รายงานวิจัยฉบับสมบูรณ์

โครงการ องค์ประกอบทางเคมีจากสมุนไพรไทย ว่านดีงูเห่า โพกระดิ่ง และมะนาวผี

โดย นางสาวฉวี เย็นใจ และคณะ

มีนาคม 2562

สัญญาเลขที่ RSA5980022

รายงานวิจัยฉบับสมบูรณ์

โครงการ องค์ประกอบทางเคมีจากสมุนไพรไทย; ว่านดีงูเห่า โพกระดิ่ง และมะนาวผี

โดย นางสาวฉวี เย็นใจ มหาวิทยาลัยขอนแก่น

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย และมหาวิทยาลัยขอนแก่น

สารบัญ

หัวข้อ	หน้า
บทคัดย่อ	1
รายละเอียดผลการดำเนินงานของโครงการ	4
บทนำ	7
การทดลอง	8
ผลการทดลอง	13
สรุปผลการทดลอง	25
ภาคผนวก	28
รายงานการเงิน	29
สำเนาสมุดบัญชีเงินฝาก	31

บทคัดย่อ

จากการแยกสกัดรากว่านดีงูเห่าได้สารบริสุทธิ์ 18 สาร โดยมีสารใหม่ 2 สารได้แก่ toddayanin (**1**) และ 8*S*-10-O-demethylbocconoline (**2**) พบว่าสาร **3** แสดงฤทธิ์ต้านเซลล์มะเร็งช่องปาก เต้านมและมะเร็งปอดในระดับปานกลาง

สารเบนโซไทรามีนชนิดใหม่ชื่อ atalantums A–G (**19**–**25**) และสารที่ทราบโครงสร้างแล้ว 5 สาร สามารถแยกได้จากเปลือกผลมะนาวผี เมื่อนำสารทั้งหมดไปทดสอบฤทธิ์ต้านมะเร็งท่อน้ำดี KKU-M214, KKU-M213 และ KKU-M156 พบว่าสาร **23** แสดงค่า IC_{50} เท่ากับ 1.97 ± 0.73 μM กับเซลล์ KKU-M156 ซึ่งแรงกว่าสารมาตรฐาน ellipticine ถึง 4.7 เท่า สาร **19** แสดงฤทธิ์ฆ่าเซลล์มะเร็ง KKU-M214 ด้วยค่า IC_{50} เท่ากับ 3.06 ± 0.51 μM ซึ่งใกล้เคียงกับสารมาตรฐาน 5-fluorouracil

สารไลโมนอยด์ชนิดใหม่ 3 สารได้แก่ limonophyllines A-C (**31**, **34** และ **35**) และพบสารที่ทราบโครงสร้างแล้ว 2 สาร (**32** และ **33**) พบสารประเภทอะคริโดนอัลคาลอยด์ 11 สาร (**36**-**46**) ทั้งหมดแยกได้จากลำต้นของมะนาวผี เมื่อทดสอบฤทธิ์ทางชีวภาพพบว่าสาร **42**, **44** และ **46** แสดงฤทธิ์ต้านเซลล์มะเร็ง KKU-M156 โดยมีค่า IC_{50} ระหว่าง 3.39 ถึง 4.1 μg/ml แต่กับเซลล์มะเร็งตับ (HepG2) แสดงค่า IC_{50} ระหว่าง 1.43 ถึง 8.4 μg/ml

ในการแยกสกัดใบมะนาวผีพบสารประเภทฟลาโวนอยด์ชนิดใหม่ชื่อ atalantraflavone (**47**) และยังพบสารที่ทราบโครงสร้างแล้ว 8 สาร จากการทดสอบฤทธิ์ต้านเอนไซม์อะซีทิลโคลินเอสเทอร์เรส พบว่าสาร lupalbigenin แสดงเปอร์เซ็นต์การยับยั้งที่ 79% โดยออกฤทธิ์ที่ดีกว่า tacrine ถึง 1.4 เท่า

ในการแยกเมล็ดโพกระดิ่งพบลิกแนน 11 สาร (**59**-**66**) สารเหล่านี้แสดงฤทธิ์ต้านเซลล์มะเร็งท่อน้ำดีและมะเร็งตับในระดับปานกลางถึงดี จากนั้นนำสาร deoxypodophyllotoxin ซึ่งเป็นสารหลัก มาดัดแปลงโครงสร้างด้วยวิธีทางเคมีอินทรีย์ได้ทั้งสิ้น 12 อนุพันธ์ เมื่อทดสอบฤทธิ์ทางชีวภาพ พบว่าสาร **68**, **74**, **75** และ **78** แสดงฤทธิ์ต่อเซลล์มะเร็ง KKU-100 และ HepG2 ด้วยค่า IC_{50} ระหว่าง 0.42 ถึง 2.01 μM ซึ่งแรงกว่าสารตั้งต้น deoxypodophyllotoxin สำหรับสาร **75** แสดงค่า IC_{50} เท่ากับ 0.75 และ 0.46 μM ต่อเซลล์ KKU-100 และ HepG2 ตามลำดับ จะเห็นได้ว่าสาร **75** น่าจะเป็นสารต้นแบบในการพัฒนาเป็นสารต้านมะเร็งต่อไป

Abstract

Chemical investigation of the roots of *Toddalia asiatica* led to the isolation and purification of 18 compounds including two new compounds, toddayanin (**1**) and 8*S*-10-O-demethylbocconoline (**2**). Coumarin **3** showed moderate cytotoxicity against KB, NCI-H187 and MCF-7 cell lines.

