oxidative properties. These compounds are considered as cancer chemopreventive agents because they can prevent the formation of reactive oxygen and nitrogen species, which play important roles in carcinogenesis.[10] Thus, it is possible that polyphenols in the extract might provide potential effect on A549 cells. However, further investigation on purified compounds from aqueous leaves extract on lung cancer cell should be elucidated. This might be a promising therapeutic agent targeting lung cancer and/or colon cancer.

Acknowledgements

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Kaempferol Suppresses Cell Proliferation and Migration in KKU-100 Human Cholangiocarcinoma Cells

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Abstract

Cholangiocarcinoma (CCA), a malignant tumor of the bile duct, is one of the major health problems of the populations in Northeast of Thailand. The disease is particularly recognized to be very difficult for diagnosis with poor prognosis to current radiotherapy and chemotherapy. Searching for novel and effective natural products used for the cancer prevention or treatment of CCA is urgently needed. The purpose of the study was to investigate the anticancer activity of kaempferol (KFR), a natural flavonoid widely distributed in fruits and vegetables, in KKU-100 human CCA cells. The effect of KFR on the long-term period of KKU-100 cell growth and proliferation was performed using the clonogenic assay. The result observed at 10 days post-treatment showed that KFR(20-80μM) for 48 h significantly reduced the colony forming ability of KKU-100 cells. The effect of KFR on the cell migration of KKU-100 cells was investigated using the wound healing assay. The result observed at 24, 48 and 72 h post-treatment indicated that KFR at 20 μM delayed KKU-100 cell migration in a time-dependent manner. Our findings suggest that KFR has significant anticancer activity on CCA *in vitro* via suppressing cell proliferation and migration. Further studies investigating potential use of KFR for the cancer prevention or treatment of CCA are warranted.

Keywords: kaempferol, cholangiocarcinoma, cell proliferation, cell migration

แคมป์เฟอรอลยับยั้งการเพิ่มจำนวนและการเคลื่อนที่ของเซลล์มะเร็งท่อน้ำดีมนุษย์ชนิด **KKU-100**

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บทคัดย่อ

มะเร็งท่อน้ำดี เป็นมะเร็งที่เกิดขึ้นบริเวณท่อทางเดินน้ำดี นับเป็นปัญหาสุขภาพที่สำคัญของประชากรภาคตะวันออกเฉียงเหนือของไทยการวินิจฉัยมะเร็งชนิดนี้ทำได้ยากและมีการพยากรณ์โรคไม่ดี โดยมีการตอบสนองต่ำต่อการรักษาด้วยการฉายรังสีและยาเคมีบำบัด ดังนั้นการค้นหาสารธรรมชาติที่มีประสิทธิภาพป้องกันหรือรักษามะเร็งท่อน้ำดีจึงมีความจำเป็นเร่งด่วน งานวิจัยนี้มีจุดประสงค์เพื่อศึกษาฤทธิ์ต้านมะเร็งของแคมป์เฟอรอล (สารฟลาโวนอยด์ที่พบมากในพืชผักและผลไม้) ต่อเซลล์มะเร็งท่อน้ำดีมนุษย์ชนิด KKU-100 ด้วยวิธี clonogenic assay พบว่าการให้แคมป์เฟอรอลที่ความเข้มข้น 20-80ไมโครโมลาร์เมื่อสัมผัสเซลล์มะเร็งท่อน้ำดีเป็นระยะเวลา 48 ชม. สามารถลดการสร้างโคโลนีของเซลล์ได้อย่างมีนัยสำคัญ เมื่อศึกษาผลต่อการเคลื่อนที่ของเซลล์ด้วยเทคนิค wound healing assay พบว่าแคมป์เฟอรอลที่ความเข้มข้น 20ไมโครโมลาร์สามารถยับยั้งการเคลื่อนที่ของเซลล์มะเร็งท่อน้ำดีเมื่อถูกสัมผัสยาเป็นเวลา 24 48 และ 72 ชม. ผลการศึกษาครั้งนี้แสดงว่าแคมป์เฟอรอลสามารถต้านมะเร็งท่อน้ำดีในหลอดทดลองได้อย่างมีนัยสำคัญโดยยับยั้งการเพิ่มจำนวนและการเคลื่อนที่ของเซลล์ดังนั้นจึงควรทำการศึกษาต่อไปเพื่อแสดงศักยภาพของแคมป์เฟอรอลในการป้องกันหรือรักษามะเร็งท่อนำดี

คำสำคัญ: แคมป์เฟอรอล, มะเร็งท่อน้ำดี, การเพิ่มจำนวนของเซลล์, การเคลื่อนที่ของเซลล์

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Introduction

Cholangiocarcinomas (CCA) or bile duct cancers is a type of cancer that arises from the neoplastic transformation of cholangiocytes within the bile ducts, both inside and outside the liver. Over the past few decades, several studies have shown that the incidence and mortality rate of CCA have been increasing worldwide. There is a high incidence of CCA in Southeast Asian countries due to chronic endemic parasitic infection with liver flukes. Obviously, the highest incidence of CCA has been reported in Northeastern Thailand.[1] CCA is illustrated by poor prognosis, late diagnosis, and lack of response to both chemotherapy and radiation therapy.[2] Additionally, surgical resection is the only intervention to offer a chance of treatment but the five-year survival rate is very low.[2, 3] Therefore, improved treatments for this type of cancer are urgently needed.

Kaempferol (KFR), a member of the flavonols, is abundantly present in tea, grapes, broccoli, and berries.[4, 5] It has been reported to invoke cytotoxic activities in several human cancer cell lines such as breast cancer,[6] ovarian cancer,[7] lung cancer,[8] leukemia,[9] prostate cancer,[10] and colon cancer.[11] However, the cytotoxic activities of kaempferol against CCA has not been validated. The purpose of this study was to determine the antiproliferative and antimigrative effects of KFR in KKU-100 human CCA cells.

Materialsand Methods

Materials

Kaempferol (KFR) was obtained from Sigma Chemical (St. Louis, MO, USA). Crystal violet was purchased from Fluka Chemika (Buchs, Switzerland). Reagents for cell culture were purchased from Gibco BRL Life Technologies (Grand Island, NY, USA).

Cell line and cell culture

The human CCA cell line used in this study was KKU-100, which was kindly obtained from Prof. Dr. Banchob Sripa. This cell line was routinely cultured in Ham's F12 media, supplemented with sodium bicarbonate, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), at pH 7.3, 100 U/mL penicillin, 100 µg/mL streptomycin and 10% fetal bovine serum (FBS), and maintained under an humidified atmosphere containing 5% CO_2 at 37°C. Cells were subcultured every 3 days using 0.25% trypsin-EDTA.

Clonogenic assay

The assay was carried out according to the previously described method.[12] KKU-100 cells (2.5×10^5) were seeded into 6-well plates and allowed to grow for 24h. The cells were then treated with different concentrations of KFR (20-80µM) for 48 hr. After treatment, cells were trypsinized and counted.Only 600 viable cells were seeded in 6-well plates and the cells were grown without KFR for another 10 days. Based on this technique,a single cell formed a colony consisting of 50 or more cells. The cell colonies were stained with crystal violet and the number of colonies were counted.

Wound healing assay

The wound healing assay was used to assess the cell migration ability using the previously described method.[12] KKU-100 cells (1.5×10^5) were seeded into 24-well plates and incubated overnight. A scratch wound was made with a sterile 200 µL pipette tip. After washing with phosphate-buffered saline to remove any detached cell, cells were incubated with 20 µM KFR. A series of images of the scratched wound were taken during the 0-72 h. period. The closing of scratched wound, which represented the migration process, was determined by capturing of the denuded area along the scratch using Image-Pro Plus software (Media Cybernetics, LP, USA). The percentage of wound closure was calculated by dividing the width of denuded area by the width at time 0.

Statistical analysis

All results are presented as the mean±SEM. Statistical comparison between control and treatment group was performed using Student's t-test. Statistical significance was set at $p<0.05$.

Results

Effects of KFR on colony formation ability of KKU-100 CCA cells

The clonogenic assay was performed determine the effects of KFR on long-term viability and replicative ability or clonogenicity of CCA cells. Treatment with KFR caused a decline in colony forming ability of KKU-100 cells (Figure 1A and 1B).

Effects of KFR on CCA cell migration of KKU-100 CCA cells

Enhanced cell migration and invasion are important aspects of cancer phenotypes.In this study, the effects of KFR on cell migratory activity of KKU-100 cells were examined using wound healing assay. As illustrated in Figure 2A and 2B, KFR significantly suppressed cell migration of KKU-100 CCA cells.
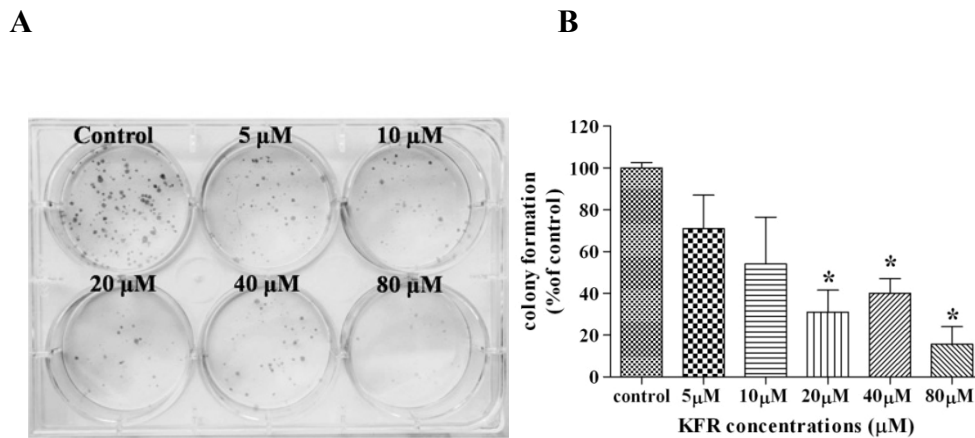
A

B

Figure 1. Effects of KFR on colony formation ability of CCA cells. KKU-100 cells were treated with 20μM KFR for 48 hr. After treatment, only viable cells were further grown in full media without KFR for 10 days. Then, cells were stained with 0.5% crystal violet and photographed. (A) Figures shown are representative of three independent experiments. (B) The bar shows the percentage of colony formation relative to the control. Each bar represents the mean±SEM averaged from three independent experiments. * $p<0.05$ compared with control.

A

B
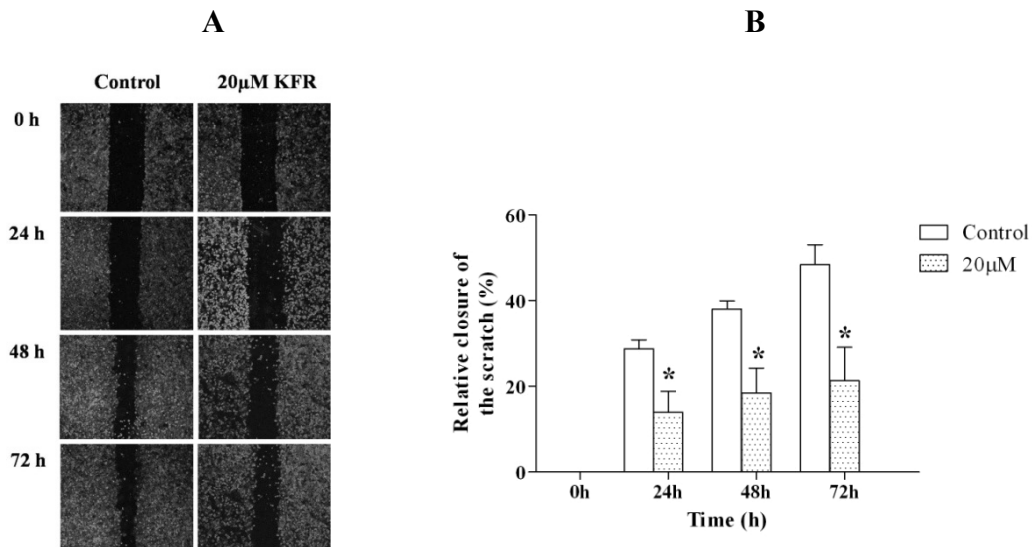
Figure 2. Effects of KFR on CCA cell migration. (A) Scratched wounds of monolayer KKU-100 cells were treated with 20 μM KFR. Cell migration during a period 0-72 h was monitored under phase-contrast microscopy (x4 magnification). Representative images of wound healing were obtained at the time of the scratch,24, 48 and 72h later. (B) The graph shows the level of cell migration into the wound scratch quantified as the percentage of wound closure. The data are mean±SEM averaged from three independent experiments (triplicate in each experiment).* $p<0.05$ compared with control.

Discussion

The most important characteristic of a cancer cell is the ability to sustain proliferation and resistance to cancer cell death.[13] Thus, we investigated the effect of KFR on colony formation ability of KKU-100 CCA cells. The result observed showed that treatment with KFR at micromolar concentration significantly suppressedthe colony forming ability of KKU-100

cells, which implies that KFR could mitigate the long-term viability and replicative ability of CCA cells. Consistently, a previous study reported that KFR at 50, 100 and 150 μM doses significantly inhibited colony formation of renal cell carcinoma cells, which is associated with cell cycle arrest at G2/M phase.[14] Possibly, KFR may also impede the cell cycle progression of CCA, this regulatory effect of KFR

should be further validated. As a result, KFR appear to be a promising drug candidate for control the growth of CCA cells.

Metastasis, which is initiated by the migration and invasion of cells into the surrounding vasculature, is the primary cause of morbidity and mortality in cancer patients.[15] Indeed, a compound with the ability to block the metastasis-associated steps could be a potential candidate for cancer chemoprevention and chemotherapy. In this study, the results demonstrated that KFR could inhibit the migratory property of CCA cells. Result were consistent with a previously report for the inhibitory effect on the migration of glioblastoma cells[16] and bladder cancer cells.[17] These observations, taken together, suggest broad spectrum of activity of KFR against various types of cancers.

Conclusion

KFR exhibited effective antiproliferative activity against CCA cells *in vitro*. In addition, KFR also retarded cell migration ability of KKU-100 human CCA cells. These data revealed KFR might have potential role for CCA chemoprevention and chemotherapy. Further *in vivo* investigations *in vivo* are needed before KFR can become a new drug candidate in the prevention and/or treatment of CCA.

Acknowledgement

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F15 | Apoptotic-Inducing Effect of Luteolin on Human Cholangiocarcinoma Cells

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Abstract

Cholangiocarcinoma (CCA) is one of devastating cancers with a very poor prognosis and is associated with a high mortality. At present, the development of new strategies for prevention and treatment of CCA is highly desired. Several dietary polyphenols from natural sources are known to possess anticancer activities. In this study, the cytotoxic effect of luteolin on human CCA cells (KKU-100) and the underlying mechanisms mediating its effect were investigated. Sulphorhodamine B assay showed that luteolin had a potent cytotoxicity on CCA cells with IC_{50} (concentration that inhibits cell growth by 50%) value of 6.8 ± 1.2 µM (mean ± SEM) at 48 h. CCA cell apoptosis was clearly detected with fluorescent dye staining and flow cytometry using FITC-annexin V/PI staining. To investigate whether luteolin-induced CCA cell apoptosis is mediated through mitochondrial pathway, the mitochondrial transmembrane potential and activity of caspase-9 were assessed. The results show that luteolin induced mitochondria depolarization of CCA cells as evident in the shift in JC-1 fluorescence from red to green. In addition, luteolin activated caspase-9 in KKU-100 cells. These results indicate that luteolin could induce apoptosis of CCA cells through the intrinsic mitochondrial pathway, demonstrating its potential as a chemotherapeutic agent for CCA therapy.

Keywords: luteolin, cholangiocarcinoma, apoptosis, mitochondrial pathway, caspase

ผลของ luteolin ต่อการเหนี่ยวนำการตายแบบ apoptosis ในเซลล์มะเร็งท่อน้ำดี

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บทคัดย่อ

มะเร็งท่อน้ำดีเป็นหนึ่งในโรคมะเร็งที่มีความรุนแรงซึ่งมีการพยากรณ์โรคที่ไม่ดีและมีความสัมพันธ์กับอัตราการตายสูง การพัฒนาหาสารหรือวิธีการรักษาใหม่เพื่อป้องกันและรักษามะเร็งท่อน้ำดีจึงมีความจำเป็นอย่างยิ่ง สารในกลุ่มโพลีฟีนอลจาก ธรรมชาติหลายชนิดมีฤทธิ์ต้านมะเร็งได้ ในงานวิจัยนี้ได้ทำการศึกษาฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งท่อน้ำดีของมนุษย์ชนิด KKU-100 และกลไกในการออกฤทธิ์ฆ่าเซลล์ของสาร luteolin การทดลองด้วยวิธี sulphorhodamine B พบว่า luteolin มีความ เป็นพิษต่อเซลล์มะเร็งท่อน้ำดี โดยมีค่าความเข้มข้นที่ยับยั้งการมีชีวิตรอดของเซลล์ได้ 50% (IC_{50}) เท่ากับ 6.8 ± 1.2 µM (ค่าเฉลี่ย ± ความคลาดเคลื่อนมาตรฐาน) ที่เวลา 48 ชั่วโมง นอกจากนี้สาร luteolin ยังชักนำให้เซลล์มะเร็งท่อน้ำดีเกิดการตาย แบบ apoptosis ด้วยการวิเคราะห์โดยย้อมด้วยสารเรืองแสง AO/EB และการย้อมด้วย FITC-annexin V/PI และวิเคราะห์โดย

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วิธี flow cytometry เพื่อตรวจสอบว่าสาร luteolin มีผลชักนำการเกิด apoptosis ผ่านทางวิถีไมโตคอนเดรียหรือไม่ ผู้วิจัยได้
ตรวจวัดค่าศักย์ไฟฟ้าของเยื่อหุ้มไมโตคอนเดรียของเซลล์ และค่าการทำงานของเอนไซม์ caspase-9 พบว่า luteolin ชักนำให้
เกิดการสูญเสียศักย์ไฟฟ้าของเยื่อหุ้มไมโตคอนเดรียโดยสังเกตจากมีการเปลี่ยนแปลงของสี JC-1 จากสีแดงไปเป็นสีเขียว
นอกจากนี้ยังมีผลกระตุ้นการทำงานของ caspase-9 ในเซลล์ KKU-100 ด้วย ผลการทดลองทั้งหมดแสดงให้เห็นว่า luteolin
สามารถชักนำให้เซลล์มะเร็งท่อน้ำดีมีการตายแบบ apoptosis ผ่านทางวิถีไมโตคอนเดรีย ดังนั้น luteolin น่าจะมีประสิทธิภาพ
ในการใช้เพื่อเป็นสารต้านมะเร็งท่อน้ำดี

คำสำคัญ: ลูทีโอลิน, มะเร็งท่อน้ำดี, อะพอพโทซิส, วิถีไมโตคอนเดรีย, คาสเปส

Introduction

Cholangiocarcinoma (CCA) or bile duct cancer is an epithelial cell malignancy arising from varying locations within the biliary tree. The most contemporary classification based on anatomical location includes intrahepatic, perihilar, and distal CCA.[1] The incidence and mortality rates from this disease markedly increase worldwide. CCA is highly prevalent in Thailand, with the highest incidence in the northeast region.[2] At the late stage of CCA, patients tend to be resistant to chemotherapy.[3] Therefore, it is necessary to explore alternative treatment options for treatment of CCA patients. Programmed cell death, or apoptosis, plays a crucial role in the elimination of unwanted cells without eliciting an inflammatory response to maintain tissue homeostasis.[4] The ability of cancer cells to resist toward apoptosis is a hallmark of most cancer types.[5] Therefore, apoptotic induction is the important mechanism for cancer suppression.

Luteolin, 3′,4′,5,7-tetrahydroxyflavone, belongs to a group of flavonoids. It is naturally found in vegetables and fruits such as celery, parsley, broccoli, onion leaves, carrots, peppers, cabbages, apple skins, and chrysanthemum flowers.[6] Luteolin has been shown to exhibit several biological activities such as antioxidant, anti-inflammatory, antimicrobial and anticancer.[7] The anticancer activities of luteolin are mediated through various mechanisms including inhibition of cancer cell proliferation, induction of apoptosis, anti-angiogenesis, and anti-metastasis.[6] Although several studies have elucidated significant anticancer effect of luteolin against various types of cancer, its effect against CCA remains to be fulfilled. In this study, the apoptosis-inducing activity of luteolin on human CCA (KKU-100) cells and the underlying molecular mechanism responsible for its effects were investigated.

Materials and methods

Chemicals and reagents

Luteolin, sulphorhodamine B (SRB), acridine orange, ethidium bromide, propidium iodide (PI) were obtained from Sigma Chemical (St. Louis, MO, USA). 5,5′,6,′-Tetrachloro-1,1′,3,3′- tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) was obtained from Clayman Chemical (Ann Arbor, MI, USA). BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit II was purchased from BD Biosciences (San Jose, CA, USA). Caspase-9 substrate I (Ac-LEHD-AFC) was purchased from Calbiochem (EMD Millipore, Billerica, MA, USA).

Cell culture

A human CCA cell line derived from human tumor tissue with poorly differentiated adenocarcinoma, KKU-100, was kindly provided by Prof. Banchob Sripa, Department of Pathology, Faculty of Medicine, Khon Kaen University. Cells were grown in Ham's F12 medium supplemented with sodium bicarbonate, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; pH 7.3), 100 U/mL penicillin, 100 µg/mL gentamycin sulphate, and 10% fetal calf serum and maintained under 5% CO_2 in air at 37 °C. The cells were subcultured every 3 days using 0.25 % trypsin–EDTA.

Cytotoxicity assay

The cytotoxicity of luteolin on CCA cells was examined by the SRB assay. Briefly, KKU-100 cells were seeded onto 96-well culture plates at the density of 7,500 cells/well in 100 µL of Ham's F12 media and left overnight. After exposure of cultured cells to luteolin at various concentrations for 48 h, the culture medium was removed. Cultured cells were fixed with ice-cold 10% trichloroacetic acid for 1 h at 4 °C, and subsequently stained with 0.4% SRB in 1% acetic acid for 30 min. Excess dye was removed by rinsing several times with 1% acetic acid, and protein-bound dye was dissolved with 10 mM Tris base solution for determination of absorbance with microplate reader with a filter wavelength of 570 nm. Cell cytotoxicity was expressed in term of percentage of untreated control absorbance following subtraction of mean background absorbance. The 50% inhibitory concentration (IC_{50}) concentration was calculated from the dose-response curves.

Acridine orange/ Ethidium bromide (AO/EB) staining

AO/EB staining was used to determine apoptotic and necrotic cells. Briefly, KKU-100 cells were seeded onto 96-well culture plates at the density of 7,500 cells/well and allowed to attach overnight. On the next day, the medium was removed and the cells were treated with various concentrations of luteolin for 48 h. At the end of the treatment, cultured cells were rinsed with PBS and stained with AO and EB (each 1 μg/mL). The fluorescent images were captured using a Nikon Eclipse TS100 inverted microscope with excitation and long-pass emission filters of 480 and 535 nm, respectively. The numbers of viable, early apoptotic, late apoptotic and necrotic cells were enumerated and calculated as the percent cells over a total number of cells in the same area.

Detection of apoptosis using flow cytometry

KKU-100 cells were seeded in six-well plate at the density of 2.5×10^5 cells and allowed to attach overnight. Cells were incubated with 5, 10, and 25 μM luteolin for 48 h. Thereafter, the cells were washed twice with cold PBS and added with Annexin V–FITC and PI dye. Cells were incubated for 15 min at room temperature and then analyzed by flow cytometer (BD Biosciences, San Jose, CA). The percentage of cells in the different stages was evaluated using BD FACSDiva™ Software (BD Biosciences, San Jose, CA).

Determination of mitochondrial transmembrane potential

The assay of mitochondrial transmembrane potential ($\Delta\Psi_m$) was performed by using the cationic, lipophilic dye, JC-1 staining. In Brief, KKU-100 cells were seeded at the density of 7.5×10^3 cells and left overnight. After treatment with 25 μM luteolin for 24 h, fluorescent signals were read using the Gemini XPS fluorescent plate reader with the excitation and emission wavelengths of 485 and 538 nm, respectively. Images were captured using a Nikon Eclipse TS100 inverted microscope. JC-1 forms J-aggregates in healthy mitochondrial matrix, which can be visualized as red fluorescence. In depolarized mitochondria, JC-1 effluxes to the cytoplasm and exists as monomers with green fluorescence. The shift of red to green fluorescence is an indicative of depolarization of $\Delta\Psi_m$.

Determination of caspase - 9 activity

After treatment with luteolin for 12 h, the cultured cells were trypsinized, washed in PBS, and adjusted to 10^6 cells for each reaction. Cell pellet was lysed with cell lysis buffer on ice for 10 min and then centrifuged. The supernatant was transferred to individual black microplate wells. The sample in each well was mixed with reaction buffer (containing 10% glycerol, 0.5 mM EDTA, 20 mM HEPES (pH 7.0), and DTT 10 mM) and fluorogenic Ac-LEHD-AFC, a caspase-9 substrate (50 μM). Reaction mixtures were incubated for 4 h at 37°C in dark and fluorescent signals were read using the Gemini XPS fluorescent plate reader with the excitation and emission wavelengths of 355 and 538 nm, respectively.

Statistical analysis

Statistical comparison between the control and treatment groups was performed using Student's t-test. The level of significance was set at $p < 0.05$.

Results

Cytotoxic effect of luteolin on CCA cells

The results showed that luteolin exerted potent cytotoxic effect on KKU-100 cells in a dose-dependent manner with the IC_{50} value of 6.8 ± 1.2 μM (Figure 1).

Luteolin induced CCA cell apoptosis

The results showed that the relatively large proportion of cells underwent apoptosis after luteolin treatment in a dose-dependent manner (Figure 2).

To further confirm the apoptotic-inducing effect of luteolin on CCA cells, the cells were stained with annexin V-FITC and PI, followed by flow cytometry analysis. A representative result of flow cytometry is presented in Figure 3. The results showed that the numbers of apoptotic cells were dramatically increased after treatment with 25 μM of luteolin for 48 h.

Luteolin induced depolarization of mitochondrial transmembrane potential ($\Delta\Psi_m$)

A decrease in J-aggregated form/ monomer form ratio is indicative of depolarized cells. The results are shown in Figure 4. The number of cells with depolarized mitochondrial membranes was found to significantly increase in luteolin-treated cells.

Luteolin increased caspase-9 activity in CCA cells

The results demonstrated that caspase-9 activity was significantly increased in KKU-100 cells after luteolin treatment (Figure 5).
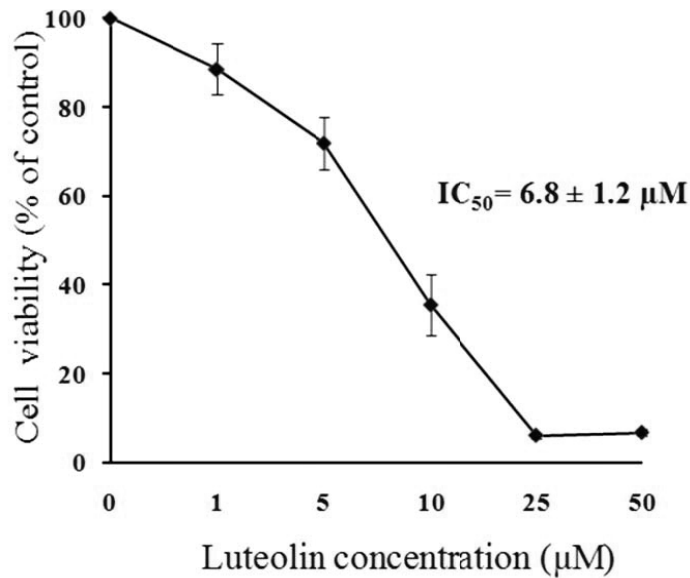
Figure 1. Cytotoxic effect of luteolin on CCA cells. KKU-100 cells were treated with various concentrations of luteolin for 48 h. Cells were analyzed for the cytotoxicity using SRB assay. The results are presented as percentage of control. Each value represents the mean ± SEM of three independent experiments.
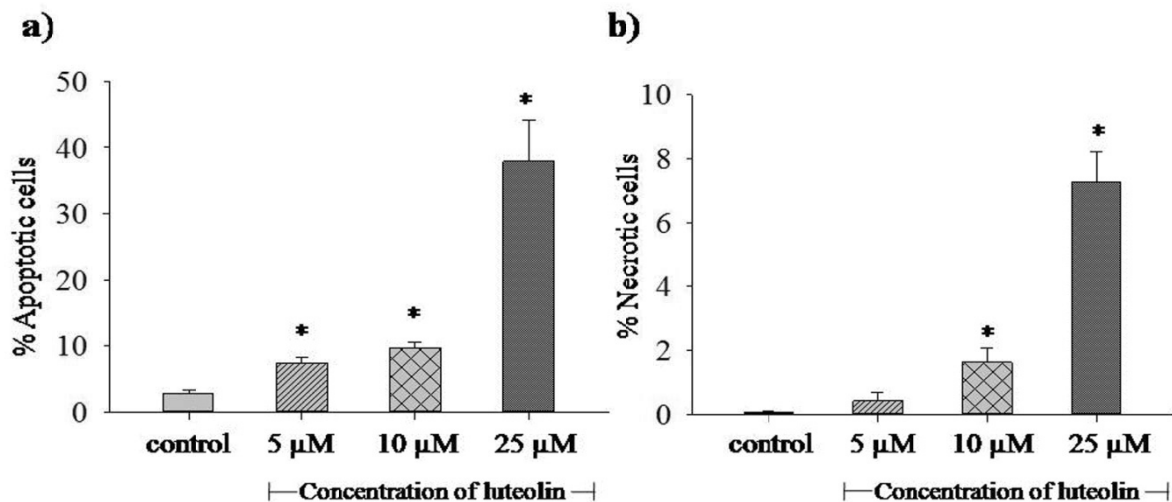

Figure 2. Luteolin induced apoptosis and necrosis of CCA cells assessed by AO/EB staining. KKU-100 cells were treated with various concentrations of luteolin (0-25µM) for 48 h. The number of apoptotic and necrotic cells were counted. The data are expressed as percentage of apoptotic **(a)** and necrotic cells **(b)** over total number of cells in the same area. The data are the mean ± SEM of three independent experiments. *$p < 0.05$ vs control group
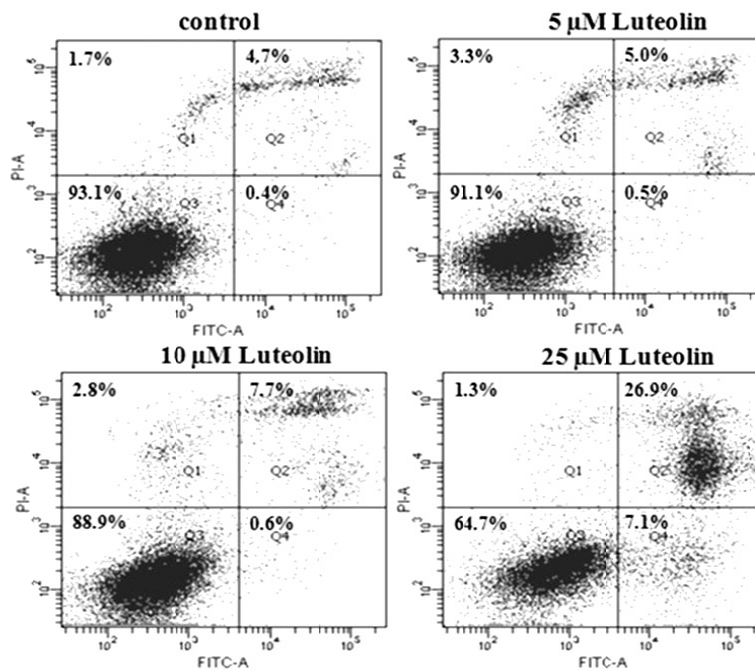
Figure 3. Luteolin induced apoptosis of CCA cells determined by flow cytometry using annexin V-FITC/PI staining. KKU-100 cells were treated with various concentrations of luteolin 0-25 μM for 48 h. Representative profiles from two independent experiments are shown and the percentage of cells in each phase are shown within the quadrant. The lower right quadrant (Q4) depicts the percentage of early apoptotic cells (annexin V-FITC-stained cells) and the upper right quadrant (Q2) represents the percentage of late apoptotic/necrotic cells (annexin V-FITC- and PI-stained cells)
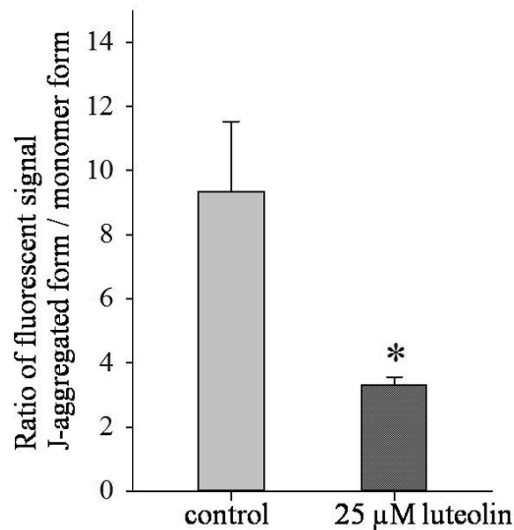
Figure 4. Luteolin induced the loss of mitochondrial transmembrane potential. KKU-100 cells were seeded at the density of 7.5×10^3 cells per well and incubated with 25 μM luteolin for 24 h. The fluorescent signals were read using the Gemini XPS fluorescent plate reader with the excitation and emission wavelengths of 485 and 538 nm, respectively. The results are mean ± SEM of three independent experiments. *$p < 0.05$ vs control group.
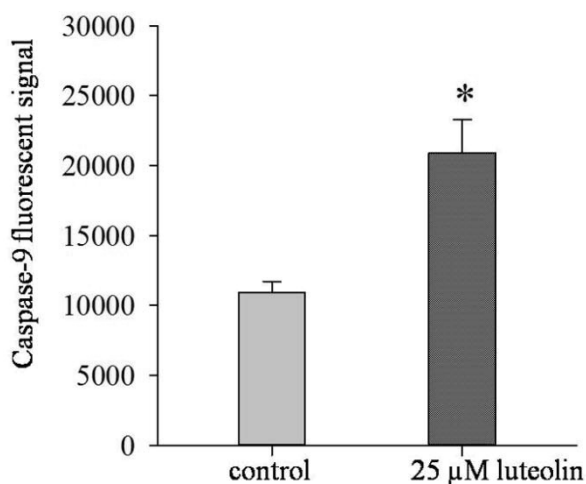
Figure 5. Luteolin induced activation of caspase-9. KKU-100 cells were treated with 25 μM luteolin for 12 h. The total cell lysates were assayed for caspase activity using specific fluorogenic substrate. Each bar represents the mean ± SEM of three experiments. *$p<0.05$ vs control

Discussion

In the present study, we have investigated the apoptotic-inducing effect of luteolin against human CCA cells. The effect of luteolin-induced apoptosis has been previously reported in several cancer cells such as oral squamous cancer cells[8], neuroblastoma brain tumor cells[9], non-small cell lung cancer cells.[10] Consistently, luteolin significantly induced apoptosis of CCA KKU-100 cells.

Mitochondrial transmembrane potential gives a valuable indicator of cells' health and functional status.[11] Once permeability transition pores in the mitochondrial inner membrane opened, it can cause mitochondrial depolarization, resulting in ATP depletion and cell death.[12] In the present study, it has been found that luteolin could induce the loss of mitochondrial membrane integrity of CCA cells. In addition, the activity of caspase-9, which is an important enzyme involved in the intrinsic pathway of apoptosis induction, was significantly increased in CCA cells after luteolin exposure. These results reveal that luteolin-induced CCA cell apoptosis is partly mediated via caspase-dependent mitochondrial signaling pathway. Therefore, luteolin could be a potential chemotherapeutic agent for CCA treatment. Further research must be carried out to fully investigate its mechanism of actions.

Conclusion

Luteolin exhibits apoptotic-inducing effect against human CCA (KKU-100) cells which is mediated partially by intrinsic mitochondrial pathway. This may provide a new therapeutic agent in the treatment of CCA.

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F16 Optimization of Microcarrier Cell Culture for Free Radical Detection in Microglia Cell Line

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Abstract

Microcarrier cell culture is an effective technique of cell culturing that provides cell attach on a spherical surface and produces higher yield than conventional or monolayer cell culture. This study aims to optimize microcarrier conditions, including cell culture vessel, cell density and microcarrier beads concentration, for a microglia cell line, Highly Aggressive Proliferating Immortalized (HAPI) cells. Activation of microglia leads to production of various cytokines and free radicals that involves in pathogenesis of neuroinflammatory diseases such as Alzheimer's disease. The results showed that cell viability on microcarrier beads was higher up to 2 fold in cell culturing condition with silicone-coated glass petri dish than 6-well plate. The optimum cell culture condition was 3.0×10^5 cells/mL seeding on 4 mg/ml microcarrier beads at 24 hr incubation period. Cell morphology as observed by SEM and inverted microscope demonstrated the monolayer with normal morphology on microcarrier surface. This microcarrier culture condition will be applied to study real-time detection of free radical production in the intact microglia cells.

Keywords: microcarrier cell culture, microcarrier beads, HAPI cell

การศึกษาความเหมาะสมของการเลี้ยงเซลล์บน microcarrier สำหรับการตรวจหาอนุมูลอิสระในเซลล์ไมโครเกลีย

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บทคัดย่อ

เทคนิคการเลี้ยงเซลล์บน microcarrier เป็นเทคนิคที่มีประสิทธิภาพอย่างยิ่งต่อการเลี้ยงเซลล์ชนิดยึดเกาะ โดยสามารถเลี้ยงเซลล์ให้ยึดเกาะบนพื้นผิววัตถุทรงกลมหรือเม็ด microcarrier ได้ ซึ่งเป็นการเพิ่มพื้นที่ผิวการยึดเกาะจึงสามารถเพิ่มจำนวนเซลล์ได้มากกว่าการเลี้ยงเซลล์แบบวิธีดั้งเดิม อีกทั้งการเลี้ยงเซลล์ด้วยเทคนิคนี้ยังสามารถใช้ในการศึกษารูปร่างและกิจกรรมต่าง ๆของเซลล์ในขณะที่เซลล์ยังมีชีวิตอยู่ได้อีกด้วย การศึกษาครั้งนี้ได้ใช้เซลล์ไมโครเกลียของหนู

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ได้แก่ Highly Aggressive Proliferating Immortalized cells หรือ HAPI โดยเซลล์ชนิดนี้จะหลั่งไซโตไคน์และอนุมูลอิสระเมื่อ
ถูกกระตุ้น ซึ่งเกี่ยวข้องกับการเกิดโรคความเสื่อมทางระบบประสาท เช่น อัลไซเมอร์ เป็นต้น จากผลการวิจัยพบว่า การเลี้ยง
เซลล์ในจานแก้วเพาะเชื้อที่เคลือบผิวด้วยซิลิโคน สามารถเพิ่มจำนวนเซลล์มีชีวิตได้มากกว่าการเลี้ยงในภาชนะพลาสติก
หกหลุมที่เคลือบผิวเช่นเดียวกันได้ถึงสองเท่า โดยปริมาณความหนาแน่นของเซลล์ที่เหมาะสมคือ $3x10^5$ เซลล์ต่อมิลลิลิตร
ปริมาณความเข้มข้นของเม็ด microcarrier ที่ 4 มิลลิกรัมต่อมิลลิลิตร และใช้เวลาการเลี้ยง 24 ชั่วโมง นอกจากนี้ยังได้ศึกษา
รูปร่างของเซลล์ที่ยึดเกาะบนเม็ด microcarrier โดยใช้กล้องจุลทรรศน์ชนิดหัวกลับและกล้องจุลทรรศน์อิเล็กตรอนชนิดส่องกราด
พบว่าการเลี้ยงเซลล์ที่ 24 ชั่วโมง ไม่ทำให้เซลล์มีการเปลี่ยนแปลงรูปร่างไปจากการเลี้ยงเซลล์แบบวิธีดั้งเดิม ดังนั้นในการศึกษา
วิจัยครั้งนี้จึงสามารถหาความเหมาะสมของการเลี้ยงเซลล์บนเม็ด microcarrier และสามารถนำไปใช้ในการหาอนุมูลอิสระในเซลล์
ขณะมีชีวิตได้ในลำดับต่อไป

คำสำคัญ: เทคนิคการเลี้ยงเซลล์บน microcarrier, เม็ด microcarrier, เซลล์ HAPI

Introduction

Microglia cells are mononuclear phagocytic cells that play a role in first line of defense in central nervous system (CNS). Activation of microglia cells relates to production of inflammatory cytokine including TNF-α, IL-1β and reactive oxygen/nitrogen species (ROS/RNS). Since ROS/RNS plays a key role in pathogenesis of neurodegenerative disorders, the detection of ROS/RNS is crucial for studying in brain injury such as Alzheimer's disease and Parkinson's disease.[1-2]

Highly Aggressive Proliferating Immortalized cells (HAPI) are immortalized, high proliferation and non-genetically modified microglia cell line that was developed for *in vitro* study of neuroinflammatory disease. The characterizations of HAPI cells were described previously by Cheepsunthorn *et al.*[3] and Horvath *et al.*[4]

Microcarrier cell culture is a technique of cell culturing on spherical shape called "microcarrier beads". This technique promotes anchorage-dependent or intact cells to grow in suspension by attach on microcarrier beads that increase higher yield of cell line culture and usually use in the industry for biological material such as vaccine, vector and monoclonal antibody. Moreover they become a technique for vital cell study in term of structure, growth, and differentiation.[5-6] Microcarrier beads which widely use for a wide variety of attachment cell line is Cytodex-1™ which contain positively charge of N,N-diethylaminoethyl (DEAE) that optimal for cell attach and growth but the beads can stick on the glass surface when culturing in a glass vessel, therefore, silicone-coated surface is recommended to solve this problem. Moreover the siliconized coating surface shows hydrophobic property that is not suitable for cell attaching on the wall of cell culture vessel.[7]

In previous study, Suntivich[8] has cultured HAPI cells on microcarrier beads and has demonstrated that this technique was applicable for detection of free radical induced by Fenton-like reaction. Therefore, this study aims to optimize the microcarrier culture condition including cell culture vessel, cell density and microcarrier beads concentration. The optimum condition should increase cell viability on microcarrier beads and, thus, increase the sensitivity for studying real time free radical detection in vital cells.

Materials and Methods

Microcarrier preparation

Cytodex-1™ microcarrier beads (GE Healthcare, Uppsala, Sweden) were hydrated in Ca^{2+}, Mg^{2+}-free 1X PBS (50-100 ml/g cytodex-1™) for 24 hr at room temperature with gentle agitation. Hydrated microcarrier beads were then sterilized by autoclaved 115°C for 15 min, 15 psi and resuspended in Dulbecco's modified Eagle's medium-low glucose (DMEM) without phenol red (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2.5% heat-inactivated fetal bovine serum (FBS, PAA Lab, Queensland, Australia).

Microcarrier cells culture

HAPI cells were kindly provided by Prof. James R. Conor (Department of Neuroscience and Anatomy, Hershey, Medical Center, Hershey, PA). The cells were plated either in silicone-coated 6-well plate or 90 mm glass petri dish with sigmacote® (Sigma-Aldrich, St. Louis, MO, USA) at various density between $2-5x10^5$ cells/mL and 0.5, 1, 2 and 4 mg/ml cytodex-1™. Cell density was determined by counting cell with a hemocytometer.

Cell viability testing on microcarrier cell culture

Cell viability was determined using Presto blue reagent (Invitrogen Corporation, San Diego, USA). HAPI cells-attached-microcarrier beads were gentle resuspended, transferred 100 µl to 96-well plate.

The 10 µl of presto blue reagent was added and incubated for 1 hr at 37 ºC under 5% CO_2 and 95% air. The absorbance was measured at 562 and 630 nm via aicroplate reader (Bio-Tek Instruments, Winooski, VT, USA).

Cell morphology

HAPI cells-attached-microcarrier beads were observed at 24, 48 and 72 hr under inverted microscope (Nikon Eclipse TE2000-s, USA).

Scanning electron microscopy (SEM)

HAPI cells-attached-microcarrier beads were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2-7.4 at 4ºC overnight and then washed with 0.1 M sodium cacodylate buffer at 4ºC, 15 min for 3 times. The sample was dehydrated with series of ethanol (30% - 100%) at 4ºC, 15 min for 2 times. The sample then was dried by Hitachi HCP-2 critical point dryer (Hitachi Ltd., Japan) and coated with platinum/palladium for 4 min and observed with a Hitachi S2500 scanning electron microscope (Hitachi Ltd., Japan).