Seven new benzoyltyramines, atalantums A–G (**19**–**25**), and five known compounds were isolated from the peels of *Atalantia monophylla*. All compounds were examined for cytotoxicity against the cholangiocarcinoma cell lines KKU-M214, KKU-M213, and KKU-M156. Compound **23** exhibited the strongest cytotoxicity against KKU-M156 cells, with an IC_{50} value of 1.97 ± 0.73 μM, an approximately 4.7-fold higher activity than that of the ellipticine standard. Compound **19** displayed strong cytotoxicity against KKU-M214 cells, with an IC_{50} value of 3.06 ± 0.51 μM, nearly equal to that of the 5-fluorouracil standard.

Three new limonoids, limonophyllines A-C (**31**, **34** and **35**), along with two known limonoids (**32** and **33**) and 11 acridone alkaloids (**36**-**46**) were isolated from the stems of *Atalantia monophylla*. Compounds **42**, **44** and **46** displayed cytotoxicity against KKU-M156 cell line with IC_{50} ranging from 3.39 to 4.1 μg/ml while cytotoxicity against HepG2 cell line with IC_{50} ranging from 1.43 to 8.4 μg/ml.

A new flavonoid, atalantraflavone (**47**) as well as eight known compounds were isolated from the leaves of *Atalantia monophylla* (L.) DC. It was found that isoflavonoid **51**, lupalbigenin, showed 79% inhibition to AChE by the modified Ellman's method and was 1.4 fold stronger than the tacrine standard.

Eleven lignans (**59**-**66**) were isolated from the seed of *Hernandia nymphaeifolia*. Most of the lignans exhibited strong to moderate cytotoxicity against cholangiocarcinoma KKU-M156 and HepG2 cell lines. Deoxypodophyllotoxin was used as the starting material for structural modification. Twelve lignan derivatives were synthesized from deoxypodophyllotoxin. Cytotoxicity evaluation against cholangiocarcinoma, KKU-100 and HepG2 cell lines, showed that compounds **68**, **74**, **75** and **78** exhibited stronger cytotoxicity than the starting material, **56**, with IC_{50} ranging from 0.42 to 2.01 μM. Compound **75** displayed interesting activity by showing IC_{50} values of 0.75 and 0.46 μM against KKU-100 and HepG2 cell lines, respectively.

From these observation, **75** seems to be useful as a lead compound for the development of anticancer agents.

รายละเอียดผลการดำเนินงานของโครงการ

1. สำหรับหัวหน้าโครงการวิจัยผู้รับทุน

1.1 การดำเนินงาน ☑ ได้ดำเนินงานตามแผนที่วางไว้

1.2 รายละเอียดผลการดำเนินงานของโครงการ

1. สรุปย่อ

วัตถุประสงค์

1. สกัดแยกและพิสูจน์โครงสร้างทางเคมีขององค์ประกอบทางเคมีของพืชสมุนไพร ว่านดีงูเห่า โพกระดิ่ง และมะนาวผี
2. ทดสอบฤทธิ์ทางชีวภาพของสารที่แยกได้ ต่อเชื้อรา เชื้อมาลาเรีย เชื้อวัณโรค และฤทธิ์ฆ่าเซลล์มะเร็ง
3. ดัดแปลงโครงสร้างทางเคมีของสารหลักที่แยกได้ โดยใช้ปฏิกิริยาทางเคมีอินทรีย์ และทดสอบฤทธิ์ทางชีวภาพ

การดำเนินงานวิจัย

1. แยกสกัดสมุนไพรว่านดีงูเห่า มะนาวผี และเมล็ดโพกระดิ่ง ด้วยตัวทำละลาย เฮกเซน เอทิลอะซีเตท และเมทานอล จากนั้นทำสารให้บริสุทธิ์ด้วยวิธีทางโครมาโทกราฟี และตกผลึก
2. หาโครงสร้างทางเคมีของสารบริสุทธิ์ทั้งหมดด้วยวิธีทางสเปกโทรสโกปี ได้แก่ ^{1}H NMR, ^{13}C NMR, 2D NMR, IR, MS และวิธีอื่นเช่น การบิดแสงระนาบเดียว CD
3. ดัดแปลงโครงสร้างของลิกแนนที่ได้จากโพกระดิ่งด้วยปฏิกิริยาทางเคมีอินทรีย์
4. ทดสอบฤทธิ์ของสารบริสุทธิ์ในการฆ่าเซลล์มะเร็งเช่น มะเร็งท่อน้ำดี มะเร็งปอด มะเร็งเต้านม มะเร็งช่องปาก และฤทธิ์ต้านอนุมูลอิสระ

ระยะเวลาดำเนินการ 3 ปี

ผลงานตีพิมพ์ ตลอด 3 ปี

1. Sombatsri, A., Thummanant, Y., Sribuhom, T., Wongphakham, P., Senawong T., Yenjai C. Atalantums H-K from the peels of *Atalantia monophylla* and their cytotoxicity Natural Product Research, 2019, DOI:10.1080/14786419.2019.1576042
2. Posri, P., Suthiwong, J., Takomthong, P., Wongsa, C., Chuenban, C., Boonyarat, C., Yenjai, C. A new flavonoid from the leaves of *Atalantia monophylla* (L.) DC, Natural Product Research, 2018, DOI:10.1080/14786419.2018.1457667
3. Suthiwong, J., Wandee, J., Pitchuanchom, S., Sojikul, P., Kukongviriyapan, V., Yenjai, C*. Cytotoxicity against cholangiocarcinoma and HepG2 cell lines of lignan

derivatives from *Hernandia nymphaeifolia*. Medicinal Chemistry Research, 2018, 27, 2042-2049.