Statistical analysis

The data were presented as mean ± SEM. The statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Bonferroni's test using Graphpad prism version 5 (GraphPad Prism software, San Diego, CA, USA) and p-values of $p < 0.05$ were considered as significance.

Results

Optimization of microcarrier cell culture

In order to obtain the maximum yield of microglia attached on microcarrier beads, the surface type and ratio of cells to microcarrier were evaluated.

On silicone-coated 6-well plate, HAPI cells were seeded at cell density of 3×10^5 cells/ml (6.23×10^4 cells/cm^2 bottom area) with various concentrations of microcarrier beads (0.5, 1.0, 2.0 and 4.0 mg/ml). Cell viability on microcarrier beads was measured at 24, 48 and 72 hr. The results showed that the maximum value of cell viability ($37.3 \pm 0.6\%$) was observed in the culture with 4.0 mg/ml cyodex-1^{TM} (Figure 1).

On silicone-coated glass petri dish, HAPI cells were seeded with various cells density (2×10^5, 3×10^5, 3.5×10^5, 4×10^5 and 5×10^5 cells/ml) and with difference concentrations of microcarrier beads (0.5, 1.0, 2.0 and 4.0 mg/ml). Cell viability on microcarrier beads were measured at 24, 48 and 72 hr and the results were shown in Figure 2 and Table 1.
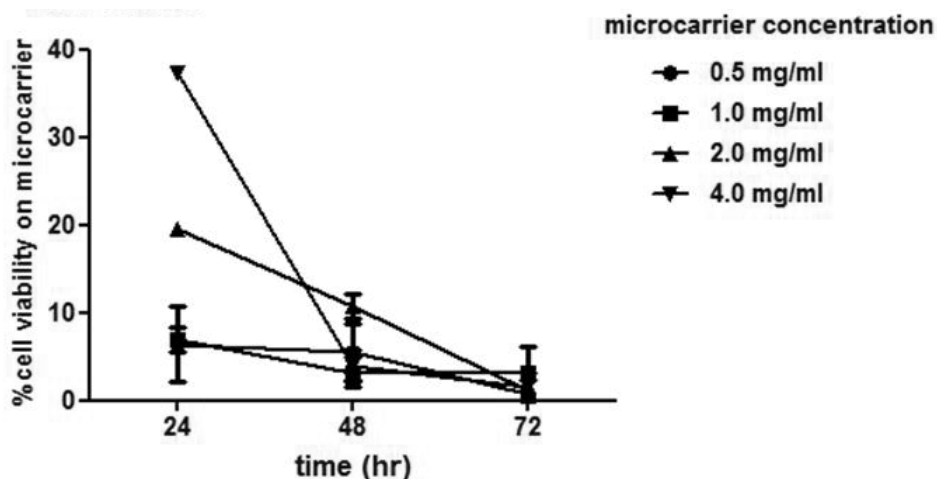
Figure 1.   HAPI cells at density of 3×10^5 cells/ml were seed on difference concentration of microcarrier beads (0.5, 1.0, 2.0 and 4.0 mg/ml). Cell viability was measured at 24, 48 and 72 hr. Data were presented as mean ± SEM (n=3)

The maximum value of cell viability on microcarrier beads was observed in the culture with 4.0 mg/ml cyodex-1[TM] at all cell density condition. The glass petri dish showed a better yield of microglia attached on microcarrier than that of 6-well plate. At cell density of $3x10^5$ cells/ml that were seeded on 4 mg/ml cytodex-1[TM] incubated for 24 hr showed significantly increased in cell yield comparing to all cell density conditions except $3.5x10^5$ cells/ml (Figure 2f).

HAPI cells morphology attached on microcarrier beads

Morphology of HAPI cells attached on microcarrier was observed using inverted microscope (Figure 3) and scanning electron microscope (Figure 4). The results demonstrated the monolayer HAPI cells on the microcarrier beads. The microcarrier culture did not affect cell morphology. The cells were round with no or less process, present bright refringency and small-dark nucleus. However, increasing incubation time showed cell contraction-attached on microcarrier beads with dark refringency and % cell viability also decreased.
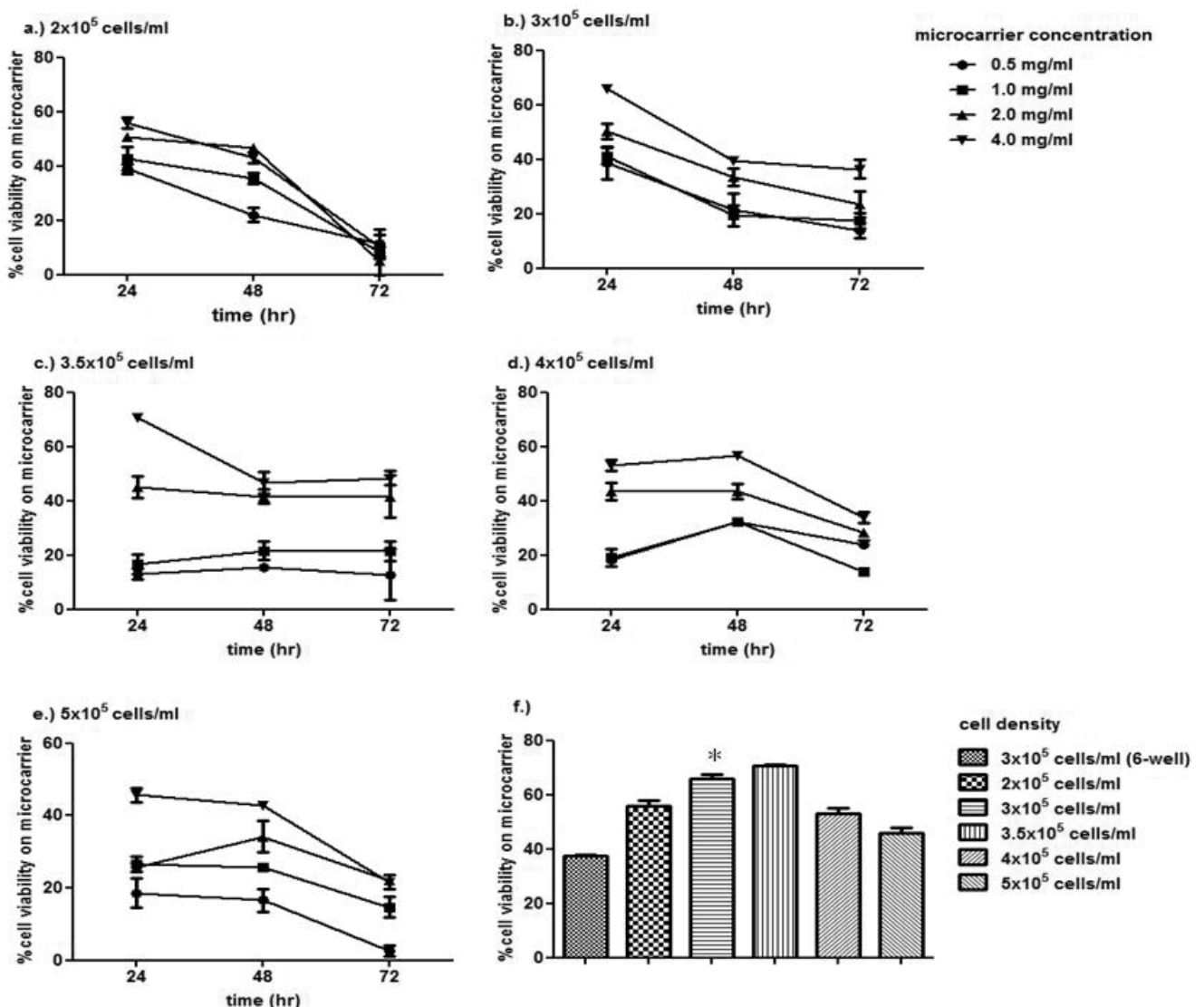
Figure 2. HAPI cells were plated in silicone-coated glass at density of $2x10^5$(a.), $3x10^5$(b.), $3.5x10^5$(c.), $4x10^5$(d.) and $5x10^5$(e.) cells/ml on difference concentrations of microcarrier beads (0.5, 1.0, 2.0 and 4.0 mg/ml). Cell viability was measured at 24, 48 and 72 hr. Data were compared between cells that were plated in 6-well plate and glass petri dish at 24 hr incubation time (f.). Data were presented as mean ± SEM (n=3). *$p<0.05$ when compared to other conditions.

Table 1. Cell viability on microcarrier beads which were placed on 4.0 mg/ml cytodex-1 in glass petri dish at 24 hr. (p<0.05)

Cells density		%cell viability
cells/ml	cells/cm^2	on microcarrier beads (mean ± SEM)
2x10^5	1.5x10^4	55.7 ± 2.0
3x10^5	5.6x10^4	66.0 ± 1.3
3.5x10^5	6.6x10^4	70.7 ± 0.4
4x10^5	7.5x10^4	52.9 ± 2.1
5x10^5	9.4x10^4	45.7 ± 1.9

Figure 3. Microscopic observations of HAPI cells which were plated in monolayer cell culture method (a.) and seeded on microcarrier beads at 24 hr (b.), 48 hr (c.) and 72 hr (d.).

380μm
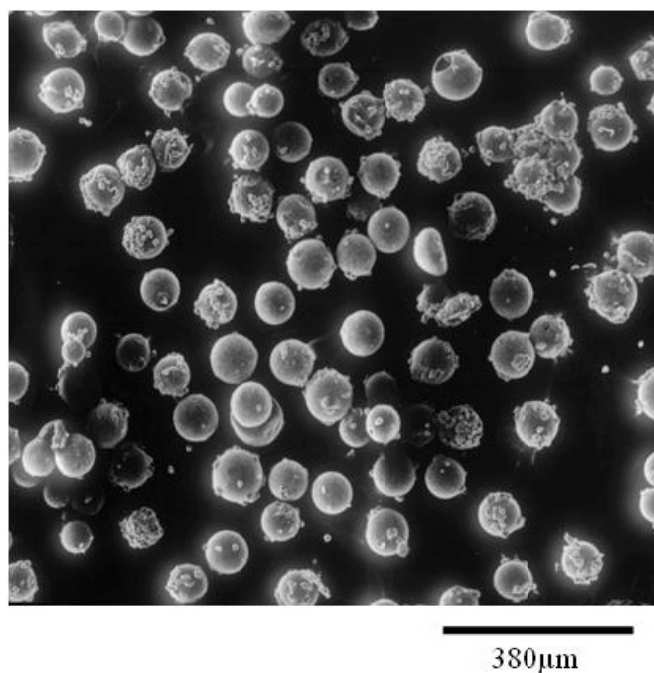
Figure 4. Scanning electron microscopy of HAPI cell-attached on microcarrier beads

Discussions

Our results demonstrated that silicone-coated glass petri dish was suitable for microcarrier cell culture that yielded nearly 2 fold of cell viability, compared with silicone-coated 6-well plate at the same cell density. The siliconized with sigmacote® on the glass may cause hydrophobic surface that not suitable for cell attachment, therefore they promote cell attach on microcarrier beads more than a bottom of culture vessel.

In monolayer or conventional cell culture, HAPI cell morphology is normally round with no or less process, present bright refringency and small-dark nucleus. HAPI cell is sensitive in morphological change when activate with stimuli substance including the percentage of fetal bovine serum (% FBS). Decreasing from 10% FBS-DMEM or 5% FBS with low sugar-DMEM, HAPI cells showed more process and phagocytosis.[3-9] However, phase contrast and SEM-images indicated that HAPI cells conserved their normal morphology under the condition of cell culture medium supplemented with 2.5% FBS. We estimated that within 24 hr the cells covered about 70% of microcarrier surface. The longer incubation period may cause the overgrowth of the cells on the surface that indicated by increasing dark refringency cells and decreasing of cell viability.

Conclusion

This study, the microcarrier cell culture condition for a microglia cell line was optimized. The culture condition was cell density of $3x10^5$ cells/mL and 4.0 mg/ml cyodex-1 and incubated for 24 hr on silicone-coated glass petri dish. With this condition the cells still maintained their morphological characterization. This culture condition will further apply for real time free radical detection in vital cells.

Acknowledgements

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F17 Identification of ERK1/2- Mediated Autophagy Induction: a Mechanism Underlies the Neuroprotective Effect of Quercetin

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Abstract

Quercetin is a ubiquitous flavonoid in fruits and vegetables. In addition to antioxidant, it possesses wide range of activities, including anti-inflammatory, anti-bacterial, anti-diabetics, anti-cancer, lipid lowering, and recently, autophagy induction. Autophagy induction is linked to the neuroprotective effect of quercetin. ERK1/2 is a vital pathway that governs various cellular processes, including, cell survival and proliferation. ERK1/2 activation is also observed in quercetin treatment; thus, we aim to investigate whether this pathway plays active role in autophagy induction, SH-SY5Y cells were used in this study. Quercetin at 0.1-5 µM did not affect the cell viability, in which, the cell viability is between 99 – 107 percent of the control. This concentration range was subsequently used for the experiments. PD184352, a MEK1/2 inhibitor, was used to study the effect of ERK1/2 inhibition. Treatment with PD184352 reduced the cell viability in a dose-dependent manner - with 70% of the control at 0.25 µM. At this concentration, suppression of phospho-ERK1/2 occurred at 50%. Treatment of the cells with 2 µM of quercetin after exposure to 0.25 µM of PD184352 restored the viability of cells to 88%. Interestingly, ERK1/2 inhibition by PD184352 led to inhibition of autophagy, demonstrated by the reduction of LC3-II expression, and quercetin treatment can significantly attenuated the effect of PD184352. Our results suggested that the ERK1/2 activation cascade is upstream of autophagy induction, and that the neuroprotective effect of quercetin mediates through this axis. ERK1/2 could be the hub for quercetin to exert diverse biological effects.

Keywords: quercetin, autophagy, ERK1/2 MAPK, neuroprotective effect

การศึกษาวิถี ERK1/2 กับการเหนี่ยวนำกระบวนการกินตัวเอง: กลไกภายใต้ฤทธิ์ปกป้องเซลล์ประสาทของเควอซิติน

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บทคัดย่อ

เควอซิติน (quercetin) เป็นสารในกลุ่มฟลาโวนอยด์พบได้ในผักและผลไม้ นอกจากจะเป็นสารต้านอนุมูลอิสระแล้ว ยังมีฤทธิ์ด้านการอักเสบ ต้านจุลชีพ ต้านเบาหวาน ต้านมะเร็ง ลดระดับไขมันในเลือด และ ล่าสุด สามารถกระตุ้นกระบวนการกินตัวเอง (autophagy) จากการศึกษาที่ผ่านมาพบว่าการเหนี่ยวนำให้เกิดกระบวนการกินตัวเองสัมพันธ์กับฤทธิ์ปกป้องเซลล์ประสาท (neuroprotective effect) ของเควอซิติน วิถี ERK1/2 เป็นวิถีถ่ายทอดสัญญาณที่สำคัญกับการอยู่รอดและการแบ่งตัวของเซลล์มี

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รายงานว่าวิถี นี้ถูกกระตุ้นให้ทำงานด้วยเควอซิติน ซึ่งน่าสนใจว่าวิถี ERK1/2 มีความสัมพันธ์กับฤทธิ์เหนี่ยวนำกระบวนการกิน ตัวเองของเควอซิตินหรือไม่ และนี่คือจุดประสงค์หลักของงานวิจัยนี้ซึ่งทำการทดลองในเซลล์เพาะเลี้ยง SH-SY5Y จากการทดลอง พบว่าสารเควอซิตินในความเข้มข้นตั้งแต่ 0.1-5 μM ไม่เป็นพิษต่อเซลล์ โดยเซลล์รอดชีวิตร้อยละเก้าสิบเก้าถึง ร้อยเจ็ดของกลุ่ม ควบคุม งานวิจัยนี้ใช้สาร PD184352 ยับยั้งเอนไซม์ MEK1/2 เอนไซม์นี้ออกฤทธิ์กระตุ้นการทำงานของ ERK1/2 พบว่าเมื่อเซลล์ ได้รับ PD184352 การรอดชีวิตของเซลล์ลดลง โดยอัตราการรอดชีวิตสัมพันธ์กับปริมาณความเข้มข้นของสาร PD184352 ที่ความ เข้มข้น 0.25 μM การรอดของเซลล์ลดเหลือร้อยละเจ็ดสิบของกลุ่มควบคุม และการทำงานของ ERK 2/1 ลดเหลือร้อยละห้าสิบ ซึ่งดูได้จากการลดลงของระดับการแสดงออกของ phospho-ERK1/2 เมื่อเซลล์ได้รับเควอซิตินในขนาดความเข้มข้น 2 μM เซลล์ รอดชีวิตเพิ่มขึ้นเป็นร้อยละแปดสิบแปด การยับยั้งการทำงานของ ERK1/2 ส่งผลต่อไปถึงการยับยั้งกระบวนการกินตัวเองโดย พบว่าโปรตีน LC3-II ซึ่งเป็นตัวบ่งชี้สถานะของกระบวนการกินตัวเองลดลง และพบว่าสารเควอซิตินสามารถเพิ่มระดับการ แสดงออกของโปรตีน phospho-ERK 2/1และลดพิษต่อเซลล์ของ PD184352 ลงได้ ผลการทดลองนี้ชี้ให้เห็นว่าเควอซิตินออก ฤทธิ์ปกป้องเซลล์ประสาทผ่านวิถี ERK1/2 และชักนำให้เกิดกระบวนการ autophagy โดยวิถีของ ERK1/2 อาจเป็นกุญแจสำคัญใน การอธิบายถึงฤทธิ์ทางชีววิทยาต่างๆ ของสารเควอซิติน ซึ่งต้องมีการศึกษาถึงกลไกในระดับโมเลกุลในเชิงลึกต่อไป

คำสำคัญ: เควอซิติน, autophagy, ERK1/2 MAPK, neuroprotective effect

Introduction

Quercetin is a ubiquitous natural flavonoid with potent anti-oxidative activity. It also exerts diverse biological effects including anti-bacterial, anti-inflammatory, neuroprotective, anti-apoptotic, lipid lowering, anti-diabetes, and anti-cancer activities.[1, 2] Recently, it was shown that quercetin induces autophagy in gastric carcinoma cells.[3] Neuroprotective effects of quercetin have been demonstrated in several models of Parkinson's disease (PD)[1,4,5] and could attribute to several mechanisms, such as, anti-oxidant, anti-inflammation, inhibition of excitotoxicity, and attenuation of toxic alpha-synuclein fibrillization.[6] Thus, quercetin and its derivatives have received high attention as potential effective therapeutic agents for neurodegenerative disorders. PD is the common neurodegenerative movement disorder that affects about 1% of the population older than 60 years.[7]

PD could either occur as sporadic or familial form, as a result of genetic mutations or exposure to environmental insults. Presence of intracytoplasmic inclusion bodies termed Lewy bodies (LB) and loss of dopaminergic neurons in substantia nigra pars compacta (SNC) are the pathological hallmarks of the disease.[7] LB is composed of abnormal protein aggregates, in which, α-synuclein protein is the major component.[8] Impaired cellular degradation of α-synuclein, either from genetic mutation or oxidative damage, leads to abnormal aggregation of the protein, in which cannot be removed by conventional ubiquitin-proteasome system (UPS).[9, 10]

Autophagy is the cellular self-digestion process that plays basic role in degradation and turnover of proteins and organelles. It can be induced upon exposure to environmental stress, leading to either cellular adaptation or cell death.[11]

So far, three types of autophagy have been identified; macroautophagy, microautophagy, and charperone-mediated autophagy (CMA). Several studies have linked autophagy to PD[12, 13], in which, macroautophagy is responsible for clearance of mutant or aggregated α-synuclein proteins.[14] In this context, autophagy serves as protective function and can be modulated for therapeutic purpose. Recent study identified the association between autophagy and ERK1/2 MAPK pathway by anatomical co-localization of phopho-ERK1/2 to mitochondria and autophagosome.[15] The ERK1/2 MAPK pathway is a sequential phosphorylation cascades that is activated by growth factor and cytokines and is implicated in diverse cellular functions.[16] Thus, ERK1/2 could be the platform that link autophagy with different signaling pathways and stimuli. Previous study in our laboratory reported that autophagy-mediated clearance of α-synuclein underlies the neuroprotective effect of quercetin (Kanyapreedakul T, 2014; unpublished). Interestingly, we observed that activation of ERK1/2 pathway occurs after quercetin treatment. Thus, this study aims to investigate whether autophagy induction effect of quercetin is mediated through ERK1/2 activation.

Materials and Methods

Cell culture

Human dopaminergic neuroblastoma SH-SY5Y cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in Eagle's minimum essential medium (MEM)/ Ham's F-12 (1:1), supplemented with 10% fetal bovine serum, 10 mg/ml pencillin, 10 U/ml streptomycin, 1

mM sodium pyruvate, and 1 mM non-essential amino acid. Cells were incubated in humidified 37 °C, 5% of CO_2 and 95% air. The cells were routinely subcultured using enzymatic digestion with 1.25XTryPLE.

MTT assay

SH-SY5Y cells were seeded on 96 well plates at density of 3×10^4 cells/well and grown for 24 hr. Cells were treated with various conditions according to the experimental designs. The MTT (3-(4,5-dimethythiazole-2-yl)-2,5-diphenyltetrazole bromide) reagent (1 mg/ml MTT in PBS) was added in each well and incubated at 37 °C for 3 hr. The solution was removed and the crystal formazan product was solubilized with 100 µl DMSO. The optical density was measured at 570/620 nm using a microplate reader (Bio-Tek instrument, Winooski, VT, USA).

Western blot analysis

SH-SY5Y cells were seeded on 6 well plates at a density of 9×10^5 cells/well for 24 hr. prior to the experiments. After the cells were treated with various conditions according to the experimental designs, the cells were washed and lysed with ice-cold RIPA lysis buffer. The lysates were centrifuged at 14,000 rpm for 20 minutes at 4^oC. Protein supernatant was collected and the concentration was determined with Lowry method. Thirty micrograms (30 µg) of protein were separated on 13.5% SDS-PAGE. The proteins from the gel were transferred to 0.45 µm PVDF membranes. Then the membranes were incubated with rabbit polyclonal antibody to LC3I/IIB (1:2000), p-ERK1/2 (1:1000) and mouse monoclonal antibody to β-actin (1:5000) at 4°C overnight. The membranes were washed and incubated with corresponding horse radish peroxidase (HRP)-conjugated secondary antibodies for one hour at room temperature. The protein bands were visualized by autoradiography using enhanced chemiluminescence (ECL plus™) and western blotting detection reagents. The intensity of bands was analyzed using Image J software (NIH, Bethesda, MD, USA). β-actin was used as internal control.

Statisical analysis

All values were expressed as mean±S.E.M. of at least three independent experiments in which triplicate samples were performed. Experimental data were analyzed using one-way ANOVA followed by Tukey comparison test using Graph Pad Prism 5 software. The statistical significance is indicated when p value is 0.05 or less.

Results

Effect of quercetin on the viability of SH-SY5Y cells

To determine the cytotoxicity of quercetin on SH-SY5Y cells, the cells were treated with various concentrations of quercetin ranging from 0.1 - 10 µM for 24 hr. As shown in Figure 1, quercetin at the concentration of 0.1-5 µM was not toxic to the cells. The percentages of cell viability were 98.91±0.57, 105.4±3.79, 106.2±3.09, 107±1.71, and 106.6±0.68% of control at 0.1, 0.5, 1, 2, and 5 µM of quercetin, respectively. While at 10 µM the viability of the cells was significantly decreased to 75.69±2.35% of control. The concentrations at the non-toxic range were used in the subsequent experiments.

Effect of PD184352 on viability of SH-SY5Y cells

To determine the effect of PD184352 on viability of SH-SY5Y cells, cells were treated with various concentrations of PD184352 ranging from 0.05 to 50 µM for 24 hr. PD184352 dose-dependently decreased the viability of cells. At 0.25 µM, the cell viability was reduced to 71.48±0.55% of control (Figure 2). This concentration was used in the subsequent experiments.

Quercetin attenuates the cytotoxic effect of MEK1/2 inhibitor

To determine the effect of quercetin on the cell viability after exposure to toxic dose of PD184352, SH-SY5Y cells were treated with PD184352 prior to treatment with quercetin. As shown in Figure 3, PD184352 at 0.25 µM decreased the cell viability to 70.93±1.11% of control. Treatment with quercetin increased the cell viability in a dose-dependent manner. At 2 µM of quercetin, the cell viability was significantly increased to 87.9±3.48% of control.
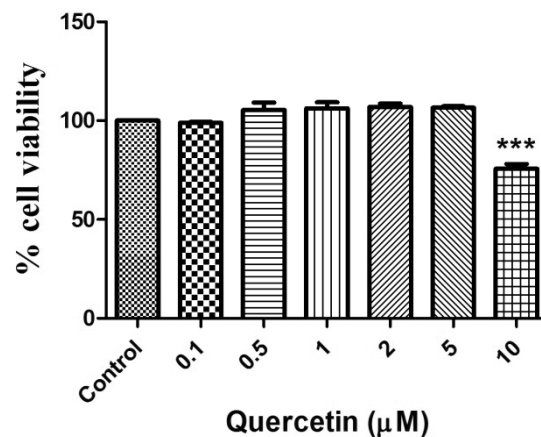
Figure 1. Effect of quercetin on the viability of SH-SY5Y cells. Cells were treated with various concentrations (0.1-10 μM) of quercetin for 24 hr. Cell viability was assessed using MTT assay and results were presented as the percentage of untreated control. *** $p < 0.001$ compared to the untreated control
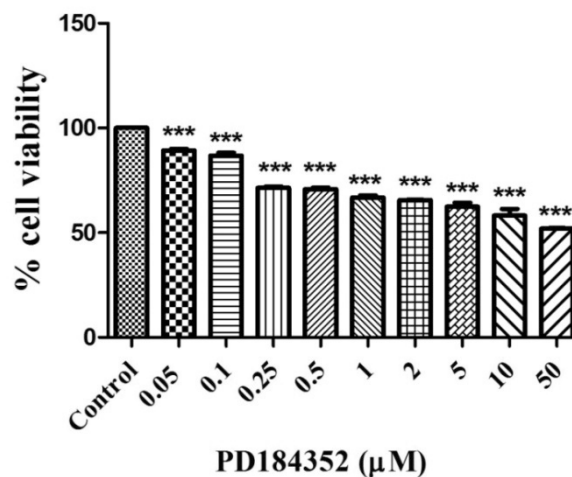

Figure 2. Effect of PD184352 on viability of SH-SY5Y cells. Cells were treated with various concentrations of PD184352 (0.05 to 50 μM) for 24 hr. Cell viability was assessed using MTT assay and results were expressed as the percentage of untreated control. *** $p < 0.001$ compared to the untreated control

Inhibition of phospho-ERK1/2 expression by PD184352

To determine the time-course effect of PD184352 on the expression of phospho-ERK1/2, cells were treated with PD184352 at the concentration of 0.25 μM for 15 min, 30 min, 1 hr, 3 hr. and 6 hr. It was found that the maximum inhibition of p-ERK1/2 expression was evident at 0.5 hr. or 30 min after the treatment (data not shown), therefore, this time-point of treatment with PD184352 was used in the subsequent experiment.

To determine the effect of PD184352 on the level of p-ERK1/2 expression, the cells were treated with various concentrations of PD184352 for 30 min. As shown in Figure 4, PD184352 dose-dependently inhibited the expression of p-ERK1/2. At 0.25 μM,

PD184352 significantly decreased the expression of p-ERK1/2 to 52.05±0.02% of control.

Effect of ERK1/2 inhibition and quercetin on LC3-II expression

To determine the effect of quercetin on the expression of p-ERK1/2 and LC-3-II proteins, the cells were treated with 0.25 μM PD184352 for 30 min prior to treatment with quercetin at 0.5, 1, and 2 μM for 1 hr before determination of p-ERK1/2 and LC3-II expression. β-actin was used as a reference. Inhibition of ERK1/2 activation with PD184352 resulted in reduction of LC3-II expression. On the contrary, treatment with quercetin attenuated the effect of PD184352 on p-ERK1/2 expression, causing increase in phospho-ERK1/2 and LC3-II expressions.
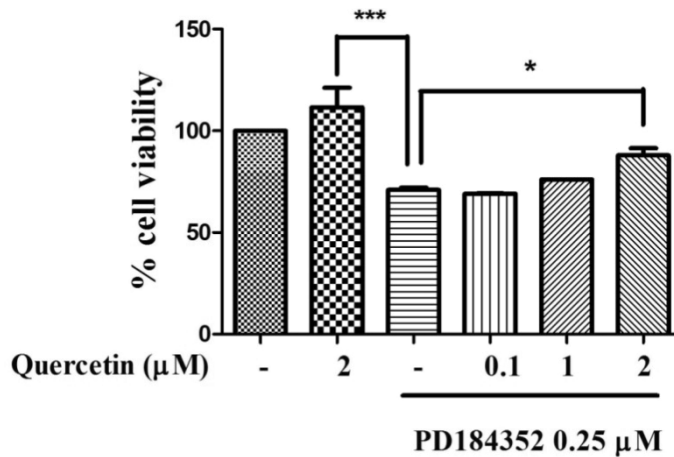
Figure 3. Effect of various doses of quercetin on cell viability after pretreated the cells with 0.25 µM PD184352 for 30 min before exposure to quercetin (0.1, 1 and 2 µM) for 24 hr. Cell viability was assessed by MTT assay. Results were presented as the percentage of untreated control. * p<0.05 compared to the group of PD184352 treated only, *** $p < 0.001$ compared between the PD184352 treated group and quercetin treated group.
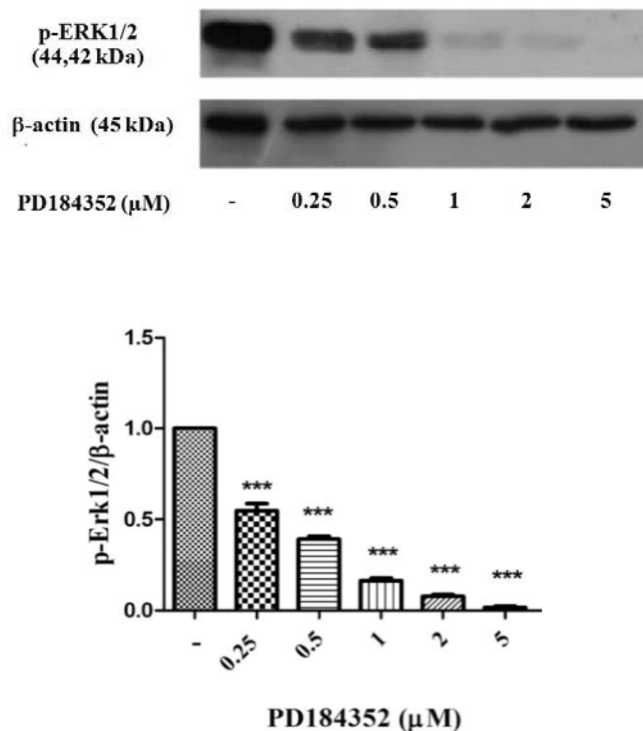

Figure 4. Expression of phospho-ERK1/2 after treatment with PD184352. Cells were treated with various concentrations of PD184352 (0.25 to 5 µM) for 30 min. Expression of phospho-ERK1/2 was determined by western blot analysis. β-actin was used as a reference. The results were expressed as the fold of untreated control. *** $p < 0.001$ compared to the untreated control.
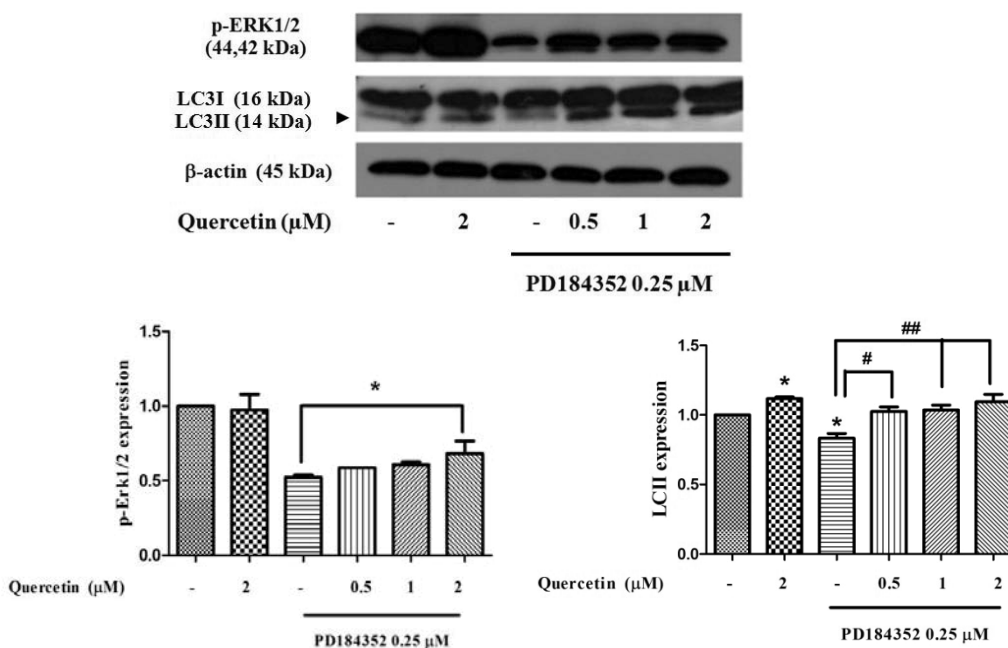
Figure 5. Expression of phospho-ERK1/2 and LC3-II in SH-SY5Y cells after treatment with PD184352 and quercetin. SH-SY5Y cells were treated with PD184352 for 30 min before exposure to quercetin at 0.5 to 2 μM for 1 hr. β-actin was used as a reference. $*$ $p < 0.05$ compared to the untreated control, $\#$ $p < 0.05$, $\#\#$ $p < 0.01$ compared to PD 184352 treated groups.

Discussion

It was found in this study that non-toxic doses of quercetin were ranging from 0.5 to 5 μM while quercetin at the concentration of 10 μM was toxic to the SH-SY5Y cells. Therefore, this aspect has to be considered in the consumption or application of high doses of the natural compound for therapeutic purposes. To elucidate whether ERK1/2 activation involves in the autophagy induction, PD184352, MEK1/2 inhibitor was used to inhibit phospho-ERK1/2 expression in SH-SY5Y cells before exposure to quercetin. Treatment with PD184352 decreased the cell viability in a dose-dependent manner, suggesting that ERK1/2 is essential to the survival of SH-SY5Y cells. However, the toxic effect of PD184352 on the cell viability was rescued by treatment with quercetin.

Reduction of phospho-ERK1/2 expression by PD184352 was confirmed by western blot analysis. At 0.25 μM, PD184352 markedly inhibited the expression of p-ERK1/2 to 52.05±0.02% of control. We further elucidated that ERK1/2 involves in autophagy induction, since reduction in LC3-II expression was evident after treated the cells with PD184352, suggesting the direct effect of ERK1/2

on autophagy. Interestingly, quercetin can restore the levels of p-ERK1/2 and LC3-II expressions in PD184352 treated cells. The result of this study is in line with the previous study that flavonoids activate ERK1/2 pathway in PC12 cell line.[17] Our study is the first to provide the evidence that quercetin exerts its neuroprotective effect by ERK1/2 mediated induction of autophagy. Further studies to confirm the findings and to elucidate the molecular mechanism are needed.

Conclusion

This study demonstrated that inhibition of ERK1/2 causes reduction in cell viability as well as attenuation of autophagy. Treatment with quercetin results in activation of ERK1/2, as demonstrated by the increase in level of phospho-ERK1/2 expression and subsequent induction of autophagy.

Acknowledgements

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F18 — Molecular Dynamics Simulationsof Binding of Anti-Alzheimer's Drugs to Human Acetylcholinesterase

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Abstract

Alzheimer's disease (AD) is a major public health problem worldwide due to an increase in elderly population. AD is a progressive neurodegenerative disorder characterized by loss of memory, cognitive decline, and dementia. The current pharmacotherapy of the early stages of AD is mainly dependent on cholinesterase inhibitors. Donepezil (DON) and galantamine (GAL) are selective inhibitors of acetylcholinesterase (AChE). In this report, we studied the interactions of DON and GAL with human AChE (hAChE) using molecular dynamics (MD) simulations. Our results indicated that DON and GAL formed hydrogen bonds with the residue H447 of hAChE, and the binding affinity of hAChE with DON wasmore potent than GAL. The calculation of binding free energies showed that the binding energy of hAChE/DON was better than hAChE/GAL, which was consistent with the experimental data.

Keywords: acetylcholinesterase inhibitor, Alzheimer'sdisease, donepezil, galantamine, moleculardynamics simulation

การจำลองแบบทางพลวัตเชิงโมเลกุลของการจับของยาที่ใช้รักษาโรคอัลไซเมอร์ต่อเอนไซม์อะซีทิลโคลีนเอสเทอเรสของมนุษย์

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บทคัดย่อ

โรคอัลไซเมอร์เป็นปัญหาสาธารณสุขที่สำคัญในหลายประเทศทั่วโลกอันเนื่องมาจากการเพิ่มขึ้นของประชากรผู้สูงอายุ โรคอัลไซเมอร์เกิดจากความเสื่อมของเซลล์ประสาทที่ดำเนินไปอย่างต่อเนื่องทำให้เกิดการสูญเสียของความจำ การลดลงของเชาว์ปัญญา และภาวะสมองเสื่อม ยาที่ใช้รักษาโรคอัลไซเมอร์ระยะแรกในปัจจุบันส่วนใหญ่ออกฤทธิ์ยับยั้งที่เอนไซม์โคลีนเอสเทอเรส ยา donepezil และ galantamineออกฤทธิ์ยับยั้งที่จำเพาะต่อเอนไซม์อะซีทิลโคลีนเอสเทอเรส ในรายงานนี้ ได้ศึกษาอันตรกิริยาระหว่างยากับเอนไซม์อะซีทิลโคลีนเอสเทอเรสของมนุษย์โดยการจำลองแบบทางพลวัตเชิงโมเลกุล ผลการทดลองบ่งบอกว่ายา donepezil และ galantamine เกิดพันธะไฮโดรเจนกับกรดอะมิโนฮีสติดีนตำแหน่ง 447 ของเอนไซม์ และความสามารถในการจับของเอนไซม์กับยา donepezil ดีกว่ายา galantamine ผลจากการคำนวณพลังงานอิสระของการยึดจับแสดงให้เห็นว่าค่าพลังงานการยึดจับของเอนไซม์กับยา donepezil ดีกว่ายา galantamine ซึ่งมีค่าสอดคล้องกับค่าที่ได้จากการทดลอง

คำสำคัญ:ตัวยับยั้งของเอนไซม์อะซิทิลโคลีนเอสเทอเรส,โรคอัลไซเมอร์, donepezil galantamine, การจำลองแบบทางพลวัตเชิงโมเลกุล

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Introduction

Alzheimer's disease (AD)is one of the most common causes of dementia involved in neurodegenerative brain disorder. AD accounts for more than half of all dementia cases in elderly people over the age of 65.[1]The progression of disease results in severe memory loss, cognitive impairment, and behavioral changes.[2] These symptoms interfere the normal daily lifestyle and quality of life in AD patients.[3]Moreover, it has been found that a significant loss of cholinergic neurons was present in the AD brain. As a result, the incapability of cholinergic transmission with remarkable decline of acetylcholine (ACh) could lead to AD symptoms.[4]Thus, one of the rationale for AD treatment is to reduce the rate of ACh breakdown by inhibition of acetylcholinesterase (AChE) that plays a major role in hydrolysis of ACh[5].Currently, two available anti-Alzheimer's drugs, donepezil (DON) and galantamine (GAL), are selective inhibitors of AChE. Based on X-ray crystal structures, different binding modes between DON and GAL in the active site of human AChE (hAChE) led to significant difference of inhibitory activity against the hAChE.[6] The half maximal inhibitory concentration (IC_{50}) values obtained from *in vitro* experiment showed that the inhibitory effect of DON (IC_{50} = 23.1 nM) on hAChE was about 85 times more potent than that of GAL (IC_{50} = 2,010 nM).[7]However, the results from X-ray structures could not explain the binding dynamics of drugs. Therefore, in the present study, we investigated binding modes and dynamic behaviors, which could lead to differences in their binding affinities using molecular dynamics (MD) simulations. This work could be useful for better understanding interactions between hAChE and its inhibitors.

Methods

The starting geometries of the hAChEcomplexed with DON and GAL were taken from the Protein Data Bank (PDB), entry codes 4EY7 and 4EY6, respectively.[6] The complexes were studied by MD simulations using the AMBER12 software package[8]coupled with the AMBER ff03 force field[9], while the partial atomic charges and empirical force field parameters for the atoms in two inhibitors were prepared by the standard procedures.[10] Briefly, each inhibitor was optimized using Gaussian09 program[11]to obtain the most stable structure.The optimized structure was then calculated the electrostatic potential (ESP) charges around its molecule. Afterwards, its restrained electrostatic potential (RESP) charges were obtained by charge fitting procedure using the antechamber module of the AMBER12 software. The MD procedures were performed as follows. The missing hydrogen atoms of hAChE and its inhibitor were addedusing the LEaP module of AMBER12. Ionizable amino acids, consisting of lysine (K), arginine (R), aspartic acid (D), glutamic acid (E), and histidine (H) was assigned using the PROPKA 3.1 program.[12]Possible intramolecular disulfide bonds[13] were also applied for maintaining the stability of protein structure. Added hydrogen atoms were minimized by steepest descent (SD) and conjugated gradient (CG) of 2,000 and 1,000 steps, respectively, to relieve bad contact of hydrogen atoms. Each complex was immersed in a cubic box of TIP3P water molecules[14] with spacing distance of 10 Å from protein surface and a chloride counter ion was added randomly to keep the entire system neutral. Then, the minimization of water molecules alone was performed and followed by the whole system minimization with the SD (2,000 steps) and CG (1,000 steps), respectively, to obtain the optimum conformation for MD simulation. The MD simulation with periodic boundary condition using *NPT* ensemble and time step of 2 fs were performed. The non-bonded interaction calculationwas truncated within a 12 Å residue-base cutoff. The particle mesh Ewald (PME) summation method[15] was applied to account for long-range electrostatic interactions.The SHAKE algorithm[16]was used to eliminate bond-stretching freedom for all atoms involving hydrogenwith a time step of 2 fs. The systems were heated up to 298 K with the relaxation time of100 ps and then simulated at the same temperature for 12 ns. The root mean square displacement (RMSD) and hydrogen bond occupancy between protein and inhibitor were analyzed by the ptraj module, while the binding free energy (ΔG_{bind}) was calculated by the MM-GBSA method.[17]On the basis of this approach, it combined the molecular mechanical energies with the calculation of the free energy of solvation. The generalized Born (GB) method was used to calculate the electrostatic contributions to the solvation free energy with a numerical solver.
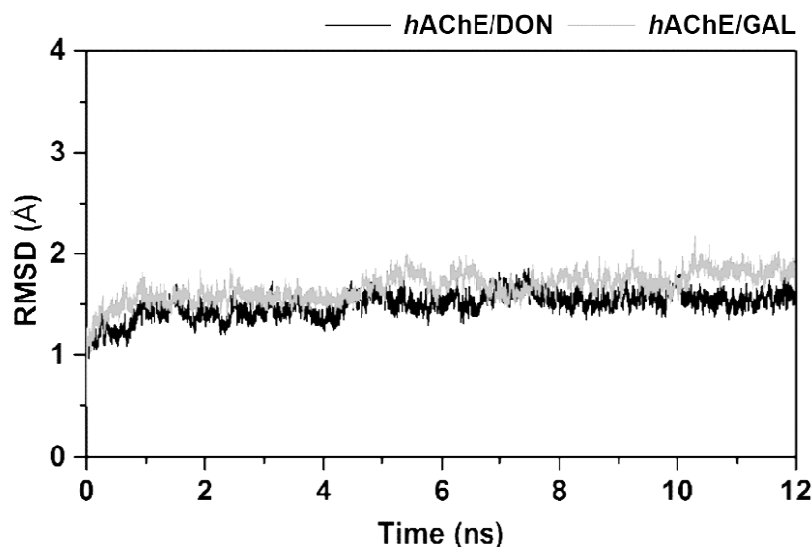
Figure 1. RMSD plots for the backbone atoms of the hAChE/DON (black) and hAChE/GAL (light gray) complexes.