4. Sombatsri, A., Thummanant, Y., Sribuhom, T., Boonmak, J., Youngme, S., Phusrisom, S., Kukongviriyapan, V., Yenjai, C. New limonophyllines A-C from the stem of *Atalantia monophylla* and cytotoxicity against cholangiocarcinoma and HepG2 cell lines, Archives of Pharmacal Research, 2018, 14(4). 341-347.
5. Suthiwong, J., Boonloh, K., Kukongviriyapan, V., Yenjai, C*. Cytotoxicity against Cholangiocarcinoma and HepG2 Cell Lines of Lignans from *Hernandia nymphaeifolia*, Natural Product Communication, 2018, 13 (1), 61-63.
6. Sukieum, S., Sang-aroon W., Yenjai C*. Coumarins and alkaloids from the roots of *Toddalia asiatica,* Natural Product Research, 2018, 32(8), 944-952.
7. Sribuhom, T., Boueroy, P., Hahnvajanawong, C., Phatchana, R., Yenjai, C*. Benzoyltyramine alkaloids atalantums A-G from the peels of *Atalantia monophylla* and their cytotoxicity against cholangiocarcinoma cell lines, Journal of Natural Products, 2017, 80, 403–408.
8. Hirunwong, C., Sukieum, S., Phatchana, R., Yenjai C*. Cytotoxic and antimalarial constituents from the roots of *Toddalia asiatica.* Phytochemistry Letters, 2016, 17, 242-245.
9. Phatchana, R., Thongsri, Y., Yenjai, C*. Canangalias C-H, juvenile hormone III analogues from the roots of *Cananga latifolia.* Fitoterapia, 2016, 114, 45-50.

หมายเหตุ รายการที่ 1-2 เป็นผลงานตีพิมพ์ในปีที่ 3
รายการที่ 3-6 เป็นผลงานตีพิมพ์ในปีที่ 2
รายการที่ 7-9 เป็นผลงานตีพิมพ์ในปีที่ 1

กิจกรรมอื่นๆที่เกี่ยวข้อง

การเชื่อมโยงทางวิชาการกับนักวิจัยไทย

1 รศ.ดร. จริยา หาญวจนวงศ์ ภาควิชาจุลชีววิทยา คณะแพทย์ศาสตร์ มหาวิทยาลัยขอนแก่น จ.ขอนแก่น

2. ผศ.ดร. ยอดหทัย ทองศรี ภาควิชาเทคนิคการแพทย์ คณะสหเวชศาสตร์ มหาวิทยาลัยนเรศวร ต.ท่าโพธิ์ อ.เมือง จ.พิษณุโลก

3. ศ.ดร. วีรพล คู่คงวิริยะพันธุ์ ภาควิชาเภสัชวิทยา คณะแพทย์ศาสตร์ มหาวิทยาลัยขอนแก่น จ.ขอนแก่น

บทนำ

มะนาวผี เป็นไม้ยืนต้นในวงศ์ Rutaceae มะลิว (เชียงใหม่), กรูดเปรย (จันทบุรี), นางกาน (ขอนแก่น), กรูดผี (สุราษฎร์ธานี), ขี้ติ้ว จ๊าลิ้ว (ภาคเหนือ), กะนางพลี กะนาวพลี มะนาวพลี (ภาคใต้) ต้นสีน้ำตาลเข้ม มีหนามแหลมตามต้น เนื้อไม้สีเทา ใบเดี่ยว มีกลิ่นเหมือนมะนาว ดอกช่อ ออกที่ปลายกิ่ง กลีบดอกสีเหลือง ฐานรองดอกเป็นรูปถ้วย สีเขียว เกสรตัวผู้สีเหลือง ผลเดี่ยว รูปไข่ ปลายผลมีปุ่มออกมาเล็กน้อย สีเขียวอมเหลือง มีต่อมน้ำมันที่ผิว นิยมใช้ทำเครื่องเรือนต่าง ๆ ผลมีลักษณะกลมหรือรี เป็นผลขนาดเล็ก ผิวผลเรียบเป็นสีเขียวอ่อนหรือเทา มีขนาดเส้นผ่านศูนย์กลางประมาณ 2-4 เซนติเมตร ผิวผลหนาคล้ายหนังและมีต่อมน้ำมันเป็นจุดหนาแน่น ที่ปลายผลมีก้านเกสรเพศเมีย ติดทน ผลภายในเป็นกลีบคล้ายผลส้ม ภายในมีเมล็ดจำนวนน้อย ลักษณะของเมล็ดเป็นรูปรีสีขาว โดยจะออกดอกและเป็นผลในช่วงเดือนธันวาคมถึงเดือนเมษายน โดยจะพบได้ตามป่าชายหาด ชายฝั่ง ป่าเต็งรัง ป่าดิบแล้ง บนเขาหิน ที่ระดับความสูงตั้งแต่ใกล้ระดับน้ำทะเลจนถึงประมาณ 800 เมตร สรรพคุณทางยาของมะนาวผีพบว่าใช้รักษาโรครูมาติซั่ม อาการอัมพาต น้ำมันจากผลใช้รักษาระบบทางเดินหายใจ (Basa, 1975; Bunyapraphatsara, 1999; Panda, et al., 2004)

จากการสืบค้นข้อมูลด้านองค์ประกอบทางเคมี พบว่ารากและลำต้นของมะนาวผีประกอบไปด้วยสารประเภทอะคริโดนอัลคาลอยด์ คูมาริน ไตรเทอร์ปีน สเตียรอยด์ และฟลาโวนอยด์ อย่างไรก็ตามผลของมะนาวผียังไม่พบรายงานการวิจัยขององค์ประกอบทางเคมี