Table 1. Percentage of hydrogen bond occupations calculated from MD simulations

System	Hydrogen bond interaction	% hydrogen bond occupation
hAChE/DON	$^+$N–H···NE2@H447	99
	F295@N–H···O	92
hAChE/GAL	O–H···O@H447	54
	$^+$N–H···OD2@D74	28
	S203@O–H···O	14
	$^+$N–H···OD1@D74	14

··· represents hydrogen bonds

Results

System stability

To obtain information about the dynamic stability after equilibration of the two hAChE/inhibitor complexes, the RMSD of each system relative to the initial structure for backbone atoms (C, N, and CA) versus simulation time was measured. The results are shown in Figure 1. The RMSD values of backbone atoms for the hAChE/DON complex reached equilibrium ~4.5 ns and fluctuated at ~1.4 Å. The equilibrium time and conformational fluctuation for the hAChE/GAL complex were found at ~6.7 ns and ~1.7 Å, respectively. The RMSD calculation showed that both hAChE/DON and hAChE/GAL complexes reached equilibrium at ~7 ns. Hence, the two investigated systems were further analyzed the MD trajectories from 7 to 12 ns.

Hydrogen bond of hAChE/inhibitor complex

To measure hydrogen bond formation, the hydrogen bonds can be calculated in term of the percentage of hydrogen bond occupation in accordance with the two geometric criteria of (1) a proton donor and acceptor distance ≤ 3.5 Å and (2) a donor–H···acceptor

bond angle $\geq 120^{o}$[18, 19] The percentage of hydrogen bond occupationsis presented in Table 1 and the schematic views of hydrogen bond interactions are shown in Figure 2.The hydrogen bond occupations with the values of >75%, 50-75%, and <50% are defined as strong, medium, and weak hydrogen bonds, respectively. The results showed that the hAChE in complex with DON has the higher percentage of hydrogen bond occupation than hAChE/GAL complex

Binding free energy calculation

In order to predict the binding free energies of complex in solution, the MM-GBSA method was performed. The experimental value of free energy of binding (ΔG_{bind}^{exp}) was calculated using the formula: $\Delta G_{bind} \approx RT \ln IC_{50}$, where $R = 1.987 \times 10^{-3}$ kcal/mol and $T = 298$ K. The IC_{50} values of DON and GAL are 2.31×10^{-8} M and 2.01×10^{-6} M, respectively.[7] The calculated binding free energy (ΔG_{bind}) and ΔG_{bind}^{exp} are shown in Table 2. Our results indicated that the favorable binding free energy from the MM-GBSA approach of DON (–27.9 kcal/mol) was better than GAL (–15.2 kcal/mol).
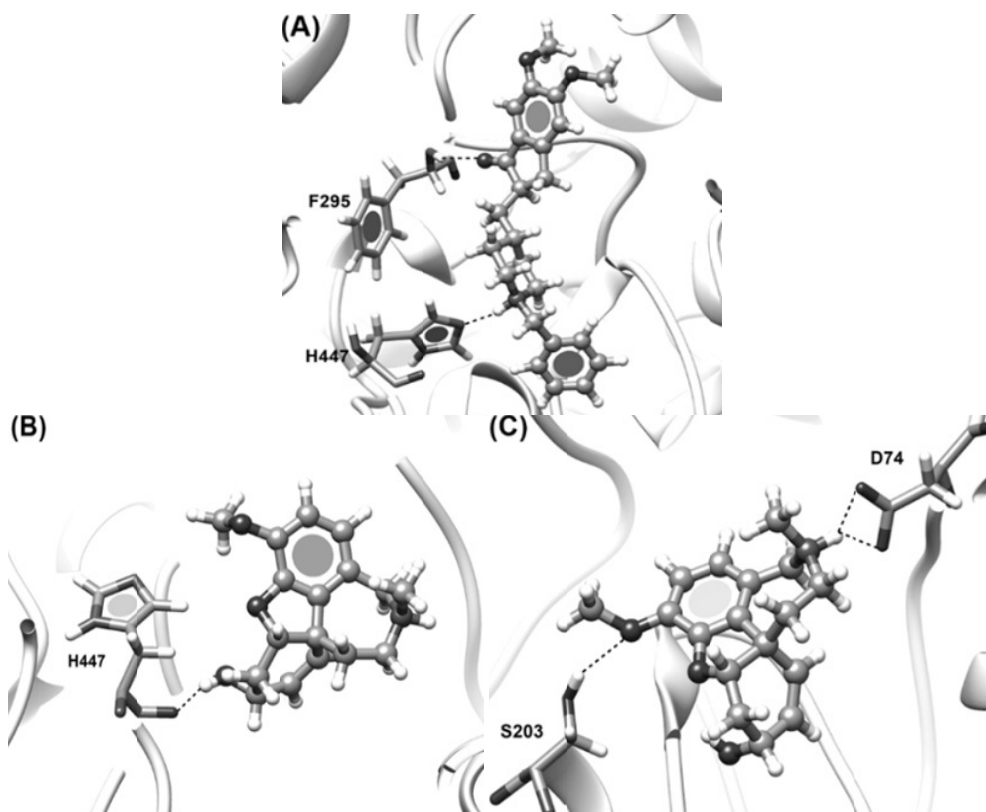
Figure 2. The schematic views of hydrogen bond interactions (dotted lines) demonstrated from the MD snapshot between the hAChE and two hAChE inhibitors: (A) DON and (B and C) GAL

Table 2. Binding free energy of hAChE in complex with DON and GAL.

Binding free energy (kcal/mol)	DON	GAL
ΔG_{bind}	-27.9 ± 7.4	-15.2 ± 4.8
ΔG_{bind}^{exp}	-10.4	-7.8

Discussion

This is the first MD simulation study of the binding of DON and GAL to hAChE. Our results showed that the catalytic residue H447 of hAChE is necessary for binding of DON and GAL. Moreover, the binding between hAChE and DON revealed two strong hydrogen bonds, while the binding of GAL to hAChE demonstrated three weak and one medium hydrogen bonds. The results suggested that binding affinity of DON was more potent than GAL. For binding free energy calculation, it indicated that the binding free energy of hAChE/DON was better than hAChE/GAL, which was consistent with the experimental data.[7] In addition to hydrogen bond, other types of interactions including π-π interaction and van der Waals force play an important role in the binding of both drugs to hAChE. Therefore, the π-π and van der Waals interactions will be further investigated.

Conclusion

In this study, the binding dynamics of DON and GAL with hAChE were investigated using MD simulation. Our results showed that the catalytic residue H447 of hAChE is important for binding of DON and GAL. The calculation of binding free energies revealed that the binding affinity of hAChE/DON was more potent than hAChE/GAL, which was in consistence with the experimental data.[7]

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F19 Molecular Dynamics Simulation of Donepezil Binds to Human Butyrylcholinesterase

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Abstract

Molecular dynamics (MD) simulation is a computational method which investigates the structural dynamics of biological molecules and their ligands. In this study, MD simulations were carried out for human butyrylcholinesterase (hBuChE) and donepezil complex, in order to predict its binding free energy. At the equilibrium, potential interactions of donepezil to hBuChE are based on the strong hydrogen bond between donepezil piperidinium moiety and carbonyl oxygen of hBuChE His438. Moreover, the binding free energy of hBuChE-donepezil complex was evaluated from three methods: Molecular Mechanic Poisson-Boltzmann Surface Area (MM-PBSA), Molecular Mechanic Onufriev's Generalized Born Surface Area (MM-Onufriev's GBSA), and Molecular Mechanic Tsui's Generalized Born Surface Area (MM-Tsui's GBSA). Our results showed the calculated binding free energy from MM-PBSA best resembles the experimental value.

Keywords: binding free energy, donepezil, human butyrylcholinesterase, molecular dynamics simulation

การศึกษาแบบจำลองเชิงพลวัตการจับของยาโดเนเพซิลต่อเอนไซม์บิวทิริลโคลีนเอสเทอร์เรสของมนุษย์

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บทคัดย่อ

การจำลองเชิงพลวัติเป็นเทคนิคทางคอมพิวเตอร์ซึ่งสามารถใช้ในการจำลองการเคลื่อนไหวของโครงสร้างชีวโมเลกุลเชิงซ้อน การศึกษาแบบจำลองเชิงพลวัติของสารประกอบเชิงซ้อนระหว่างเอนไซม์บิวทิริลโคลีนเอสเทอร์เรสของมนุษย์กับยา donepezilเพื่อคำนวณค่าพลังงานการยึดจับของยาและเอนไซม์ เมื่อระบบเข้าสู่สมดุลแล้ว การจับกันของยา donepezil กับเอนไซม์บิวทิริลโคลีนเอสเทอร์เรสของมนุษย์นั้นเกิดจากพันธะไฮโดรเจนที่แรงระหว่างหมู่ piperidinium ของยา donepezil กับคาร์บอนิลออกซิเจนของกรดอะมิโนฮีสทิดีนตำแหน่ง 438 นอกจากนั้นทำการประเมินพลังงานอิสระของการยึดจับโดยใช้วิธีการคำนวณ สามวิธีการได้แก่ Molecular Mechanic Poisson-Boltzmann Surface Area (MM-PBSA) Molecular Mechanic Onufriev's Generalized Born Surface Area (MM-Onufriev's GBSA) และ Molecular Mechanic Tsui's Generalized Born Surface Area (MM-Tsui's GBSA) ผลการศึกษาพบว่าการทำนายพลังงานอิสระของการยึดจับจากวิธีการ MM-PBSA ให้ผลใกล้เคียงกับผลการทดลองมากที่สุด

คำสำคัญ: พลังงานอิสระของการยึดจับ, donepezil, เอนไซม์บิวทิริลโคลีนเอสเทอร์เรสของมนุษย์, การจำลองเชิงพลวัตระดับโมเลกุล

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Introduction

Alzheimer's disease (AD) is the most common type of dementia which is characterized by a gradual loss of cognitive function. In AD brain, the degeneration of cholinergic neurons involved in the memory leads to a reduction of acetylcholine (ACh).[1,2] Current treatment of the early stage of AD mainly depends on cholinesterase inhibitors which increase the level of ACh. These inhibitors are considered as an effective therapy for AD.[3] Several studies found that the level of acetylcholinesterase (AChE) in the brain decreases along the disease progression, while level of butyrylcholinesterase (BuChE) increases.[4] Donepezil is an anti-AD drug which binds to AChE and BuChE. Currently, molecular dynamics (MD) simulations are widely used to study the interaction at the molecular level between protein and ligand in aqueous solution. It have provided detailed information on the fluctuations and conformational changes of proteins. These methods are able to determine the structure, dynamics and thermodynamics of biological molecules and their ligands. Recently, there is no report on MD study of donepezil and human BuChE (hBuChE). Therefore, we studied the binding of hBuChE in complex with donepezil using MD simulations.

Methods

MD simulations were performed by AMBER12 package.[5] The optimization of ligand was preparedusing Gaussian09 program.[6] Pymol program.[7] was used to visualize the protein structure. The AMBER03 force field[8] was used as molecular mechanic parameter for enzyme, while general amber force field (GAFF)[9] was used for ligand.

Protein preparation

Ligand-free hBuChE crystal structure was chosen from the protein data bank (PDB). There are three available ligand-free structures, namely1P0I[10], 2PM8[11], 4AQD[12] In this study, 1P0I was chosen because its crystal has high X-ray resolution. Missing residues (ASP378, ASP379, and ASN455) were added to the structure.Mutated residues(GLN17, GLN481, and GLN486) were changed back to the native ASN. The modified structure of hBuChE 1P0I was assigned protonation for each residue at physiological pH using H^{++}program.[13]

Ligand preparation

The donepezil was modelled as a protonation state in corresponding to the physiological pH 7.4. Its structure was optimized by Gaussian09 software under the Hartree-Fock method using 6-31G(d) level of basis set. Finally, Restrained Electrostatic Potential (RESP) was used to fit the ligand charges.

Complex preparation

Human AChE (hAChE)-donepezil complex structure is available in protein data bank (PDB ID: 4EY7). hAChE and hBuChE share 65% amino acid sequence identity and have similar molecular forms and active center structure, thus; hAChE-donepezil complex was superimposed to the hBuChE (modified 1P0I). Finally, hAChE was removed from the superimposed structure, leaving hBuChE-donepezil complex.

Molecular dynamics simulation

Minimization of hydrogen atoms was performed using Sander program of AMBER package with 10 Å of cutoff distance for 1,000 cycles with steepestdescent (SD) and followed by 2,000 cycles of conjugate gradient (CG). Truncated octahedral box of TIP3P[14] water model was used to immerse the system with a minimal solute–wall distance of 10 Å from protein surface. To neutralize the system, four atoms of chloride ions were added as counter ions. Afterwards, only solvents were minimized with 12 Å cutoff distance under constant volume condition for 3,000 cycles (1,000 cycles for SD and 2,000 cycles for CG) to release bad contact between solute and solvent, and all atoms were then minimized.

To avoid the bias of random initial velocity generation, system was heated from 0 K to 298 K using Berendsen thermostat.[15] The particle mesh Ewald method (PME)[16] with 12 Å of cutoff was used to relieve long range electrostatic interactions, and any bond involvingin hydrogen atoms was constrained using SHAKE algorithm.[17]

After heating, the simulation condition was changed from constant volume to isothermal–isobaric (*NPT*) ensemble. The isotropic position scaling was used to maintain the pressure to 1 bar with a relaxation time of 1 ps.

Root-mean-square deviation (RMSD) of atomic positions

The RMSD calculation was performed by setting the starting model as a reference, and then calculating the distance between atomic positions that change along the simulation time against the reference positions, using eq.1.

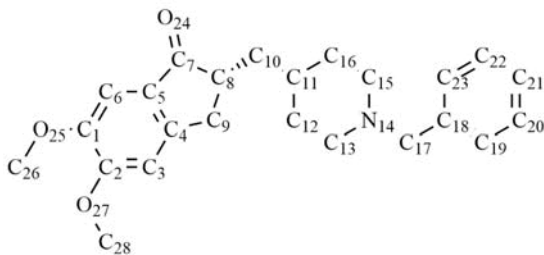$$RMSD = \sqrt{\frac{\sum_{i=0}^{N} m_i (X_i - Y_i)^2}{M}} \qquad (1)$$

Figure 1. Structure of donepezil

Where N is the number of atoms, m_i is the mass of atom i, X_i is the coordinate vector for target atom i, Y_i is the coordinate vector for reference atom i, and M is the total mass.

Ligand torsion angle measurement

There are four flexible torsion angles of donepezil, including C9-C8-C10-C11, C8-C10-C11-C16, C13-N14-C17-C18, and N14-C17-C18-C19, as shown in Figure 1. To define the flexibility and stability of donepezil, the torsion angles were measured every 2 ps.

Hydrogen bond

Hydrogen bond formation between proton donor and acceptor depicted as Donor-H·····Acceptor was determined every 2 ps along the 9-14 ns of simulation time using two criteria:

1. The angle between three atoms must be greater than 120 degree.
2. The distances of hydrogen bond must be less than 3.5 Å.

The strong, medium, and weak hydrogen bond are defined by hydrogen bond occupations with the values of >75%, 50-75%, and <50%, respectively.

Binding free energy calculation

In this study, the binding free energy of hBuChE-donepezil complex was calculated from the difference in free energy changes between bound and unbound states of an enzyme-ligand complex (eq.2).

$$\Delta G_{binding} = \Delta G_{complex} - (\Delta G_{enzyme} + \Delta G_{ligand}) \quad (2)$$

The free energy was divided into two energy contributions: enthalpy (ΔH) and entropy ($T\Delta S$). The calculation of enthalpy in gas phase was performed by the Molecular Mechanics (MM), and the solvation enthalpy was calculated by Poisson-Boltzmann Surface Area (PBSA) method, while entropy term was calculated by normal mode analysis (NMA)(eq. 3).

$$\Delta G = (\Delta E_{MM} + \Delta G_{solvation}) - T\Delta S \quad (3)$$

To calculate enthalpy contribution, three methods of energy estimation, namely MM-PBSA, MM-Onufriev's GBSA, and MM-Tsui's GBSA were used. The binding free energy was calculated using mm_pbsa.pl perl script of AMBER12 package for 50 snapshots (9-14 ns). The internal and external dielectrics constant were set to 1 and 80, respectively, and MolSurf program was used to calculate solvent accessible surface area (SASA) which is in a part of nonpolar solvation contributions. Other parameters of each method are described as follows.

MM-PBSA method[18]; the atomic radius of topology files of complex, enzyme, and ligand were set to default value of parse atomic radius. The grid spacing for MMPBSA method was set to 0.25 Å, and the dielectric constants of the boundary grid edges were set to either internal or external dielectric constant depending on whether the mid points of the grid edges were inside or outside the solute surface. The resolution of solvent accessible arcs was set to 0.125 Å. Surface tension and their offset were set to 0.00542 kcal/mol and 0.92 kcal/mol respectively. MM-PBSA method, MM-Onufriev's GBSA method[19], and MM-Tsui's GBSA method[20] were performed.

Normal Mode Analysis (NMA)[21] was used for entropic calculation in order to obtain the translational, rotational, and vibrational energies.

The mean of binding free energy ($\Delta \bar{G}_{binding}$) was calculated using eq.4.

$$\Delta \bar{G}_{binding} = \Delta \bar{H} - T\Delta \bar{S} \quad (4)$$

The experimental binding free energy ($\Delta G_{binding}^{exp}$) was calculated using the mean of binding free energy from a thermodynamic formula (eq.5)[22]. The standard derivation of $\Delta G_{binding}^{exp}$ was estimated using the first order Taylor's series expansion around the $\Delta G_{binding}^{exp}$ point (eq.6).

$$\Delta G_{binding}^{exp} = RTlnK_i \quad (5)$$

$$\sigma_{\Delta G_{binding}^{exp}} = \frac{RT\sigma_{K_i}}{K_i} \quad (6)$$

Where K_i is inhibition constant, $\Delta G_{binding}^{exp}$ is experimental binding free energy, R is gas constant (1.986 kcal/mol·K), T is temperature (298 K), σ_{K_i} is the standard deviation of K_i, and $\sigma_{\Delta G_{binding}^{exp}}$ is the standard derivation of $\Delta G_{binding}^{exp}$.
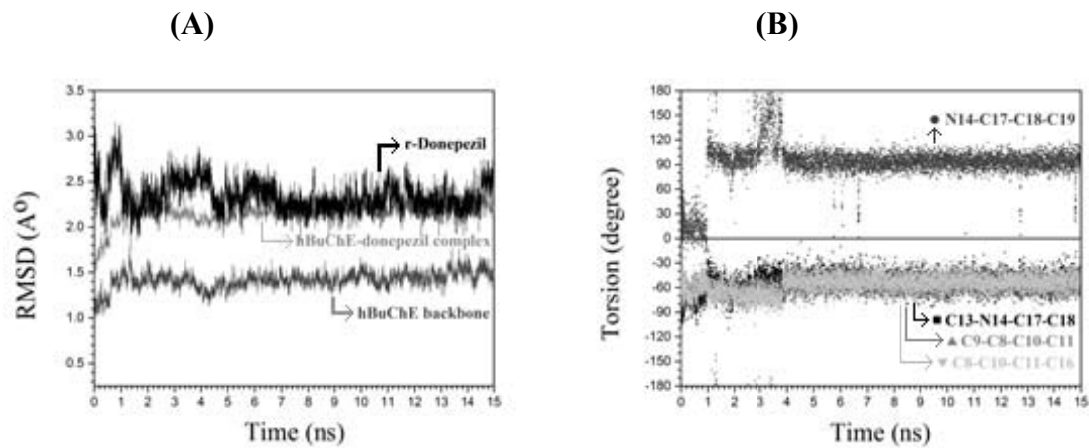
(A)   **(B)**

Figure 2.   Geometrical parameters calculated through 15 ns of MD simulations of the hBuChE-donepezil complex, (A) RMSD, (B) Torsion angle.

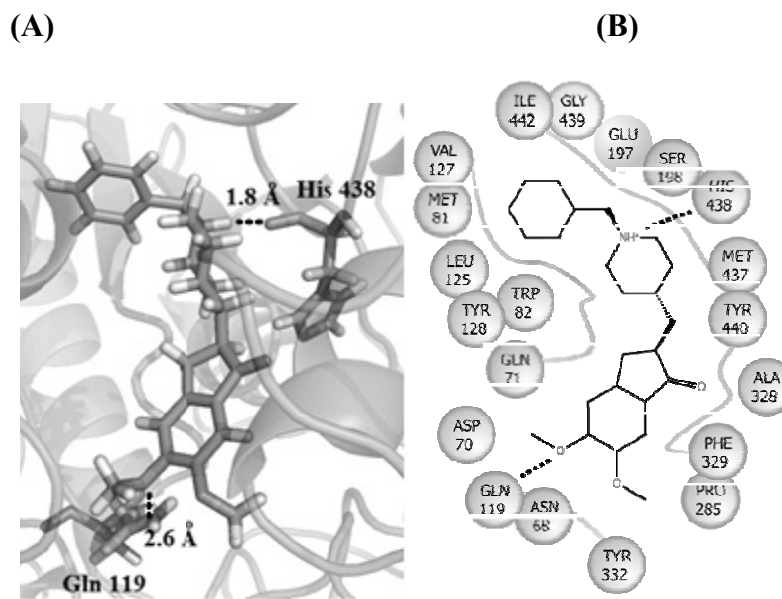**(A)**   **(B)**

Figure 3.   Binding mode of hBuChE-donepezil complex at the equilibrium. (A) The average hydrogen bond interaction from 9 to 14 ns; (B) 2D diagram of interaction between hBuChE and donepezil showing hydrogen bond (dashed line)

Table 1 The hydrogen bond obtained from 9 to 14 ns.

D⋯A	Occupation (%)	Distance (Å)	Angle (degree)
His438:O⋯HN:527Don	100	1.8±0.1	163±8
Don527:O⋯HN:119Gln	19.49	2.6±0.4	137±12

Where D→A represent donor and acceptor atom. Don is donepezil. Distance and angle are reported in terms of mean ±standard deviation.

Table 2 The binding free energy calculated by MM-PBSA, MM-Onufriev's GBSA and MM-Tsui's GBSA.

	Experiment[23]	MM-PBSA	MM-Onufriev's GBSA	MM-Tsui's GBSA
Binding free energy (ΔG) (kcal/mol)	-7.7 ± 0.1	-7.0± 6.1	-21.9 ±6.3	-31.5±6.2

Results are reported in term of mean ± standard deviation.

Results

Molecular dynamics simulation

The RMSD revealed that time to equilibrium of the complex, hBuChE backbone atoms and donepezil atoms was~8 ns (Fig.2A), while the torsion angles of donepezil ligand reached equilibrium after 4 ns (Fig.2B).

Binding mode and Hydrogen bond

The hydrogen bond was investigated every 2 ps. Figure 3 indicated that the strong hydrogen bond between NH^+ group of piperidinium moiety of the drug and carbonyl oxygen of catalytic histidine 438 residue was observed. Another weak hydrogen bond occurs between methoxy group and the amide group of the glutamine 119 residue. The hydrogen bond parameters are shown in the Table1.

Binding free energy

The binding free energies were calculated by MM-PBSA, MM-Onufriev's GBSA, and MM-Tsui's GBSA methods. The results are summarized in table 2.

The K_i of donepezil was taken from *in vitro* experiment.[23] The K_i value was converted to binding free energy using eq. 5. The results indicated that value from the MM-PBSA method is correlated well with experimental data.

Discussion

The RMSD results revealed that the simulation system reached equilibrium after 8 ns.

Therefore, the hBuChE-donepezil complex was then analyzed the MD trajectory from 9 ns to 14 ns. The torsion angle calculation indicated that the structure of donepezil is quite stable, because its torsion angle did not change at the early simulation time (~4 ns). The hydrogen bond between electron donor carbonyloxygen of histidine 438 backbone and –NH– group of donepezil is important for the binding ofhBuChE-donepezil complex. In agreement with Hou T. *et. al.*[24], our results demonstrated that the MM-PBSA method is better than MM-GBSA in calculating absolute binding free energy. The calculation of binding free energy suggested that the MM-PBSA is the suitable method for comparing to experimental binding free energy.

Conclusion

In this study, we determined the binding mode and binding free energy of hBuChE-donepezil complex using MD simulation. At equilibrium, donepezil forms a weak and a strong hydrogen bond with hBuChE, suggested the protonation of piperidine ring is essential for strong hydrogen bond formation. MM-PBSA is the best method to predict binding free energy of hBuChE-donepezil complex.

Acknowledgement

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F20   Reactive Oxygen Species Scavenging Property of Unripe *Carica papaya* Fruit Extract

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Abstract

Oxidative stress is one of the major causes of many diseases and disorders. Natural antioxidant with reactive oxygen species (ROS) scavenging property, however, is possibly able to prevent oxidative stress. *Carica papaya* fruit juice has been reported to exert antioxidant activity and cytoprotective effect in various types of cell. Thus, this present study aimed to investigate the ROS scavenging activityof unripe *C.papaya* fruit juice (CPW). Inhibition of deoxyribose degradation mediated by hydroxyl radical (·OH) was measured to show the ability of CPW to scavenge ·OH compared with antioxidant standards. CPW exerted a considerable ·OH scavenging activity (IC_{50} = 785.81 ± 33.82 µg/mL).Trolox equivalent antioxidant capacity and N-acetyl cysteine equivalent antioxidant capacity of CPW were 282.72 ±10.66 mg trolox/g CPW and 440.49 ±79.08 mg NAC/g CPW, respectively. However, CPW did not exert the hypochlorous acid scavenging activity assessed by determining amount of remaining TNB. These findings suggest that CPW could effectively scavenge ·OH radical that could be attributed to their phenolic constituents. In order to comprehend activities of CPW in reduction of ROS harmfulness, it is suggested that other aspects of antioxidant effects and cytoprotective effect against ROS-induced cell death and the mechanism underlying itshould be further investigated.

Keywords: *Carica papaya*, reactive oxygen species, oxidative stress, antioxidant

การกำจัดสารออกซิไดซ์แรงสูงของสารสกัดจากผลมะละกอแขกดำดิบ

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บทคัดย่อ

การทำลายเซลล์โดยสารอนุมูลอิสระเป็นสาเหตุหนึ่งของโรคและความผิดปกติในการทำงานของร่างกายหลายประการ อย่างไรก็ดีสารต้านอนุมูลอิสระจากธรรมชาติที่มีฤทธิ์ในการกำจัดสารออกซิไดซ์แรงสูงอาจมีความสามารถในการป้องกันการ เกิดภาวะเครียดออกซิเดชันได้จากการศึกษาหลายชิ้นพบว่าสารสกัดจากผลมะละกอแขกดำ(*Carica papaya*) มีฤทธิ์ในการต้าน สารอนุมูลอิสระและยังมีฤทธิ์ปกป้องเซลล์จากสารออกซิไดซ์แรงสูงดังนั้นการศึกษานี้จึงมีวัตถุประสงค์เพื่อตรวจหา ความสามารถของสารสกัดจากผลมะละกอแขกดำดิบในการกำจัดสารออกซิไดซ์แรงสูงจากการทดลองพบว่ามะละกอแขกดำดิบ สามารถกำจัดสารอนุมูลอิสระไฮดรอกซิลได้ โดยมีค่า IC_{50}เท่ากับ 785.81 ± 33.82 ไมโครกรัม/มิลลิลิตร ซึ่งมีค่าเทียบเท่ากับสารต้านอนุมูล

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อิสระมาตรฐาน Trolox เท่ากับ 282.72 ± 10.66มิลลิกรัมของ trolox ต่อกรัมของมะละกอแขกดำดิบและ N-acetyl cysteine เท่ากับ 440.49 ± 79.08 มิลลิกรัมของ NAC ต่อกรัม ของมะละกอแขกดำดิบอย่างไรก็ตามจากการศึกษาพบว่ามะละกอแขกดำดิบ ไม่มีฤทธิ์ในการกำจัดกรดไฮโปคลอรัสผลการศึกษาชี้ให้เห็นว่าสารสกัดนี้มีประสิทธิภาพในการกำจัดสารอนุมูลอิสระไฮดรอกซิลได้ โดยฤทธิ์ดังกล่าวอาจเป็นผลจากสารฟีนอลิกที่พบได้ในผลของมะละกอแขกดำดิบ นอกจากนั้นควรทำการทดลองฤทธิ์ต้านอนุมูล อิสระของสารสกัดมะละกอแขกดำดิบ รวมไปถึงฤทธิ์ในการปกป้องการตายของเซลล์จากการเหนี่ยวนำของสารออกซิไดซ์แรงสูง และกลไกที่เกี่ยวข้องเพิ่มเติมต่อไปเพื่อสนับสนุนฤทธิ์ดังกล่าวของสารสกัดมะละกอแขกดำดิบ

คำสำคัญ:มะละกอแขกดำ, สารออกซิไดซ์แรงสูง, ภาวะเครียดออกซิเดชัน, อนุมูลอิสระ

Introduction

Physiological reactive oxygen species (ROS) level is controlled by its production and elimination, which are well balance. Accumulating evidence proves that at physiological state, ROS participate in diverse biological processes of living organisms. Examples of currently known ROS physiological roles include regulation of immune response, vascular tone, vascular permeability, and angiogenesis.[1]However, under certain circumstance, the level of ROS is increased due to imbalance of ROS production and elimination.This certain imbalance state causes increase of ROS level and is called oxidative stress. Under cellular oxidative stress conditions, production of ROS in large amount induces alteration of cellular functions and apoptosis[2] which eventually leads to cellular or organ dysfunction.

Hydroxyl radical ($\cdot$OH) is known to be the most biologically active free radical so far. It is produced *in vivo* by Fenton reaction in the presence of hydrogen peroxide (H_2O_2) and iron ions. Hypochlorous acid (HOCl) is the reactive form of chlorine, catalyzed by peroxidase enzyme, mainly neutrophil-derived myeloperoxidase *in vivo*.[3]

Due to the fact that natural products are considerably safe, consumption of fruits that are proved to have antioxidant activity should be promoted and could be used for the purpose of preventing oxidative stress-caused diseases

Carica papaya L. or papaya belongs to the Caricaceae family. They are grown in tropical areas world-wide including south-east Asia. Up to date, a number of studies showed promising results concerning antioxidant activity and cytoprotective effect of *C. papaya* in various types of cell and animals.[4-6] Thus, in this study, *C.papaya* fruit juice was chosen to investigate further into the ROS scavenging activities.

Materialsand Methods

Chemicals

2-Deoxy-D-ribose, $FeCl_3$, 2-Thiobarbituric acid (TBA), and Sodium borohydride were purchased from Sigma-Aldrich.Troloxwas purchased from Aldrich. Ethylenediaminetetraacetic acid (EDTA) trisodium salt, L-ascorbic acid sodium salt, 30% Hydrogenperoxide, NaOCl, and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma. N-Acetyl-L-cysteine (NAC) was purchased from Flukachemika. Trichloroacetic acid (TCA) was purchased from Merck.

Preparation of Carica papaya fruit extract (CPW)

Unripe fruits of *Carica papaya* were purchased from local market in KhonKaen, Thailand. Fresh fruits were peeled and seeds were removed before passing through an automatic juice extractor to obtain juice. Afterward, the juice was centrifuged at 3,000 g, 4°C for 10 minutes to separate remaining fruit pulps. The supernatant was then lyophilized and kept at -40°C in the light protecting containers. The working aqueous stock solutions of CPW were freshly prepared before each experiment by dissolving fruit powder in distilled water.

Determination of hydroxyl radical scavenging activity

The assay was performed following a standard deoxyribose degradation method with a slight modification.[7] Reaction mixture contained, in a final volume of 1 mL, 5.6 mM2-deoxy-D-ribose, 0.2 mM EDTA trisodium salt, 0.2 mM $FeCl_3$, 0.2 mM sodium-L-ascorbate, 100 mM phosphate buffer (pH7.4), and CPWor antioxidant standards in various concentrations as references. After incubation at 50°C for 20 minutes, 5.6% TCA (w/v) and 3.2% TBA (w/v) were added and the mixture was then heated in a water bath at 100°C for 15 minutes. The absorbance of the mixture was measured at 532 nm in triplicate. The percent inhibition was calculated from the following equation:

$$\%\text{inhibition} = \frac{(\text{OD of negative control} - \text{OD of sample})}{\text{OD of negative control}} \times 100$$

Determination of hypochlorus acid scavenging activity

The hypochlorus acid (HOCl) scavenging activity of CPW was assessed by determining amount of remaining 2-nitro-S-thiobenzoic acid(TNB).[8]HOCl was freshly prepared before an experiment by adjusting the pH of NaOCl solution to 6.2 with 0.6 M H_2SO_4. The concentration of HOCl was subsequently determined by measuring the absorbance at 235 nm using the molar absorption coefficient of 100 $M^{-1}cm^{-1}$. TNB was prepared from 1 mM DTNB solution in 50 mM potassium phosphate buffer (pH 6.6) containing 5 mM EDTA in addition to 20 mM sodium borohydride. The mixture was incubated at 37°C for 30 minutes. The concentration of TNB was then determined by measuring the absorbance at 412 nm using the molar absorption coefficient of 13600 $M^{-1}cm^{-1}$. The assay was performed at room temperature. The assay mixture contained a 40 μM TNB solution, with or without CPW or standard antioxidants (i.e. ascorbic acid, NAC, and trolox). The absorbance of the mixture was measured at 412 nm before and 5 minutesafter the addition of 40 μ MHOCl. The percent TNB remaining was calculated from the following equation:

$$\%TNB remaining = 100 - \left[\frac{(\text{OD of negative control} - \text{OD of sample})}{\text{OD of negative control}} \times 100 \right]$$

Statistical analysis

The results were presented as the mean ±SD or SEM as indicatedGraphPad Prism version 5.03 software was used to analyze the data. All data groups were compared with one-way analysis variance (ANOVA) followed by Dunnett's multiple comparison test. The statistically significant level was considered at p-value < 0.05.

Results

Hydroxyl radical scavenging activity

Inhibition of deoxyribose degradation mediated by hydroxyl radical (·OH) in a mixture of Fe^{3+}-EDTA, H_2O_2, and ascorbic acid was measured to show the ability of CPW to scavenge ·OH compared with antioxidant standards. Hydroxyl radical scavenging activities of trolox, NAC, and CPW showed in dose-dependent manner. CPW exerted a considerable ·OH scavenging activity (IC_{50} = 785.81 ± 33.82 μg/mL), while trolox and NAC have IC_{50} of 164.70 ± 14.75 μg/mL and 292.62 ± 19.71 μg/mL, respectively as shown in Table 1.Trolox equivalent antioxidant capacity and N-acetyl cysteine equivalent antioxidant capacityof CPW were 282.72 ±10.66 mg trolox/g CPW and 440.49 ±79.08 mg NAC/g CPW, respectively At concentration of 1000 μg/ml, the percentage inhibition values were 87.8 ±0.87%, 84.8 ±3.25%, and 61.12 ± 2.28% for trolox, NAC, and CPW, respectivelyas shown in Figure 1.

Hypochlorus acid scavenging activity

In this assay, hypochlorous acid (HOCl) reactively induce oxidation of TNB (λ_{max} = 412) into DTNB (λ_{max} = 325). In the presence of HOCl scavenger, therefore, it inhibits the TNB oxidation. The remaining of TNB was measured to evaluate the ability to inhibit TNB oxidation of CPW and other antioxidant standards as shown as Figure 2. N-acetyl cysteine exhibited a potent HOCl scavenging activity (IC_{50} = 14.15 ±4.10 μg/ml). Similarly to ascorbic acid, which has IC_{50} = 104.48 ±13.90 μg/ml. Trolox and CPW, however, did not exhibit the scavenging activity.

Table 1. IC_{50} values of CPW, trolox, and NAC to inhibit degradation of 2-deoxy-D-ribose mediated by hydroxyl radical.

Sample	IC_{50}*
CPW	785.81 (33.82)
Trolox	164.70 (14.75)
NAC	292.62 (19.71)

* Data are presented as mean (SD); n = 3

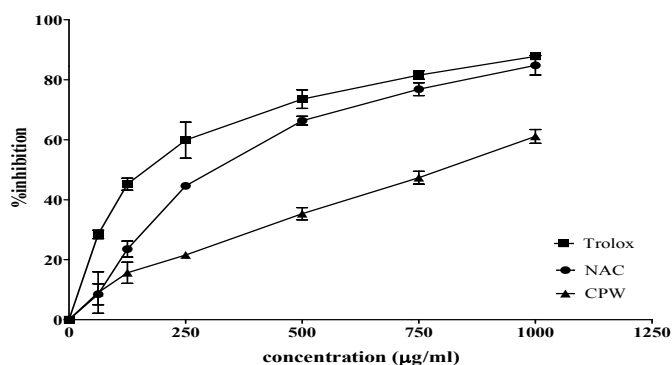\Figure 1. Hydroxyl radical scavenging activity assay. Effects of trolox, N-acetyl cysteine (NAC), and CPW on the scavenging of ·OH. The data represent the percentage inhibition of deoxyribose degradation. The results are mean ±SEM (n = 3).
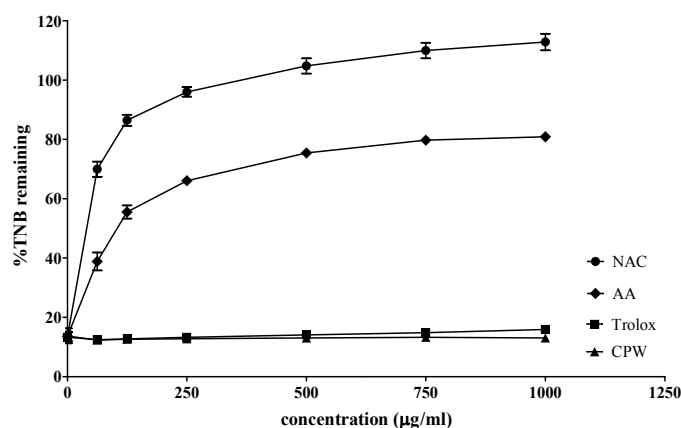

Figure 2. Hypochlorus acid scavenging activity assay. Scavenging effects of ascorbic acid (AA), trolox, N-acetyl cysteine (NAC), and CPW on hypochlorus acid (HOCl). The data are expressed as mean ±SEM of percentage of 5-thio-2-nitrobenzoic acid (TNB) remaining (n = 4).

Discussion

It is now well established that excessive accumulation of reactive oxygen species or oxidative stress is a key cause of cellular damage by interfering with cellular physiological functions and inducing cell death[2], which is implicated in the progression of many diseases and disorders. Growing number of studies show that many natural products have an ability to protect human cell from oxidative insults based on their antioxidant activity. Extract of unripe *C. papaya* has drawn our attention due to its antioxidant property in different cell types and animal experiments.[4-6] Therefore, in this present study, we investigated ·OH and HOCl scavenging activity of CPW.

Hydroxyl radical is one the most reactive and harmful ROS, generated *in vivo* by Fenton and Haber-Weiss reactions. The mixture of Fe^{3+}, EDTA, ascorbic acid, and H_2O_2 at pH 7.4 was incubated to form hydroxyl radicals, which further damage 2-deoxy-2-

ribose and generate malondialdehyde (MDA) like product. This compound then reacts with TBA at low pH and forms a pink chromogen (λ_{max} = 535 nm).[7] By adding CPW in the system removes generated hydroxyl radical and consequently reduces pink chromogen formation. Our data indicated that aqueous extract of CPW exhibited a marked dose-dependent ·OH scavenging activity. An efficient ·OH scavenging activity of CPW would benefit particular sites, where Fe^{2+}/Fe^{3+} are abundantly available such as circulating system and atherosclerotic lesion.[9] Hypochlorus acid, however, could not be scavenged by CPW. This finding might limit beneficial effects of CPW at inflammation sites, where myeloperoxidase enzyme from local neutrophils and macrophages can produce large amount of HOCl.

Certain ROS scavenging activity of CPW could be attributed to their phenolic constituents. Characterization of the unripe *C. papaya* fruit extract by gas chromatography showed that the major phenolic

compounds of the flesh are epigallocatechin, ferulic acid, synaptic acid, and 2-phenylethyl-beta-D-glucoside.[10] In general, phenolic compounds that consist of aromatic rings and hydroxyl side chain(s) effectively scavenge ROS by donating H^+ atom to free radical and become free radicals themselves but they rapidly stabilize by the resonance delocalization of the electron within the aromatic ring.

It is suggested that other aspects of antioxidant effects of CPW and cytoprotective effect against ROS-induced cell death and the mechanism underlying it, including effect on endogenous antioxidant enzyme protein expression should be further investigated to comprehend activities of CPW in reduction of ROS harmfulness.

Conclusion

The present study highlights effective ·OH radical scavenging of the unripe fruit extract of *C. papaya*. This action would be beneficial to certain sites, where there are abundant of Fe^{2+}/Fe^{3+}. Hydroxyl radical scavenging activity of CPW could be attributed to their phenolic constituents. However, cytoprotective effect of CPW and relevant mechanism are suggested to be further studied.

Acknowledgements

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F21 Comparison of the Activity of DPPH Scavenging of Crude Extract Propolis in Extraction Solvents

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Abstract

Propolis is a resinous substance collected by honeybee from various plant sources. Ethanol is usually use for propolis extract because ethanolic extract propolis always gives polyphenolic compound and flavonoid substances that showvarious biological activities. The objective of this study is to extract the propolis in several solvents: hexane, dichloromethane, ethanol and water. Furthermore, their biological properties and chemical components were investigated. Among four extracts, water extract propolis showed the lowest percentage yield (0.05% (w/w)). Hexane, dichloromethane, ethanol were as well as in the percentage yield which were 19.66% (w/w), 22.56% (w/w) and 18.68% (w/w), respectively. The ability of extract for inhibit free radical were done by DPPH scavenging assay. At 2,000 μg/mL, ethanolic extract can inhibit DPPH radical about 84 percent and 80 percent for dichloromethane extract. Hexane extract can inhibit DPPH radical about 60 percent and water extract can inhibit DPPH radical 33 percent.The components of crude extract were separated by TLC technique in mobile phase hexane: ethyl acetate: acetic acid (60:40:1% (v/v)). Chromatogram of hexane, dichloromethane, ethanol showed the similar pattern about 4 spots on TLC plate but water extract showed only one spot on TLC plate. These results reveal that ethanol is the good solvent for propolisextraction because the ability to scavenge DPPH is higher than other extracts.

Keywords: propolis, free radical, extraction solvent, thin layer chromatography

การเปรียบเทียบฤทธิ์ต้านอนุมูลอิสระของสารสกัดพรอพอลิสที่สกัดด้วยตัวทำละลาย

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บทคัดย่อ

พรอพอลิสเป็นผลิตภัณฑ์ธรรมชาติที่ได้จากผึ้ง สารสกัดจากพรอพอลิสในเอทานอลมีสารสำคัญเป็นองค์ประกอบมี ฤทธิ์ทางชีวภาพที่น่าสนใจ เช่นต้านการอักเสบ ต้านอนุมูลอิสระ ต้านแบคทีเรีย เป็นต้น การศึกษานี้มีวัตถุประสงค์เพื่อ เปรียบเทียบฤทธิ์ต้านอนุมูลอิสระของสารสกัดพรอพอลิสที่สกัดด้วยตัวทำละลายเฮกเซน ไดคลอโรมีเทน เอทานอลและน้ำ รวมทั้งวิเคราะห์องค์ประกอบทางเคมีของสารสกัด จากการสกัดแบบลำดับส่วน พบว่าผลผลิตร้อยละของสารสกัดไดคลอโร มีเทนมีผลผลิตร้อยละมากที่สุด 22.56 เปอร์เซ็นต์โดยน้ำหนักต่อน้ำหนักรองลงมาคือสารสกัดเฮกเซน 19.66 เปอร์เซ็นต์โดย น้ำหนักต่อน้ำหนักและสารสกัดเอทานอล 18.68 เปอร์เซ็นต์โดยน้ำหนักต่อน้ำหนักส่วนสารสกัดน้ำมีผลผลิตร้อยละน้อยที่สุด

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เท่ากับ 0.05 เปอร์เซ็นต์โดยน้ำหนักต่อน้ำหนักเมื่อนำสารสกัดมาทดสอบฤทธิ์ยับยั้งอนุมูลอิสระโดยวิธี DPPH scavenging assay พบว่าสารสกัดทั้ง 4 ชนิด สามารถยับยั้งอนุมูลอิสระได้ โดยการสกัดด้วยเอทานอลที่ความเข้มข้น 2,000 ไมโครกรัมต่อมิลลิลิตรมีฤทธิ์ในการยับยั้งได้สูงถึง 84 เปอร์เซ็นต์ ซึ่งใกล้เคียงกับฤทธิ์ของสารสกัดด้วยไดคลอโรมีเทน 80 เปอร์เซ็นต์ ในขณะที่สารสกัดน้ำสามารถยับยั้งอนุมูลอิสระได้น้อยที่สุด 33 เปอร์เซ็นต์และสารสกัดด้วยเฮกเซนสามารถยับยั้งได้ 60 เปอร์เซ็นต์ เมื่อเปรียบเทียบแบบแผนโครมาโทแกรมของสารสกัดพรอพอลิสในตัวทำละลายต่าง ๆซึ่งศึกษาด้วยวิธีทินเลเยอร์โครมาโทกราฟีชนิดซิลิกาเจล 60 F_{254} พบว่าสารสกัด เฮกเซน ไดคลอโรมีเทน และเอทานอล มีโครมาโทแกรมขององค์ประกอบสารคล้ายคลึงกัน ซึ่งสามารถแยกได้ 4 จุด ขณะที่โครมาโทแกรมของสารสกัดพรอพอลิสด้วยน้ำแตกต่างจากสารสกัดอื่นเพราะพบสารเพียงจุดเดียวเท่านั้น จากการศึกษานี้แสดงให้เห็นว่าสารสกัดพรอพอลิสในทุกตัวทำละลายมีความสามารถในการยับยั้งอนุมูลอิสระและเอทานอลเป็นตัวทำละลายที่ดีที่สุดเมื่อเปรียบเทียบกับเฮกเซน ไดคลอโรมีเทน และน้ำ

คำสำคัญ : พรอพอลิส, อนุมูลอิสระ, ตัวทำละลายอินทรีย์, ทินเลเยอร์โครมาโทกราฟี

บทนำ

พรอพอลิส (propolis) เป็นผลิตภัณฑ์ธรรมชาติที่ได้จากผึ้ง ซึ่งผึ้งเก็บยางไม้จากตาหรือเปลือกของต้นไม้ และผสมกับขี้ผึ้งและสารคัดหลั่งที่ผึ้งปล่อยออกมา โดยผึ้งใช้พรอพอลิสปิดรอยรั่วและเคลือบรังให้แข็งแรง นอกจากนี้พบว่าใช้ห่อหุ้มซากศัตรูที่ตายในรัง เพื่อไม่ให้เกิดการเน่าเหม็นในรังผึ้ง[1] พรอพอลิสมีสารหลายชนิดเป็นองค์ประกอบ เช่น พอลีฟีนอลิกเทอร์พีนอยด์สเตอรอยด์ และกรดอะมิโน แต่อย่างไรก็ตามความแตกต่างทางด้านภูมิประเทศ ภูมิอากาศ โดยเฉพาะทางด้านพันธุ์พืชในแต่ละพื้นที่เป็นปัจจัยสำคัญที่ส่งผลทำให้เกิดความแตกต่างขององค์ประกอบทางเคมีของพรอพอลิสในแต่ละพื้นที่นั้นเอง[2] จากสาเหตุนี้ทำให้พรอพอลิสจากทวีปยุโรป อเมริกาเหนือและใต้ เอเชียและแอฟริกามีองค์ประกอบทางเคมีที่แตกต่างกัน[1] พบว่าพรอพอลิสจากยุโรปและจีนมีฟลาโวนอยด์เป็นองค์ประกอบหลัก[3] ในขณะที่พบสารกลุ่มเทอร์พีนอยด์และอนุพันธ์กรดคูมาริกเป็นองค์ประกอบหลักของพรอพอลิสที่ได้จากบราซิล[4] ซึ่งองค์ประกอบทางเคมีที่สำคัญเหล่านี้ถูกสกัดด้วยตัวทำละลายน้ำและเอทานอลนั้นมีฤทธิ์ทางชีวภาพที่น่าสนใจเช่น ต้านการอักเสบ ต้านอนุมูลอิสระ ต้านแบคทีเรีย เป็นต้น[5]

จากการศึกษาที่ผ่านมาพรอพอลิสมักจะถูกสกัดด้วยตัวทำละลายเอทานอลซึ่งเป็นตัวทำละลายที่เป็นที่นิยมเพราะให้สารสำคัญหลายชนิดและสารสกัดสามารถแสดงฤทธิ์ทางชีวภาพได้ดีอย่างเช่น ต้านอนุมูลอิสระ ต้านแบคทีเรีย ต้านการอักเสบเป็นต้น เมื่อทำการสกัดพรอพอลิสด้วย เอทานอลจะทราบดีว่าพรอพอลิสจากประเทศไทยจะมี 2ลักษณะคือส่วนสารละลายใส อาจจะมีสีน้ำตาลเข้ม อาจเรียกส่วนแรกว่าส่วนที่ละลายได้ดีในน้ำหรือชอบน้ำ (hydrophilic) และส่วนที่มีลักษณะเหมือนไข อาจเรียกว่า ส่วนที่ละลายได้ดีในไขมัน

(lipophilic)พรอพรอลิสจากเชียงใหม่ที่ใช้ในการทดลองมักแสดงสัดส่วนระหว่างส่วนที่ชอบน้ำและส่วนที่ละลายในไขมันที่เท่ากัน[6] ส่วนที่มีลักษณะเป็นไขเรามักจะทิ้งไม่ได้นำมาใช้ ทั้งนี้เพราะตรวจวัดทำการทดสอบได้ยากแต่อย่างไรก็ตามพรอพอลิสจากอิหร่านที่สกัดด้วยเฮกเซนและไดคลอโรมีเทนมีความสามารถในการยับยั้งเชื้อราได้ดีเช่นกัน[7] ในขณะที่พรอพอลิสที่สกัดด้วยไดคลอโรมีเทนมีองค์ประกอบทางเคมีที่แตกต่างจากที่สกัดด้วยเมทานอล[8] ซึ่งแสดงให้เห็นว่าพรอพอลิสยังมีสารในกลุ่มอื่นที่สามารถแสดงฤทธิ์ทางชีวภาพได้ ซึ่งยังไม่มีรายงานที่ศึกษาถึงสารสกัดพรอพอลิสด้วยตัวทำละลายอื่นๆรวมทั้งฤทธิ์ทางชีวภาพ ดังนั้นการศึกษานี้จึงมีความสนใจศึกษาองค์ประกอบทางเคมีและฤทธิ์ทางชีวภาพของสารสกัดพรอพอลิสที่สกัดด้วยตัวทำละลายต่างกัน

วัสดุและวิธีการ

การสกัดสารจากพรอพอลิส (propolis)

พรอพอลิสซื้อมาจากร้านค้าในอำเภอร้องกวาง จังหวัดแพร่ นำพรอพอลิส (propolis) 20 กรัม หั่นให้เป็นชิ้นเล็กๆ บดให้ละเอียดจากนั้นสกัดด้วยตัวทำละลายเฮกเซน ไดคลอโรมีเทน เอทานอลและน้ำตามลำดับปริมาตร 200 มิลลิลิตร และสกัดด้วย ultrasonic bath ที่อุณหภูมิห้อง เป็นเวลา 30 นาที หลังจากนั้นนำส่วนผสมที่ได้มากรองด้วยกระดาษกรอง Whatman No.1 โดยใช้กรวยกรองสุญญากาศและเก็บส่วนของสารละลายมาระเหยตัวทำละลายออกด้วยเครื่องระเหยแห้ง (rotary evaporator) นำสารสกัดที่ระเหยตัวทำละลายแล้วนำมาชั่งน้ำหนักและเก็บให้พ้นแสงที่อุณหภูมิห้องเพื่อใช้ในการทดลองต่อไป

การศึกษาองค์ประกอบทางเคมีของพรอพอลิสโดยเทคนิคทินเลเยอร์โครมาโทรกราฟี

เตรียมสารสกัดพรอพอลิสที่ความเข้มข้น 1 มิลลิกรัมต่อมิลลิลิตร จุดสารสกัดลงบนแผ่น TLC plate ชนิด silica gel 60 F254 จากนั้นนำแผ่น TLC ไปแช่ใน mobile phase ซึ่ง mobile phase ประกอบด้วย hexane : ethyl acetate : acetic acid) 60:40:1 เปอร์เซ็นต์ปริมาตรต่อปริมาตรตรวจสอบการเคลื่อนที่ของสารด้วยไอของไอโอดีน (iodine vapor)

การศึกษาฤทธิ์ในการต้านอนุมูลอิสระของสารสกัดจากพรอพอลิส (propolis)

ศึกษาโดยวิธี DPPH radical-scavenging เตรียมความเข้มข้นของสารสกัดเฮกเซน ไดคลอโรมีเทน เอทานอล และน้ำที่ความเข้มข้น 100-2000 ไมโครกรัมต่อมิลลิลิตร นำสารสกัดปริมาตร 0.3 มิลลิลิตร เติมตัวทำละลาย ปริมาตร 2.4 มิลลิลิตร หลังจากนั้นเติมสารละลาย DPPH ที่มีความเข้มข้น 150 ไมโครโมลาร์ ปริมาตร 0.3 มิลลิลิตรเขย่าสารละลายทั้งหมดให้เข้ากันและตั้งทิ้งไว้ในที่มืดเป็นเวลา 30 นาที จากนั้นนำไปวัดค่าการดูดกลืนแสงที่ 517 นาโนเมตรนำค่าที่ได้มาคิดเปอร์เซ็นต์การยับยั้งอนุมูลอิสระได้ดังนี้

$$\% \text{ DPPH radical scavenging activity} = \frac{\{A_0 - (A_1 - A_s)\}}{A_0} \times 100$$

เมื่อ A_0 คือ ค่าการดูดกลืนแสงของ DPPH

    A_1 คือ ค่าการดูดกลืนแสงของสารสกัดพรอพอลิสที่ทำปฏิกิริยากับ DPPH

    A_s คือ ค่าการดูดกลืนแสงของสารสกัดพรอพอลิส

ผลการทดลอง

ลักษณะของสารสกัดพรอพอลิสด้วยตัวทำละลายเฮกเซน ไดคลอโรมีเทน เอทานอล และน้ำ

พรอพอลิสที่ใช้ในการทดลองได้มาจาก ตำบลหนองผึ้ง อำเภอสารภี จังหวัดเชียงใหม่ มีสีน้ำตาลเข้มจนเกือบเป็นสีดำ ไม่มีกลิ่น มีลักษณะแข็งและเหนียว พบว่าสารสกัดพรอพอลิสด้วยเฮกเซนมีสีเหลือง สารสกัดพรอพอลิสด้วยไดคลอโรมีเทนมีสีน้ำตาลเข้ม สารสกัดเอทานอลมีสีเหลืองอ่อน และสารสกัดน้ำมีสีน้ำตาล สารสกัดแต่ละชนิดเมื่อนำมาคำนวณหาผลผลิตร้อยละ พบว่าสารสกัดไดคลอโรมีเทนมีผลผลิตร้อยละมากที่สุดเท่ากับ 22.56 เปอร์เซ็นต์โดยน้ำหนักต่อน้ำหนักและสารสกัดเฮกเซนมีค่าเท่ากับ 19.66 เปอร์เซ็นต์โดยน้ำหนักต่อน้ำหนักสารสกัดเอทานอลมีค่าเท่ากับ 18.68 เปอร์เซ็นต์โดยน้ำหนักต่อน้ำหนักส่วนผลผลิตร้อยละของสารสกัดน้ำมีค่าน้อยที่สุดเท่ากับ 0.05 เปอร์เซ็นต์โดยน้ำหนักต่อน้ำหนัก

การศึกษาความสามารถในการยับยั้งอนุมูลอิสระของสารสกัดพรอพอลิส

วิธี DPPH scavenging assay เป็นวิธีทดสอบความสามารถในการยับยั้งอนุมูลอิสระของสารสกัดพรอพอลิสด้วยตัวทำละลายเฮกเซน ไดคลอโรมีเทน เอทานอลและน้ำ ตามลำดับ พบว่าสารสกัดพรอพอลิสทั้ง 4 ชนิดสามารถยับยั้งอนุมูลอิสระ DPPH ได้ซึ่งความสามารถในการยับยั้งอนุมูลอิสระเพิ่มขึ้นเมื่อความเข้มข้นของสารสกัดมากขึ้นด้วย (รูปที่ 1) พบว่าสารสกัดเอทานอลที่ความเข้มข้น 2,000 ไมโครกรัมต่อมิลลิลิตรมีฤทธิ์ในการยับยั้งได้สูงสุดถึง 84 เปอร์เซ็นต์ รองลงมาคือฤทธิ์ของสารสกัดไดคลอโรมีเทน 80 เปอร์เซ็นต์ และสารสกัดเฮกเซน 60 เปอร์เซ็นต์ ขณะที่สารสกัดน้ำสามารถยับยั้งอนุมูลอิสระได้น้อยที่สุดเท่ากับ 33 เปอร์เซ็นต์ นอกจากนี้สารสกัดไดคลอโรมีเทนและเอทานอลมีฤทธิ์ในการยับยั้งได้ใกล้เคียงกันในทุกความเข้มข้นของสารสกัด
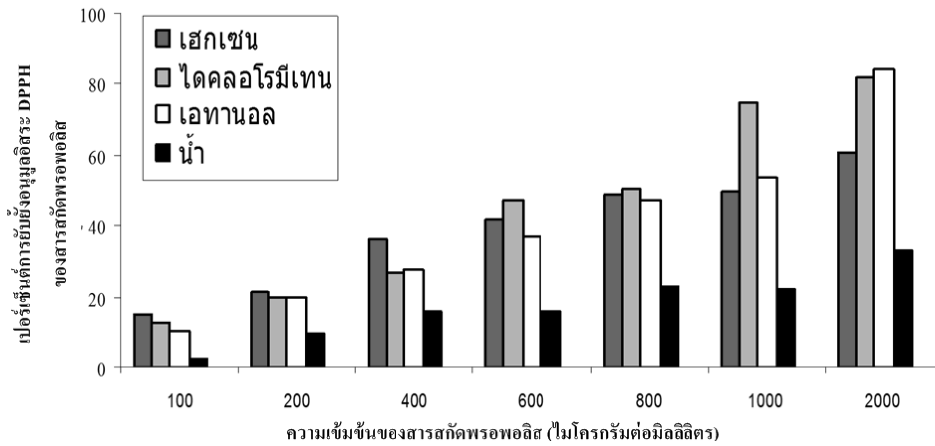
รูปที่ 1 แสดงเปอร์เซ็นต์การยับยั้งอนุมูลอิสระ DPPH ของสารสกัดพรอพอลิสด้วยตัวทำละลายเฮกเซน ไดคลอโรมีเทน เอทานอล และน้ำ

การศึกษาองค์ประกอบทางเคมีของสารสกัดพรอพอลิสด้วยวิธีทินเลเยอร์โครมาโทกราฟี

จากการศึกษาองค์ประกอบทางเคมีของสารสกัดพรอพอลิสโดยการแยกด้วยชนิด silica gel 60 F$_{254}$ จากนั้นนำแผ่น TLC ไปแช่ใน mobile phase ซึ่ง mobile phase ประกอบด้วย hexane : ethyl acetate : acetic acid) 60:40:1 เปอร์เซ็นต์ปริมาตรต่อปริมาตรตรวจสอบการเคลื่อนที่ของสาร

ด้วยไอของไอโอดีน (iodine vapor) พบว่าสารสกัดทั้งสี่ชนิดแสดงโครมาโทแกรมดังรูปที่ 2 พบว่าสารสกัดพรอพอลิสด้วยไดคลอโรมีเทนและสารสกัดพรอพอลิสด้วยเอทานอลแสดงโครมาโตแกรมที่มีความคล้ายคลึงกัน ขณะที่สารสกัดเฮกเซนมีความแตกต่างเล็กน้อยจากสารสกัดอื่นๆในขณะที่สารสกัดด้วยน้ำเกิดโครมาโทแกรมขึ้นเพียงจุดเดียว
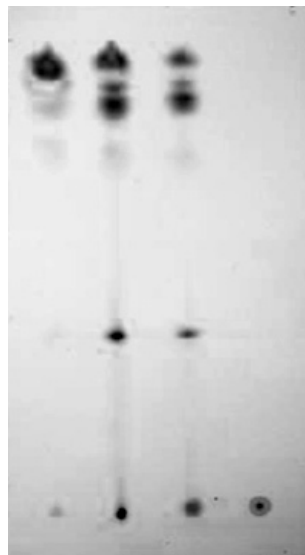
ตัวทำละลาย   1   2   3   4

รูปที่ 2 แสดงโครมาโทแกรมการวิเคราะห์องค์ประกอบของสารสกัดในตัวทำละลายเฮกเซน ไดคลอโรมีเทน เอทานอล และน้ำ ชนิด silica gel 60 F$_{254}$ จากนั้นนำแผ่น TLC ไปแช่ใน mobile phaseประกอบด้วย hexane : ethyl acetate : acetic acid (60:40:1 เปอร์เซ็นต์ปริมาตรต่อปริมาตร) ตรวจสอบการเคลื่อนที่ของสารด้วยไอของไอโอดีน ตัวทำละลายเฮกเซน (หมายเลข 1), ตัวทำละลายไดคลอโรมีเทน (หมายเลข 2), ตัวทำละลายเอทานอล (หมายเลข 3), ตัวทำละลายน้ำ (หมายเลข 4)

วิจารณ์ผลการทดลอง

ดังนั้นการศึกษานี้ได้สกัดพรอพอลิสด้วยตัวทำละลายที่ไม่มีขั้วจนถึงมีขั้วตามลำดับได้แก่ เฮกเซน ไดคลอโรมีเทน เอทานอล และน้ำ เราคาดหวังว่าจะลดตัวรบกวนหรือไขในชั้นของสารสกัดพรอพอลิสในเอทานอลและน้ำ และน่าจะทำให้สารสกัดสามารถแสดงฤทธิ์ในการต้านอนุมูลอิสระได้มากขึ้น รวมทั้งตรวจสอบฤทธิ์ต้านอนุมูลอิสระของพรอพอลิสในตัวทำละลายเฮกเซน และไดคลอโรมีเทนเมื่อนำมาแยกสารด้วยเทคนิคทินเลเยอร์โครมาโทกราฟพบว่าสารสกัดพรอพอลิสสามชนิด ได้แก่ เฮกเซน ไดคลอโรมีเทน และเอทานอล สามารถแยกองค์ประกอบของสารได้ ขณะที่สารสกัดพรอพอลิสด้วยน้ำแสดงจุดโครมาโทแกรมเพียงจุดเดียว มีรายงานว่าพรอพอลิสที่สกัดด้วยน้ำได้สารจำพวกกรดอะมิโนกรดฟีนอลิกเอสเทอร์ของกรดฟีนอลิกฟลาโวนอยด์กรดซินเนมิก และกรดคาเฟอิก[9] แต่อย่างไรก็ตามพัทยาและสุพรรณี[6] ซึ่งใช้พรอพอลิสจากแหล่งเดียวกัน รายงานว่าสารสกัดด้วยน้ำของพรอพอลิสมีสารโพลีฟีนอลิกและฟลาโวนอยด์เป็นองค์ประกอบ อาจเป็นเพราะเฟสเคลื่อนที่ที่ใช้ในการทดลองนี้อาจจะเหมาะสมกับการแยกสารที่มีขั้วน้อยหรือไม่มีขั้ว จึงทำให้ไม่สามารถแยกสารที่น่าจะมีขั้วมากของสารสกัดน้ำของพรอพอลิสในสารสกัดพรอพอลิสด้วยเฮกเซนรายงานว่ามีองค์ประกอบสำคัญได้แก่สารประเภทไขผึ้ง bromfenvinphos[10] และ สารอื่น pentacyclic triterpenoid alkanoates เป็นกลุ่มสารที่ฤทธิ์ทางชีวภาพที่น่าสนใจ[11] นอกจากนี้สารสกัดพรอพอลิสไทยด้วยเฮกเซนมีสาร cardanol และ cardol ซึ่งมีฤทธิ์ต้านการเพิ่มจำนวนและมีความเป็นพิษต่อเซลล์มะเร็ง[12] นอกจากนี้ยังรายงานว่าพรอพอลิสที่สกัดด้วยไดคลอโรมีเทนสามารถแสดงฤทธิ์ดังกล่าวได้เช่นเดียวกัน[12]

จากการศึกษานี้ พบว่าสารสกัดพรอพอลิสทั้ง 4 ชนิด แสดงฤทธิ์ยับยั้งอนุมูลอิสระ DPPH ได้ต่ำกว่าสารสกัดพรอพอลิสด้วย 70% v/v เอทานอลเพียงชนิดเดียวที่ความเข้มข้น 1000 ไมโครกรัมต่อมิลลิลิตร สามารถยับยั้งอนุมูล

อิสระ DPPH ได้ 90 เปอร์เซ็นต์ ได้รายงานไว้ในพัทยาและสุพรรณี[9] เป็นไปได้ว่าเมื่อมีการสกัดด้วยตัวทำละลายแบบลำดับส่วน อาจทำให้มีสารที่สามารถแสดงฤทธิ์เสริมกันกับสารสกัดจากเอทานอลได้ถูกสกัดออกไปอยู่ในสารสกัดไดคลอโรมีเทนหรือเฮกเซนจึงทำให้ความสามารถในการยับยั้งอนุมูลอิสระลดลงสอดคล้องกับงานวิจัยของ Umthong[12] กล่าวว่าสารสกัดอย่างหยาบจากพรอพอลิสอาจมีสารอื่นๆ และเพิ่มฤทธิ์ของสารสกัดพรอพอลิสได้ ขณะเดียวกันอาจเป็นไปได้ว่าความเข้มข้นของสารออกฤทธิ์ในสารสกัดแบบหยาบมีจำนวนน้อยลงเพราะการสกัดแบบลำดับส่วน จึงทำให้สารสกัดพรอพอลิสด้วยเอทานอลมีความสามารถยับยั้งอนุมูลอิสระได้น้อยลง เป็นที่น่าสนใจว่าสารสกัดพรอพอลิสในตัวทำละลายน้ำมีความสามารถยับยั้งอนุมูลอิสระได้คงที่เมื่อเทียบกับงานวิจัยที่ผ่านมาของพัทยาและสุพรรณี[6]

สรุปผลการทดลอง

จากผลการศึกษาการสกัดสารพรอพอลิสจากตำบลหนองผึ้ง อำเภอสารภี จังหวัดเชียงใหม่โดยใช้ตัวทำละลายต่างชนิดกัน คือ เฮกเซน ไดคลอโรมีเทน เอทานอล และน้ำ จากการศึกษาฤทธิ์ในการยับยั้งอนุมูลอิสระของสารสกัดพรอพอลิสโดยวิธี DPPH scavenging assay พบว่าสารสกัดทั้งสี่ชนิดสามารถยับยั้งอนุมูลอิสระได้ สารสกัดเอทานอลมีประสิทธิภาพในการยับยั้งอนุมูลอิสระได้สูงกว่าสารสกัดเฮกเซน ไดคลอโรมีเทน และน้ำ

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Liquid Chromatography Technique for Fractionation, Identification and Biological Activity Analysis of Salivary Gland Extract of *Aedes aegypti*

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Abstract

Mosquito-borne diseases are ones of the most important world health problem. The pathogens are transmitted during blood feeding which the processes could be modified by mosquitoes' salivary components. This study aimed to develop a separation technique that is able to maintain biological activity of salivary/salivary gland proteins for further biological analysis and identification. Here, size exclusion liquid chromatography was applied to fractionate salivary gland proteins, and the antiplatelet activity was used as a test model to test activity of the fractions. One hundred salivary glands of *Aedes aegypti* were extracted and fractionated using ZORBAX GF-250 (4.6 x 250 mm, particle size 4 μm) as a stationary phase and 100 mM sodium phosphate buffer pH 7.0 as mobile phase. The eluents were collected every 1 min intervals to study anti-platelet activity. The results showed that the fraction eluted at 12 min reversed ADP-induced platelet aggregation *in vitro*. The molecular weight of this fraction was 97 kDa. To identify the protein, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. A target protein band was observed with molecular weight of 63 kDa. This band was in-gel tryptic digested and analyzed via liquid chromatography-tandem mass spectrometry (LC-MS/MS). Apyrase, an enzyme with antiplatelet activity, was identified using MASCOT search engine. In conclusion, the biological activity of salivary glands extract was conserved after size-exclusion chromatography.

Keywords: *Aedes aegypti,* antiplatelet, salivary gland, size-exclusion

การใช้เทคนิค liquid chromatography ในการแยก บ่งชี้ชนิด และศึกษาฤทธิ์ทางชีวภาพของสารสกัดจากต่อมน้ำลายยุงลาย (*Aedes aegypti*)

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บทคัดย่อ

โรคติดต่อที่มียุงเป็นพาหะเป็นปัญหาทางสาธารณสุขที่สำคัญ การแพร่เชื้อของยุงเกิดขึ้นระหว่างการดูดเลือดของยุง ซึ่งองค์ประกอบของน้ำลายยุงนั้นเป็นตัวแปรของการแพร่เชื้อและมีผลต่อระบบภูมิคุ้มกันของร่างกาย การศึกษานี้มีวัตถุประสงค์ เพื่อพัฒนาวิธีการแยกองค์ประกอบในสารสกัดจากน้ำลายหรือต่อมน้ำลายยุงยังคงฤทธิ์ทางชีวภาพภายหลังการแยก ในที่นี้ใช้

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วิธี size exclusion liquid chromatography ในการแยกโปรตีนจากต่อมน้ำลายยุง และใช้การตรวจวัดการเกาะกลุ่มของเกล็ดเลือด เพื่อเป็นต้นแบบในการทดสอบความคงตัวของฤทธิ์ทางชีวภาพ การศึกษาดำเนินการโดยนำต่อมน้ำลายยุงจำนวน 100 ต่อมมาสกัดและแยกบน ZORBAX GF-250 (ขนาด 4.6 x 250 มิลลิเมตร, อนุภาคขนาด 4 ไมครอน) เป็นเฟสคงที่ และโซเดียมฟอสเฟสบัฟเฟอร์เป็นเฟสเคลื่อนที่ และเก็บตัวอย่างที่แยกทุก 1 นาที จากการศึกษาพบว่าตัวอย่างที่แยกที่ 12 นาที มีฤทธิ์ในการลดการเกาะกลุ่มของเกล็ดเลือดที่กระตุ้นด้วย ADP ซึ่งน้ำหนักโมเลกุลคำนวณได้คือ 97 กิโลดาลตัน เมื่อนำสารสกัดจากต่อมน้ำลายยุงมาแยกด้วย SDS-PAGE พบว่าโปรตีนที่สนใจอยู่ที่ 63 กิโลดาลตัน จากการวิเคราะห์ด้วย LC-MS/MS ตามด้วยการสำรวจหาชนิดโปรตีนจากโปรแกรม Mascot ระบุว่า โปรตีนนั้นคือ apyrase ซึ่งเป็นเอนไซม์ที่มีฤทธิ์ต้านการเกาะกลุ่มของเกล็ดเลือด ดังนั้นจึงสรุปว่าฤทธิ์ทางชีวภาพของสารสกัดจากต่อมน้ำลายยุงยังคงสภาพเมื่อแยกด้วย size exclusion liquid chromatography

คำสำคัญ: ยุงลาย, การเกาะกลุ่มของเกล็ดเลือด, ต่อมน้ำลาย, การแยกตามขนาด

Introduction

Mosquito-borne diseases are the most important world health problem in both human and animals.[1] Mosquitoes transmit not only the pathogens during blood feeding but also their salivary proteins to host. Salivary proteins have various activities to counteract host hemostatic system, blood coagulation, platelet aggregation and vasoconstriction, for enhancing blood-feeding and virus transmission efficiently.[2-3] Separation and identification of salivary and salivary gland proteins are required for further studying of the mechanisms of blood feeding, transmission of diseases as well as host-immune responses.[4]

In this study, we utilized size exclusion liquid chromatography for fractionation of protein from salivary glands of *Aedes aegypti*, the most important vector of virus infectious diseases, for example, yellow fever and dengue fever.[5] We proposed that with size exclusion liquid chromatography, the fractionated proteins maintain their biological activities. In order to evaluate this propose, antiplatelet activity was used as a test model. Furthermore, the target protein of salivary glands extract was identified using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Materials and Methods

Salivary gland extraction

Salivary glands of *Aedes aegpyti* were obtained from Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University. The salivary gland extract (SGE) was prepared by homogenization of 100 salivary glands in 1 ml of cold phosphate saline buffer. The extract was subjected to one -80°C freeze-thaw cycle and centrifuged at 4°C at 5000 g for 10 min. The clear supernatant was collected and kept at -80°C.

Fractionation of salivary gland extract by size exclusion liquid chromatography

SGE was fractionated by size using a gel-filtration column (ZORBAX GF-250 column, 4.6 x 250 mm, particle size 4 μm) with isocratic mobile phase consisted of 100 mM of NaH_2PO_4/Na_2HPO_4 buffer pH 7.0. The HPLC system consisted of auto sampler, Water 2695 separation module, Water 2996 photodiode array detector operated at 210 nm with Empower 2 software (Waters corporation, Milford, USA). The flow rate was 0.2 ml/min and injection volume was 60 μl.

The fractions of SEG were collected at 1 min intervals from 0 to 25 min. Concentration and molecular weight of protein fractions were determined by using gel filtration standard (Bio-Rad Laboratories Ltd, California, USA). The stock standard was dissolved in 1 ml and then prepared working standard by diluting to 30-fold. The 2.5 μl of the standard was injected to HPLC system as above described.

Identification of salivary gland extract by LC-MS/MS

SGE was separated by 12.5% SDS-PAGE, gel electrophoresis and stained with Coomassie brilliant blue (Bio-Rad Laboratories Ltd, California, USA). The molecular weight of protein bands were estimated by molecular mass markers (BLUelf Prestained Protein Ladder; GeneDireX, LasVegas, USA). The target band was excised and digested (Trypsin singles, proteomics grade, Sigma-Aldrich, Missouri, USA) without reduction and alkylation steps as described previously by Shevchenko et al.[6] The dried extract samples were stored -20°C and dissolved with 2% acetonitrile and 0.01% formic acid before analysis by LC-MS/MS.

Analysis was performed by using electrospray-ionization (ESI)-ion trap (Esquire HCT, Bruker). Chromatographic part used C-18 reversed-phase capillary column (Polaris C18-A, 75 x 2.1

mm, particle size 5 μm) with a flow rate of 0.15 ml/min. The gradient profile mobile phase was a linear gradient from 90% A (5% acetonitrile, 0.1% formic acid) to 40% B (80% acetronitrile, 0.1% formic acid) in 15 min and maintained at isocratic conditions (40% B) for 30 min before turning to initial conditions within 1 min and equilibrated for 14 min. The electrospray capillary voltage was set to 2.3 kV. MS scan began at 100 m/z to 3000 m/z. For fragmentation, amplitude was set at 0.65 V. Mass data collected during LC-MS/MS were analyzed by Data analysis 4.1 (Bruker). Finding compound of mass data were performed by using the MASCOT search engine (Matrix Science Ltd., London, UK).

Platelet aggregation assay

Platelet aggregation was assayed by turbidimetric method. A 10 ml of freshly blood in 3.8% sodium citrate was used for platelet preparation. Blood sample was centrifuged at 800 rpm for 10 min to obtain platelet-rich plasma (PRP). Part of PRP was further centrifuged at 3500 rpm for 10 min to obtain platelet-poor plasma (PPP). PPP was used to adjust PRP in ratio 1:1

PRP samples (290 μl) with fractionated SGE (5 μl) were incubated for 1 min. Then samples were incubated further for 1 min at 37°C. Baseline was set for 1 min then samples were stirred at 1000 rpm for 1min. Platelets were stimulated by adding of 5 μl of 8 μM of ADP. The intensity of light transmission over 5 min was then measured using an aggregometer (Chrono-log Model 560CA, Chrono-Log). Changes in light transmission were recorded during stirring of sample. Percentage of platelet aggregation was calculated compared to the base line.

Results

Fractionation of salivary gland extract by size-exclusion liquid chromatography

Chromatogram of *Aedes aegypti* SGE is shown in Figure 1a, the four major peaks were eluted at 12.0, 13.2, 14.3 and 15.6 min. In order to calculate the molecular weight of each fraction, a gel filtration standard with molecular weight range 1350-670,000 Da was used for constructing a molecular weight standard curve. Chromatogram of gel filtration standard contains thyroglobulin, γ-globulin, ovalbumin, myoglobin and vitamin B 12. The retention time of each eluent was 9.6, 11.8, 13.1, 14.0 and 15.8 min, respectively (Figure 1b). The standard curve plotting between log MW and retention time was linear with the coefficient of determination (r^2) 0.9707 (Figure 1c).

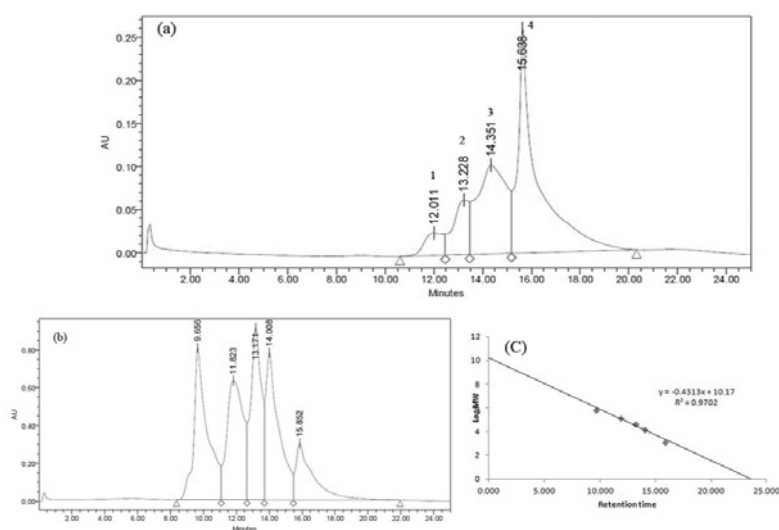Molecular weights of *Aedes aegypti* SGE was calculated and shown in Table 1.

Figure 1. Chromatogram of *Aedes aegypti* salivary gland extract (a), gel filtration standard (b), and molecular weight standard curve (c).

Table 1. Retention time and molecular weight of *Aedes aegypti* salivary proteins.

Peak no.	Retention time (min)	Molecular weight (kDa)
1	12.0	97.6
2	13.2	29.2
3	14.3	9.5
4	15.6	2.6
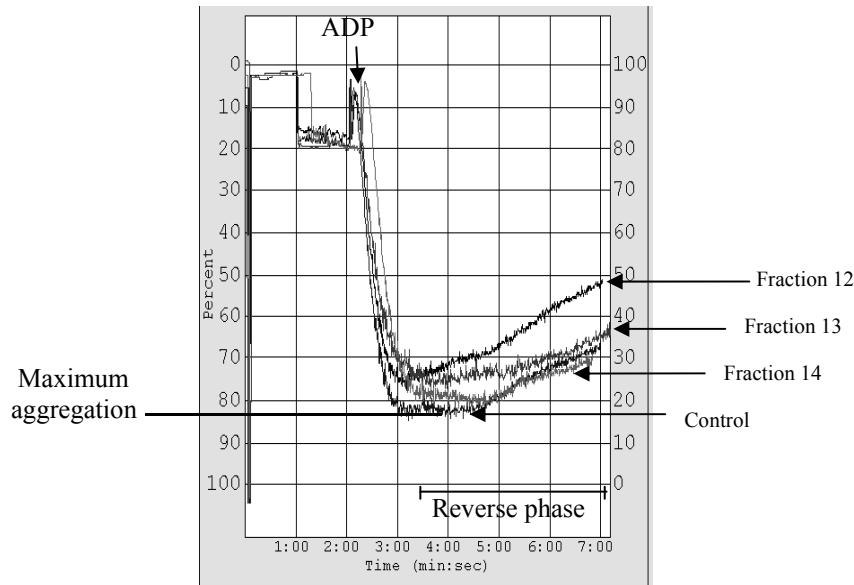
Figure 2.　Effect of fractionated SGE on ADP induced-platelet aggregation.

Antiplatelet activity of Aedes aegypti SGE

Fractions of SGE were collected and the eluent at 12, 13 and 14 min were tested for antiplatelet activity. PRP sample was incubated with each fraction of SGE at 37 °C for 1 min. Platelet aggregation was induced by 8 μM ADP. The results showed that, without SGE, ADP induced platelet aggregation accounted for 65% and following by reverse aggregation. At the end of measurement, platelet aggregation was 50%.

The fraction eluted at 13 and 14 min showed no effect on ADP induced platelet aggregation. Although, the fraction eluted at 12 min slightly decreased the value of maximum aggregation (55%), it rapidly reversed about 25% of platelet aggregation (Figure 2). Therefore, fraction eluted at 12 min showed antiplatelet activity by reversing platelet aggregation process.

Identification of Aedes aegypti SGE by LC-MS/MS

The platelet aggregation assay showed that protein at fraction eluted at 12 min had antiplatelet activity. The molecular weight was estimated to be about 97 kDa by the size exclusion chromatography. To identify the protein composition, SGE was separated by SDS-PAGE, and the protein band nearly 97 kDa was targeted, digested and analyzed by LC-MS/MS (Figure 3). Raw data was analyzed further to identify this protein by using MASCOT search engine. Result of searching showed that protein hit was apyrase with score 453 (Table 2).
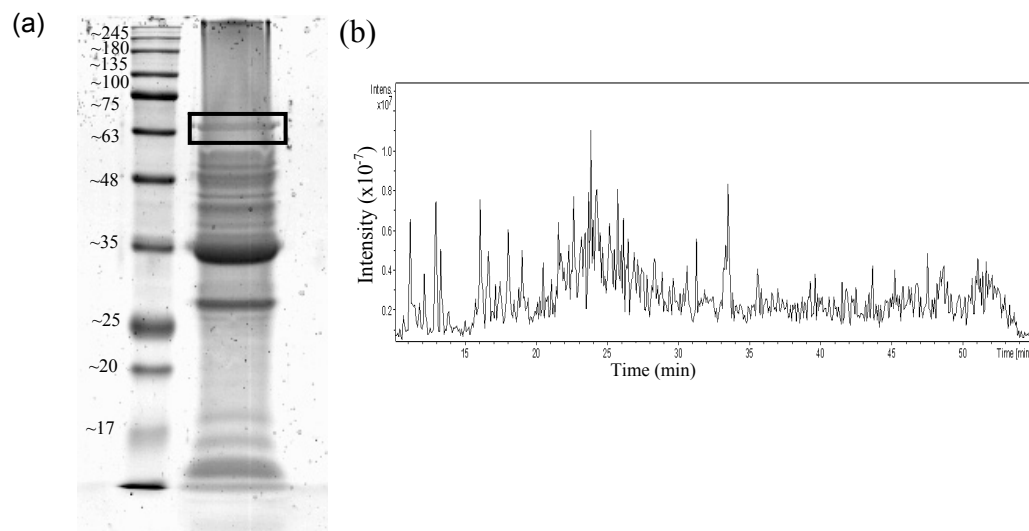
Figure 3. Separation of SGE by SDS-PAGE and stained with Coomassie Brilliant Blue (a). Base peak chromatogram of 63 kDa protein of SGE (b)

Table 2. Identified protein by MASCOT from salivary gland profiles of *Aedes aegypti*

Protein name	Molecular mass (Da)	Score	Protein sequence coverage	Sequence
Apyrase	62715	453	32 %	R.TGPLDSDVFK.N
				K.VTLSNAVEAVR.R
				K.GLAPYLAELNK.E
				R.LTLYFDEEGEVK.N
				K.DKVEGPYPTLVESK.N
				K.EAEYYIVVPSYLADGK.D
				K.EGIPTIVANLVMNNDPDLK.S
				K.ITNGDIIEAAPFGSTADLIR.L
				K.GADIWDVAEHSFALDDEGR.T
				K.NPIYLNAGDNFQGTLWYNLLR.W
				K.IAAEAGDDIDVIVGAHSHSFLYPDSK.Q

Discussion

Salivary gland protein and salivary protein play a key role not only in enhancing blood-feeding but also pathogen transmission and host-immune response. Fractionation of protein from salivary gland or saliva is required to clarify the function of salivary composition.

Here the size exclusion liquid chromatography was applied to fractionate the protein. Principally, the technique is based on the size or molecular weight of protein. However, shape and hydrophobicity also influence the separation.[7] This may explain the difference in the molecular weight obtained from size exclusion liquid chromatography and SDS-PAGE.

Our results indicated that the fractions of protein maintain their biological activity. Here we used antiplatelet activity as a model to evaluate biological activity. Platelet aggregation is the first line of defense to avoid blood loss during tissue injury.[8] Several agonists such as ADP and collagen induce platelet activation. Mosquitoes are necessary to produce antiplatelet agents to facilitate a blood meal.[9] Ribeiro, 1987 studied the effect of *Aedes agypti* saliva from salivation on platelet aggregation. He found that mosquito saliva inhibits platelet aggregation. To date, there have been reported that salivary protein of *Aedes aegypti* contains apyrases, which have ability to inhibit platelet aggregation by hydrolyzing di-and tri-phosphate.[10]

Regarding to the platelet aggregation profile, the protein fraction eluted at 12 min caused reverse platelet aggregation. This effect may due to inhibition of ADP release or insufficient concentration of ADP. Result from MASCOT search engine report that the target protein was apyrase, an

ADP-hydrolysing enzyme. Therefore, the results obtained from biological assay and mass spectrometry were consistent.

Conclusion

Aedes aegypti salivary proteins fractionated from the size exclusion liquid chromatography was able to maintain antiplatelet activity which is one of biological activities of salivary protein. Therefore, the liquid chromatography techniques will be further developed to fractionation and identify the bioactive compounds in salivary gland extract. Furthermore, this study may lead to understanding the transmission mechanism of mosquitoes and their therapeutic.

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F23 Preliminary Study on the Effects of *Andrographis paniculata* on Platelet Aggregation in Thai Healthy Volunteers

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Abstract

Andrographis paniculata (Burm.F) Wall ex Nees has been used in Thai traditional medicine to relieve common cold, fever, and diarrhea for centuries. Its bioactive compound, andrographolide, showed anti-platelet activities in animal study models. However, the effect of *A. paniculata* has not been reported on human platelet functions. Therefore, we have investigated the effects of *A. paniculata* on platelet activity in ten healthy volunteers. *A. paniculata* was taken 3 x 2 g within one day (total 6 g/day). Platelet aggregation induced by epinephrine (Epi), adenosine diphosphate (ADP) and collagen (Col) was studied before and after *A. paniculata* administration 2 and 24 hr. Thromboxane B_2 (TXB_2) level in these aggregated platelets was also assessed. It was found that ADP-induced platelet aggregation was decreased while Epi-induced platelet aggregation was increased after *A. paniculata* ingestion. By considering platelet activity to disaggregation, normal aggregation and hyperaggregation groups, platelet aggregation in normal aggregation group was decreased whereas in disaggregation group was increased. However, TXB_2 levels were not significantly different. This study, therefore, provided the preliminary scientific data for the clinical study of *A. paniculata* on platelet aggregation in human.

Keywords: *Andrographis paniculata,* platelet aggregation, TXB_2, epinephrine, ADP

การศึกษาเบื้องต้นเกี่ยวกับผลของยาสมุนไพรฟ้าทะลายโจรต่อการทำงานของเกล็ดเลือดในอาสาสมัครสุขภาพดี

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บทคัดย่อ

ยาสมุนไพรฟ้าทะลายโจรมีสรรพคุณบรรเทาอาการเจ็บคอ แก้หวัด ลดไข้ และท้องเสียที่นิยมใช้กันมานาน การศึกษาในสัตว์ทดลองพบว่าแอนโดรกราโฟไลด์เป็นสารสำคัญที่มีผลยับยั้งการจับกลุ่มของเกล็ดเลือด อย่างไรก็ตามยังไม่เคยมีการศึกษาในคน ผู้วิจัยจึงทำการศึกษาผลของยาสมุนไพรฟ้าทะลายโจรต่อการจับกลุ่มของเกล็ดเลือดในอาสาสมัครสุขภาพดี 10 คน โดยให้อาสาสมัครรับประทานยาสมุนไพรฟ้าทะลายโจรครั้งละขนาด 2 กรัม 3 เวลาหลังอาหารภายใน 1 วัน (รวม 6 กรัม/วัน)

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วิเคราะห์เปรียบเทียบผลของสมุนไพรฟ้าทะลายโจรต่อการจับกลุ่มของเกล็ดเลือด รวมทั้งระดับทรอมโบแซนบี 2 ในเกล็ดเลือด ระหว่างก่อนรับประทานยาและหลังรับประทายาที่เวลา 2 และ 24 ชั่วโมงพบว่าการจับกลุ่มของเกล็ดเลือดที่ถูกกระตุ้นด้วย อะดีโนซีนไดฟอสเฟตลดลง ในขณะที่การจับกลุ่มของเกล็ดเลือดที่ถูกกระตุ้นด้วยอีพิเนฟรินเพิ่มขึ้นหลังรับประทานสมุนไพรฟ้า ทะลาย เมื่อพิจารณาตามลักษณะการจับกลุ่มของเกล็ดเลือดซึ่งแบ่งเป็นผู้ที่มีการจับกลุ่มของเกล็ดเลือดน้อยกว่าปกติ ปกติ และ มากกว่าปกติ พบว่าการจับกลุ่มของเกล็ดเลือดในผู้มีการจับกลุ่มของเกล็ดเลือดแบบปกติจะลดลง แต่ในผู้มีการจับกลุ่มของ เกล็ดเลือดแบบน้อยกว่าปกติจะเพิ่มขึ้น อย่างไรก็ตามพบว่าไม่มีผลต่อระดับทรอมโบแซนบี 2 ในเกล็ดเลือด การศึกษาครั้งนี้ เป็นข้อมูลเบื้องต้นที่สำคัญของการศึกษาสมุนไพรฟ้าทะลายโจรต่อเกล็ดเลือดในทางคลินิกต่อไป

คำสำคัญ: ฟ้าทะลายโจร, การจับกลุ่มของเกล็ดเลือด, ทรอมโบแซนบี 2, อีพิเนฟริน, อะดีโนซีนไดฟอสเฟต

Introduction

Androgprahis paniculata (Burm.F) Wall ex Nees has long been used to relieve sore throat, fever and common cold in Asia and Scandinavia.[1] *A. paniculata* is well known as Fah Talai Joan in Thailand. It was included in The National List of Essential Medicines 2013 to relief the symptoms of common cold and non-infectious diarrhea.[2] Active ingredients of *A. paniculata* exhibit therapeutic effect in many clinical and experimental studies.[3] Andrographolide is one of the major active compounds of *A. paniculata*. Andrographolide plays an important role in the inflammatory process, cyclooxygenase (COX) enzyme expression.[4,5] Moreover, in an *in vitro* study, andrographolide inhibited human platelet aggregation-induced by platelet-activating factor (PAF)[6] and inhibited mice platelet aggregation induced by thrombin.[7] Platelets are essential for primary hemostasis and also play an important pro-inflammatory role. Its activation may lead to the exposure of many adhesive molecules and inflammatory cytokines.[8] If *A. paniculata* exhibits antiplatelet effects, it is possibly be harmful to the physiological condition of hemostasis. However, there is still no clinical study about the effects of *A. paniculata* on human platelet aggregation. This preliminary study is planned to evaluate the effects of *A. paniculata* on platelet activity such as platelet aggregation induced by platelet agonists in healthy volunteers.