ว่านดีงูเห่า เป็นไม้เถารอเลื้อย ลำต้นมีหนาม พบได้ทั่วไปในประเทศไทย จีน อินเดีย และแอฟริกา มีสรรพคุณรักษาอาการปวดเมื่อย รักษาไข้หวัด ปวดท้อง และแก้อาการฟกช้ำ ผลใช้รักษาไข้มาลาเรีย แก้ไอ ราก ใช้ช่วยระบบย่อยอาหาร ในส่วนของใบ ใช้รักษาโรคปอด จากการสืบค้นข้อมูล พบว่าต้มไม้ชนิดนี้มีองค์ประกอบทางเคมีเป็นสารประเภทคูมาริน ไตรเทอร์ปีน ฟีแนนทริดีนอัลคาลอยด์ และน้ำมันหอมระเหย มีผู้ศึกษาฤทธิ์ทางชีวภาพต่างๆเช่น การต้านมะเร็งชนิด U-937 ฤทธิ์ต้านเบาหวาน ต้านอนุมูลอิสระ และต้านแบคทีเรีย

โพกระดิ่ง เป็นไม้ต้นขนาดกลาง สูง 10-20 เมตร ลำต้นแตกกิ่งต่ำ เปลือกสีเทาเป็นมัน ใบเดี่ยว เรียงเวียนสลับ รูปไข่กว้างแกมรูปสามเหลี่ยม ปลายใบแหลม โคนใบกลม ก้านใบยาว 6-20 เซนติเมตร ติดเลยโคนใบเข้ามาในแผ่นใบเล็กน้อย ดอกออกเป็นช่อ แตกแขนงสั้นๆ ตามซอกโคนใบใกล้ยอดหรือที่ยอด ดอกแยกเพศอยู่ในช่อเดียวกัน ออกเป็นกลุ่ม กลุ่มละ 3 ดอก ตรงกลางเป็นดอกเพศเมียขนาบด้วยดอกเพศผู้ ดอกเพศเมียมีกลีบดอกสีขาว 4 กลีบ มีฐานรองรับไข่เป็นรูปถ้วยและมีขนาดโตขึ้นรองรับผล ผลสดกลม สีดำ เมล็ดกลมมีเนื้อนุ่มคล้ายฟองน้ำหุ้ม พบทางภาคใต้ของไทย ขึ้นตามป่าชายหาดบนหาดทรายของเกาะและฝั่งทะเลด้านทะเลอันดามัน ออกดอกและผลเดือน กันยายน –พฤศจิกายน

ที่มาและความสำคัญ

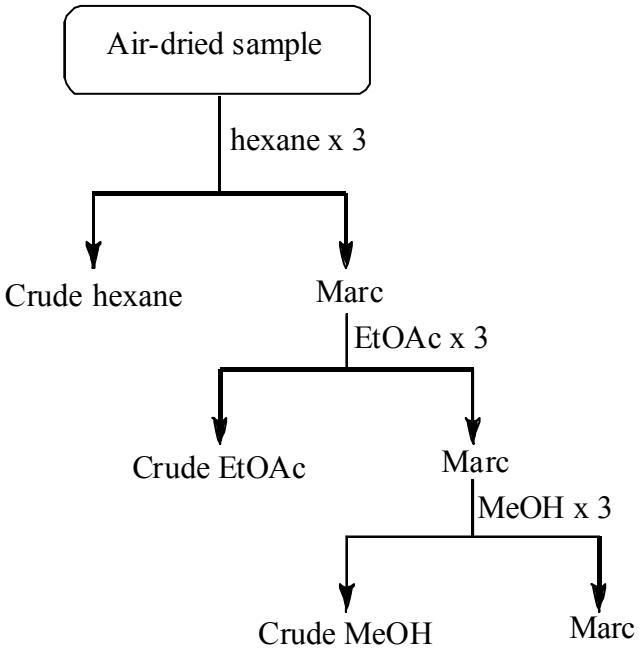
สมุนไพรไทยจัดเป็นทรัพยากรที่สำคัญของประเทศ และเป็นสิ่งสำคัญที่ช่วยในการดูแลรักษาสุขภาพของประชากรชาวไทยมาเนิ่นนานและต่อเนื่อง สังคมในปัจจุบันมีการพัฒนาทางเทคโนโลยีอย่างรวดเร็ว

วิถีชีวิตของคนไทยเร่งรีบขึ้น การรับประทานอาหารที่ดีต่อสุขภาพ การได้รับสารพิษทั้งทางตรงและทางอ้อม เป็นเหตุให้ประชาชนประสบปัญหาด้านสุขภาพมากขึ้น สำหรับประเทศไทยเองต้องสูญเสียเงินตราต่างประเทศไปเป็นจำนวนมาก เพื่อนำเข้ายาจากต่างประเทศ ฉะนั้นการนำเอาสมุนไพรไทยมาพัฒนาเพื่อให้เป็นยารักษาโรคจึงจำเป็นต่อเศรษฐกิจของชาติเป็นอย่างมาก จากกระแสความนิยมสมุนไพรในปัจจุบัน ทั้งในลักษณะยารักษาโรค อาหารและเครื่องดื่มเสริมสุขภาพ ตลอดจนการพอกและอบด้วยสมุนไพร ทำให้ควรจะมีการศึกษาค้นคว้าหาองค์ความรู้ด้านต่างๆ ของสมุนไพรให้กว้างขวางมากยิ่งขึ้น เพื่อให้มีข้อมูลทางวิทยาศาสตร์ ซึ่งจะนำมาใช้เป็นหลักฐานอ้างอิงที่เชื่อถือได้ของสรรพคุณต่างๆ ของสมุนไพรเหล่านั้น เพื่อจะได้ส่งผลให้มีการใช้สมุนไพรอย่างถูกต้อง ปลอดภัย น่าเชื่อถือ และได้ประโยชน์สูงสุด ตลอดจนส่งผลให้มีการพัฒนาสมุนไพรไทยอย่างต่อเนื่องและยั่งยืน