Materials and Methods

Chemicals and study drug

Vacutainer citrate tube was purchased from BD (New Jersey, USA). Epinephrine (Epi), adenosine diphosphate (ADP) and collagen (Col) were purchased from Helena Laboratory (USA).

EIA kit for thromboxane B_2 (TXB_2) was purchased from GE Healthcare (USA). The highest quality chemicals and reagents were purchased from Sigma-Aldrich (MO, USA or Germany).

A. paniculata 500 mg capsules were obtained from Manufacturing Unit of Herbal Medicine and Products, manufactured under GMP by Ayurved Thamrong School, Center of Applied Thai Traditional Medicine (CATTM), Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. The powders contain 3% of andrographolide (unpublished data from the unit of *A. paniculata* preparation). Therefore, one capsule of *A. paniculata* contains about 15 mg of andrographolide.

Subject design

The study protocol and related materials were reviewed and approved by Siriraj Institutional Review Board (SIRB) of Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. Ten subjects were enrolled in this study including 5 male and 5 female, age between 25 to 60 years old. They were healthy based on medical history, physical examination, clinical chemistry, hematologic screening and urine pregnancy test in female volunteers. Subjects were instructed to abstain from using any drugs that are known to affect platelet aggregation for at least 2 weeks before the study. They were also instructed to abstain from alcohol and smoking during the study. All participants provided verbal and written informed consent. According to National List Essential Medicines in Thailand[2], the participants received 3 x 2 g capsules of *A. paniculata* after meals within 1 day (total 6 g/day). Since pharmacokinetic study following 60 mg andrographolide oral administration revealed maximum plasma level of approximately 1.9 µM at 1.5-2 hr.[9] This plasma level is found enough to reveal any anti-PAF effects.[6, 9] Therefore, blood was taken in the morning before (0 hr.) and after 2 and 24 hr of *A. paniculata* administration to study platelet aggregation.

Human platelet aggregation assay

Citrated whole blood was centrifuged at 250 x g for 10 min at room temperature to prepare platelet rich plasma (PRP). Some PRP was further centrifuged at 4500 x g for 2 min at room temperature to prepare platelet free plasma (PFP) for setting as a blank. Platelet aggregation was determined using Born's technique[10] in aggregometer (AggRAM™, Helena, USA). Epi, ADP and Col were used as platelet agonists. Briefly, PRP in a silicone-treated glass cuvette was pre-incubated at 37 °C for at least 3 min. Then aggregation was induced by addition of each agonist while stirring at 600 rpm. The reaction was allowed to proceed for 5 min. The maximum amplitude of platelet aggregation was expressed as percentage of a difference between light transmission of aggregated PRP and of PFP. Activated and none activated PRP were immediately kept to evaluate TXB_2 level.

The classification of platelet status

Currently, there is no consensus on how to evaluate platelet function. Each laboratory follows its own practice. In our laboratory, platelet aggregation status was divided into three patterns, disaggregation (dis), normal aggregation (normal) and hyperaggregation (hyper), depend on the response of platelets to the panel of agonists.[11,12] Briefly, disaggregation and hyperaggregation are defined for primary phase of 25 µM Epi-induced aggregation and secondary phase of 1 µM Epi-induced aggregation, respectively, whereas normal aggregation is defined for platelet aggregation in a concentration-dependent manner.

Enzyme immunoassay for TXB_2 (a stable metabolite of TXA_2)

TXB_2, a stable metabolite of TXA_2 in supernatants of activated and none activated platelets were measured using an enzyme immunoassay commercial kit (TXB_2 EIA kit, GE Healthcare). Briefly, at the end of the platelet aggregation test, activated and none activated PRP was centrifuged at 4500 x g at 4° C for 10 minutes. Supernatant was collected and TXB_2 was assayed according to the manufacturer's instruction.[13]

Statistical analysis

The results were presented as mean ± standard error of mean (SEM). Data analysis was performed using GraphPad Prism version 5.03. Two-way ANOVA was tested to find any differences of parameters among groups followed by Bonferroni's post hoc test. P-value less than 0.05 were considered statistically significant.

Results

Demographic data of subjects

Baseline parameters for blood chemistry and complete blood count were within normal range for all volunteers (Table 1). Data were presented as mean ± SEM.

The effects of A. paniculata on platelet aggregation

The overall results showed that patterns of platelet aggregation were not changed for the entire of the study except one subject that disaggregation was changed to slightly normal aggregation. Epi-induced platelet aggregation (25 µM) was significantly increased after 24 hr while ADP- induced was significantly decreased after 2 and 24 hr of A. paniculata administration (Figure 1B and 1C). By considering platelet status, subjects existing normal aggregation and disaggregation exhibited significantly changed in Epi-induced aggregation in the opposite direction (Figure 1A and 1B).

By gender considering, platelet aggregation induced by 1 µM Epi was significantly higher in female than that in male at the entry hour (Figure 2A). After A. paniculata administration, aggregation induced by 1 µM Epi was still higher while aggregation induced by 5 µM ADP was significantly lower after 2 hr (Figure 2A, 2C). However, neither male nor female exhibited any significantly changed in each agonist-induced aggregation after 2 and 24 hr of A. paniculata administration.

Effects of A. paniculata on TXB_2 in platelets

Overall results of A. paniculata on TXB_2 in supernatant of aggregated PRP did not reveal any significant change. However, according to platelet status, decreased TXB_2 was found in subject existing normal platelet aggregation (Figure 3B).

Table 1. Baseline of healthy volunteers were included in the study

Baseline	Male (n = 5)	Female (n = 5)	Normal Range
Age (year)	31.8±6.2	41.2±15.5	-
Body weight (kg)	71.1±16.7	53.6±6.5	-
High (cm)	170.0±7.1	156.0±6.5	-
Body mass Index (kg/m^2)	24.4±4.0	22.1±3.5	18-24
Hemoglobin (g/dL)	15.1±1.6	13.3±0.4	12.0-18.0
Hematocrit (%)	45.4±3.8	40.3±1.5	37-52
WBC (x10^3/µL)	6.9±4.4	8.1±5.3	4.0-11.0
Platelet count (x10^3/µL)	197.4±83.6	262.4±63.1	150-440
FBS (mg/dL)	89.0±7.8	86.4±5.5	74-100
Total cholesterol (mg/dL)	187.0±59.9	230.4±18.7	<200
Creatinine (mg/dL)	1.0±0.1	0.70±0.01	0.51-0.95
AST (U/L)	23.3±2.1	20.4±4.2	0-32
ALT (U/L)	21.0±4.6	19.0±15.0	0-33
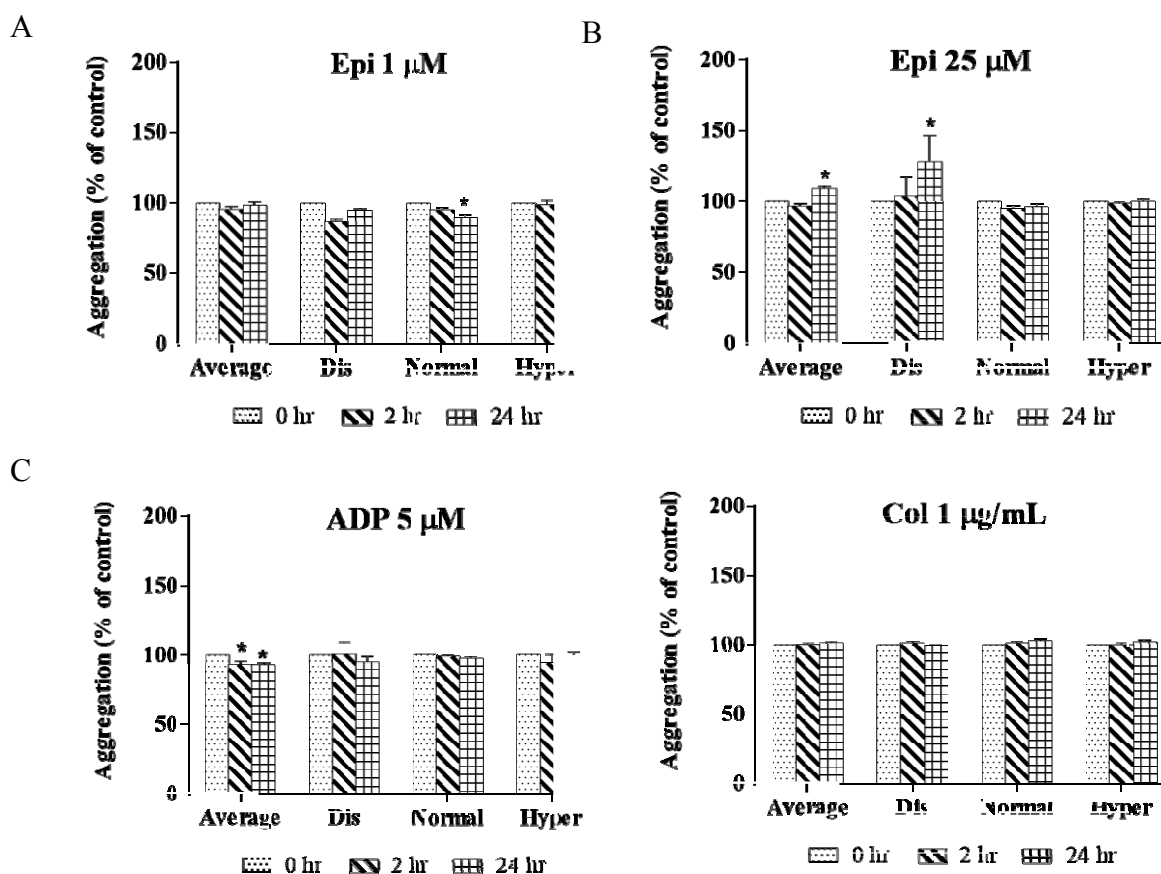
Figure 1. The effect of *A. paniculata* on platelet aggregation induced by 1 µM Epi (A), 25 µM Epi (B), 5 µM ADP (C) and 1 µg/mL Col (D). Percentage of aggregation after 2 and 24 hr of *A. paniculata* administration were compared to those of 0 hr as percent of control. Data were shown as mean ± SEM. Average is mean value of 10 subjects which were classified to Dis (n=2), Normal (n=2) and Hyper (n=6). *p <0.05 compared to 0 hr.
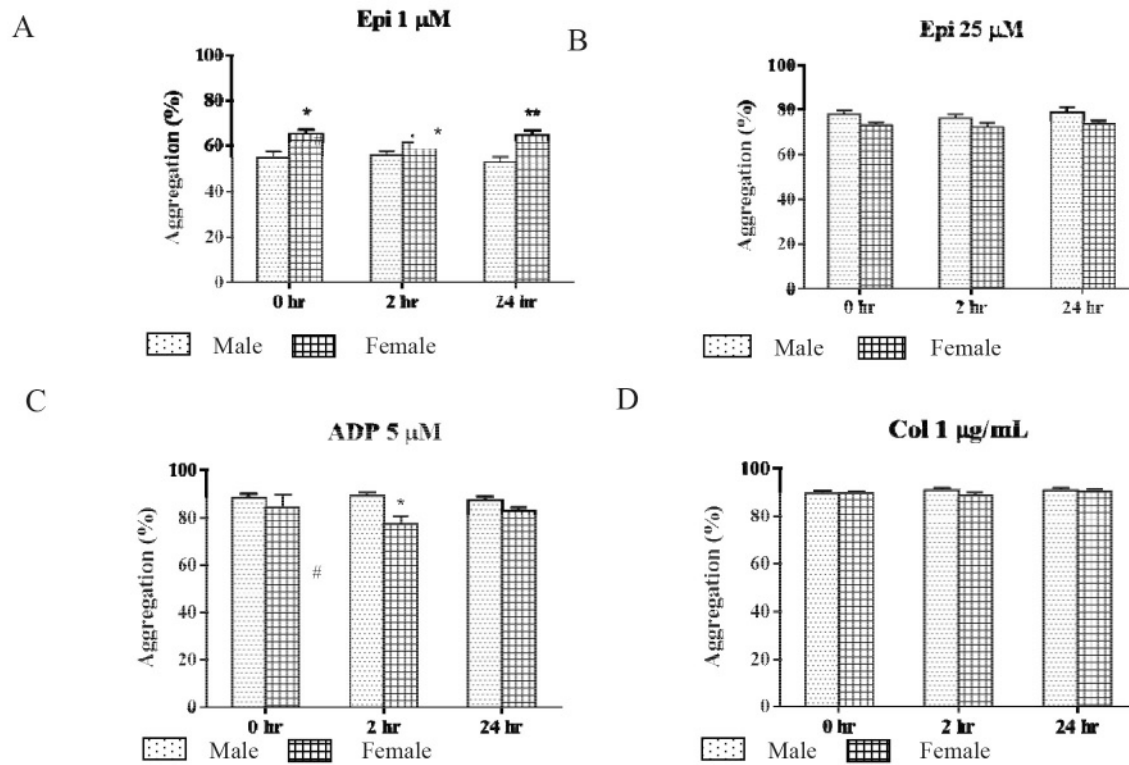
Figure 2. The percentage of platelet aggregation induced by 1μM Epi (A), 25 μM Epi (B), 5 μM ADP (C) and 1 μg/mL Col (D) in male and female of 0 hr, 2 hr and 24 hr after *A. paniculata* administration were shown as mean ±SEM. $*p < 0.05$ when compared between male and female at each time point. $^{\#}p < 0.05$ when compared to 0 hr within sex group.
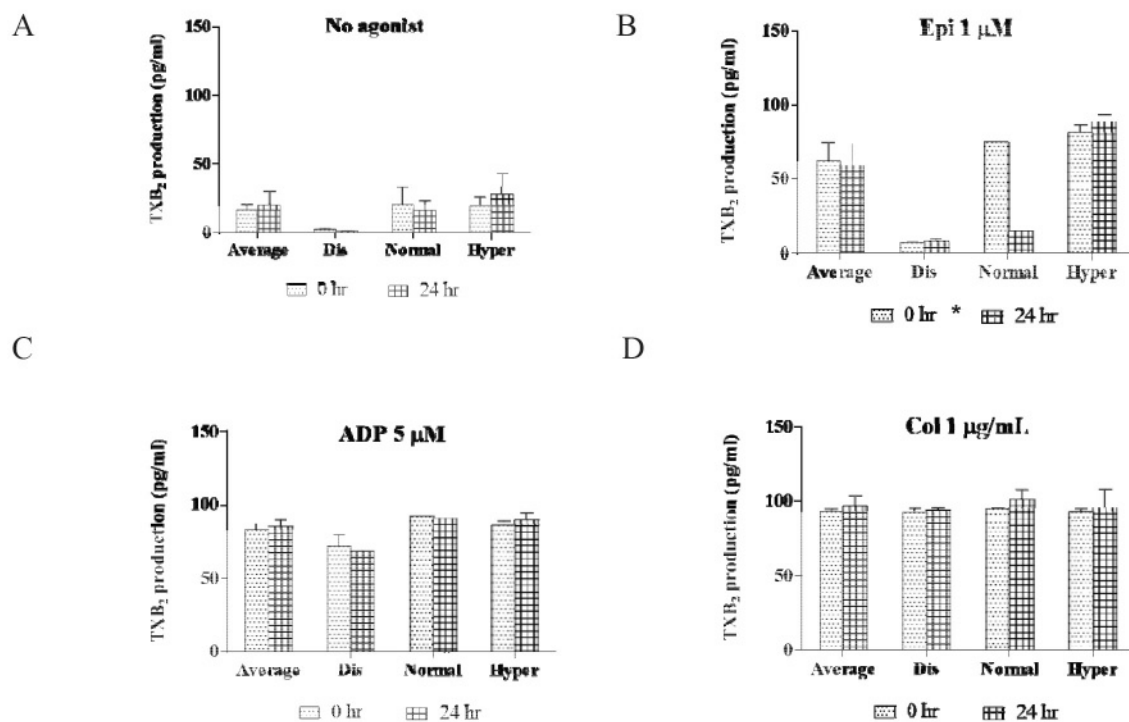
Figure 3. The effects of *A. paniculata* on TXB_2 level in platelets. TXB_2 was assessed in supernatants from PRP after induction by no agonist (A) or 1 μM Epi (B), 5 μM ADP (C) and 1 μg/mL Col (D) using EIA kit. Data were shown as mean ± SEM. Average is mean value of 10 subjects which were classified to Dis (n=2), Normal (n=2) and Hyper (n=6). *n=1

Discussion

The effects of *A. paniculata* on platelet aggregation induced by Epi, ADP or Col were studied, *ex vivo*, in healthy volunteers. Currently, there is no consensus on how to evaluate platelet function while each laboratory follows its own practice. For this study, three types of aggregation patterns were classified to be disaggregation, normal aggregation and hyperaggregation according to the studies of Ketsa-ard *et al*, 1991[11] and Akarasereenont *et al*, 2006.[12] They were found that, about 60 % of subjects exhibited platelet hyperaggregation at the study entry hour. These agreed to the study of Yee *et al*, 2006.[14] After *A. paniculata* administration, these patterns were not changed except one subject that disaggregation was changed to normal aggregation. The percentage of 25 µM Epi-induced aggregation in disaggregation group was, therefore, increased after *A. paniculata* administration (Figure 1B). Surprisingly, the percentage of 1 µM Epi-induced aggregation in normal aggregation group was decreased (Figure 1A). At this time, no conclusive explanation has been offered for this finding, further study on platelet aggregation with well-controlled design of a larger group should be performed. However, for the overall results, average percentage of ADP-induced platelet aggregation was significantly decreased while of Epi-induced was significantly increased and of Col-induced was not significantly changed after *A. paniculata* administration. The variation in the effects of *A. paniculata* on platelets aggregation among different aggregation pattern groups and among different agonists may be due to the multifactorial mechanisms of platelet aggregation. Moreover, they are likely to involve with different platelet receptors in each pathway.[15] *A. paniculata* may differently react with each receptor on platelets leading to variability in platelet aggregation response.[16,17] However, the effects may not stable if receptor was reversibly reacted.[18] Moreover, substantial interindividual variation in drug response among subjects may differently govern drug metabolizing enzyme. Genetic variations may be recognized as one of important factors to alter effects of *A. paniculata* on platelets.[19] This is probably the reasons that our finding did not show the inhibitory effect of *A. paniculata* on platelets as found in the previous studies.[6,7] In addition, the studied protocol was also quite different. Firstly, the formulation of *A. paniculata* in this study is crude powder in capsule not an extraction. Secondly, platelet agonists are Epi, ADP and Col but not thrombin, PAF or calcium ionophore A23187. Finally, the effect of *A. paniculata* on platelet aggregation is an *ex vivo* study not an *in vitro* study. The other possible influencing factor on platelets is sex difference because sex-related differences in platelet reactivity have been reported, with female having more reactive platelets than that in male.[20] In this study, platelet aggregation at the study entry hour in female was also significantly higher than that in male. After *A. paniculata* administration, platelet aggregation in male was significantly higher instead. Moreover, female, not male, revealed significant decreased in platelet aggregation, possibly indicated that platelets of female are more reactive than that of male. Given the role of COX pathway, COX-1 generally serves physiologic, housekeeping functions, such as generation of pro-aggregatory TXA_2 by platelets.[21] In this study, TXB_2, a stable metabolite of TXA_2, was also investigated using EIA kit. Overall result of TXB_2 production did not significantly change after *A. paniculata* administration. However, TXB_2 level of one subjects existing normal aggregation was clearly decreased while of another one was missed due to unavailable specimen. This decreased TXB_2 level probably due to the decreased platelet aggregation found in normal aggregation group. So, this study indicated that *A. paniculata* has probably a small effect on platelets preferably in subjects existing normal platelet aggregation pattern.

Conclusion

This study revealed the preliminary data of *A. paniculata* effects on platelet aggregation induced by Epi, ADP and Col in human volunteers. Individual variability of platelet response to *A. paniculata* administration was found. It had led to difficulties in interpreting the effect of *A. paniculata*. Moreover, it may also support the concept of personalized medicine based on platelet reactivity. Further study with well-controlled design of a larger group may be necessary for the conclusions. However, modulation of platelet reactivity by gender difference or products of COX pathway, TXA_2, should be considered for *A. paniculata* usage such as some pathological processes with suspicious of bleeding tendency

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F24 Effects of the Aqueous Extract of Dark Purple Glutinous Rice Variety Luem Pua Tea on Gastrointestinal Movement in Experimental Animals

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Abstract

Previous reports have indicated many beneficial effects of phenolic compounds from plants on the gastrointestinal tract. The aim of this study is to investigate the effects of aqueous extract of dark purple glutinous rice var. Luem Pua tea on gastrointestinal motility both *in vivo* and *in vitro* experiments. Whole gut transit time and intestinal transit rate in mice were measured using the charcoal test. Either water (control), tea extract (equivalent to 2 or 5 g of rice/kg body weight) or ferulic acid (100 mg/kg) was administered orally to the animals. Thirty minutes later, charcoal suspension was administered orally. For whole gut transit time, time after charcoal administration until first observation of defecated charcoal was recorded as a whole gut transit time (min). Intestinal transit rate was determined at 30 min after charcoal administration by measured distance traveled by the charcoal head from the pylorus as well as total length of the small intestine and expressed as % intestinal transit rate. The results showed that the tea extract at both 2 and 5 g/kg, but not ferulic acid, could significantly reduce whole gut transit time in mice. The tea extract at either 2 or 5 g/kg, and ferulic acid could significantly increase intestinal transit rate when compared to the control. In isolated guinea pig ileum, the tea extract induced the contraction of guinea pig ileum in a dose dependent manner while ferulic acid at concentration upto 20 nmol/ml could not. The results suggested that aqueous extract of rice tea from dark purple glutinous rice variety Luem Pua has prokinetic action and might be useful for the treatment of decreasing bowel activity such as constipation.

Keywords: dark purple rice Variety Luem Pua tea, whole gut transit time, intestinal transit rate, isolated ileum

ผลของสารสกัดจากชาข้าวเหนียวดำพันธุ์ลืมผัวที่มีผลต่อการบีบตัวของทางเดินอาหารในสัตว์ทดลอง

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บทคัดย่อ

จากการศึกษาก่อนหน้านี้มีข้อมูลว่า สารกลุ่ม polyphenolics จากพืชหลายชนิดมีผลต่อการเคลื่อนไหวของทางเดินอาหารทำให้ลำไส้บีบตัวมากขึ้น การศึกษาครั้งนี้มีจุดประสงค์เพื่อศึกษาผลของสารสกัดด้วยน้ำของชาข้าวเหนียวดำพันธุ์ลืมผัวที่มีผลต่อการบีบตัวของทางเดินอาหาร โดยศึกษาทั้งใน *in vivo และ in vitro* การศึกษาระยะเวลาที่ค้างอยู่ในทางเดินทางอาหารและเพิ่มอัตราการเคลื่อนที่ในลำไส้ ทำในหนูเม้าส์โดยใช้ charcoal test หนูได้รับการป้อนด้วย น้ำ (กลุ่มควบคุม), สารสกัดจากชาข้าวฯ (เทียบเท่ากับน้ำหนักสารสกัดจากชาข้าวฯ 2 หรือ 5 g/kg) หรือ ferulic acid ขนาด 100 mg/kg หลังการป้อน 30 นาที จึงป้อน charcoal และบันทึกระยะเวลาที่ค้างอยู่ในทางเดินทางอาหารได้แก่เวลาหลังป้อน charcoal จนกระทั่ง charcoal ออกมาในอุจจาระ ส่วนอัตราการเคลื่อนที่ในลำไส้ หาโดยการวัดระยะทางการเคลื่อนที่ของก้อน charcoal ในลำไส้เล็กเปรียบเทียบกับความยาวของลำไส้เล็กทั้งหมดเป็นร้อยละ

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ผลการทดลองพบว่า สารสกัดจากชาข้าวฯ ทั้งขนาด 2 และ 5 g/kg สามารถลด ระยะเวลาที่ค้างอยู่ในทางเดินทางอาหารและเพิ่ม อัตราการเคลื่อนที่ในลำไส้ ได้อย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มควบคุม ส่วน ferulic acid สามารถเพิ่ม อัตราการเคลื่อนที่ในลำไส้ แต่ไม่มีผลเปลี่ยนแปลงระยะเวลาที่ค้างอยู่ในทางเดินทางอาหาร การศึกษาในลำไส้เล็กส่วน ileum จากหนูตะเภา พบว่า สารสกัด จากชาข้าวฯ สามารถทำให้เกิดการหดตัวของลำไส้ได้แบบขึ้นกับความเข้มข้น ในขณะที่ ferulic acid ความเข้มข้นถึง 20 n mol/ml ยังไม่เห็นผล จากผลการศึกษาครั้งนี้เสนอแนะว่าสารสกัดจากชาข้าวเหนียวดำพันธุ์ลืมผัว มีฤทธิ์ในการเพิ่มการเคลื่อนตัวของ ทางเดินอาหาร และน่าจะเป็นประโยชน์ในการนำสารสกัดจากชาข้าวฯ ไปใช้ในการรักษาความผิดปกติในระบบทางเดินอาหารได้ เช่น ช่วยแก้ไขอาการท้องผูก เป็นต้น

คำสำคัญ : ชาข้าวเหนียวดำพันธุ์ลืมผัว, ระยะเวลาที่ค้างอยู่ในทางเดินทางอาหาร, อัตราการเคลื่อนที่ในลำไส้, ลำไส้เล็กแยกของ หนูตะเพา

Introduction

Constipation is one of common problems of gastrointestinal tract motility disorders. Common symptoms of constipation include infrequent bowel movements (less than three bowel movements a week) and difficult passage of hard stool.[1] and cause delayed transit through the stomach, small intestine or colon.[2] Constipation maybe associated with psychological disturbances and the reverse is true as well. Some studies referred that physical inactivity, water intake and constipation are risk factors for colorectal cancer.[3] In addition, lifestyle modifications, such as consuming significant health care resources that are high fiber, drinking more water and exercise may help to reduce constipation.

Dark purple rice variety Luem Pua (Oryza sativa var. glutinosa) is a special variety of glutinous rice which has been reported to have high content of polyphenolic compounds, especially anthocyanins and gamma-oryzanol (mixture of ferulic acid). Additionally, among many varieties of black rice, Luem Pua rice has been reported to have very high antioxidant activity. Interestingly, several recent works reported that antioxidant agents could prevent and improve many diseases such as anti-inflammation[4], cancer[5], cardiovascular diseases[6] and gastrointestinal motility disorders.[7] With high antioxidant activity of Luem Pua rice, this experiment aimed to investigate the effects of the aqueous extract of tea made from dark purple glutinous rice variety Luem Pua on gastrointestinal movement in male mice *(in vivo)* and the contraction of isolated guinea pig ileum *(in vitro)*.

Materials and Methods

Animals

Male ICR mice, 6 weeks and weighing 20 to 40 g for *in vivo* experiment and male guinea pigs (300-500 g) for *in vitro* experiment were used and purchased from the National Laboratory Animal Center, Mahidol University, Salaya, Nakhon prathom. Mice were housed in stainless cages and maintained in an air-conditioned room (25 ± 1 ^{o}C) with a 12 h light: 12 h dark cycle. The animals fed with a standard chow diet (Chareon Pokapan Co. Ltd., Thailand) and drinking water *ad libitum*. All procedures in this study were approved by the Animal Ethics Committee of Khon Kaen University, based on the Ethic of Animal Experimentation of National Research Council of Thailand (Record No. AEKKU 75/2556).

Extraction of dark purple rice variety Luem Pua tea

Dark purple rice variety Luem Pua tea (Antho-Aro Tea) was received from Phitsanulok Rice Research Center, Wangthong, Phitsanulok. The rice was extracted with hot distilled water (10 g/100 ml) for 5 minutes and then filtered through multiple layers of gauze and cotton wool. The procedure was repeated five times. The filtrate was collected and lyophilized. Dried powdered Luem Pua rice tea extract (LP) was kept at 4 ^{o}C in a tight container, protected from light and be dissolved in distilled water on the day of the experiment. The percent yield of the extract was 3.34.

In vivo experiments

Whole gut transit time: All mice were fasted over night (18 h) before experiments, Mice, 10 mice in each group, were orally administered with distilled water (as a control), ferulic acid (100 mg/kg body weight) or LP (equivalent to 2 or 5 g of rice/kg body weight). Thirty minutes later mice were gavaged with 1 ml of charcoal suspension (12 g charcoal and 2 g gum acacia were ground in a mortar and suspended in 130 ml distilled water). Then each animal was transferred to separate cage that its bottom was covered with white sheet. First observation of defecated charcoal recorded as a whole gut transit time.

Intestinal transit rate: All mice were fasted over night (18 h) before experiments, Mice, 10 mice in each group, were orally administered with distilled water (as a control), ferulic acid (100 mg/kg body weight) or LP (equivalent to 2 or 5 g of rice/kg body weight). Thirty minutes later mice were gavaged by charcoal suspension (1 ml/animal). Mice were killed at 30 minutes after charcoal administration by cervical dislocation. Small intestine was removed. The extent of charcoal propulsion in the small intestine was measured as distance from the pyloric sphincter to the leading

edge of the charcoal and total length of pyloric sphincter to caecum and calculated for intestinal transit rate as follow;

Intestinal transit rate % = $\dfrac{\text{distance traveled by charcoal}}{\text{total length of the small intestine}}$ x 100

In vitro experiment

Isolated guinea pig ileum: The guinea pig ileum preparation was set up in a 25 ml organ bath containing Tyrode's solution at 37°C, aerated with 95% O_2 and 5% CO_2. The ileum was set with a transmural electrode connected to an electrical stimulator. Muscle tone was recorded by using isotonic transducer and polygraph recorder.

Statistical analysis

Data were analyzed using the statistical Sigma Stat. Data are presented as mean ± S.E.M. Differences between groups were assessed by one-way analysis of variance (ANOVA), followed by Dunnet's t-test for the difference between groups. A p-value of less than 0.05 was considered as statistically significant.

Results

In vivo experiments
Whole gut transit time

The results of this study were shown in Figure 1. Whole gut transit time or time for first observation of defecated charcoal, in the control and mice received either 100 mg/kg ferulic acid, 2 g/kg LP (LP2) or 5 g/kg LP (LP5) were 229.43 ± 20.14, 214.63 ± 18.78, 162.45 ± 15.79 and 165.15 ± 17.12 minutes, respectively. It was clearly seen that whole gut transit times were significantly shorter in the groups treated with LP when compared to the control group. No significant change on whole gut transit time could be seen in the group treated with ferulic acid.

Intestinal transit rate

Figure 2 shows the effects of FA and LP on intestinal transit rate. Intestinal transit rates of the control, FA, LP2 and LP5 were 27.39 ± 2.56, 57.66 ± 3.73, 79.24 ± 5.72 and 69.07 ± 4.89 %, respectively. It was clearly seen that ferulic acid and LP, at either doses, could significantly increase intestinal transit rate when compared to the control group. Interestingly, intestinal transit rate of the animals received 2 g/kg LP was significant higher than the FA-treated group.

In vitro experiment

Electrical stimulation (60 volts) and acetylcholine (0.052 µg/ml) could stimulate contraction of ileum muscle as shown in Figure 3. Adding LP into the bath at 0.5,1 or 1.5 mg/ml also induced contraction of the ileum in a concentration-dependent manner. However, ferulic acid at 20 nmol/ml had no effect on the contraction of the ileum (Figure 3).
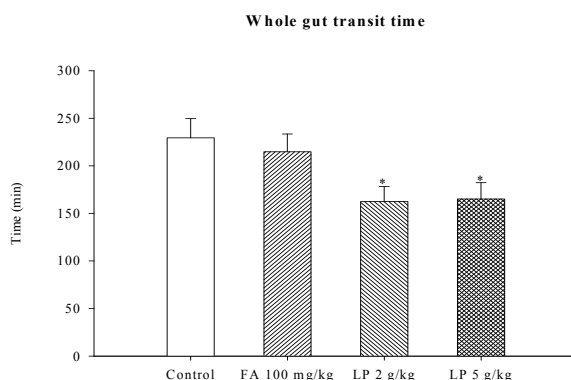

Figure 1. The effects of ferulic acid and LP on whole gut transit time in mice. Data were expressed as mean ± S.E.M. * significant different when compared to the control, FA = ferulic acid; LP = Luem Pua rice tea extract.
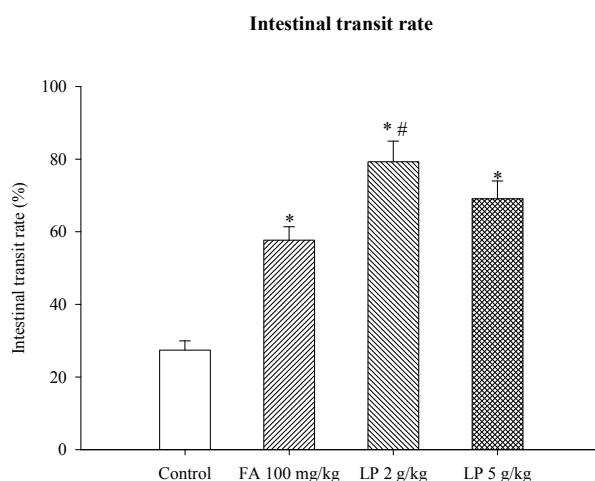
Intestinal transit rate

Figure 2. The effects of ferulic acid and LP on intestinal transit rate in mice. Data were expressed as mean ± S.E.M. * significant different when compared to the control group; # significant different when compared to the FA group, FA = ferulic acid; LP = Luem Pua rice tea extract.
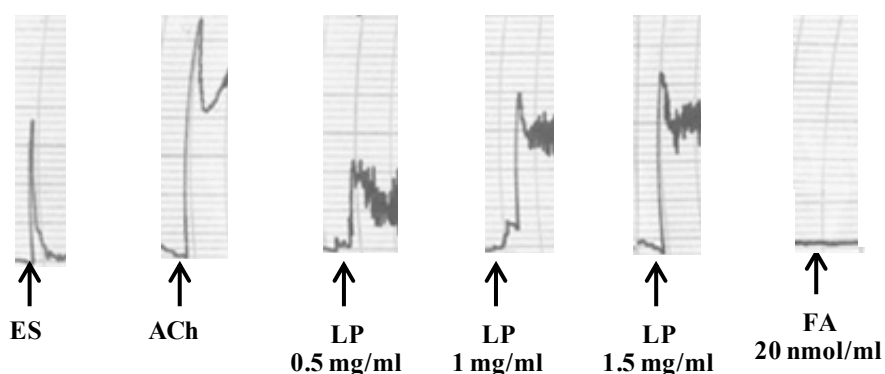

Figure 3. The responses of guinea pig ileums to electrical stimulation (ES) at 60 volts,　acetylcholine (ACh) 0.052 µg/ml, Luem Pua rice tea extract (LP) at 0.5, 1 or 1.5 mg/ml and ferulic acid (FA) at 20 nmol/ml.

Discussion

The results of this study showed that aqueous extract of dark purple glutinous rice variety Luem Pua tea could help improve gastrointestinal motility both in *in vivo* and *in vitro* models. Many studies have suggested the use of many natural products such as black tea and green tea to increase gastrointestinal motility[8] and prevent constipation.[9] Ferulic acid, one of the phenolic compounds commonly found in many kinds of plant including rice, has been shown to have gastrokinetic effect.[10] However, in this study, in mice, ferulic acid at the dose tested, could increase intestinal transit rate but had no effect on whole gut transit time. Surprisingly, in our preliminary study, at the dose up to 20 nmol/ml of ferulic acid, no effect on isolated guinea pig ileum was seen and need to be further clarified. Nevertheless, the different pattern of effects among LP and ferulic acid suggest that the active substances in LP might not be only ferulic acid. Luem Pua rice is a rich source of anthocyanins, especially cyanidin-3-glucoside. Having strong antioxidant and anti-inflammatory activities, cyanidin-3-glucoside reduced cytokine-induced inflammation in intestinal cells[11] and help improve the contractile function of the ileum. Detecting the active principles and clarify the mechanism of actions of those substances in LP will be performed.

Conclusion

In summary, our results showed that dark purple rice variety Luem Pua tea could improve peristalsis as observed by whole gut transit time, intestinal transit rate and isolated ileum. These results suggest the use of Luem Pua tea as one of the nutraceutical for gastrointestinal motility disorder, especially for constipation

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Effects of Fresh Betong Watercress Juice and Phenethyl Isothiocyanate on Serum ALT and AST in Paracetamol-Induced Hepatotoxicity in Rats

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Abstract

Paracetamol is one of the major causes of liver toxicity that found in various countries. Betong watercress, vegetables containing phenethyl isothiocyanate (PEITC), can reduce paracetamol toxicity. The objective of this study was to investigate the effects of fresh Betong watercress juice (FBWCJ) and PEITC in paracetamol-induced hepatotoxicity. Rats were randomly divided into 8 groups (n=6) as the following. Group I: rats were obtained distilled water. Group II: the animals were obtained single dose 30 % (w/v) gum acacia (10 mL/kg). Group III: rats were obtained corn oil (10 mL/kg) once a day for 3 days. Group IV: the animals were obtained single dose APAP (1.5 g/kg). Group V: the animals were obtained PEITC (50 mg/kg) once a day for 3 days. Group VI: the animals were obtained PEITC (50 mg/kg) once a day for 3 days and simultaneously obtained 1.5 g/kg APAP at 24 h later. Group VII: the animals were obtained 8 g/kg fresh Betong watercress juice (FBWCJ) three times a day for 3 days. Group VIII: the animals were obtained 8 g/kg fresh Betong watercress juice (FBWCJ) three times a day for 3 days and simultaneously obtained 1.5 g/kg APAP at 24 h later. Forty-eight hours after complete a treatment in each groups, serum samples were collected. The results showed that serum ALT levels in PEITC and FBWCJ followed with APAP treated groups were significantly decreased when compared with APAP treated group ($p < 0.05$). The level of serum AST in group VI was significantly decreased when compared with APAP treated group ($p < 0.05$). However the level of serum AST in group VIII was not significantly different compared with APAP treated group. In conclusion, PEITC and FBWCJ are effective for reduced paracetamol-induced liver toxicity.

Keywords: Betong watercress, phenethyl isothiocyanate, paracetamol, hepatotoxicity

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ผลของน้ำคั้นผักน้ำเบตงสดและเฟนเอทิลไอโซไธโอไซยาเนทต่อระดับเอนไซม์ ALT และ AST ในหนูแรทที่ถูกชักนำให้เกิดความเป็นพิษต่อตับด้วยพาราเซตามอล

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บทคัดย่อ

พาราเซตามอล (APAP) เป็นสาเหตุที่สำคัญที่ทำให้เกิดความเป็นพิษต่อตับที่พบในหลายประเทศ ผักน้ำเบตงเป็นผักที่มีสารเฟนเอทิลไอโซไธโอไซยาเนท (PEITC) อาจลดความเป็นพิษต่อตับที่เกิดจาก APAP ได้ ดังนั้นงานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาผลของน้ำคั้นผักน้ำเบตงสด (FBWCJ) และ PEITC ในหนูแรทที่ถูกชักนำให้เกิดความเป็นพิษต่อตับจากพาราเซตามอล แบ่งสัตว์ทดลองออกเป็น 8 กลุ่ม กลุ่มละ 6 ตัว กลุ่มที่ 1 ให้น้ำกลั่น กลุ่มที่ 2 ให้ 30 % กัมอะคาเซีย ทางปากครั้งเดียว กลุ่มที่ 3 ให้น้ำมันข้าวโพด ทางปากวันละครั้ง 3 วัน กลุ่มที่ 4 ให้ APAP ขนาด 1.5 ก/กก. ทางปากครั้งเดียว กลุ่มที่ 5 ให้ PEITC ขนาด 50 มก./กก. ทางปาก วันละครั้งติดต่อกัน 3 วัน กลุ่มที่ 6 ให้ PEITC ขนาด 50 มก./กก. ทางปาก วันละครั้งติดต่อกัน 3 วัน 24 ชม.

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ต่อมาให้ APAP ขนาด 1.5 ก/กก. ทางปาก ครั้งเดียว กลุ่มที่ 7 ให้ FBWCJ ทางปากขนาด 8 ก/กก. วันละ 3 ครั้งติดต่อกัน 3 วัน กลุ่มที่ 8 ให้ FBWCJ ทางปากขนาด 8 ก/กก. วันละ 3 ครั้งติดต่อกัน 3 วัน 24 ชม.ต่อมา ให้ APAP ขนาด 1.5 ก/กก. ทางปาก ครั้งเดียว ทำการเก็บตัวอย่างเลือดที่เวลา 48 ชั่วโมง ภายหลังการให้สารและยาครบตามกลุ่มการทดลองที่ระบุไว้เพื่อวัดระดับเอนไซม์ อะลานีน อะมิโนทรานสเฟอเรส (ALT) และเอนไซม์แอสปาร์เตต อะมิโนทรานสเฟอเรส (AST) ผลการวิจัยพบว่า ระดับ ALT ในกลุ่มที่ 6 และ 8 ต่ำกว่าอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับกลุ่มที่ได้รับ APAP เพียงอย่างเดียว ($p < 0.05$) ระดับ AST ในสัตว์ทดลองกลุ่มที่ 6 ต่ำกว่าอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับกลุ่มที่ได้รับ APAP เพียงอย่างเดียว ($p < 0.05$) สรุปได้ว่า PEITC และ FBWCJ มีฤทธิ์ ลดความเป็นพิษต่อตับที่เกิดจากพาราเซตามอล

คำสำคัญ: ผักน้ำเบตง, เฟนเอทิลไอโซไทโอไซยาเนท, พาราเซตามอล, ความเป็นพิษต่อตับ

Introduction

Paracetamol (acetaminophen; N-acetyl-*p*-aminophenol, APAP) is widely used as an analgesic and antipyretic drug. APAP is one of the major causes of liver toxicity that was found in various countries including Thailand. U.S. Food and Drug administration reported that 48% of acute liver failure was caused by paracetamol.[1] U.S. Food and Drug administration has been advised the maximum amount of APAP in a prescription tablet, capsule, or other dosage unit limited to 325 mg since January 14, 2014. FDA requested this action to protect consumers from the risk of severe liver damage which can result from taking too much APAP. In therapeutic doses, APAP is safe, but in overdose it can cause severe hepatic necrosis.[2] APAP is rapidly absorbed from the gastrointestinal (GI) tract with peak concentrations achieved within 90 minutes of a therapeutic dose. APAP distributes rapidly and evenly throughout most tissues and fluids.[3] APAP is extensively metabolized. The major metabolic pathways are the glucuronidation and sulfation. The minor fraction of the drug is converted to a highly reactive alkylating metabolite which is inactivated with reduced glutathione and excreted in the urine as cysteine and mercapturic acid conjugates. Overdoses of APAP causes acute hepatic necrosis as a result of depletion of glutathione and of binding of the excess reactive metabolite to vital cell constituents.[4] These damage can be prevented by inhibits cytochrome P450 2E1 (CYP2E1) in phase I, increased glutathione (GSH) level and induced glutathione *S*-transferase (GST) in phase II.

The clinical and biochemical signs APAP induce hepatotoxicity are shown at least 24 hours postingestion.[5, 6] The most common marker used to confirm the liver injury for APAP overdose is the activity of certain hepatocellular enzyme, ALT and AST in the blood.[7] Histopathology and AST and ALT clinical chemistries suggested 1,500 mg/kg APAP produced phenotypes of early, full, and recovery stages of liver injury at 6, 24, and 48 hours, respectively.[8]

Watercress (*Nasturtium officinale* R. Br.) is a leafy vegetable of the family Cruciferae which include cabbage, cauliflower, Brussels sprouts, broccoli and kale. This plant grows in cool and flowing water about 2 to 3 inches deep, in a partially shaded area.[9] In Thailand, watercress can be found at Betong District of Yala Province. It is claimed that this plant is originally from France and has been brought to grow in China and then in Thailand by Chinese immigrants. Watercress is the richest of glucosinolate nasturtiin, which is hydrolysed by myrosinase, an enzyme that is activated upon crushing the vegetables by chopping or chewing, producing phenethyl isothiocyanate (PEITC).[10,11] The previous studies showed that PEITC inhibited CYP2E1 in phase I and also enhanced GST in phase II resulting increase in APAP detoxification.[12,13]

Therefore, the objective of this study was to investigate the effects of fresh Betong watercress juice and PEITC on serum ALT and AST that are markers of hepatotoxicity in rats after APAP exposure.

Materials and Methods

Chemical

Paracetamol (purity > 98 %), PEITC, α-ketoglutaric acid, dl-aspartic acid , dl-alanine and sodium pyruvate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium dihydrogen orthophosphate dehydrate was purchased from Fisher Scientific UK Limited (Leicestershire, UK). Sodium phosphate dibasic AR and 2,4-Dinitrophenylhydrazine, A.R. was purchased from HiMedia Laboratories Pvt. Ltd (LBS Marg, Mumbai, India). Sodium hydroxide anhydrous was purchased from Carlo Erba Reagent (Milan, Italy). Acacia was purchased from Ajax Finechem Pty Ltd. (Auckland, New Zealand).

Plant preparation

Fresh Betong watercress was obtained from a local farm in Betong district, Yala province. The specimen was cleaned thoroughly and divided into portions. A portion of 50 g of Betong watercress was chopped and add with 40 mL of distilled water. The mixture was blended for 3 min into a fine paste using a kitchen mini blender and homogenized for 3 min with homogenizer (IKA T10 Basic Ultra-Turrax Homogenizer system). The vegetable paste was filtered with cheesecloth and squeezed to release the juice into a large beaker. After filtrated the total volume of Betong watercress juice is 70 mL. The amount of Betong watercress in distilled water is 50 g/70 mL. Then fresh Betong watercress juice (FBWCJ) was feed to rats.

Animal

Male Wistar rats weighing between 200 and 250 g were obtained from the Southern Laboratory Animal Facility, Prince of Songkla University, Hatyai, Sonkhla, Thailand. The experimental protocol was approved by the Animal Ethic Committee, Prince of Songkla University (Ref. 17/57). During the experimental period, the animals were maintained under a controlled environment (temperature of 25 ± 2 ºC, light/dark cycle of 12/12 hr) with food and water *ad libitum*. Before dosing, the animals were fasted overnight (12 hr) but freely access to water.

Experimental design

Rats were randomly divided into 8 groups as follows:

Group I: The animals were obtained distilled water.

Group II: The animals were obtained single dose of 10 mL/kg of 30 % (w/v) of gum acacia.

Group III: The animals were obtained 10 mL/kg of corn oil once a day for 3 days.

Group IV: The animals were obtained single dose of 1.5 g/kg APAP in 30 % gum acacia.

Group V: The animals were obtained 50 mg/kg PEITC in corn oil once a day for 3 days.

Group VI: The animals were obtained 50 mg/kg PEITC in corn oil once a day for 3 days and simultaneously obtained 1.5 g/kg APAP at 24 hr later.

Group VII: The animals were obtained 8 g/kg fresh Betong watercress juice (FBWCJ) three times a day for 3 days.

Group VIII: The animals were obtained 8 g/kg fresh Betong watercress juice (FBWCJ) three times a day for 3 days and simultaneously obtained 1.5 g/kg APAP at 24 h later.

Blood samples were collected 48 hr after obtained various types of treatment. Serum was kept at 4 °C and separated by centrifugation at 1600 x g for 10 min.

The dose of APAP[8] and PEITC[14] were followed with previous reported.

Determination of alanine transferase and aspartate transaminase

ALT and AST catalyze the transfer of amino group from alanine and aspartate to α-ketoglutarate which products are pyruvate and oxaloacetate, respectively. The reactions are as follows:

α-ketoglutarate + alanine $\longrightarrow$ pyruvate + glutamate

α-ketoglutarate + aspartate $\longrightarrow$ oxaloacetate + glutamate
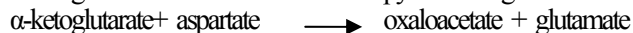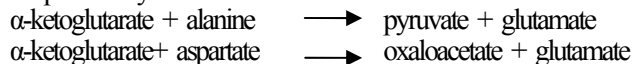
Then products (pyruvate and oxaloacetate) occurred react with 2, 4 dinitrophenyl hydrazine to form a brown product, phenylhydrazone. The absorbance at 505 nm was then determined.[15]

Procedure

Measurement procedure of ALT and AST standard

After 100 μL of distilled water was put into each five test tube, 500, 450, 400, 350 and 300 μL of the desired substrate were added into these tubes, respectively. Then the pyruvate)0, 50, 100, 150 and 200 μL(was add to the test tubes, respectively. After the content was mixed and then incubated at 37 °C, exactly 30 min for ALT, or 60 min for AST activity measurements, then tubes were removed from the water-bath. Then 500 μL of 2, 4 dinitrophenyl hydrazine reagent was added into each test tube, which was mixed and incubated at room temperature for 30 min. Next 5 mL of 0.4 N sodium hydroxide was added; then a rubber stopper was inserted, and the contents were mixed by inversion. At the end of exactly 30 min, the optical density of the solution is measured at 505 nm.

Measurement procedure of ALT and AST in serum

500 μL of the desired substrate was pipetted into test tubes. The serum (100 μL) was added into the tubes and incubated at 37 °C, 30 min for ALT, or 60 min for AST. Then the tubes were removed from the water-bath. Upon the addition of 500 μL of 2, 4 dinitrophenyl hydrazine reagent, the mixtures were mixed and let stand at room temperature for 30 min. Next 5 mL of 0.4 N sodium hydroxide was added. At the end of exactly 30 min, the optical density of the solution was measured at 505 nm.

Data and Statistical analysis

Data are present as means ± standard error of the mean (SEM). Data were analyzed using one-way analysis of variance (ANOVA) followed by least square difference (LSD) using SPSS software. Statistically significant differences were defined as $p < 0.05$.
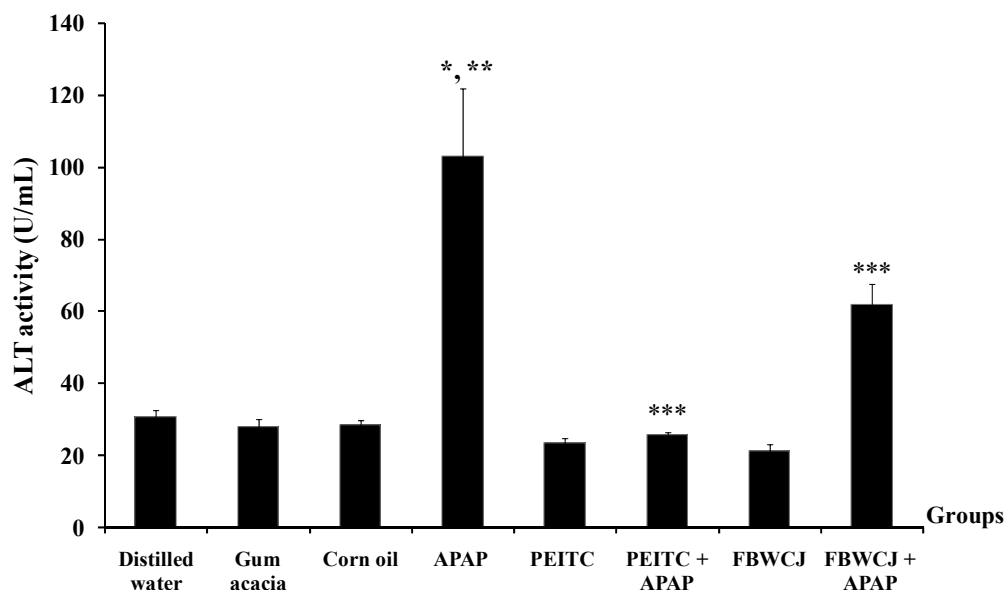
Figure 1. Effects of phenethyl isothiocyanate (PEITC) and fresh Betong watercress juice (FBWCJ) on serum ALT in rats. Results are expressed as mean $\pm$ SEM of six rats and data were analyzed by one-way ANOVA followed by LSD. Significant levels were defined as $p < 0.05$.
*significant difference when compared with distilled water group ($p < 0.05$).
**significant difference when compared with 30 % gum acacia group ($p < 0.05$).
***significant difference when compared with APAP group ($p < 0.05$).

Results

Effects of phenethyl isothiocyanate (PEITC) and fresh Betong watercress juice (FBWCJ) on serum ALT in rats

The effects of PEITC and FBWCJ on serum ALT in rats were investigated and were represented in Figure 1.

From the results were shown that ALT level in distilled water group range from 13.00 – 59.60 U/mL, in 30 % gum acacia treated group range from 24.42 – 38.11 U/mL, in corn oil treated group range from 24.33 – 33.78 U/mL were not significant difference. ALT level in APAP treated group was significantly increased when compared with 30 % gum acacia (102.96 $\pm$ 18.98 U/mL vs. 27.97 $\pm$ 2.13 U/mL; $p < 0.05$). The enzyme levels in PEITC and FBWCJ treated group (23.58 $\pm$ 1.27 U/mL, 21.37 $\pm$ 1.82 U/mL, respectively) were not significantly

difference when compared with corn oil (28.59 $\pm$ 1.32 U/mL) and distilled water group (30.85 $\pm$ 1.84 U/mL), respectively. The ALT level in PEITC followed by APAP treated group was significantly decreased when compared with APAP 1.5 g/kg treated group (25.77 $\pm$ 0.59 U/mL vs. 102.96 $\pm$ 18.98 U/mL; $p < 0.05$). The enzyme level in FBWCJ followed by APAP treated group was significantly decreased when compared with APAP treated group (61.92 $\pm$ 5.59 U/mL vs. 102.96 $\pm$ 18.98 U/mL; $p < 0.05$) (Figure 1).

Effects of phenethyl isothiocyanate (PEITC) and fresh Betong watercress juice (FBWCJ) on serum AST in rats

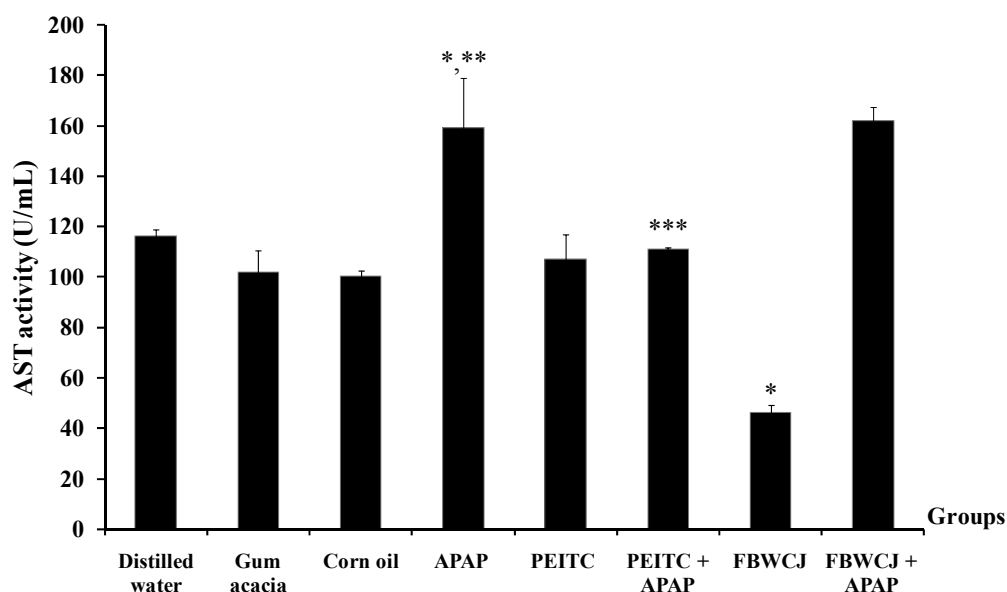The effects of PEITC and FBWCJ on serum AST in rats were investigated and represented in Figure 2.

Figure 2. Effects of phenethyl isothiocyanate (PEITC) and fresh Betong watercress juice (FBWCJ) on serum AST in rats. Results are expressed as mean ± SEM of six rats and data were analyzed by one-way ANOVA followed by LSD. Significant levels were defined as $p < 0.05$.
*significant difference when compared with distilled water group ($p < 0.05$).
**significant difference when compared with 30 % gum acacia group ($p < 0.05$).
***significant difference when compared with APAP group ($p < 0.05$).

The results showed that AST levels in 30 % gum acacia treated group and corn oil treated group were not significantly different when compared with distilled water group. AST level in APAP treated group was significantly increased when compared with 30 % gum acacia (158.89 ± 19.83 U/mL vs. 101.80 ± 8.52; $p < 0.05$). The level of AST in PEITC treated group was not significantly difference when compared with corn oil (107.15 ± 9.61 vs 100.29 ± 2.13). AST level in FBWCJ treated group was significantly decreased when compared with distilled water group (46.12 ± 3.03 U/mL vs.116.29 ± 2.46 U/mL; $p < 0.05$). The AST level in PEITC followed with APAP treated group was significantly decreased when compared with APAP (110.81 ± 0.84 U/mL vs 158.89 ± 19.83 U/mL; $p < 0.05$). The AST level in FBWCJ followed by APAP treated was not significantly difference when compared with APAP treated group (161.86 ± 5.30 U/mL vs 158.89 ± 19.83 U/mL) (Figure 2).

Discussion

APAP is one of the most commonly used medicines with a high safe profile when used properly. In case of improper use including continuous taking, intention and accident, APAP can cause significant liver injury.[16] The most common marker used to confirm the liver injury for APAP overdose is the activity of certain hepatocellular enzyme, ALT and AST in the blood.[7] ALT is thought to be more specific for hepatic injury because it is present mainly in the cytosol of the liver. AST has cytosolic and mitochondrial forms and is present in tissues of the liver, heart, skeletal muscle, kidneys, brain, pancreas, lungs and in white and red blood cells. ALT is thought to be more specific than AST for hepatic injury because it is present mainly in the cytosol of the liver at low concentrations elsewhere.[17]

In this study, the results showed that single oral dose of 1.5 g/kg APAP caused hepatotoxicity in rats as indicated by the increase both in serum ALT and AST. In adult, only a small amount of N-acetyl-p-benzoquinoneimine (NAPQI), a toxic by product produced during the metabolism of the paracetamol, is produced and then inactivated by conjugation with glutathione. Previous studies at toxic doses of APAP, the normal glucuronide pathway is saturated and the production of NAPQI is increased. Eventually the depletion of glutathione occurs and increased NAPQI binds to cellular proteins and leads to hepatic injury.[18]

This present studies demonstrate that PEITC and FBWCJ reduce APAP-induced hepatotoxicity. The ALT levels were significantly decreased in pretreated with PEITC or FBWCJ followed with APAP. These results were similar to the previous study[12] that showed the protective effect of PEITC

on acetaminophen metabolism and hepatotoxicity in mice. Moreover PEITC exhibited inhibition of CYP2E1 and other isoforms *in vitro* study[19] and induction of GST activity.[13] In addition, Leclercq and co-worker demonstrated that various types of CYP450 including CYP2E1 which was responsible for activating APAP to toxic metabolite, NAPQI, were inhibited by watercress.[20] Therefore, the protective actions of PEITC and FBWCJ are possibly due to the suppression of APAP activation by mainly inhibition of CYP2E1 in phase I and induction of GST in phase II.

The results showed that the serum AST level was significantly decreased in PEITC but not altered in FBWCJ followed with APAP treatment when compared with APAP group. In some incidents, acute kidney failure may also occur during this phase, the rising hepatic ALT and AST to abnormal levels, typically caused by either hepatorenal syndrome or multiple organ dysfunction syndromes. In these cases, it has been suggested that the toxic metabolite is produced more in the kidneys than in the liver.[21] In addition, PEITC reduced not only hepatoxicity but also other organ pathologies such as lung lesions.[22] According to these findings, it implies that APAP induces more than one organ lesion and PEITC can protect not only liver but also other organs from toxicity induced by APAP. However the results of ALT and AST levels in FBWCJ suggest that possibly protects more effectively to the hepatoxicity than organ lesions.

It has been recognized that PEITC is a product of glucosinolate-myrosinase hydrolysis. Consumption of 57 g of fresh watercress resulted in a 30-67 % conversion of gluconasturtiin corresponding to 12-15 mg (72.6-90.7 μmole) of PEITC in the body.[23]

It is most likely that PEITC was occurred during FBWCJ process. During blending of the fresh plant to prepare juice, glucosinolates come in to contact with endogenous myrosinase and hydrolysis was rapidly initiated. In addition, PEITC was occurred in GI tract by the action of microflora in large intestine.[24] As measure the amount of PEITC in plant by using Jiao method[25], it was found that 8 g/kg of fresh Betong watercress contained 0.354 mg of PEITC.

Therefore, this study indicates that PEITC and FBWCJ reduce APAP-induced hepatotoxicity.

Conclusion

The results demonstrate that PEITC and FBWCJ have a protective role against APAP induced hepatic damages. The mechanism of action may be due to reducing the formation of the toxic APAP metabolite through inhibition of APAP metabolizing enzyme, CYP2E1, and induction detoxifying enzyme, GST.

Acknowledgements

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F26 Effects of Roasted Sesame Seeds on Pharmacokinetics of Paracetamol in Rats

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Abstract

Consuming sesame seeds has long been interested due to its health benefit. Sesame seed or oil has been documented to inhibit CYP 2E1. Little is known about interaction between sesame and paracetamol which is a commonly used analgesic and antipyretic drug. Therefore, this study was aimed to investigate the effects of roasted sesame seeds on pharmacokinetics of paracetamol in rats. Three groups of male Wistar rats (n=6 each) were orally received a single administration of paracetamol (25 mg/kg BW), 30% (w/v) gum acacia (10 mL/kg BW) followed by paracetamol (25 mg/kg BW), or roasted sesame seeds (1 g/kg BW) followed by paracetamol (25 mg/kg BW). Paracetamol and roasted sesame seeds were suspended with 30% gum acacia. Serial blood samples were collected during 110 min post dose. Plasma concentrations of paracetamol were determined using a reverse-phase high performance liquid chromatographic technique. Pharmacokinetic parameters were estimated based on the non-compartmental analysis using PK solutions 2.0^{TM} software. Pharmacokinetic analysis showed a significant increase in λ_z and a significant decrease in $t_{1/2z}$ of paracetamol after pretreatment with roasted sesame seeds, when compared with the group receiving paracetamol alone ($p<0.05$). The findings indicated that roasted sesame seeds enhanced the elimination of paracetamol in rats. That may lead to reduction of the pharmacological response of paracetamol *in vivo*.

Keywords: paracetamol, sesame seed, pharmacokinetics, interaction

ผลของงาดำคั่วต่อเภสัชจลนศาสตร์ของพาราเซตามอลในหนูขาว

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บทคัดย่อ

งาได้รับความสนใจมาอย่างช้านานเนื่องจากมีประโยชน์ต่อสุขภาพ มีการศึกษาว่าเมล็ดงาหรือน้ำมันงามีผลในการยับยั้งการทำงานของ CYP 2E1 แต่ยังไม่พบข้อมูลที่ศึกษาปฏิกิริยาระหว่างกันของงากับพาราเซตามอลซึ่งเป็นยาที่ใช้ในการรักษาอาการปวดและลดไข้อย่างแพร่หลาย ดังนั้นในการศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาผลของงาดำคั่วต่อเภสัชจลนศาสตร์ของพาราเซตามอลในหนู หนูเพศผู้สายพันธุ์ Wistar ถูกแบ่งออกเป็น 3 กลุ่ม กลุ่มละ 6 ตัว หนูแต่ละกลุ่มได้รับสารที่แตกต่างกันทางปากเพียงครั้งเดียว ประกอบด้วยกลุ่มที่ 1 ได้รับพาราเซตามอลขนาด 25 มิลลิกรัมต่อกิโลกรัม กลุ่มที่ 2 ได้รับร้อยละ 30 กัมอะคาเซียตามด้วยพาราเซตามอลขนาด 25 มิลลิกรัมต่อกิโลกรัม และกลุ่มที่ 3 ได้รับงาดำคั่วขนาด 1 กรัมต่อกิโลกรัมตามด้วยพาราเซตามอลขนาด 25 มิลลิกรัมต่อกิโลกรัม พาราเซตามอลและงาดำคั่วแขวนตะกอนด้วยร้อยละ 30 กัมอะคาเซีย เก็บตัวอย่างเลือดเป็นระยะเวลา 110 นาทีหลังจากได้รับยา การวิเคราะห์ความเข้มข้นของพาราเซตามอลใช้เครื่องโครมาโตกราฟฟีของเหลวสมรรถนะสูง พารามิเตอร์ทางเภสัชจลนศาสตร์วิเคราะห์โดยใช้แบบจำลองแบบไม่แบ่งส่วน คำนวณด้วยพีเคโซลูชั่นซอฟท์แวร์ ผลการวิเคราะห์ทางเภสัชจลนศาสตร์พบว่าค่าคงที่ของการกำจัดยาเพิ่มขึ้น และค่าครึ่งชีวิตในการกำจัดยา

ลดลงอย่างมีนัยสำคัญในกลุ่มที่ได้รับงาดำคั่วร่วมกับพาราเซตามอลเมื่อเปรียบเทียบกับกลุ่มที่ได้รับพาราเซตามอลเพียงอย่าง เดียว สรุปได้ว่างาดำคั่วมีผลเพิ่มการกำจัดยาพาราเซตามอลในหนูขาว จากผลดังกล่าวนี้อาจมีผลลดประสิทธิภาพการรักษาได้

คำสำคัญ: พาราเซตามอล, งาดำ, เภสัชจลนศาสตร์, ปฏิกิริยาระหว่างกัน

Introduction

Sesame (*Sesamum orientale* L.,), a member of *Pedaliaceae* family, is an important crop of the tropics and subtropics.[1] It has been called the 'queen of the oil seed crops' because of the high yield of oil and quality of the seed, oil, and meal.[2] Sesame seed consists of proteins, lipids, carbohydrates, vitamins, minerals, and antioxidants, including sesamin and sesamolin.

Sesame oil and roasted sesame seeds are widely used in various types of Asian cuisine. Sesame oil is used as seasoning in many dishes. In Japan, mainly roasted sesame seeds are consumed, and it is preferred to grind the seeds just before consumption to ensure a fresh aroma. In Europe and the United States, the roasted seeds are a popular topping for bakery products.[3] In Thailand, roasted sesame is used to make a soft drink, sesame seed confection and snack while sesame oil is a valuable cooking oil. Current flows are more health conscious. That makes sesame seed consumption popular.

Previous studies have shown that sesame has an influence on pharmacokinetics. For example, when nitroglycerin (3.5 mg/kg) was administered orally in a 20% sesame oil emulsion to rats, peak plasma concentrations of nitroglycerin were decreased but its bioavailability was unaffected.[4] Ueng *et al.*[5] reported that safrole, methylenedioxyphe-nyl in sesame oil was inhibited human cytochrome CYP 2E1 and affects the drug metabolism in human.[6] Concurrent intake of sesame with some drugs may lead to an alteration of their pharmacological responses.

Paracetamol (acetaminophen; N-acetyl-*p*-amino phenol) is an analgesic antipyretic drug. It has been commonly used due to its effectiveness and safety at its therapeutic dose. There is no report about an interaction between sesame and paracetamol. This study is therefore aimed to examine an effect of sesame seeds on pharmacokinetics of paracetamol in rats.

Materials and Methods

Chemicals

Standard paracetamol (purity>98%) and theophylline (purity>99%) which was used as an internal standard were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acacia was purchased from Ajax Finechem Pty Ltd. (Auckland, New Zealand). HPLC grade solvents, methanol and acetonitrile, and phosphoric acid were obtained from Avantor

Performance Meterials Inc. (Center Valley, PA, USA). Perchloric acid was purchased from Carlo Erba Reagenti (Milan, Italy). Sodium sulfate was purchased from VWR International Ltd. (Poole, Dorset, UK). Water was purified using a Milli-Q® water purification system.

Preparation of roasted sesame seeds

Black sesame seeds (15 g) were purchased from Ban Sop Soi Cooperatives at Muang district, Mae Hong Son province, Thailand. A 15 g portion of sesame seeds was roasted using the Cuizimate Induction Cooker (Nesco Group Co., Ltd., Bangkok, Thailand) by setting the electric power at 400 watts for 10 minutes. After roasting, the seeds were grinded with a mortar and pestle to fine powder prior to test.

Animals

The study included eight-week-old male Wistar rats (210-230 g) provided by Southern Laboratory Animal Facility, Prince of Songkla University. The animal experiments were approved by the Animal Ethic Committee, Prince of Songkla University (ref. 22/55). During the experimental period, the animals were maintained under a controlled environment (temperature of 25 ± 2 °C, light/dark cycle of 12/12 h) with food and water *ad libitum*.

Animal treatments

The animals were randomly divided into 3 groups (n = 6 each). They received different treatments as follows; group I, paracetamol (25 mg/kg BW) alone; group II, 30% (w/v) gum acacia at 15 min before paracetamol (25 mg/kg BW); and group III, roasted sesame seeds (1 g/kg BW) at 15 min before paracetamol (25 mg/kg BW). All treatments were given by gastric garvage. Paracetamol and roasted sesame seeds were suspended with 30% gum acacia. Prior to drug administration, the animals were fasted overnight but freely accessed to water. The dose of paracetamol[7] and roasted sesame seeds[8] were follows with previous reported.

Blood collection

Blood samples (500 µL) were collected heparinized tubes by clipping tail vein at 5, 15, 30, 50, 80 and 110 min after paracetamol administration. The samples were immediately centrifuged at 1600 x g for 10 min at 4°C and the plasma layer was stored at -80°C until analysis.

Extraction procedure

Plasma aliquots (100 µL) were deproteinized by adding 10 µL of 30% perchloric acid containing 100 µg/mL of internal standard (theophylline). The samples were mixed using vortex for 5 sec before centrifugation at 10000 x g for 10 min at 4 °C. The supernatant was filtered through a 0.2 µm pore size membrane filter and placed into a glass vial. The aliquot (20 µL) was injected into the HPLC system.

HPLC analysis of paracetamol in plasma
Instrumentation

The high performance liquid chromatographic system consisted of a Waters 515 HPLC pump, a Waters™ 717 plus autosampler, and a Waters 2487 Dual λ absorbance detector (Waters Corporation, Milford, MA) set at a wavelength of 254 nm. A guard column (Fortis C18, 10 x 4.6 mm, 5 µm; Clayhill Industrial Park, Neston, Cheshire, UK), and Fortis UniverSil C18 HPLC Columns (150 x 4.6 mm, 5 µm; Clayhill Industrial Park, Neston, Cheshire, UK) were used. Data were collected and analyzed using a CSW32 Chromatography Software (ALS Czech Republic s.r.o., Prague, Czech Republic).

Chromatographic conditions

The separation was performed using isocratic elution at ambient temperature. The mobile phase consisted of 7% acetonitrile and in 93% 0.05 mM sodium sulfate buffer (pH 2.2, adjusted with phosphoric acid).[9] Prior to delivery into the system, it was filtered through a 0.22 µm-nylon filter and degassed by sonication. The flow rate was 1.5 mL/min.

Data analysis

Pharmacokinetic behaviors of paracetamol for all rat groups were analyzed based on non-compartmental analysis. The parameters included peak plasma concentration (C_{max}), time to reach peak plasma concentration (t_{max}), area under the plasma concentration-time curve ($AUC_{0\rightarrow\infty}$), terminal elimination rate constant (λ_z), absorption rate constant (k_a), elimination half-life ($t_{1/2z}$), apparent total clearance (CL/F), apparent volume of distribution (V_z/F), and mean residence time (MRT). The analysis was performed using PK solutions 2.0™ software (Montrose, CO, USA).

All values were expressed as mean ± SD. Statistical analysis was performed using analysis of variance (ANOVA) followed by least significant difference (LSD) test. Statistically significant differences between groups were defined as p values less than 0.05. The software used was the SPSS version 16.0 statistical software.

Results

Plasma concentration-time curves of paracetamol in rats after an oral administration of paracetamol (25 mg/kg BW) alone, gum acacia (30%, w/v) followed by paracetamol (25 mg/kg BW), and roasted sesame seeds (1 g/kg BW) followed by paracetamol (25 mg/kg BW), are shown in Figure 1. The pharmacokinetic parameters for all treatments were described in Table 1. There were no statistical differences for t_{max}, C_{max}, k_a, $AUC_{0\rightarrow\infty}$, and CL/F values of paracetamol among different group of treatments.

$t_{1/2z}$ in rats treated with roasted sesame seeds followed by paracetamol was significantly decreased, when compared with those given paracetamol alone (23.04 ± 3.78 min vs. 30.66 ± 2.61 min; p = 0.021), or gum acacia, followed by paracetamol (23.04 ± 3.78 min vs. 39.17 ± 7.54 min; p = 0.000). However, $t_{1/2z}$ was significantly increased in rats administered gum acacia followed by paracetamol, compared with those receiving paracetamol alone (39.17 ± 7.54 min vs. 30.66 ± 2.61 min; p = 0.011).

λ_z for the group receiving roasted sesame seeds followed by paracetamol was significantly increased, when compared with the group receiving paracetamol alone (0.03 ± 0.00 min⁻¹ vs. 0.02 ± 0.00 min⁻¹; p = 0.025), or gum acacia followed by paracetamol (0.03 ± 0.00 min⁻¹ vs. 0.02 ± 0.00 min⁻¹; p = 0.001).

V_z/F was significantly decreased for the group receiving roasted sesame seeds followed by paracetamol, compared with the group receiving gum acacia followed by paracetamol (2.79 ± 0.90 L/kg vs. 4.23 ± 1.29 L/kg; p = 0.025).

In addition, MRT for the group receiving gum acacia followed by paracetamol was significantly increased, when compared with the group receiving paracetamol alone (70.80 ± 11.75 min vs. 56.93 ± 2.83 min; p = 0.006) , or roasted sesame seeds followed by paracetamol (70.80 ± 11.75 min vs. 51.02 ± 5.16 min ; p = 0.000).

The results indicated that a single oral dose pretreatment with roasted sesame seeds enhanced elimination of paracetamol orally given to the rats at the dose of 25 mg/kg BW.
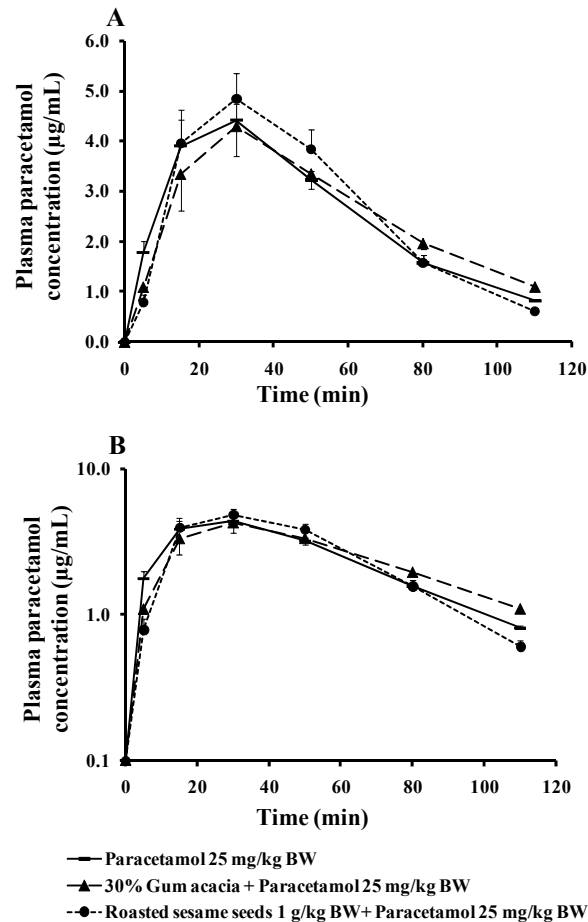
Figure 1. Mean plasma concentration-time curves of paracetamol in rats (n=6) administed paracetamol (25 mg/kg BW), 30% gum acacia followed by paracetamol (25 mg/kg BW), and roasted sesame seeds (1 g/kg BW) followed by paracetamol (25 mg/kg BW). A: Standard Plot, B: Semi-Log Plot.

Table 1. Pharmacokinetic parameters of paracetamol in rats receiving paracetamol (25 mg/kg BW), 30% gum acacia followed by paracetamol (25 mg/kg BW), and roasted sesame seeds (1 g/kg BW) followed by paracetamol (25 mg/kg BW).

Parameters	Paracetamol 25 mg/kg BW	30% Gum acacia + Paracetamol 25 mg/kg BW	Roasted sesame seeds 1 g/kg BW + Paracetamol 25 mg/kg BW
t_{max} (min)	19.60 ± 1.95	23.43 ± 6.39	20.91 ± 1.76
$t_{1/2z}$ (min)	30.66 ± 2.61	$39.17 \pm 7.54^{*}$	$23.04 \pm 3.78^{*,**}$
C_{max} (μg/mL)	4.60 ± 0.86	4.34 ± 1.76	5.09 ± 1.42
k_a (min^{-1})	0.10 ± 0.02	0.10 ± 0.05	0.07 ± 0.01
λ_z (min^{-1})	0.02 ± 0.00	0.02 ± 0.00	$0.03 \pm 0.00^{*,**}$
$AUC_{0\rightarrow\infty}$ (μg·min/mL)	315.44 ± 44.84	346.20 ± 50.03	312.67 ± 65.99
V_z/F (L/kg)	3.58 ± 0.69	4.23 ± 1.29	$2.79 \pm 0.90^{**}$
CL/F (L/min/kg)	0.08 ± 0.01	0.07 ± 0.01	0.08 ± 0.02
MRT (min)	56.93 ± 2.82	$70.80 \pm 11.75^{*}$	$51.02 \pm 5.16^{**}$

Each value represents mean ± SD of 6 animals. t_{max}: the time to reach peak plasma concentration, $t_{1/2z}$: elimination half-life, C_{max}: the peak plasma concentration, k_a: absorption rate constant. λ_z: terminal elimination rate constant, $AUC_{0-\infty}$: area under the plasma concentration-time curve, V_z/F: apparent volume of distribution, CL/F: apparent total clearance, MRT: the mean residence time.
Single asterisks (*) indicate significant difference from paracetamol group ($p < 0.05$).
Double asterisks (**) indicate significant difference from gum acacia followed by paracetamol group ($p < 0.05$).

Discussion

With the growing interest in sesame seeds and their association with a variety of health benefits, their pharmacokinetic and food-drug interaction are important issues. This study examined the effects of roasted sesame seeds on paracetamol pharmacokinetics in rats. The findings imply that roasted sesame seeds enhanced elimination of paracetamol.

Sesame lignans, such as sesamin, episesamin, sesaminol, sesaminol triglycoside (STG) and sesamolin, are major constituents containing a methylenedioxyphenyl group in sesame seed. Nabekura et al.[10] found that sesamin inhibits P-glycoprotein in KB-C2 cells. This inhibition causes accumulation of drug in the cell. Meaning as paracetamol accumulates in the hepatocyte and metabolized by responsible enzyme.

In this study demonstrated that MRT in roasted sesame seeds treated rats slightly decreased. This effect may result from the interaction of gum acacia and sesame seeds. Gum acacia enhance $t_{1/2z}$ and MRT in rats. Since, gum acacia, widely used in pharmaceutical preparations, is used as vehicle control, therefore the result indicated that this substance is not a suitable vehicle. It was reported that the coexistence of amoxicillin and gum acacia in the upper gastrointestinal tract resulted in a pharmacokinetic interaction that significantly decreased the absorption of amoxicillin; significantly decreased the C_{max} and $AUC_{0\rightarrow\infty}$ when compared with the amoxicillin alone group.[11] In this study, gum acacia does not reduce the absorption of paracetamol, but tends to increase the time to maximum plasma concentrations (t_{max}).

Conclusion

A single oral dose pretreatment with roasted sesame seeds may lead to reduction of the pharmacological response of paracetamol in vivo and this result can be the basis for the application in humans. Further research can be applied to the sesame roasted with paracetamol to reduce the toxicity of paracetamol in case of overdose.

Acknowledgements

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Effects of Roasted Sesame Seeds on Serum AST and ALT Levels in Paracetamol-Induced Hepatotoxicity in Rats

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Abstract

Sesame seed is a reservoir of nutritional components with numerous beneficial effects for humans. The bioactive components present in the seed include vital minerals, vitamins, phytosterols, polyunsaturated fatty acids, tocopherols and unique class of lignans such as sesamin and sesamolin. These compounds were found to play a role in the prevention liver toxicity from paracetamol, however, the effect of roasted sesame seed on hepatotoxicity has not been reported. Therefore, the present study investigated the effects of roasted sesame seeds on aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in paracetamol-induced hepatotoxicity in rats. Male Wistar rats were randomly divided into 5 groups (n=6) as follows: group I, distilled water; group II, 30% (w/v) gum acacia (10 mL/kg); group III, paracetamol (1,500 mg/kg); group IV, roasted sesame seeds (1,000 mg/kg); group V, roasted sesame seeds (1,000 mg/kg) plus paracetamol (1,500 mg/kg). All animals were administered by a single oral route. Serum samples were collected at 48 hours later. The results found that the level of AST and ALT in roasted sesame seeds followed with paracetamol was significantly decreased ($p<0.05$) when compared with paracetamol treated alone. It is concluded that roasted sesame seeds was effective in alleviating liver damage in rats caused by paracetamol overdose.

Keywords: roasted sesame seed, paracetamol, hepatotoxicity, aspartate aminotransferase (AST), alanine aminotransferase (ALT)

ผลของงาดำคั่วต่อระดับซีรั่ม AST และ ALT ในหนูแรทที่ถูกชักนำให้เกิดความเป็นพิษต่อตับด้วยพาราเซตามอล

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บทคัดย่อ

เมล็ดงาเป็นแหล่งคุณค่าทางโภชนาการที่มีประโยชน์ต่อมนุษย์ โดยภายในเมล็ดมีสารอาหารที่จำเป็นต่อร่างกายมากมาย เช่น แร่ธาตุ วิตามิน ไฟโตสเตอรอล กรดไขมันไม่อิ่มตัว วิตามินอี หรือ โทโคฟีรอล นอกจากนี้ยังมีสารลิกแนนที่จัดเป็นเอกลักษณ์ในงา คือ เซซามีน และเซซาโมลีน ซึ่งเป็นส่วนประกอบหลักที่พบในเมล็ดงา สารประกอบเหล่านี้มีบทบาทสำคัญ ที่สามารถป้องกันความเป็นพิษต่อตับจากยาพาราเซตามอลได้ ในขณะที่ยังไม่มีการรายงานผลของเมล็ดงาดำคั่ว ต่อการป้องกันพิษต่อตับ ดังนั้นการวิจัยนี้ได้ศึกษาผลของงาดำคั่ว ต่อระดับเอมไซม์แอสพาร์เตตอะมิโนทรานสเฟอเรส (AST) และ เอมไซม์อะลานีนอะมิโนทรานสเฟอเรส (ALT) ในซีรั่มของหนูแรท ที่ถูกชักนำให้เกิดความเป็นพิษต่อตับด้วยพาราเซตามอล แบ่งสัตว์ทดลองออกเป็น 5 กลุ่ม ดังนี้ กลุ่ม 1 สัตว์ทดลองได้รับน้ำ; กลุ่ม 2, ได้รับ 30% (w/v) กัมอะคาเซีย; กลุ่ม 3, ได้รับ พาราเซตามอล (1,500 มก./กก.); กลุ่ม 4 ได้รับงาดำคั่ว (1,000 มก./กก.) และกลุ่ม 5 ได้รับทั้งงาดำคั่ว (1,000 มก./กก.) และพาราเซตามอล (1,500 มก./กก.) โดยหนูทุกตัวจะได้รับการบริหารยาทางปากครั้งเดียว จากนั้นทำการเก็บตัวอย่างเลือดที่เวลา 48 ชั่วโมง ภายหลังการให้ยา ผลการศึกษาพบว่า ระดับ AST และ ALT ในซีรั่มของหนูที่ได้รับงาดำคั่ว และ พาราเซตามอล มีระดับลดลงอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) เมื่อเทียบกับกลุ่มที่ได้รับพาราเซตามอลเพียงอย่างเดียว ดังนั้นสรุปได้ว่างาดำคั่วมีประสิทธิภาพในการบรรเทาความเสียหายของตับในหนูแรท ที่เกิดจากการใช้ยาพาราเซตามอลเกินขนาด

คำสำคัญ: งาดำคั่ว, พาราเซตามอล, ความเป็นพิษต่อตับ, แอสพาร์เตตอะมิโนทรานสเฟอเรส, อะลานีนอะมิโนทรานสเฟอเรส

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Introduction

Paracetamol or Acetaminophen (APAP) (*N-acetyl-p-aminophenol*), is one of the most widely used analgesic and antipyretic drug. It is usually safe when administered at therapeutic dose. Typical therapeutic dose of APAP are not hepatotoxicity, because most of the APAP gets glucuronidation (40-67%) or sulfate (20-46%) with little drug bioactivation.[1] The toxicity of APAP usually occur as an over the counter of this drug. APAP causes liver injury in experimental animals and humans. Injury after large doses of APAP is resulting by the formation of the toxic electrophilic metabolite, N-acetyl-*p*-benzoquinonimine (NAPQI), which is an intermediate product of APAP oxidation by CYP2E1.[1, 2] NAPQI is form covalent adducts with hepatic proteins and causes cell death. Glutathione (GSH) plays an important role for eliminate this toxic metabolite. But high level of NAPQI causes depleted GSH level and minimized by treatments with N-acetylcysteine that enhance hepatocyte synthesis of GSH.[1, 3]

The clinical and biochemical signs APAP induce hepatotoxicity are shown at least 24 hours postingestion.[4, 5] The rising of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are one of the first biochemical signs of hepatotoxicity following APAP overdose.[6, 7] Histopathology and AST and ALT clinical chemistries suggested 1,500 mg/kg APAP produced phenotypes of early, full, and recovery stages of liver injury at 6, 24, and 48 hours, respectively.[8] In addition, the preliminary studied 1,500 mg/kg BW of APAP can induce acute liver toxicity in rats. Therefore, this dose was chosen for study.

Sesame seeds (*Sesamum orientale* L.) is a flowering plant in Pedaliaceae family. Sesame seeds were widely used in food and nutraceutical industries in many countries. It is a rich in variety nutrients and sources of edible oil.[9] Sesame seed is high in protein, vitamin B1, dietary fiber as well as an excellent source of phosphorous, iron, magnesium calcium, manganese, copper and zinc. In addition, the important nutrients in sesame seeds is sesamin and sesamolin, which are antioxidant properties.[10] Both substances belong to a group of special beneficial fibers called lignans. The roasting of sesame seeds degrades the lignan sesamolin to sesamol.[11] These components can maintain the intracellular glutathione levels.[12] That will increase the capability of liver to detoxify toxic agents. The previous studies reported the effect of lignin on hepatotoxicity[13], while the effect of roasted sesame seed on hepatotoxicity has not been reported. So, this study was conducted to determine the effects of sesame seed on AST and ALT level in APAP induced liver toxicity in rats.

Materials and methods

Chemicals and Reagents

Paracetamol (purify > 98%), alpha-ketoglutaric acid, DL-aspartic acid, DL-alanine and sodium pyruvate were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Sodium dihydrogen orthophosphate dehydrate was purchased from Fisher Scientific UK Limited (Leicestershire, UK). Sodium phosphate dibasic AR and 2,4-Dinitrophenylhydrazine, A.R. was purchased from HiMedia Laboratories Pvt. Ltd (LBS Marg. Mumbai, India). Sodium hydroxide anhydrous was purchased from Carlo Erba Reagent (Milan, Italy). Black sesame seeds (*Sesamum orientale* L.) obtained from Mae Hong Son Province, Thailand. Acacia was purchased from Ajax Finechem Pty Ltd (Auckland, New Zealand). Roasted sesame seeds and APAP suspension were prepared in 30% (w/v) gum acacia in total volume 10 mL/kg.