การทดลอง

การแยกสารด้วยตัวทำละลายอินทรีย์

นำสมุนไพรได้แก่เปลือกผลมะนาวผี รากว่านดีงูเห่า เมล็ดโพกระดิ่ง มาหั่น อบแห้ง บดละเอียดได้นำมาแช่ด้วย hexane จำนวน 2 ครั้ง เป็นเวลาครั้งละ 3 วัน โดยเติมตัวทำละลายให้ท่วม พร้อมทั้งคนอย่างสม่ำเสมอ นำสารละลายที่ได้ไประเหยแห้ง ได้สารสกัดหยาบ hexane แช่กากที่เหลือด้วย EtOAc จำนวน 2 ครั้ง เป็นเวลาครั้งละ 3 วัน นำสารละลายที่ได้ไประเหยแห้ง ได้สารสกัดหยาบ EtOAc แช่กากที่เหลือด้วย MeOH จำนวน 2 ครั้ง นำสารละลายที่ได้ไประเหยแห้ง ได้สารสกัดหยาบ MeOH โดยแสดงขั้นตอนได้ดังแผนภาพด้านล่าง



การแยกสกัดสารของว่านดีงูเห่า

ในการแยกสกัดรากว่านดีงูเห่าจำนวน 7 กิโลกรัม ได้ส่วนสกัดหยาบ hexane EtOAc และ MeOH จำนวน 147, 89 และ 121 กรัมตามลำดับ นำสารสกัดหยาบชั้น **EtOAc** จำนวน 62 กรัม ทำตามขั้นตอนดังต่อไปนี้ เมื่อนำสารสกัดหยาบไปแยกด้วย silica gel flash column chromatography (FCC) ได้ส่วนสกัดย่อย 13 ส่วนได้แก่ F1-F13 นำส่วนย่อย F3 ไปแยกด้วย FCC และใช้ hexane:EtOAc (97:3) เป็นตัวชะได้ 15 ส่วนย่อย F3.1-F3.15 เมื่อแยกส่วนย่อย F3.15 นำมาตกผลึกด้วย MeOH ได้สาร **11** (8.5 mg, 0.0001%) ส่วนย่อย F4 นำมาตกผลึกด้วย EtOAc ได้สาร **5** (340 mg, 0.0048 %) เป็นของแข็งสีเหลือง ส่วนย่อย F6 นำมาแยกต่อด้วย FCC โดยใช้ hexane:EtOAc (80:20) เป็นตัวชะ ได้ส่วนย่อย 7 ส่วนได้แก่ F6.1-F6.7 นำส่วน F6.5 มาแยกต่อด้วย FCC (hexane:EtOAc; 90:10) ได้สาร **4** (88.4 mg, 0.0013 %) สำหรับส่วนย่อย F6.7 ก็นำมาแยกด้วย FCC และชะด้วย CH_2Cl_2:hexane ได้ 5 ส่วนย่อยได้แก่ F6.7.1-F6.7.5 ส่วนย่อย F6.7.2 แยกต่อด้วย FCC และชะด้วย isocratic system ของ hexane:EtOAc (90:10) ได้สาร **10** (4.9 mg, 0.0001 %) สำหรับส่วนย่อย F6.7.5 แยกให้บริสุทธิ์ด้วย TLC using hexane:CH_2Cl_2 (70:30) ได้สาร **14** (1.1 mg, 0.00001 %) ส่วนย่อย F7 นำมาแยกด้วย FCC (hexane:EtOAc) ได้ส่วนสกัดย่อย 7 ส่วนได้แก่ F7.1-F7.7 เมื่อนำส่วนย่อย F7.1 และ F7.2 มาทำให้บริสุทธิ์ด้วยวิธี PLC โดยใช้ hexane:CH_2Cl_2 (60:40) ได้สาร **8** (6.3 mg, 0.0001 %) และ **9** (68.4 mg, 0.0010 %) ตามลำดับ เมื่อนำ F7.7 มาทำให้บริสุทธิ์ด้วย silica gel FCC และชะด้วย isocratic system ของ hexane:CH_2Cl_2:EtOAc (70:25:5) ได้สาร **3** (50.6 mg, 0.0007 %) และ **1** (3.7 mg, 0.0001 %) ส่วน F9 นำไปแยกด้วยวิธี silica gel FCC และชะด้วย isocratic system ของ hexane:CH_2Cl_2 (50:50) ได้ส่วนสกัดย่อย 10 ส่วนได้แก่ F9.1-F9.10 ส่วนย่อย F9.8 นำมาแยกด้วย FCC และแยกด้วย isocratic system ของ hexane:EtOAc (80:20) ได้ 14 ส่วนย่อย F9.8.1-F9.8.14 นำส่วนย่อย F9.8.6 มาแยกต่อด้วย FCC ชะด้วย hexane:CH_2Cl_2:EtOAc (30:65:5) ได้สารบริสุทธิ์ **2** (5.2 mg, 0.0001 %) ส่วนย่อย F9.8.8 นำไปกรองด้วย Sephadex LH-20 CC ได้สาร **16** (7.2 mg, 0.0001 %) ส่วนย่อย F9.8.11 นำไปแยกด้วย TLC โดยใช้ hexane:CH_2Cl_2:MeOH (40:58:2) ได้สาร **17** (6.1 mg, 0.0001 %) ส่วนสกัด F9.10 นำไปกรองด้วย Sephadex LH-20 CC ได้ 8 ส่วนสกัดย่อย F9.10.1-F9.10.8 สำหรับส่วนสกัด F9.10.6 นำไปทำให้บริสุทธิ์ด้วย PLC โดยใช้ hexane:CH_2Cl_2:EtOAc (45:40:15) ได้สาร **7** (26 mg, 0.0004 %) ส่วนสกัดย่อย F9.10.8 นำไปแยกด้วย CC โดยใช้ hexane:EtOAc (80:20) ได้ส่วนสกัดย่อย 5 ส่วนได้แก่ F9.10.8.1-F9.10.8.5 ส่วนสกัด F9.10.8.4 นำไปแยกด้วย FCC และชะด้วย hexane:EtOAc (70:30) ได้สารบริสุทธิ์ **18** (11.6 mg, 0.0002 %) ส่วนสกัดย่อย F10 นำไปแยกด้วย FCC และชะด้วย hexane:EtOAc (70:30) ได้ 16 ส่วนสกัดย่อย ได้แก่ F10.1-F10.16 ส่วนสกัดย่อย F10.10 นำไปแยกด้วย FCC และชะด้วย hexane:CH_2Cl_2:EtOAc (50:40:10) ได้สารบริสุทธิ์ **13** (16.9 mg, 0.0002 %) ส่วนย่อย F10.13 นำไปแยกด้วย PLC โดยใช้ CH_2Cl_2:MeOH (1:99) ได้สาร **12** (8.5 mg, 0.0001 %) ส่วนสกัด F12 นำไปทำให้บริสุทธิ์ด้วย FCC และชะด้วย CH_2Cl_2:EtOAc ได้สาร **15** (25.5 mg, 0.0004 %) และ **6** (26 mg, 0.0004 %)