Roasting of sesame seeds

Whole sesame seeds (15 g) were freshly prepared by placed in a pan and smoulder-roasted in Cuizimate induction cooker (Model: RBSIH19NEW) (Nesco Group Co., Ltd., Bangkok, Thailand) for 10 min. After roasting, the seeds were grinded with a mortar and pestle to fine powder prior to test.

Animals

Thirty (30) male albino Wistar rats weighing 220-250 g were obtained from Southern Laboratory Animal Facility, Prince of Songkla University, Hat Yai campus. Animals were housed individually with *ad libitum* access to water in environmentally controlled room with temperature 23 ± 2 °C, humidity 55%, 12 hours light/dark cycle. Animals were fasted overnight but had free access to water prior to treatment. The care and procedures adopted were approved of Institutional Animal Ethics Committee (Ref. 12/55).

Experimental design

Rats were randomly divided into 5 groups (n= 6 each). Group I, rats were given orally distilled water (10 mL/kg BW). Group II, rats were given orally 30% (w/v) gum acacia (10 mL/kg BW). Group III, rats were given orally APAP (1,500 mg/kg BW). Group IV, rats were given orally roasted sesame (1,000 mg/kg BW). Group V, rats were given orally roasted sesame (1,000 mg/kg BW) at 15 min before APAP (1,500 mg/kg BW). All animals were administered by single route. Rat's serum was collected at 48 hours later.

The dose of APAP[8] and roasted sesame seeds.[14] were follows with previous reported.

Preparation of Serum

The blood was collected from the tail vein of rats at 48 hours after the last dose of the drug. Approximately 1 mL of blood was collected into centrifuge tube and then centrifuged at 1600 ×g for 10 min at 4°C in a bench centrifuge to obtain serum. The serum samples were used for the analysis of aminotransferase activities.

Measurement of serum aminotransferase levels

Hepatic injury was assessed by measuring the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in serum. These enzymes were determined by the technique of Reitman and Frankel (1957).[15] Briefly, the reaction of mixture was contained 0.1 mL of serum and 0.5 mL of the desired substrate. After the content was mixed and then incubated at 37 °C, exactly 30 min for ALT, or 60 min for AST level measurements, respectively. Then 0.5 mL of 2, 4-dinitrophenyl hydrazine was added into each test tube and incubated at room temperature for 30 min. Then 5 mL of 0.4 N sodium hydroxide was added and measured by spectrophotometer at 505 nm. The levels of AST and ALT were expressed as U/mL.

Statistical analysis

The data of AST and ALT levels were expressed as mean ± S.E.M. Differences between the mean values were estimated using one-way analysis of variance (one-way ANOVA) followed by *post hoc* comparison by using SPSS version 16.0 statistical software. The results were considered statistically significant when $p < 0.05$.

Results

Effects of roasted sesame seeds on AST and ALT levels

The mean AST level in serum of rats after administration with difference of treatments is shown in figure 1. AST level of distilled water group and 30% gum acacia treated group were ranging from 105.8-128.47 U/mL and 71.29-140.00 U/mL, respectively. These results were shown that AST level among these groups were not significant difference. So, gum acacia has been used as a vehicle. AST level of roasted sesame seeds group was not significantly increased (99.61-122.39 U/mL) when compared with distilled water group. AST level of APAP treated group was significantly increased (129.00-205.13 U/mL) when compared with distilled water group and 30% gum acacia treated groups, while AST level of roasted sesame seeds followed with APAP group was significantly decreased (92.47- 151.13 U/mL) ($p < 0.05$) when compared with APAP treated group.

The mean ALT level in serum of rats after administration with difference of treatments is shown in figure 2. ALT levels of distilled water group was ranging from 18.63 – 27.05 U/mL. ALT levels of 30% gum acacia treated group (23.92 – 37.25 U/mL) and roasted sesame seeds group (9.90-40.40 U/mL) were not significantly difference when compared with distilled water group. ALT level of APAP treated group was significantly increased (68.88 – 176.38 U/mL) when compared with distilled water group and 30% gum acacia treated groups, while ALT level of roasted sesame seeds followed with APAP group was significantly decreased (90 – 40.40 U/mL) ($p < 0.05$) when compared with APAP treated group.
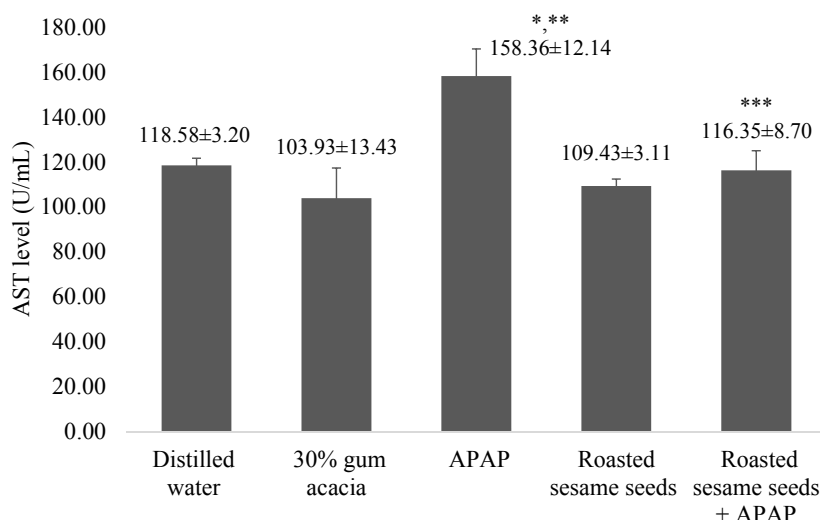

Figure 1.   Effects of roasted sesame seeds on AST level in different groups.
Data are mean ± S.E.M.
*   Significant differences as compared with distilled water group ($p < 0.05$).
**   Significant differences as compared with 30% gum acacia ($p < 0.05$).
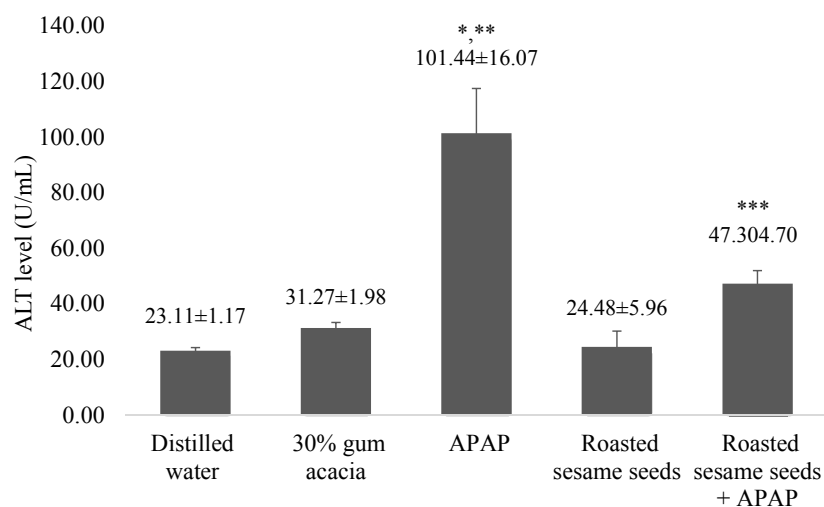*** Significant differences as compared with APAP group ($p < 0.05$).

Figure 2. Effects of roasted sesame seeds on ALT level in different groups.
Data are mean ± S.E.M.
* Significant differences as compared with distilled water group ($p < 0.01$).
** Significant differences as compared with 30% gum acacia ($p < 0.01$).
*** Significant differences as compared with APAP group ($p < 0.05$).

Discussion

Assessment of liver function can be made by estimating the level of serum AST and ALT. AST is a cellular enzyme that can be identified to some extent in the heart, skeletal muscles, kidneys, brain, pancreas and blood cells. This enzyme exists in two isoforms, cytoplasmic and mitochondrial. The ALT, as present primarily in the cytosol of the liver and it is a more specific indicator of liver inflammation.[16, 17] In the condition of hepatotoxicity, these enzymes leak into blood stream in conformity with the extent of liver damage.

In this study, roasted sesame seeds was effective in alleviating liver damage in rats caused by APAP overdose. The toxic response of liver to APAP overdose is presented by elevation of AST and ALT levels in serum. We found that single oral dose of 1,500 mg/kg APAP can elevated levels of AST and ALT in rat. The rising of these enzymes in serum mean that N-acetyl-p-benzoquinone imine (NAPQI) covalent binding to intracellular proteins results in depletion of reduced glutathione (GSH). This results in the formation of superoxide anion, which is responsible for the production of hydroxyl radical by initiating the Haber-Weiss reaction and lead to dysfunction hepatocyte.[18] Chandrasekaran and coworker[12] reported that APAP (1,000 mg/kg) alone significantly ($p<0.001$) increased superoxide anion generation after 6 hours and hydroxyl radical generation after 12 and 24 hours in the APAP group. This study also found that the elevations of AST and ALT levels were rise to 33% and 339% of distilled water group, respectively.

AST and ALT levels were not increase in roasted sesame seeds (1,000 mg/kg) group compared with distilled water group. This result indicated that roasted sesame seeds was no cause toxicity in the liver cells. Furthermore, co-administration of roasted sesame seeds and APAP was effectively in alleviating liver damage in rats, because AST and ALT levels significantly decreased when compared with APAP treated alone. By reason of, compounds found in roasted sesame seeds are consists of various antioxidants such as tocopherol, sesamin, sesamolin, and sesamol.[19] Sesamol is an effective antioxidant found mainly in roasted sesame seeds or in processed sesame oil, and it is an efficient scavenger of the entire range of ROS.[20] Chandrasekaren and coworker[12] reported that sesame oil maintained the intracellular glutathione levels, reduced reactive oxygen species levels, and inhibited lipid peroxidation in rats with APAP-induced acute liver injury. Moreover, the further study of them, which is also found sesamol protected rat liver tissue against APAP-induced acute hepatic injury that it may be inhibits hydroxyl-radical generated lipid peroxidation.[13] More recently, sesame was assumed that it could interact with the P450 isozymes and affect the drug metabolisms in human, due to having a methylenedioxyphenyl compounds which potent inhibitions or inactivated of some CYP isoforms (CYP1A2, CYP2E1 and CYP2A6).[21] In addition, 3,4-Methylenedioxyphenol (sesamol) is effective against acetaminophen-induced liver injury in rats. Chandrasekaren and coworker[22] reported that equimolar doses (1 mmol/kg) of sesamol and N-acetylcysteine significantly inhibited acetaminophen (300 mg/kg)-increased serum AST and ALT levels 6 hours post-administration. Sesamol and N-acetylcysteine maintained hepatic glutathione levels and inhibited lipid peroxidation. Therefore, it is

suggested that roasted sesame seeds dietary consumption could be a potential for reduce liver damage caused by APAP overdose.

Conclusion

In summary, roasted sesame seeds were effective in alleviating liver damage in rats caused by APAP overdose. May be, associated with the substance in roasted sesame seeds effective as antioxidant that its reduced reactive oxygen species levels and maintained the intracellular glutathione levels including to inhibitions or inactivated of Cytochrome P450 (CYP2E1) of liver tissue in rats, which, causes decreased of toxic metabolite NAPQI. This is resulted decreased of AST and ALT levels in the blood. However, there should be additional studies to mechanism of sesame, such as measure of GSH level or determine of expression CYP2E1 and histopathology.

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F28 Spasmolytic Action of White Pepper Grains (*Piper nigrum*) Methanolic Extract in Non-pregnant Rat Uterus

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Abstract

White pepper (*Piper nigrum*) is an herb widely used in Thai traditional medicine. It has been used to treat or reduce the symptoms of many disorders. One of its advantages is to relief the symptoms of dysmenorrhea. Previously, it has been reported that piperine, the main alkaloid of this plant, has inhibitory effect on cyclooxygenase-2 (COX-2) synthesis which is an enzyme responsible for the production of prostaglandins. These mediators play an important role in generating symptoms of dysmenorrhea. The aim of this study was to investigate the spasmolytic action of white pepper fruit methanolic extract in non-pregnant rat uterus and the possible mechanism(s) of its action. Piperine was also used to test its effect. The result of this study showed that the uterine contraction induced by $CaCl_2$ in Ca^{2+} free high K^+ solution (60 mM KCl) and oxytocin (1 mU/mL) was significantly ($p < 0.01$) reduced by the extract (15-150 µg/mL) or (10-100 µg/mL) respectively. In addition, the spasmolytic effect of the piperine (30 µg/mL) or diclofenac (10^{-4} M) was also tested. They were able to completely inhibit the contraction of the uterus precontracted with arachidonic acid (10^{-5} M). This study therefore suggested that the spasmolytic effect of the extract on rat uterus may act by inhibiting the entry of the extracellular calcium into muscle cells. The extract may also have an additional inhibitory action on COX-2, consequently, prostaglandins synthesis which is the main causes of dysmenorrhea. Our results strongly support the use of white pepper in traditional medicine to relief painful of dysmenorrhea.

Keywords: white pepper (*Piper Nigrum*), piperine, rat uterus, oxytocin, spasmolytic

การศึกษาผลของสารสกัดพริกไทยขาวในเมทานอลต่อการลดการหดเกร็งของมดลูกในหนูขาว

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บทคัดย่อ

พริกไทยขาวเป็นพืชสมุนไพรที่มีการใช้อย่างแพร่หลายในทางการแพทย์แผนไทย โดยได้มีการนำพริกไทยขาวมาใช้รักษาความผิดปกติหลายชนิด ประโยชน์ชนิดหนึ่งของพริกไทยขาวที่มีการนำเอามาใช้ในทางการแพทย์แผนไทยคือ ใช้ลดอาการปวดท้องในขณะมีประจำเดือน จากการศึกษาฤทธิ์ของพิเพอรีนซึ่งเป็นสารแอลคาลอยด์หลักของพริกไทยพบว่าสารนี้สามารถยับยั้งการสังเคราะห์เอนไซม์ COX-2 ซึ่งเป็นเอนไซม์ที่ใช้สร้างพรอสตาแกลนดินที่เป็นสาเหตุหลักของการปวดท้องขณะมีประจำเดือนได้ จุดประสงค์ของวิจัยครั้งนี้เพื่อศึกษากลไกของสารสกัดพริกไทยขาวในเมทานอลและพิเพอรีนต่อการลดการหดเกร็งของมดลูกในหนูขาว จากการทดลองพบว่าสารสกัดของพริกไทยในขนาด 15-150 ไมโครกรัม/มิลลิลิตร และ 10-100 ไมโครกรัม/มิลลิลิตร สามารถยับยั้งการหดตัวของมดลูกที่ถูกเหนี่ยวนำด้วยสารละลาย แคลเซียมคลอไรด์ (10^{-5}–10^{-2} โมลาร์) ในสารละลาย Ca^{2+} free high K^+

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(โพแทสเซียมคลอไรด์ 60 มิลลิโมลาร์) และ ออกซิโทซิน (1 มิลลิยูนิต/มิลลิลิตร) ตามลำดับได้อย่างมีนัยสำคัญทางสถิติ ($p < 0.01$) นอกจากนี้พบว่า สารมาตรฐานพิเพอรีน (30 ไมโครกรัม/มิลลิลิตร) และ ไดโคลฟีแนค (10^{-4} โมลาร์) สามารถยับยั้งการหดตัวของ มดลูกซึ่งถูกเหนี่ยวนำด้วยกรดอะราคิโดนิก (10^{-5} โมลาร์) ได้อย่างสมบูรณ์ การศึกษาในครั้งนี้เสนอแนะว่ากลไกการคลายตัวของ มดลูกที่เกิดจากสารสกัดของพริกไทยอาจเกิดจากฤทธิ์ที่สามารถยับยั้งแคลเซียมไม่ให้ผ่านเข้าสู่เซลล์กล้ามเนื้อ และอาจมีฤทธิ์ในการ ยับยั้งเอนไซม์ COX-2 ซึ่งเป็นเอนไซม์ที่ใช้ในการสร้างพรอสตาแกลนดินที่เป็นสาเหตุหลักในการปวดประจำเดือน ผลการทดลองใน ครั้งนี้จึงสนับสนุนการใช้พริกไทยขาวในการรักษาแบบแพทย์แผนไทยเพื่อลดอาการปวดประจำเดือน

คำสำคัญ: พริกไทยขาว, พิเพอรีน, มดลูกของหนูขาว, ออกซิโทซิน, ฤทธิ์ลดการหดเกร็ง

Introduction

White pepper *(Piper nigrum)* is a member of the Piperaceae family. It is a well-known spice considered as ''The King of spices''. It contains a pungent alkaloid piperine, found in the roots and fruits of *Piper nigrum* in both black and white pepper grains.[1] *Piper nigrum* can be used for different purposes such as human dietaries, as medicine, as preservative and as biocontrol agents.[2,3] In addition, piperine exhibits diverse pharmacological activities like antihypertensive and antiplatelets[4],antioxidant, antitumor[5], anti-asthmatics[6] , antipyretic, analgesic, anti-inflammatory, anti-diarrheal, antispasmodic, anxiolytic, antidepressants[7], hepato-protective[8], immuno-modulatory, antibacterial, antifungal, anti-thyroids, anti-apoptotic, anti-metastatic, antimutagenic, anti-spermatogenic, anti-colon toxin, insecticidal and larvicidal activities.

Previously, Kazem et al, (2007)[9] has reported spasmolytic effect of black pepper on rat uterus which could have mechanism mediated via the inhibition of voltage-dependent calcium channels and the stimulation of β-adrenoceptors. In addition, *Piper nigrum* was reported to have anti-inflammatory activity probably by an inhibition on the expression of COX-2 protein and COX-2 mRNA[10], the main causes of dysmenorrhea. Thus it is of interest to study on the mechanism of the effect of piperine involving COX-2 or other possible mechanisms which have not been investigated. The aim of the present study was to investigate the effect of methanolic extract of white pepper fruit on the non-pregnant rat uterus contractions induced by some spasmogens and the possible mechanism(s) involved.

Materials and Methods

Plant materials

White pepper grains were purchased from Herbal drug store in Hatyai District, Songkhla Province, Thailand.

Extraction

White pepper grains were powdered by an electrical grinder and the powder (1 kg) was extracted with absolute methanol for 3 days at room temperature. The solvent was then removed, stores desiccated at room temperature protected from light. The extraction was repeated for 5 times. The whole methanolic extracts were pooled, and evaporated at 50 °C under reduced pressure in Rotary Evaporator. The crude methanol extract 64.2 g (6.42%) were obtained and the yellow viscous, oil-like mixture was stored at 4 °C until used.

Drugs and chemicals

Dimethyl sulfoxide (DMSO), estradiol benzoate and arachidonic acid (AA) were purchased from Sigma chemical company (St. Louis, U.S.A). All drugs were prepared as stock solutions ($CaCl_2$ 10^{-2} M, oxytocin 1 mU/mL), AA (10^{-5} M), diclofenac sodium (10^{-4} M) in 0.1% ascorbic acid in distilled water. Estradiol benzoate was dissolved in olive oil to give a concentration of 1 mg/mL for intraperitoneal injection. Methanolic extract was dissolved in a mixture of dimethyl sulfoxide (DMSO) and tween 80 to give a stock of 20 mg/mL for use in all experiments. The final concentration of DMSO and tween 80 in the organ bath was less than 0.5% and did not affect contraction or relaxation of the uterus. NaCl and $NaHCO_3$ were purchased from Merck. KCl, $CaCl_2$ and glucose were purchased from Carlo Erba. The De Jalon solution composition (pH 7.4 and 29 °C) was NaCl, 154 mM; KCl, 5.6 mM; $CaCl_2$, 0.3 mM; $NaHCO_3$, 1.7 mM; $MgCl_2$, 1.4 mM and glucose, 5.55 mM and Locke-Ringer solution composition (pH 7.4 and 29 °C) was NaCl, 154 mM; KCl, 5.6 mM; $CaCl_2$, 2.16 mM; $NaHCO_3$, 1.7 mM; $MgCl_2$, 2.10 mM and glucose, 5.55 mM. These common salts were used to prepare physiological solutions, which were freshly prepared for each day of experiment.

Experimental Animals

All experiments were carried out using virgin female Wistar rats (200-300 g.) which were supplied from Division of Animal House, Faculty of Science, Prince of Songkla University. All experimental protocols were approved by Institutional Animals Care and Use Committee,

Prince of Songkla University (MOE 0521.11/363). They were housed in air-conditioned room (24-26 ^{o}C) with 12 hours light/dark cycle. Rats were injected intraperitoneally with estradiol benzoate dissolved in olive oil (100 µg/mL) 24 hours before the experiment.

Experimental Protocol

On the day of experiment the rats were sacrificed by cervical dislocation. From the cervical portion of each uterine horn (1-1.5 cm), a piece of uterus was dissected and immersed in an organ bath containing De Jalon-Ringer solution. This medium was gassed continuously with 95% O_2, 5% CO_2 and maintained at 37 ^{o}C throughout the experiments. The isometric contraction of the uterus was recorded using force displacement transducer and Grass polygraph. The initial tension was 2 g and the uterus was equilibrated in the medium for a period of at least 1 hour during which, the bath solution was replaced with fresh solution every 15 min. In all experiments, a uterus segment from the other horn was also set up as a vehicle control preparation to compare the effect of time and vehicle to the drug effect. The control uterus was prepared and used in the same manner as the drug treated uterus, but the relevant vehicle of the drug was added to the organ bath instead of the drug solution. Each experiment was repeated in at least 6-10 replicates.

Experimental Procedures
Effect of methanolic extract of white pepper on uterine contraction induced by CaCl$_2$ in Ca^{2+}- free high K$^+$ solution

To study the role of extracellular calcium, the uterine horns were bathed for 1 hour in De Jalon-Ringer solution with resting tension of 2 g. The solution was then replaced by Ca^{2+}- free high K$^+$ (60 mM KCl) De Jalon-Ringer solution. CaCl$_2$ was added to the organ bath cumulatively (10^{-6}-10^{-2} M) before and after tissue incubation with different concentration of the extract (15, 50, 150 µg/mL). Each uterus preparation was used only for one of the concentration of the extract.

Effect of methanolic extract of white pepper on uterine contraction induced by oxytocin

A uterine horn was bathed for 1 hour in Locke-Ringer solution with resting tension of 2 g. The uterus was contracted by the addition of oxytocin (1 mU/mL). After rhythmic contraction was stable, the extract (0.3-100 µg/mL) was added to the organ bath cumulatively. Changes in force and frequency of contraction were then recorded.

Effect of piperine or diclofenac sodium on uterine contraction induced by AA (arachidonic acid)

A uterine horn was bathed for 1 hour in De Jalon-Ringer solution with resting tension of 2 g. The uterus was precontracted by the addition of AA (10^{-5} M). After rhythmic contraction was stable, the uterus was washed 3 times with fresh De Jalon-Ringer solution to remove AA. De Jalon-Ringer solution was changed every 10 minutes until the tension of the uterus reached the baseline. Piperine (30 µg/mL) or diclofenac sodium (10^{-4} M) was then added to the medium for 20 min followed with the addition of AA (10^{-5} M) for 20 min. After then, the uterus was washed 3 times with De Jalon-Ringer solution to remove the extract and AA. De Jalon-Ringer solution was changed every 10 minutes until the tension of the uterus reached the baseline. AA (10^{-5} M) was then added to the organ bath to observe the responsiveness of the uterus to AA alone after previously exposure to piperine or diclofenac sodium.

Data analysis

Values (changes in contraction recorded in comparison with 100% contractions induced by spasmogens in the absence of the extract) were expressed as mean±SEM. Statistical comparisons were made by independent sample *t-test* or repeated-measure analysis of variance (ANOVA) and *p* values less than 0.01 were considered statistically significant.

Results

Effect of methanolic extract of white pepper on uterine contraction induced by CaCl$_2$ in Ca^{2+}- free high K$^+$ solution

Calcium chloride (10^{-5}-10^{-2} M) induced rat uterine contractions in a concentration-dependent manner ($p<0.01$). The uterine contraction curves induced by CaCl$_2$ were inhibited by pretreatment (10 min) with the extract (15, 50, 150 µg/mL, n = 6, $p < 0.01$) in a dose-dependent manner as shown in Figure 1.

Effect of methanolic extract of white pepper on uterine contraction induced by oxytocin

Methanoloc extract of white pepper at the concentration 10-100 µg/mL significantly reduced the oxytocin-induced uterine contraction. ($p < 0.01$) (Figure 2.)

Effect of piperine or diclofenac sodium on uterine contraction induced by arachidonic acid

The effect of piperine (30 µg/mL) and diclofenac sodium (10^{-4} M) on the AA-induced uterus contraction were tested in this study. The results showed that the stimulatory effect of AA (10^{-5} M) on the contraction of rat uterus was completely inhibited by the addition with piperine (30 µg/mL) or diclofenac sodium (10^{-4} M) (n=6). The stimulation by AA could be recovered after the removal of piperine or diclofenac sodium. Representative tracing of the effect of piperine or diclofenac sodium on AA-induced uterus contraction was shown in Figure 3 and Figure 4, respectively.
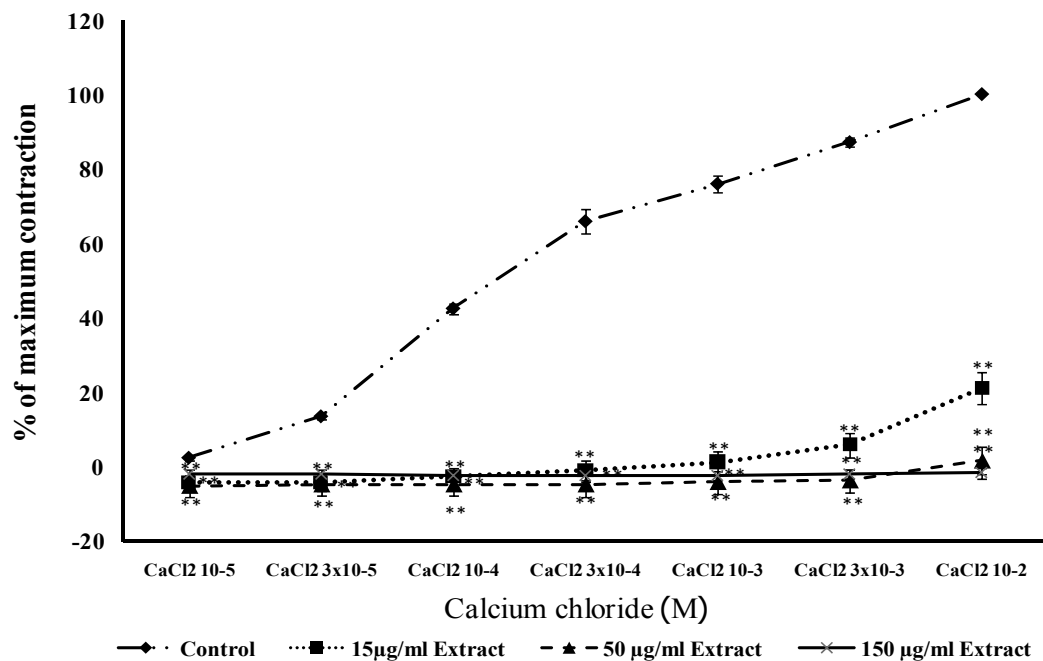
Figure 1. The spasmogenic effects of the contractile response of isolated rat uterus to cumulative concentrations of the $CaCl_2$ in Ca^{2+} free high K^+ solution in the absence or presence of extract (15, 50, and 150 µg/mL). The statistical comparisons were carried out for extract at 15, 50 and 150 µg/mL (n=6, ** $p < 0.01$).
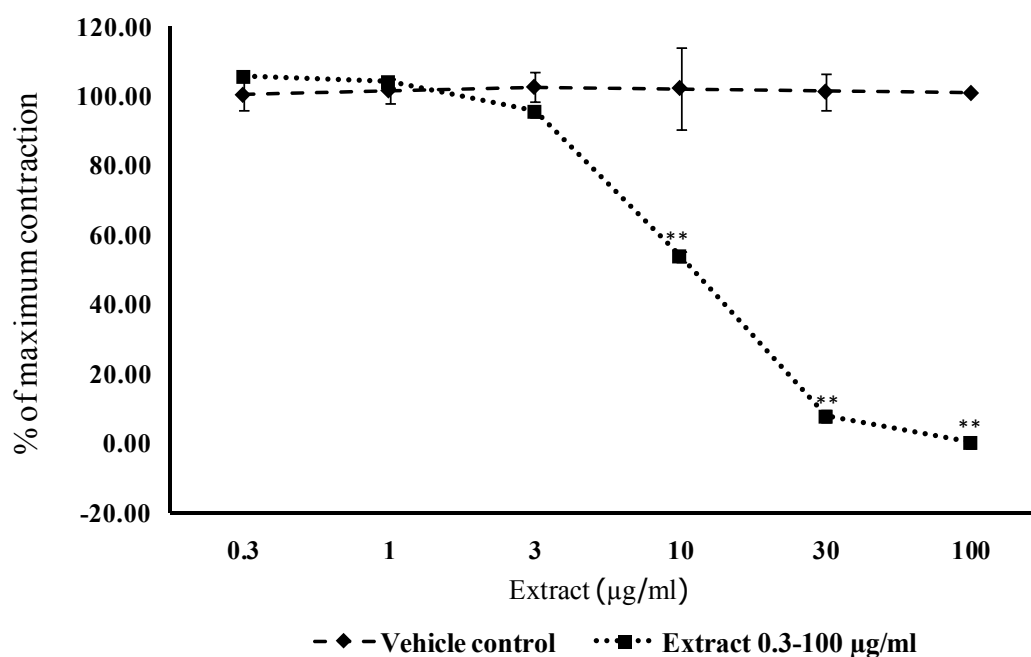
Figure 2. The relaxing effect on the rat uterus contractions induced by oxytocin in the presence or absence of the extract (0.3-100 µg/mL) (n=6, ** $p < 0.01$).
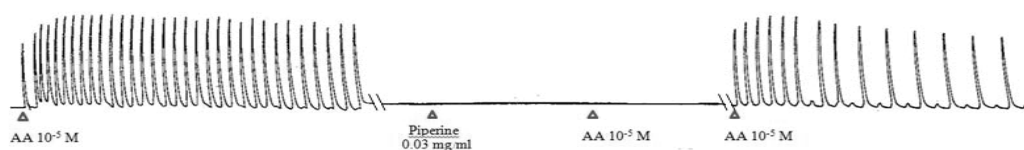
Figure 3. Representative tracing of the effect of the piperine (30 μg/mL) on the rat uterine contractions induced by arachidonic acid (AA, 10^{-5} M) (n=6).
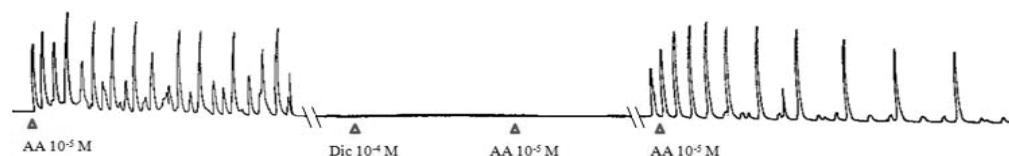
Figure 4. Representative tracing of the effect of the diclofenac (Dic, 10^{-4} M) on the rat uterine contractions induced by arachidonic acid (AA, 10^{-5} M) (n=6).

Discussion

The aim of this study is to determine the effect and mechanism of action of the methanolic extract of white pepper on uterine contraction in rat. From our preliminary finding showed that the extract had no effect on uterus contraction in isolated rat uterus. However, the extract was able to completely inhibit uterine contraction induced by $CaCl_2$ and other stimulants such as oxytocin and arachidonic acid (AA). One major finding of this study was an inhibitory effect of the extract on uterine contraction induced by $CaCl_2$ in Ca^{2+} free high K^+ solution (KCl 60 mM). The mechanism of $CaCl_2$ induced uterine contraction in Ca^{2+}- free high K^+ solution is that the high K^+ concentration could depolarizethe cell membrane.[11] This effect changed the ions conductance of the cell which in turn, activates voltage-dependent calcium channel opening, leading to calcium influx into the cells and then stimulated uterine contraction.[12] Although calcium channel was opened, the lack of calcium in Ca^{2+} free high K^+ solution (KCl 60 mM) could not stimulate the uterus to contract. The additions of $CaCl_2$ to the organ bath caused an influx of calcium into the cell via the opened calcium channel and stimulate the contraction of the uterus. The result of this study showed that the extract dose-dependently inhibited uterine contraction induced by $CaCl_2$ in Ca^{2+} free high K^+ solution (KCl 60 mM). The result suggested that the inhibitory effect of methanolic extract on $CaCl_2$ may occur by blocking the calcium channel. Oxytocin induced the isolated rat uterus to contract rhythmically in Locke-Ringer solution. Oxytocin is a powerful stimulator of contraction in the uterus while increase in force, frequency and duration of action.[13, 14] Oxytocin is known as an endogenous uterine stimulant via binding to oxytocin receptor on the membrane of the uterine smooth muscle cells. The action of oxytocin inducing the uterine contraction is due to an increase in intracellular calcium resulting from both calcium influx via plasma membrane and calcium-release from internal store.[15] It is suggested that the inhibition pathway of the extract may occur through an inhibition on calcium influx and/or calcium-release from internal storage.

In order to determine of the action of piperine, the major alkaloid of white pepper, or diclofenac sodium on COX-2, AA was used to stimulate the uterine contraction. Our result showed that piperine or diclofenac sodium was able to completely inhibit the contraction. The result from this study is consistent to a previous study[10] that piperine was able to inhibit the expression of COX-2 protein and COX-2 mRNA. It has yet to be reported the mechanism of AA on uterine muscle cells to stimulate uterine contraction. However, it is well known that AA is a major precursor of prostaglandins synthesis. It was changed to PGH_2 and then to other prostaglandins such as prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), PGE_2, PGI_2 etc. $PGF_{2\alpha}$ is known to stimulate the contraction of the rat uterus through phosphatidylinositol-signaling pathway which result in an increase of calcium release from the internal storage and may also open non-selective cation channel which could cause the depolarization of plasma membrane leading to calcium influx.[16] The step that AA is converted to PGH_2 is accelerated by cyclooxygenase which is a rate-limiting enzyme of prostaglandin biosynthetic pathway. Diclofenac, one of the nonsteroidal anti-inflammatory drugs (NSAIDs), is known to be a cyclooxygenase inhibitor that could inhibit the conversion of AA to PGH_2 and then other prostaglandins synthesis.[17] In this study, diclofenac inhibited the stimulatory effect of AA on rat uterine contraction, meanwhile the direct action of AA on the contraction of rat uterus is unknown. It is, therefore,

likely that the action of AA on the contraction of rat uterus occurred due to prostaglandin(s) which is the product(s) of the conversion of AA. Piperine (30 µg/mL) was shown to completely inhibit the stimulatory effect of AA. It is known to inhibit cyclooxygenase enzyme.[18] It is possible that piperine could inhibit the action of AA by the inhibition of cyclooxygenase and, consequently, the inhibition of prostaglandin synthesis. However, it is possible that piperine could have other action that could relax the uterus such as the inhibition of calcium entry into the uterine muscle cells.

Conclusion

The result of this study indicated that methanolic extract of white pepper is a uterine relaxant. The inhibitory effect of the extract may involve an inhibition of a rise in intracellular calcium from both calcium influx via plasma membrane and calcium release from internal storage. However, this effect may be mediated mainly through a reduction on calcium influx, possibly through voltage-dependent calcium channel. Piperine, a major alkaloid of white pepper, may play a role, at least in part, on the relaxing effect of the extract on the uterus. Piperine is known to inhibit cyclooxygenase enzyme resulting in an inhibition of prostaglandin synthesis. Taken together, it is suggested that the inhibition of the calcium entry into muscle cells and cyclooxygenase enzyme could be the primary effect of methanolic extract of white pepper and piperine to relax the uterus. Our findings strongly support the use of white pepper to relief painful of dysmenorrhea in Thai traditional medicine.

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F29 | Proliferative Effect of Astaxanthin and Astaxanthin Liposome from White Shrimp Shell in Skin Keratinocyte Cells

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Abstract

Re-epithelialisation is an important event in healing of skin wounds. Astaxanthin belongs to beta carotenoids group and has been widely used for skin protection. The purpose of this study was to investigate proliferative effect of astaxanthin and astaxanthin liposome in human skin kerationocyte (HaCaT) cells by exposing the cells to various concentration of astaxanthin and astaxanthin liposome (0, 0.125, 0.25, 0.5, 1, 5 and 10 μg/mL) for 24, 48 and 72 hrs. The result showed that exposure to astaxanthin and astaxanthin liposome at 0.25, 0.5, 1, 5 and 10 μg/mL after 72 hrs significantly increased the proliferation of keratinocyte cells when compared to the control. This result suggests that astaxanthin and astaxanthin liposome may promote repair of skin wounds by facilitating keratinocyte cells proliferation.

Keywords: astaxanthin, wound healing, keratinocyte, proliferation

ผลของแอสตาแซนธินและแอสตาแซนธินลิโปโซมต่อการแบ่งตัวเพิ่มจำนวนของเซลล์คีราติโนไซต์

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บทคัดย่อ

กระบวนการสร้างเยื่อบุผิวใหม่เป็นกระบวนที่มีความสำคัญในการหายของบาดแผลบริเวณผิวหนัง แอสตาแซนธินจัดเป็นสารกลุ่มเบต้าแคโรทีนอยด์ นิยมใช้ในการรักษาอาการผิดปกติของผิวหนัง การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของแอสตาแซนธินและแอสตาแซนธินไลโปโซมต่อการแบ่งตัวเพิ่มจำนวนของเซลล์คีราติโนไซต์โดยได้รับแอสตาแซนธินและแอสตาแซนธินไลโปโซมที่ความเข้มข้น 0, 0.125, 0.25, 0.5, 1, 5, และ 10 ไมโครกรัมต่อมิลลิลิตร เป็นเวลา 24, 48 และ 72 ชั่วโมง พบว่าการได้รับแอสตาแซนธินและแอสตาแซนธินไลโปโซม ที่ความเข้มข้น 0.25, 0.5, 1, 5, และ 10 ไมโครกรัมต่อมิลลิลิตร ที่ระยะเวลา 72 ชั่วโมง มีผลเพิ่มการแบ่งตัวเพิ่มจำนวนของเซลล์คีราติโนไซต์อย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มควบคุม จากผลการศึกษานี้สรุปได้ว่าทั้งแอสตาแซนธินและแอสตาแซนธินไลโปโซมอาจชักนำการหายของบาดแผลบริเวณผิวหนังโดยส่งเสริมให้เกิดการแบ่งตัวเพิ่มจำนวนของเซลล์คีราติโนไซต์ที่ผิวหนัง

คำสำคัญ: แอสตาแซนธิน, การหายของบาดแผล, เซลล์คีราติโนไซต์, การแบ่งตัวเพิ่มจำนวนของเซลล์

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Introduction

Astaxanthin (3,3'-dihydroxy-beta,betacarotene -4,4'-dione) is a reddish pigment that belongs to a group of chemicals called carotenoids. It occurs naturally in certain algae and causes pink or red color in salmon, trout, lobster, shrimp, and other seafood.[1] Astaxanthin does not have any pro-oxidative nature like β-carotene however its potent anti-oxidative property exhibits on the cell membrane.[1] Many studies have revealed pharmacological effects of astaxanthin which are related to antioxidant[2] and anti-inflammatory effects[3], immunomodulation[4] and skin protection.[5–7] The skin protection of astaxanthin is recently investigated. It remarkably suppressed hyper-pigmentation, inhibited melanin synthesis and photoaging which consequently led to a decrease in matrix metalloproteinase-1 and skin fibroblast elastase/neutral endopeptidase expression.[6,7]

Recently, liposomes have been used as a drug carrier systems for topical delivery, due to an enhance drug penetration into the skin[8–11], promote therapeutic effectiveness[12,13], decrease side-effects[12] and sustained release of dermally active components.[14]

When a cutaneous injury occurs, the wound heals via a dynamic series of physiological events, including coagulation, granulation tissue formation, re-epithelialisation, and extracellular matrix remodeling. The keratinocyte is the predominant cell type in the epidermis. The migration and proliferation of keratinocytes play a key role in wound healing following injury.[15,16] The purpose of this study was to investigate proliferative effect of astaxanthin and astaxanthin liposome in human skin kerationocyte (HaCaT) cells.

Materials and Methods

Drug and chemicals

Astaxanthin and astaxanthin liposome was kindly provided from Associate Professor Dr. Wanwimol Klaypradit, Department of Fishery Product, Faculty of Fishery, Kasetsart University, Bangkok, Thailand. All other chemicals and solvents used throughout this study were analytical grade reagents. Astaxanthin liposome was prepared using a lipid hydration method. Ethanol solutions containing 20% w/w astaxanthin were dried to a thin film under vacuum using a rotary evaporator. The properties of liposome encapsulated astaxanthin were measured by the transmission electron microscope.

Experimental cell culture

Human skin keratinocyte (HaCaT) cells were obtained from CLS cell line service, Germany. HaCaT cells were cultured in complete Dulbecco's Modified Eagle Medium (DMEM) medium containing 10% fetal bovine serum, 2 mM L-glutamine and 1% antibiotics (100 U/mL penicillin and 100 μg/ml streptomycin). Cells were incubated at 37 ºC and 5% CO_2 under 95%

humidifier for 24 hrs before exposed to astaxanthin and astaxanthin liposome at various concentration.

Cell proliferation assay

HaCaT cells were harvested with 0.25% trypsin and resuspended to a final concentration of $1x10^4$ cells/mL. Two hundred microlitres of cell suspension were added into each well of 96-well cell culture plate. After 24 hrs of incubation, cells were treated with astaxanthin and astaxanthin liposome at concentrations of 0, 0.125, 0.25, 0.5, 1, 5 and 10 μg/mL and further incubated for 24, 48, and 72 hrs. After each incubation period, cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[17] MTT is mostly reduced by active mitochondria in living cells. An increase in cellular MTT reduction could be an index of cell proliferation. Briefly, MTT was dissolved in phosphate buffered saline, pH 7.4 at a concentration of 5 mg/mL. MTT solution was added to the cell culture to the final concentration of 0.5 mg/mL. After 2 hrs of incubation, medium was removed and the remaining insoluble formazan was dissolved in 100 μL DMSO and the optical density was measured spectrophotometrically at 570 nm.

Results and Discussion

Effect of astaxanthin and astaxanthin liposome on proliferation in HaCaT cells

To examine proliferative effect of astaxanthin and astaxanthin liposome, HaCaT cells were cultured for 24 hrs and then incubated with astaxanthin and astaxanthin liposome at various concentrations (0, 0.125, 0.25, 0.5, 1, 5 and 10 μg/mL) and durations (24, 48 and 72 hrs). Cells proliferation was determined by using MTT assay. The result showed that treatment with astaxanthin at concentration of 0.25, 0.5, 1, 5 and 10 μg/mL significantly increased keratinocyte cell proliferation when compared to control. The percentage of cell viability were 114.02±2.63, 114.89±2.35, 113.28±1.50, 113.31±0.92 and 113.28±3.02% of the corresponding controls after 72 hrs of astaxanthin exposure at 0.25, 0.5, 1, 5 and 10 μg/mL, respectively (Figure 1). However, at 24 and 48 hrs incubation was not significantly different from control. It is believed that one of the underlying mechanisms of wound repair is increased the proliferation of keratinocyte cells. Thus, increasing cell proliferation is the key of wound treatment. In this study, we demonstrated that astaxanthin and astaxanthin liposome induces keratinocyte proliferation. Keratinocyte proliferation is one of the key processes for normal wound epithelialisation and closure.[16] Astaxanthin increased the proliferation of keratinocyte cells which the mechanism was to inhibit reactive oxygen species[2,5,7], and anti-inflamatory activity.[3,5]

Similarly, 72 hrs incubation with astaxanthin liposome at 0.25, 0.5, 1, 5 and 10 µg/mL significant increased keratinocyte cell proliferation to 124.09±2.99, 125.42±5.60, 121.68±4.34, 126.99±6.62 and 126.14±5.79 %, respectively when compared to control (Figure 2). Interestingly, similar results were also found in the treatment with astaxanthin liposome, since astaxanthin liposome may have the potential to enhance drug penetration into the keratinocyte cell. To the best of our knowledge, this is the first study to show that astaxanthin and astaxanthin liposome induces proliferation in human skin keratinocyte cells. Our results indicate that astaxanthin and astaxanthin liposome may induce re-epithelialization via proliferation of keratinocytes.
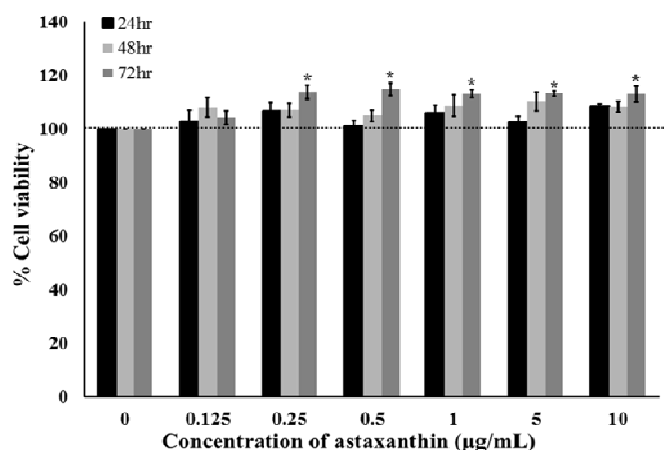
Figure 1.   Proliferative effects of astaxanthin on skin keratinocyte cells. HaCaT cells were treated with various concentrations (0.125–10 µg/mL) of astaxanthin for 24, 48, and 72 hrs. Cell proliferation was determined by MTT assay. Data are expressed as mean ± SEM of four independent experiments. Differences among groups were evaluated by one-way ANOVA followed by Tukey *post hoc* testing. * $p < 0.05$ compared to control (0 µg/mL of astaxanthin).
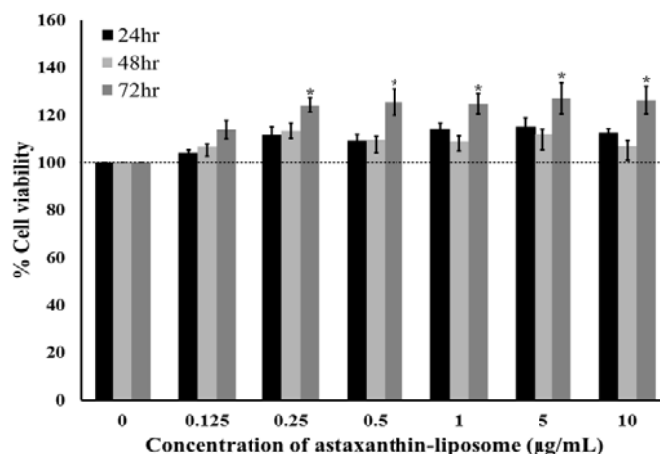
Figure 2.   Proliferative effects of astaxanthin liposome on skin keratinocyte cells. HaCaT cells were treated with various concentrations (0.125–10 µg/mL) of astaxanthin liposome for 24, 48, and 72 hrs. Cell viability was determined by MTT assay. Data are expressed as mean ± SEM of four independent experiments. Differences among groups were evaluated by one-way ANOVA followed by Tukey *post hoc* testing. * $p < 0.05$ compared to control (0 µg/mL of astaxanthin liposome).

Conclusion

In conclusion, this study demonstrated that treatment with astaxanthin and astaxanthin liposome increased keratinocyte proliferation. Further study to explore another mechanistic pathway to explain the proliferation and migration effect of astaxanthin was suggested.

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F30   Comparative Evaluation of LAL Kinetic Turbidimetric and Recombinant Factor C Methods for Measuring Endotoxin in Intravenous Pharmaceutical Products

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Abstract

Endotoxin was a biological pyrogen obtained from a lipid A-part of outer membrane lipopolysaccharide (LPS) in gram-negative bacteria. When endotoxin entered intravenously to blood stream, it can stimulate the immune system by releasing of inflammatory cytokines leading to fever, multiple organ failure (MOF) and multiple organ dysfunction syndrome (MODS). Therefore, contaminated endotoxin in intravenous pharmaceutical products can increase severity and mortality rate of patients. To reduce this problem, quantification of endotoxin contaminated in sterile pharmaceutical products is a regulation in quality control processes. Practically, endotoxin was quantified using Limulus amebocyte lysate (LAL) based assays: gel clot, turbidimetric and chromogenic methods. Recently, a new assay using recombinant Factor C (rFC) has become available for measuring endotoxin. The aim of study is to comparatively evaluate the LAL kinetic turbidimetric and rFC endpoint fluorescence methods for measuring endotoxins in intravenous pharmaceutical samples. Statistically, the Wilcoxon matched-pairs signed rank test ($p < 0.05$) showed that both methods for endotoxin estimates were not significantly different for testing samples. However, data points of endotoxin estimated by rFC method were less deviate from the zero-line which represented a different value from 0.495 EU/ml of standard endotoxin adding to the samples when compared with the LAL kinetic turbidimetric method. Moreover, the accuracy (%recovery) of endotoxin measurements by the rFC endpoint fluorescence method was higher than the kinetic turbidimetric method. The results conclude that the rFC endpoint fluorescence method may be an interesting technique for endotoxin or pyrogen testing in intravenous pharmaceutical products in the near future.

Keywords: endotoxin, recombinant factor C, LAL turbidimetric, intravenous pharmaceutical products

การประเมินเชิงเปรียบเทียบวิธีแอลเอแอลไคเนติกส์ เทอร์บิดิเมทริกซ์และรีคอมบิแนนท์แฟคเตอร์ ซี เพื่อวัดชีวพิษภายในตัวในเภสัชภัณฑ์ที่ให้ทางหลอดเลือดดำ

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บทคัดย่อ

ชีวพิษภายในตัวคือสารก่อไข้ทางชีวภาพที่ได้จากส่วนลิพิดเอของผนังด้านนอกลิพอพอลิแซ็กคาไรด์ในแบคทีเรียแกรมลบ เมื่อชีวพิษภายในตัวเข้าสู่กระแสเลือด จะสามารถกระตุ้นระบบภูมิคุ้มกัน โดยการหลั่งสารไซโตไคน์ที่กระตุ้นการอักเสบ ทำให้เกิดไข้ การทำงานของอวัยวะหลายชนิดล้มเหลว และกลุ่มอาการอวัยวะทำหน้าที่ผิดปกติ ดังนั้นชีวพิษภายในตัวที่

ปนเปื้อนในเภสัชภัณฑ์ที่ให้ทางหลอดเลือดดำ จะเพิ่มความรุนแรงและอัตราการตายของผู้ป่วย เพื่อลดปัญหานี้ การหาปริมาณ
ชีวพิษภายในตัวที่ปนเปื้อนในเภสัชภัณฑ์ปราศจากเชื้อเป็นข้อกำหนดในกระบวนการควบคุมคุณภาพ ในทางปฏิบัติชีวพิษ
ภายในตัวถูกวัดปริมาณโดยใช้การทดสอบ Limulus amebocyte lysate (LAL) คือวิธี gel clot turbidimetric และ
chromogenic เมื่อเร็ว ๆ นี้ การทดสอบแบบใหม่ด้วย recombinant Factor C (rFC) ถูกนำมาใช้ จุดมุ่งหมายของการศึกษานี้
คือการประเมินเชิงเปรียบเทียบของวิธี LAL kinetic turbidimetric และ rFC endpoint fluorescence เพื่อวัดปริมาณชีวพิษ
ภายในตัวในตัวอย่างเภสัชภัณฑ์ที่ให้ทางหลอดเลือดดำ ในทางสถิติ Wilcoxon matched-pairs signed rank test (P < 0.05)
แสดงถึงทั้งสองวิธีสำหรับประมาณการชีวพิษภายในตัวนั้นไม่แตกต่างอย่างมีนัยสำคัญในตัวอย่างที่ใช้ทดสอบ อย่างไรก็ตาม
ข้อมูลประมาณการชีวพิษภายในตัวโดยวิธี rFC เบี่ยงเบนจากเส้น 0 น้อยกว่า ซึ่งแสดงถึงค่าความต่างจาก 0.495 EU/มล.
ของชีวพิษภายในตัวมาตรฐานที่เติมลงในตัวอย่างเมื่อเปรียบเทียบกับวิธี LAL ยิ่งไปกว่านั้นค่าความถูกต้อง (% recovery)
ของการวัดตัวด้วยวิธี rFC นั้นสูงกว่าวิธี LAL ผลการวิจัยสรุปได้ว่าวิธี rFC เป็นเทคนิคที่น่าสนใจสำหรับการทดสอบชีวพิษ
ภายในตัวหรือสารก่อไข้ในเภสัชภัณฑ์ที่ให้ทางหลอดเลือดดำในอนาคตอันใกล้นี้

คำสำคัญ: ชีวพิษภายในตัว, รีคอมบิแนนท์แฟคเตอร์ ซี, แอลเอแอลไคเนติกส์ เทอร์บิดิเมทริกซ์, เภสัชภัณฑ์ที่ให้ทางหลอดเลือด

Introduction

Bacterial endotoxin is a biological pyrogen found in the outer membrane of gram-negative bacteria.[1] The structure of outer membrane is a lipopolysaccharide (LPS) complex which composed of three distinct regions: lipid A, core oligosaccharide and endotoxic O-specific antigen. An amphiphilic LPS is heat resistant and pH-stable molecule.[2] Endotoxin causes a fever when enter intravenously to the human body. It stimulates the immune system by releasing inflammatory cytokines and probably leading to multiple organ failure (MOF) and multiple organ dysfunction syndrome (MODS).[1] Therefore, the contamination of endotoxin in sterile pharmaceutical products can increase severity and mortality rate of patients who consume these products. To overcome these problems, detection and quantification of endotoxin are required as a quality control step for all parenteral pharmaceutical products.

Nowadays, the approved endotoxin measurements are gel-clot, chromogenic and LAL kinetic turbidimetric methods.[3] These techniques are based on limulus amebocyte lysate (LAL), an aqueous blood cell extracted from the horseshoe crab.[4] The basis of gel-clot method is clotting reaction of the lysate reagent in the presence of endotoxin.[5] The advantages of gel-clot method are the simplest, less expensive and the most widely used for endotoxin qualitative assay. However, the disadvantage is low sensitivity and can give false-negative and false-positive results depending on performer.[4,6] For two photometric quantitative techniques of endotoxin measurement, the chromogenic method quantifies endotoxin by measuring the chromophore released from a suitable chromogenic peptide by the reaction of endotoxins with lysate. On the other hand, the LAL kinetic turbidimetric method measures the rate of increase in turbidity of reaction products.[3] The advantages of LAL kinetic turbidimetric method are sensitive, easy to perform and less time consuming. However, this method can be exposed to possible interferences and clotting enzyme was heat labile.

Recently, the fluorescence technique, recombinant Factor C (rFC) assay (PyroGene™) was developed to determine endotoxin in protein solution.[7] The rFC protein composes of multiple binding sites for endotoxin which is catalytically activated by trace amounts of endotoxin. When activated by endotoxin binding, recombinant rFC acts to cleave a synthetic substrate resulting in the generation of fluorogenic compound in the assay mixture. The fluorescent signal is in proportion to the endotoxin concentration in the sample. The advantages of rFC method are rapid, convenient, reliable, perpetual, economical, and environmentally friendly.[6] Animals have not been used in testing or reagent manufacturing hence, redeeming diminishing horseshoe crabs from extinction. Because rFC is not derived from natural horseshoe carb blood, thus it is a standardized source of recombinant LAL. Moreover, rFC does not cross talk with contaminants like β-glucan, hence false positive and elevated results can be abolished.[6]

The aim of this study is to measure the amount of endotoxin in intravenous pharmaceutical samples using gel-clot, LAL kinetic turbidimetric and rFC endpoint fluorescence methods. The comparison of two photometric quantitative techniques, LAL kinetic turbidimetric and rFC methods was statistically evaluated to reveal the possible use of rFC endpoint fluorescence as an alternative method that does not require an animal source for endotoxin and pyrogen testing.

Materials and Methods

Preparation of sample solutions

The samples used in this study were three-batches of five-intravenous pharmaceutical products (see Table 1). The samples were initially prepared by dissolving the finished products using LAL Reagent Water (LRW) to a final concentration as described by manufacturer. Each of prepared samples was diluted at an appropriate dilution less than the Maximum Valid Dilution (MVD) value, the maximum allowable dilution of a sample at which the endotoxin limit can be determined (see Table 1). The labeled lysate sensitivity and the inhibition/enhancement tests on the sample solutions were performed as described under preparatory protocols of test kits in order to confirm that both the precision and validity of the test methods were acceptable for further use (see Table 1).

The diluted samples were then added 11 µl of *E. coli* O55:B5 standard (5 EU/ml) as described in positive product control protocol[9], to gain a final concentration of 0.495 EU/ml. The amount of endotoxin in samples were subsequently quantified using gel-clot, LAL kinetic turbidimetric and rFC methods. The diluted samples without adding of standard endotoxin were used as control blank for the assay.

Endotoxin testing reagents

PYROGENT™ Gel Clot LAL Assay, PYROGENT™–5000 Assay, PyroGene™ Recombinant Factor C Assay and LAL Reagent Water were purchased from Lonza Walkersville Inc. (Walkersville, MD, USA). All glassware was baked at 180 °C for 4 hour to render them free of endotoxin.

Gel-clot method

Escherichia coli O55:B5 standard endotoxin was prepared in LAL Reagent Water to obtain final concentration of 0.25 EU/ml. The test was performed by transferring 100 µl of sample into 10×75 mm test tube, followed by adding 11 µl of standard endotoxin (0.25 EU/ml). Then, 100 µl of lysate was added to each tube. The reaction was incubated in Heating block (Dry-Bath) at 37 °C for 60 minutes.[8] Each tube was gently mixed by tube-inversion and the result was then determined. The positive result showed a gelation when endotoxin was found. In the absence of endotoxin, gelation was not occurred.

Kinetic turbidmetric method

To prepare standard curve for the LAL kinetic turbidimetric method, *E. coli* O55:B5 standard endotoxin was diluted in LAL Reagent Water to obtain concentrations of 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 EU/ml. Then, 100 µl of standard endotoxin dilutions were loaded into 96-well plate in triplicates. For assayed samples, 11 µl of standard endotoxin (5 EU/ml) was loaded into wells and subsequently added 100 µl of appropriately diluted samples (see Table 1). Samples without adding of standard endotoxin were used as control blank.

Finally, 100 µl of reconstituted lysate was added to wells. The plate was incubated in the microplate reader (BioTek ELx808 equipped with WinKQCL™ Software) at 37 ºC for 60 °C. The kinetic reactions were measured using absorbance at 340 nm.[9]

rFC endpoint fluorescence method

Various concentrations of *E. coli* O55:B5 standard endotoxin and diluted samples were prepared as described in the kinetic turbidmetric method. The 96-well plate was pre-incubated at 37ºC for 10 °C. During the pre-incubation, the rFC reagent was freshly prepared by gentle mixing of rFC, substrate, and buffer (ratio 1:5:4). After the 10 min, 100 µl of the rFC reagent was dispensed into each well. The plate was finally incubated in the microplate reader (BioTek FLx800 equipped with WinKQCL™ Software) at 37ºC for 60 °C. The spectroscopic fluorescence was measured using the excitation/emission wavelengths at 380/440 nm.[10] For the lot of rFC reagent used, the sensitivity setting of the plate reader was adjusted. To do this step, 100 ml of 5 EU/ml endotoxin standard diluted in LAL Reagent Water was dispensed into wells and pre-incubating for 10 min. Then, 100 ml of the rFC reagent was added to each well.

Statistical Analysis

The amount of endotoxin in samples obtained from the kinetic turbidmetric and rFC endpoint fluorescence methods were compared and analyzed using the Wilcoxon matched-pairs signed rank test; significance was set at $p < 0.05$. The deviations of measured endotoxin potency from the standard endotoxin (0.495 EU/ml) were graphed as the scatter plot for each individual measurement. The accuracy and precision of two methods were represented by the values of % recovery and % Coefficient of Variation (% CV), respectively.

Results

Determination of contaminated endotoxin in the samples

The amount of contaminated endotoxin existing in samples of intravenous pharmaceutical products was determined using gel-clot, kinetic turbidmetric and rFC endpoint fluorescence methods. The results showed that the amounts of endotoxin in all samples were passed as requirement of USP endotoxin limit values as shown in Table 2.

Determination of adding standard endotoxin (0.495 EU/ml) in the samples

The determination of endotoxin amount in the diluted samples adding with standard endotoxin (0.495 EU/ml) was measured by rFC endpoint fluorescence and kinetic turbidmetric methods. The results are illustrated in Figure 1. The error bars are standard deviations from the assay in triplicate. It was found that the amounts of standard endotoxin in the samples determined by rFC endpoint fluorescence method were closer to 0.495 EU/ml than those determined by kinetic turbidmetric methods.

Table 1. The maximum valid dilution (MVD) value and appropriate dilution of each samples using for gel-clot, LAL kinetic turbidimetric and rFC endpoint fluorescence methods.

| Samples (Conc.) | Test for bacterial endotoxins | | | | | |
| | Gel-colt method | | Turbidimetric method | | rFC method | |
	MVD	Appropriate Dilution	MVD	Appropriate Dilution	MVD	Appropriate Dilution
Ciprofloxacin 2mg/ml	1:8	1:8	1:100	1:100	1:200	1:100
Levofloxacin 5mg/ml	1:28	1:25	1:350	1:100	1:700	1:100
Ondansetron 4mg/2ml	1:158	1:100	1:1980	1:100	1:3960	1:100
Meropenem 50mg/ml	1:50	1:50	1:650	1:200	1:1250	1:200
Lidocaine 10mg/ml	1:50	1:50	1:1100	1:50	1:2200	1:50

Table 2. Amount of contaminated endotoxin existing in intravenous pharmaceutical samples assayed by gel-clot, kinetic turbidmetric and rFC endpoint fluorescence methods.

| Samples | Methods | | | | |
	Endotoxin limit* EU/mg	Gel-clot EU/mg	Turbidimetric EU/mg	rFC EU/mg	Results
Ciprofloxacin 2mg/ml Lot.1	0.5	<0.5	<0.05	<0.25	Pass
Ciprofloxacin 2mg/ml Lot.2	0.5	<0.5	<0.05	<0.25	Pass
Ciprofloxacin 2mg/ml Lot.3	0.5	<0.5	<0.05	<0.25	Pass
Levofloxacin 5mg/ml Lot.1	0.7	<0.625	<0.2	<0.1	Pass
Levofloxacin 5mg/ml Lot.2	0.7	<0.625	<0.2	<0.1	Pass
Levofloxacin 5mg/ml Lot.3	0.7	<0.625	<0.2	<0.1	Pass
Ondansetron 4mg/2ml Lot.1	9.9	<0.625	<0.05	<0.25	Pass
Ondansetron 4mg/2ml Lot.2	9.9	<0.625	<0.05	<0.25	Pass
Ondansetron 4mg/2ml Lot.3	9.9	<0.625	<0.05	<0.25	Pass
Meropenem 50mg/ml Lot.1	0.125	<0.125	<0.04	<0.02	Pass
Meropenem 50mg/ml Lot.2	0.125	<0.125	<0.04	<0.02	Pass
Meropenem 50mg/ml Lot.3	0.125	<0.125	<0.04	<0.02	Pass
Lidocaine 10mg/ml Lot.1	1.1	<0.125	<0.05	<0.025	Pass
Lidocaine 10mg/ml Lot.2	1.1	<0.125	<0.05	<0.025	Pass
Lidocaine 10mg/ml Lot.3	1.1	<0.125	<0.05	<0.025	Pass

* Endotoxin limit from United States Pharmacopeia (USP)
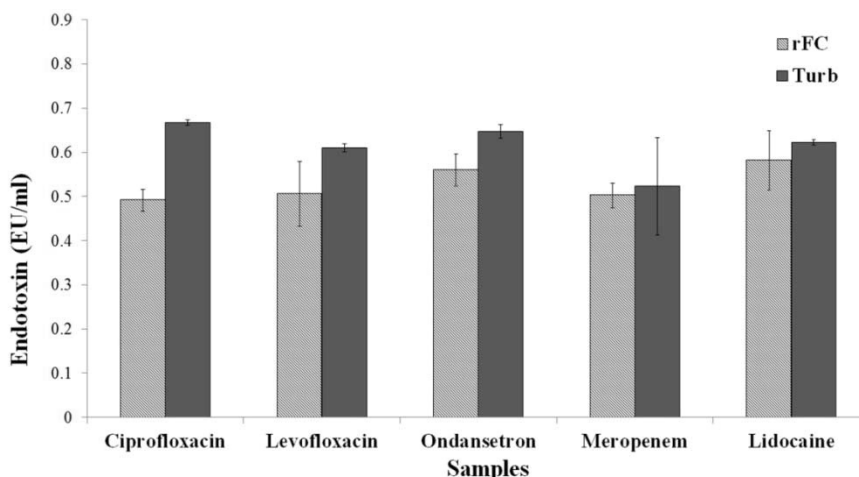
Figure 1. Amount of endotoxin in intravenous pharmaceuticals were measured by rFC endpoint fluorescence method (rFC) and kinetic turbidmetric method (Turb) . The average results for each method were graphed. The error bars are standard deviations from the assay in triplicate.

Table 3. Statistical analysis of endotoxin determination by rFC endpoint fluorescence and kinetic turbidmetric methods, using the Wilcoxon matched–pairs signed ranks test ($p < 0.05$).

Samples	Methods		
	rFC* EU/ml	Turbidimetric* EU/ml	Wilcoxon matched– pairs signed ranks (p)
Ciprofloxacin 2mg/ml	0.4913(0.4639-0.5132)	0.6671(0.6592-0.6711)	0.109
Levofloxacin 5mg/ml	0.5057(0.4325-0.5781)	0.6099(0.5997-0.6151)	0.109
Ondansetron 4mg/2ml	0.5597(0.5265-0.5983)	0.6467(0.6325-0.6637)	0.109
Meropenem 50mg/ml	0.5022(0.4816-0.5333)	0.5229(0.4586-0.6511)	1.000
Lidocaine 10mg/ml	0.5814(0.5221-0.6541)	0.6217(0.6152-0.6275)	0.285

*Mean of endotoxin amount (range of measured endotoxin)

Method comparison for endotoxin determination in the samples adding with standard endotoxin (0.495 EU/ml)

The amount of endotoxin in the samples adding with standard endotoxin (0.495 EU/ml) from rFC endpoint fluorescence and kinetic turbidmetric methods were further analyzed using statistical tools. The two methods were compared and analyzed using Wilcoxon matched-pairs signed rank test. For each sample of each method, the mean and range of endotoxin amounts were indicated (see Table 3). The results showed that all samples presented the large p values ($p > 0.05$) of two methods comparison for the Wilcoxon matched-pairs signed rank test (not permitting rejection of the null hypothesis of no difference). Thus, the amount of endotoxin in all samples obtained from rFC endpoint fluorescence and kinetic turbidmetric methods were not significantly different.

In Figure 2, the endotoxin amount in each individual lot of samples measured by the two different methods was subtracted from 0.495 EU/ml, the final concentration of standard endotoxin added. The deviations were then graphed as scatter plot. The result showed that most of the data points obtained from the rFC endpoint fluorescence method for each sample were divergently scattered and less deviated along the zero line when compared to those of kinetic turbidmetric methods.

The accuracy and precision of two methods using for determination of endotoxin in samples adding with standard endotoxin (0.495 EU/ml) were analyzed by the values of % recovery and %CV, respectively (Table 4). Except for meropenem, %CV of endotoxin measured by LAL kinetic turbidimetric method was lower than rFC endpoint fluorescence method. For %Recovery, rFC endpoint fluorescence method gave high values of %Recovery when compared to those of LAL kinetic turbidimetric method, except for ondansetron
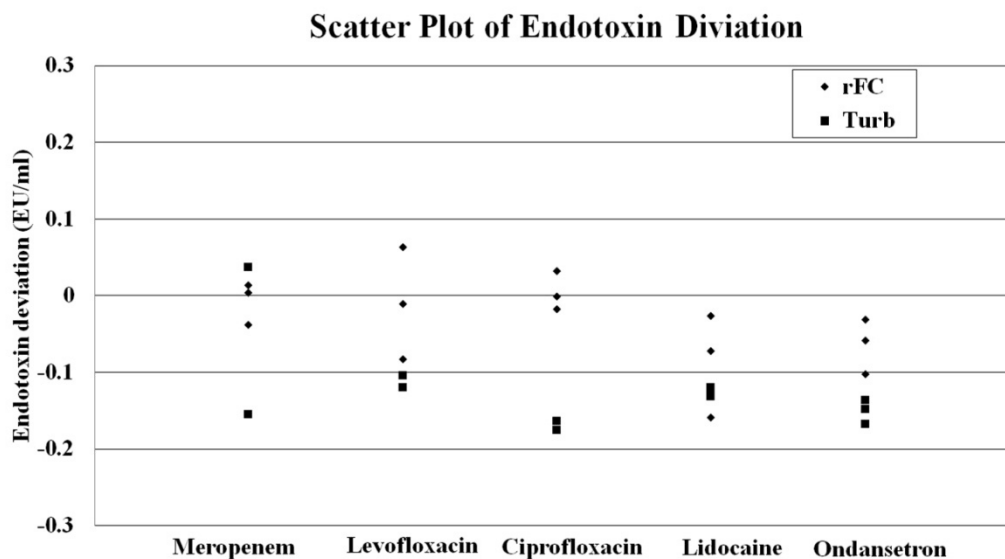
Figure 2. Scatter plot of endotoxin amount for each individual lot of sample deviated from 0.495 EU/ml of standard endotoxin measured by two different methods.

Table 4. Coefficient of Variation (% CV) and % Recovery of rFC endpoint fluorescence and kinetic turbidmetric methods.

| Samples | %CV | | %Recovery | |
| | Methods | | Methods | |
	rFC	Turbidimetric	rFC	Turbidimetric
Ciprofloxacin 2mg/ml	5.11	1.03	99.25	134.77
Levofloxacin 5mg/ml	14.40	1.46	102.16	123.23
Ondansetron 4mg/2ml	6.47	2.44	113.08	103.65
Meropenem 50mg/ml	5.46	21.23	101.45	105.64
Lidocaine 10mg/ml	6.36	0.99	117.46	125.6

Discussions

To explore the possibility for using the rFC endpoint fluorescence as an alternative method for endotoxin and pyrogen testing, the amount of endotoxin in prepared samples were determined by rFC endpoint fluorescence method in comparison with approval gel-clot and LAL kinetic turbidimetric standard methods. The level of contaminated endotoxin in testing samples were initially determined and found to pass as requirement for endotoxin limit of the United States Pharmacopeia 37 (USP 37) for all testing methods: gel-clot, LAL kinetic turbidimetric and rFC endpoint fluorescence methods. Thus, all samples were appropriate to be used for method comparison study. The *E. coli* O55:B5 standard endotoxin was added to samples to attain a final concentration of 0.495 EU/ml. The amount of standard endotoxin in those prepared samples were then measured by rFC endpoint fluorescence and LAL kinetic turbidimetric methods. The Wilcoxon matched-pairs signed rank test indicated that the two different methods for endotoxin measurement were not significantly different. Our result agreed with previous report indicating that the Limulus ameabocyte lystate (LAL) and rFC endotoxin estimates were highly correlated.[11] This result indicated that both LAL kinetic turbidimetric and rFC endpoint fluorescence methods can be practically used for determination of endotoxin in our testing samples. However, the potency of endotoxin in samples was quite high in comparison to 0.495 EU/ml when measured by LAL kinetic turbidimetric method. On the other hand, the endotoxin potency was much satisfied when measured by the rFC endpoint fluorescence method. This was similar to previous report for measuring endotoxin in house dust using LAL tubidimetric and rFC methods[11] The authors showed that LAL assay gave higher endotoxin estimates compared with rFC. This was further proven by scatter plot of endotoxin deviation from 0.495 EU/ml, the concentration of adding standard endotoxin. The data points clearly

confirmed that the amount of endotoxin for each individual lot of samples from rFC endpoint fluorescence method were less deviate from the zero-line even though they were more divergent. As previously described, rFC has similar sensitivity and potentially greater specificity for measuring endotoxin when compared with a Limulus ameabocyte lystate (LAL) .[11]

Our results generally revealed that rFC endpoint fluorescence method provided a high accuracy for endotoxin estimates as represented by %recovery which were approximate 100%. The reason is that the rFC is more specific for LPS detection.[7] However, the precision of kinetic turbidmetric methods for endotoxin estimates in testing samples was almost higher than rFC methods as indicated by value of %CV. Nevertheless, %recovery values were in the range of 50%-200% as recommended in the USP37 and %CV values were in the acceptable range of 10% for turbidemetric method and 25% for rFC method.

Conclusions

In this study, the LAL kinetic turbidimetric and rFC endpoint fluorescence methods gave comparable results for endotoxin estimates of intravenous pharmaceutical products. Our results conclude that rFC and LAL are reasonably comparable, the endotoxin estimates are correlated and either assay can be used for determination of endotoxin contamination in pharmaceutical products. Due to the probable variation of natural LAL from lot-to-lot, the endotoxin estimates may prove to be a less consistency. The rFC is a cloned protein produced by tissue culture. It exhibits a lot-to-lot consistency, endotoxin specificity, statistically more robust, greater security of supply and no animals used in testing or reagent manufacturing hence, redeeming diminishing horseshoe crabs from extinction. To these reasons, we conclude that rFC endpoint fluorescence is more challenge method for endotoxin or pyrogen testing in intravenous pharmaceutical products. However, additional data on the performance of rFC endpoint fluorescence method for endotoxin estimates in many lots and types of pharmaceutical products are needed to be further studied and confirmed.

Acknowledgements

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F31   Time-Kill Study of the *In Vitro* Activity of Fosfomycin against *Escherichia coli* ATCC Strain

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Abstract

In this study, we evaluated the *in vitro* antimicrobial activity of fosfomycin against *Escherichia coli* (*E. coli*) ATCC 25922 from the time-kill curves. The minimum inhibitory concentration (MIC) of fosfomycin against *E. coli* ATCC 25922 determined by agar dilution method was 32 µg/mL. The fosfomycin concentrations used in the time-kill study were 8 (0.25×MIC), 16 (0.5×MIC), 32 (1×MIC), 64 (2×MIC), 128 (4×MIC), 256 (8×MIC) and 512 (16×MIC) µg/mL. Then each concentration of fosfomycin was added to inoculate after incubation at 37 °C for 2 hr. The effects of fosfomycin against *E. coli* ATCC 25922 were evaluated at 0, 1, 2, 4, 6, 8, 12 and 24 hr. The amounts of bacteria were counted by adapted droplet-plate method. The time-kill curve between amounts of bacteria and time was performed. The results showed that fosfomycin was highly effective against *E. coli* ATCC 25922. Fosfomycin exhibited bactericidal effects at the concentration of 64 (2×MIC), 128 (4×MIC), 256 (8×MIC) and 512 (16×MIC) µg/mL with the concentration-dependent killing pattern.

Keywords: Fosfomycin, *Escherichia coli*, time-kill curves

การศึกษาฤทธิ์ฆ่าเชื้อกับเวลาของฟอสโฟมัยชิน ต่อเชื้อเอสเชอริเชียโคไล สายพันธุ์มาตรฐาน

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บทคัดย่อ

การวิจัยครั้งนี้เป็นการศึกษาฤทธิ์ต้านจุลชีพทางห้องปฏิบัติการของยาฟอสโฟมัยชินต่อเชื้อเอสเชอริเชียโคไลสายพันธุ์มาตรฐานโดยใช้ข้อมูลที่ได้จากกราฟการฆ่าเชื้อกับเวลาเมื่อหาค่าความเข้มข้นต่ำสุดที่สามารถยับยั้งการเจริญเติบโตของเชื้อ (MIC) ของฟอสโฟมัยชินต่อเชื้อเอสเชอริเชียโคไลสายพันธุ์มาตรฐาน ด้วยวิธี agar dilution มีค่า 32 มคก./มล. จึงกำหนดความเข้มข้นของฟอสโฟมัยชินที่ใช้ในการสร้างกราฟการฆ่าเชื้อกับเวลาคือ 8 (0.25×MIC), 16 (0.5×MIC), 32 (1×MIC), 64 (2×MIC), 128 (4×MIC), 256 (8×MIC) และ 512 (16×MIC) มคก./มล. จากนั้นเติมยาฟอสโฟมัยชินลงในเชื้อที่เพาะบ่มไว้ที่อุณหภูมิ 37องศาเซลเซียส มาแล้ว 2 ชั่วโมงเก็บตัวอย่างที่เวลา 0 (เชื้อยังไม่ได้สัมผัสกับยา), 1, 2, 4, 6, 8, 12 และ 24 ชั่วโมงหลังสัมผัสยานำมาหาจำนวนของเชื้อด้วยวิธี droplet-plate สร้างกราฟฆ่าเชื้อจากความสัมพันธ์ของปริมาณเชื้อกับเวลาผลการวิจัยพบว่าฟอสโฟมัยชินมีฤทธิ์ฆ่าเชื้อ (bactericidal) เอสเชอริเชียโคไลสายพันธุ์มาตรฐานที่ความเข้มข้น 64 (2×MIC), 128 (4×MIC), 256 (8×MIC) และ 512 (16×MIC) มคก./มล. โดยมีรูปแบบการออกฤทธิ์ต้านจุลชีพเป็นแบบ concentration-dependent killing pattern กล่าวคือความสามารถในการฆ่าเชื้อจะเพิ่มขึ้นตามความเข้มข้นของยาที่เพิ่มขึ้น

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Introduction

The bacterial time–kill curves have been proposed to offer detailed information about the antibacterial efficacy as a function of both time and antibiotic concentration.[1] Time–kill curves of many antibacterial agents have been studied in both *in vitro* kinetic models and animal infection models, including beta-lactam antibiotics, fluoroquinolones, aminoglycosides and macrolides.[2,3] *E. coli* is a gram-negative bacillus, one of the most frequent causes of many common bacterial infections worldwide. The National Antimicrobial Resistance Surveillance Center, Thailand (NARST) found *E. coli* has emerged of Extended-spectrum ß-lactamase (ESBL) producing strains studies in 2010.[4] In ESBL producing strains, the resistance to beta-lactam is frequently associated with resistance to other antibiotics, Due to the absence of new antimicrobial agents against ESBL producing strain, fosfomycin have been to evaluated in ESBL producing strains. Fosfomycin is a phosphonic acid derivative with a broad spectrum of antibacterial activity against Gram-positive and Gram-negative bacteria. The mechanism of action of fosfomycin is cytoplasmic enzyme (pyruvyltransferase) inhibition which active during first step of bacterial cell wall (peptidoglycan) synthesis.[5]

Currently, there are limited studies related to the *in vitro* activities of fosfomycin. The purpose of this study was to investigate theantimicrobial activity of fosfomycin by evaluating *in vitro* time–kill curves against *E. coli* ATCC 25922 before study with *E. coli* ESBL producing strains.

Materials and Methods

Drugs and microorganisms

Fosfomycin purchased from Wako Pure Chemical Industries, Ltd., Japan. The *E. coli* ATCC 25922 (American Type Culture Collection) was obtained from the Faculty of Medicine, Chulalongkorn University, King Chulalongkorn Memorial Hospital, Thailand. Fresh isolates were sub-culture on Mueller-Hinton agar plate for 18-20 hr at 37°C prior to each experiment.

Media preparation

Cation-adjusted Mueller-Hinton Broth (CaMHB: Fluka, Bucsh, Switzerland) and Mueller-Hinton agar (MHA: Oxoid Ltd, Basingstoke, Hamsphire, England)

were prepared according to the package manufacturer's instructions.

Bacterial inoculation

The bacterial inoculum was prepared from colonies incubated overnight on MHA plates and grown in CaMHB about 2 to 4 hr. Then suspended microorganism in sterile 0.9% saline solution to obtain the turbidity as 0.5 McFarland standard (Remel microbiology Products, Lenexa, KS, USA) approximately 1.5 x10^8 CFU/mL for *E. coli.*

MIC determination

The MIC is defined as the lowest concentration of antimicrobial agent that completely inhibits the growth of the organism as detected by the unaided eye. *In vitro* susceptibility test was performed according to the Clinical and Laboratory Standards Institute (CLSI) by the agar dilution method.[6] Briefly, MHA supplemented with 25 µg/ml of glucose-6-phosphate[6,7] (Sigma Chemical Co., St. Louis, MO, USA) was used. The inoculated plates were incubated at 37°C for 16 to 20 hr.

Time-kill study

The activity of fosfomycin was evaluated by time-killstudies. *In vitro* kinetic model was used to investigate the antibacterial efficacy of constant drug concentrations for 24 hr. The model consisted of a 75 mL vented-cap tissue culture flask with a canted neck (Corning Incorporated, NY, USA), containing 30 mL of CaMHB media incubated at 37°C. An aliquot of a suspension (100 µL) of initial inoculation (equivalent to 0.5 McFarland scale) was added to the *in vitro* model. The model was incubated for 2 hr before adding different fosfomycin concentrations to produce the log growth phase of bacterium.[8]

Time-kill studied was conducted with different and escalating fosfomycin concentrations. These concentrations were prepared at least 7 concentrations including minimum inhibition of bacterial growth (0.25×MIC, 0.5×MIC, 1×MIC), efficient bacterial killing (2×MIC, 4×MIC) and maximum bacterial killing (8×MIC, 16×MIC). CaMHB with bacteria and without antibiotics were served as a control. The effects of fosfomycin against *E. coli* ATCC 25922 were evaluated at 0, 1, 2, 4, 6, 8, 12 and 24 hr. Bactericidal activity was defined as a ≥ 3 log10 decrease in CFU/ml comparedwith the starting colony.[9]

Table 1. MIC values and concentrations in the time–kill curve experiments

Strains	MIC (µg/ml)	Tested concentrations (µg/mL)
E. coli ATCC 25922	32	8(0.25×MIC), 16(0.5×MIC), 32(1×MIC), 64(2×MIC),128(4×MIC), 256(8×MIC), 512 (16×MIC)
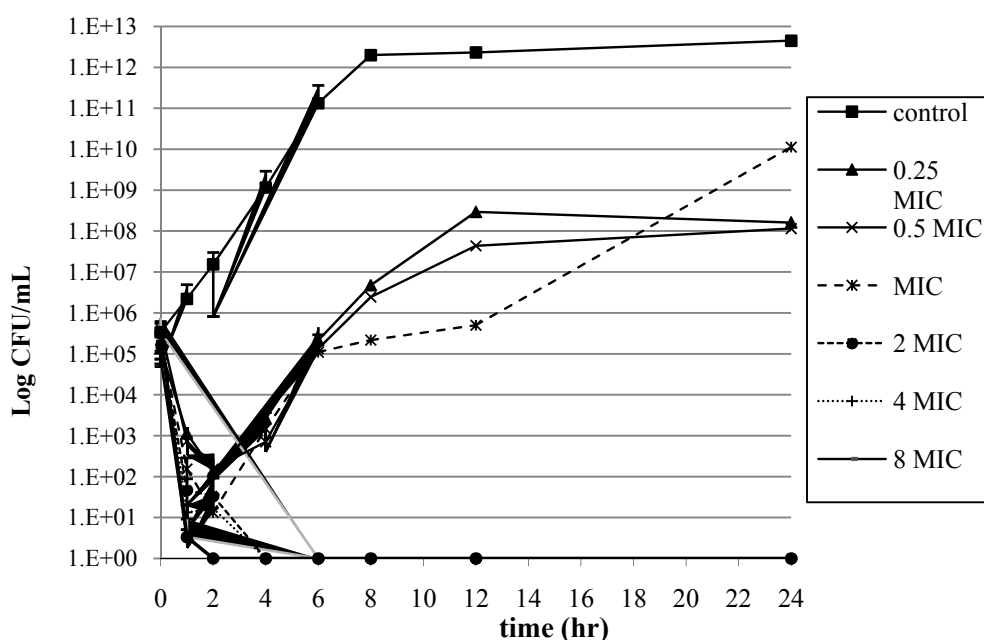
Figure 1. Time-kill curves of fosfomycin against *E. coli* ATCC 25922. Concentrations tested were 0.25×, 0.5×, 1×, 2×, 4×, 8× and 16×MIC of fosfomycin. Viable bacterial counts were determined over 24 hr of incubation.

Bacterial quantification

Bacterial counts were determined by plating 50 µL of the serial 10-fold dilutions on MHA plates, using an adapted droplet-plate method.[8] Briefly, agar plates were divided into four quadrants. Withan micropipette, 5×10 µL droplets of the chosen dilution were equidistantly plated onto one of the quadrants. A duplicate was plated onto the adjacent quadrant. Then, the plates were incubated at 37 °C for 16-24 hr before reading. The procedure was repeated at least three times. The controls with bacteria without drug were run simultaneously. Following incubated, the number of CFUs was counted in each duplicate quadrant at each time-point and averaged.

Results

The MIC values and fosfomycin concentrations used in the time–kill curve experiments against *E. coli* ATCC 25922 are summarized in Table 1. The range of fosfomycin concentrations tested in the time-kill study was from 8 to 512 µg/mL for *E. coli* ATCC 25922. The time courses of bacterial burden associated with entire fosfomycin concentrations range showed in Figure 1. Values are presented as means and standard deviations (mean ± SD). The experiments were performed in triplicate.

At concentrations of minimum inhibition of bacterial growth (0.25×MIC, 0.5×MIC, 1×MIC) showed with inhibition of bacterial growth for 2 hr(as shown in Fig.1) and there were regrowth after 4 hr. As expected, efficient bacterial killing by fosfomycin (2×MIC, 4×MIC) exhibited rapid bactericidal activity with a reduction in bactericidal count of 3-4 log in the first 2hr compared with the control (Fig.1) and no re-growth for 24 hr. The concentrations of maximum bacterial killing (8×MIC, 16×MIC) showed very rapid bactericidal activity in the first 1 hrwith complete killing. From result of the maximum killing curves at range 8×MIC-16×MIC indicates that the concentration 8×MIC showed maximum bacterial killing

Discussion

The result from studied showed the test strain was highly susceptible to fosfomycin (MIC = 32μg/ml). In the time-kill studied, fosfomycin demonstrated bacteriostatic effect agains*E. coli* ATCC 25922 for most of the concentrations tested (2×, 4×, 8×, 16× and 32×MIC) The result of this study confirmed that of Mazzei T et al.[10] andRoussos N et al.[11] Our study has shown that fosfomycin produced bactericidal effect over 24 hr with concentration of 8×MIC (256μg/ml).

Our study had certain limitations : in this study, we evaluate only the *E. coli* ATCC 25922, the data is less anddiffered from other studied.

Therefore, further studies will be studies with clinical isolated strain or ESBL-producing *E. coli* isolates.

Conclusion

Our results suggested that Fosfomycin has good *in vitro* antibacterial activity against *E. coli* ATCC 25922 . It showed a highbactericidal activities, as well as concentration-dependent killing pattern.

Acknowledgements

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𝒱ermectin

Ivermectin 6 mg

- *Broad spectrum antiparasite*
- *Highly effective against Strongyloidiasis*

Indications

Ivermectin is an anthelmintic agent used in the treatment of strongyloidiasis (*Strongyloides stercoralis*)in humans.

Therapeutic dosage

Adults

Oral: 200 µg/kg bodyweight as a single dose

DOSAGE GUIDELINES FOR STRONGYLOIDIASIS

Body Weight (kg.)	Single dose Number of tablets
15-24	½
25-35	1
36-50	1½
51-65	2
66-79	2½
> 80	200 µg/kg

Children

Ivermectin is not given to children weighing less than 15 kg.

Contraindications

Ivermectin is contraindicated in persons with an immediate hypersensitivity to the drug.

Drug Interactions

Preliminary in vivo studies demonstrate that ivermectin can enhance some of the pharmacological actions of diazepam.
Ivermectin should be use with caution in combination with p-glycoprotein transport system inhibitor or inducer such as amidarone, carvedilol, clarithromycin, cyclosporine, erythromycin, itraconazole, ketoconazole, quinidine, ritonavir, tamoxifen, verapamil, amprenavir, clotrimazole, phenothiazines, rifampin, St. John's wort.
Concomitant administration of alcohol may increase ivermectin plasma level.

Adverse reactions

Dyspnea, urinary and/or fecal incontinence, confusion, stupor, lethargy, seizures, coma, asthenia/fatigue, abdominal pain, diarrhea, constipation, nausea, vomiting, vertigo, somnolence, dizziness, tremor, rash, pruritus, urticaria, tachycardia, postural hypotension.

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ใบอนุญาตโฆษณาเลขที่ ฆศ. 1336/2554

Apolets

Clopidogrel bisulfate equivalent to 75mg of clopidogrel base

Imported Finished Product from Canada

Confidence in Bioequivalence Study with Clinical Trial

in patients who planned undergoing coronary angioplasty

Conclusion

❦ Generic clopidogrel (Apolets®) 600 mg loading dose is as effective as original clopidogrel.

❦ In the patients who required rapid platelet inhibition such ACS or adhoc PCI patients, either original or generic clopidrogrel 600 mg can be used.

❦ The inhibition of platelet aggregation can achieve up to 80% at six hour after double loading dose.

Berlin