การแยกสกัดสารจากเปลือกมะนาวผี

ในการแยกสกัดเปลือกมะนาวผีจำนวน 2 กิโลกรัม ได้ส่วนสกัดหยาบ hexane EtOAc และ MeOH จำนวน 120, 150 และ 250 กรัมตามลำดับ นำสารสกัดหยาบของเปลือกผลมะนาวผีชั้นเฮกเซน มาแยกด้วยคอลัมภ์โครมาโตรกราฟี โดยใช้เฮกเซนและเอทธิลอะซีเตทเป็นตัวชะ เมื่อรวมส่วนสกัดย่อยโดยใช้วิธี TLC (Thin Layer Chromatography) เป็นตัวชี้วัด ได้ทั้งสิ้น 6 ส่วนสกัดย่อย HF1 ถึง HF9 นำ HF6 มาแยกด้วย silica gel FCC, โดยใช้ MeOH:CH_2Cl_2 (1:99) เป็นตัวชะ ได้ 3 ส่วนสกัดย่อย HF6.1–HF6.3 นำส่วน HF6.2 มาแยกด้วย FCC และชะด้วย EtOAc:hexanes (5:95) ได้สาร **29** (50.5 mg, 0.0025%) เมื่อนำ HF7 มาแยกด้วย silica gel FCC (acetone:hexanes 5:95) ได้ 5 ส่วนสกัดย่อย HF7.1-HF7.5 ส่วนย่อย HF7.4 เมื่อนำมาแยกด้วย PLC (25:75 acetone:hexanes) ได้สาร **30** (37.3 mg, 0.0019%) ส่วนย่อย HF7.5 แยกด้วย FCC (1:99 MeOH:CH_2Cl_2) ได้ส่วนย่อย HF7.5.1-HF7.5.5 นำ HF7.5.3 มาแยกด้วย reverse phase CC (1:1 H_2O:MeOH) ได้สาร **19** (9.3 mg, 0.00046%), **20** (5.9 mg, 0.0003%) และ **21** (4.2 mg, 0.00021%) การแยก HF7.5.4 ด้วย FCC (1:99 MeOH:CH_2Cl_2) ได้ 3 ส่วนย่อย HF7.5.4.1-HF7.5.4.3 จากการแยกส่วนย่อย HF7.5.4.2 ด้วย PLC (70:29:1 hexanes:actone:MeOH) ได้สาร **23** (15.2 mg, 0.00076%) และ **25** (4.8 mg, 0.00024%) ส่วนย่อย HF7.5.4.3 แยกด้วย PLC (70:29:1 hexanes:actone:MeOH) ได้สาร **22** (11.1 mg, 0.00055%) และ **24** (4.5 mg, 0.0002%) การแยกส่วนสกัด HF8 ด้วย FCC และชะด้วย MeOH:CH_2Cl_2 (2:98) และตามด้วย PLC (65:34:1 hexanes:acetone:MeOH) ได้สารบริสุทธิ์ **27** (6.2 mg, 0.0003%).

ส่วนสกัดหยาบชั้นเอทธิลอะซีเตท นำมาแยกด้วย silica gel FCC (hexanes:EtOAc) ได้ส่วนย่อย 8 ส่วน EF1-EF8 เมื่อนำ EF6 มาแยกด้วย silica gel FCC (acetone:hexanes) ได้ส่วนสกัดย่อย 5 ส่วน ได้แก่EF6.1-EF6.5 จากการแยกส่วนสกัดย่อย EF6.2 ด้วย reverse-phase CC (2:3 H_2O:MeOH) ได้สาร 28 (3.2 mg, 0.00016%) สำหรับส่วนสกัดย่อย EF6.3 เมื่อแยกด้วยวิธี PLC (3:7 acetone:hexanes) ได้สารบริสุทธิ์ **26** (10.3 mg, 0.0005%)

การแยกสกัดสารจากต้นมะนาวผี

ในการแยกสกัดต้นมะนาวผีจำนวน 6 กิโลกรัม ได้ส่วนสกัดหยาบ hexane EtOAc และ MeOH จำนวน 12, 80 และ 100 กรัมตามลำดับนำสารสกัดหยาบชั้น EtOAc มาแยกด้วยคอลัมภ์โครมาโทรกราฟี ชะด้วยตัวทำละลาย (hexane, EtOAc and MeOH) เก็บส่วนสกัดย่อยได้ 8 ส่วน (F1 ถึง F8) เมื่อนำ F2 มาแยกสกัดต่อด้วย CC (70% CH_2Cl_2:hexane) ได้ส่วยย่อย 3 ส่วน (F2.1-F2.3) นำส่วนสกัดย่อย F2.3 ไปแยกต่อด้วย FCC (80% CH_2Cl_2:hexane) ได้สาร **36** (199.8 mg, 0.0033%) และ **44** (60.6 mg, 0.001%) ส่วนสกัดย่อย F3 นำไปแยกต่อด้วย CC (CH_2Cl_2) ได้ส่วนย่อย 5 ส่วน (F3.1-F3.5) ส่วนสกัดย่อย F3.2 และ F3.5 เมื่อแยกต่อด้วย

CC (20% EtOAc:hexane) ได้สาร **45** (47.9 g, 0.0008%) และ **37** (167.4 mg, 0.0028%) ตามลำดับ ในส่วนของ F5 แยกต่อด้วย CC (CH_2Cl_2) ได้ 3 ส่วนย่อย (F5.1-F5.3) สำหรับ F5.3 แยกด้วย CC ได้ 3 ส่วน (F5.3.1-F5.3.3) พบว่าสาร **43** (19.9 mg, 0.0003%) ได้จากส่วนย่อย F5.3.1 ในส่วนของ F5.3.2 และ F5.3.3 นำมาแยกด้วย Sephadex LH-20 โดยใช้ MeOH เป็นตัวชะ ได้สาร **39** (7.9 mg, 0.0001%) และ **46** (24.1 mg, 0.0004%) ตามลำดับ ส่วนย่อย F7 แยกด้วย FCC (CH_2Cl_2) ได้ 2 ส่วน (F7.1 และ F7.2) พบว่าสาร **31** (165.0 mg, 0.0027%) ได้จากการตกผลึกของ F7.1 สำหรับ F7.2 นำไปแยกด้วย FCC (pure CH_2Cl_2) ได้ 3 ส่วนย่อย (F7.2.1-F7.2.3) ผลึกที่ตกได้ในส่วนย่อย F7.2.1 ได้แก่สาร **42** (21.7 mg, 0.0004%) ส่วนย่อย F7.2.2 แยกได้สาร **38** (66.0 mg, 0.0011%) และ **40** (19.3 mg, 0.0003%) ส่วนย่อย F7.2.3 นำไปกรองด้วย Sephadex LH-20 ได้สาร **35** (42.8 mg, 0.0007%) และ **41** (16.2 mg, 0.0003%) สำหรับส่วยย่อย F8 นำไปแยกด้วย CC (EtOAc:hexane) ได้ 2 ส่วน (F8.1 และ F8.2) เมื่อแยกด้วย gel filtration (Sephadex LH-20) ได้สาร **32** (63.6 mg, 0.0011%) สำหรับส่วนย่อย F8.2 เมื่อแยกด้วย gel filtration (Sephadex LH-20) ได้สาร **33** (10.1 mg, 0.00017%) และ **34** (9.8 mg, 0.00016%)

การแยกสกัดสารจากใบมะนาวผี

นำใบมะนาวผีแห้งที่บดละเอียด 9.0 กิโลกรัมแช่ในตัวทำละลายเฮกเซน EtOAc และ MeOH ได้สารสกัดหยาบ 3 ส่วนๆละ 126, 174 และ 685 กรัม นำสารสกัดหยาบชั้น EtOAc แยกต่อด้วย CC โดยใช้เฮกเซน EtOAc และ MeOH เป็นตัวชะ ได้ส่วนย่อย 9 ส่วน (F1 ถึง F9) นำส่วนย่อย F3 ไปแยกด้วย FCC ได้ 3 ส่วน (F3.1–F3.3) ในส่วนย่อย F3.2 แยกต่อด้วย CC ได้สาร **55** (25.7 mg, 0.0029%) นำส่วนย่อย F5 มาแยกต่อด้วย CC (MeOH:CH_2Cl_2) ได้ส่วนย่อย 2 ส่วน (F5.1 และ F5.2) พบว่าเมื่อแยกส่วนย่อย F5.1 โดย FCC (CH_2Cl_2) ได้สาร **49** (12.8 mg, 0.0014%) ในส่วนของ F5.2 นั้นนำมาแยกต่อด้วย FCC (CH_2Cl_2) ได้สารบริสุทธิ์ **48** ในส่วนของ F7 เมื่อแยกด้วย FCC (100% CH_2Cl_2) ได้ 4 ส่วนย่อย (F7.1-F7.4) นำส่วนย่อย F7.2 มาแยกต่อด้วย Sephadex LH20 (MeOH) ได้สาร **52** (20.2 mg, 0.0022%) และ **53** (18.4 mg, 0.0020%) ส่วนย่อยที่ F7.3 นำมาแยกด้วย TLC (100% CH_2Cl_2) ได้สารบริสุทธิ์ **47** (1.5 mg, 0.00002%), **51** (4.3 mg, 0.00005%) และ **54** (2.0 mg, 0.00002%) ส่วนย่อย F9 นำมาแยกด้วย FCC (5% MeOH:CH_2Cl_2) ได้สารบริสุทธิ์ **50** (7.6 mg, 0.00008%)

การแยกสกัดสารจากเมล็ดโพกระดิ่ง

นำเมล็ดโพกระดิ่งที่แห้ง บดละเอียดจำนวน 300 กรัม แช่ด้วยตัวทำละลาย EtOAc และเมทานอล ที่อุณหภูมิห้อง หลังจากการกรองและระเหยแห้ง ได้สารสกัดหยาบ 2 ส่วนจำนวน 79 และ 23 กรัม ตามลำดับ นำสารสกัดหยาบชั้น EtOAc มาแยกต่อด้วย FCC ชะด้วย hexane:EtOAc และ EtOAc:MeOH ได้ทั้งหมด 4 ส่วนย่อย (EF1-EF4) นำส่วนย่อย EF3 มาแยกด้วย Sephadex LH-20 (MeOH) และตามด้วย CC (CH_2Cl_2) ได้

ส่วนย่อย 2 ส่วนได้แก่ EF3.2.1 และ EF3.2.2 เมื่อนำส่วนย่อย EF3.2.2 มาแยกต่อด้วย FCC (20% EtOAc-hexane) ได้สารบริสุทธิ์ **56** และได้ส่วนย่อย EF3.2.2.1 และ EF3.2.2.2 ซึ่งเมื่อทำทั้งสองส่วนย่อยให้บริสุทธิ์ พบว่าได้สาร **66** และ **59** ตามลำดับ ในส่วนของ EF4 แยกได้สาร **58** และยังได้ 2 ส่วนย่อย (EF4.1 และ EF4.2) เมื่อทำการแยก EF4.2 ให้บริสุทธิ์ ด้วย CC และ PLC ได้สาร **57, 61**, **63** และ **65**

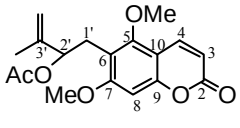
ในส่วนของสารสกัดหยาบชั้นเมทานอล เมื่อนำมาแยกด้วย CC ได้ส่วนสกัดย่อย 4 ส่วน MF1-MF4 นำ MF2 มาแยกด้วย CC ได้ 3 ส่วนย่อย MF2.1-MF2.3 พบว่า สารบริสุทธิ์ **60** และ **62** ได้มาจากการแยกส่วนย่อย MF2.2 และ MF2.3 ตามลำดับ สำหรับ MF3 เมื่อแยกด้วย CC และต่อด้วย PLC ได้สารบริสุทธิ์ **64**

เมื่อนำสาร deoxypodophyllotoxin มาเป็นสารตั้งต้นในการเตรียมอนุพันธ์ต่างๆ โดยอาศัยปฏิกิริยาทางเคมีอินทรีย์ เช่นปฏิกิริยา saponification รีดักชัน อะเซทิเลชัน และโบรมิเนชัน ได้อนุพันธ์ทั้งสิ้น 12 อนุพันธ์ หลังจากทำการทดสอบฤทธิ์ต้านมะเร็งท่อน้ำดีของเซลล์ KKU-100 และยังทดสอบกับเซลล์มะเร็งตับ (HepG2) พบว่า สารหลายอนุพันธ์แสดงฤทธิ์ที่ดี โดยมีค่า IC_{50} กับเซลล์ KKU-100 อยู่ในช่วง 0.42 ถึง 2.01 μM และสารบางชนิดยังมีแนวโน้มที่จะเป็น lead compound ในการพัฒนาต่อไปเป็นยาได้

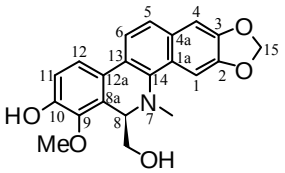
ผลการทดลอง

ว่านดีงูเห่า

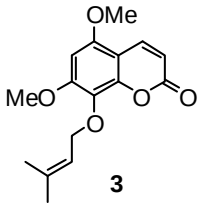
จากการแยกรากของว่านดีงูเห่า ได้สารบริสุทธิ์ทั้งสิ้น **18** สาร เมื่อหาโครงสร้างทางเคมีด้วยวิธีทางสเปกโตรสโกปี พบว่ามีโครงสร้างดังภาพข้างล่าง สารเหล่านี้ได้แก่ toddayanin (**1**) และ 8*S*-10-O-demethylbocconoline (**2**) ซึ่งเป็นสารใหม่ ส่วนสารที่ทราบโครงสร้างแล้วมี 16 สาร ได้แก่ artanin (**3**), coumurrayin (**4**), toddaculine (**5**), toddanol (**6**), toddalolactone (**7**), isopimpinellin (**8**), phellopterin (**9**), 5-methoxy-8-geranyloxypsoralen (**10**), 8-methoxydihydrochelerythrine (**11**), 8-methoxynorchelerythrine (**12**), skimmiamine (**13**), norchelerythrine (**14**), chelerythrine (**15**), oplopanone (**16**), nelumol (**17**), และ *p*-isopentenoxybenzenepropanoic acid (**18**)



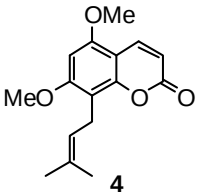
1



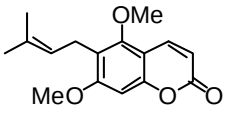
2



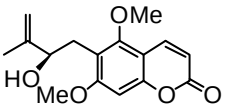
3



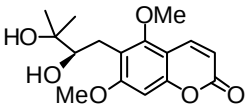
4



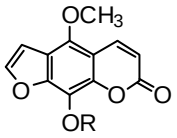
5



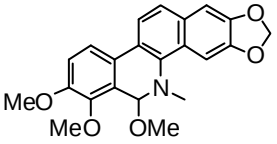
6



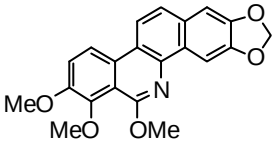
7



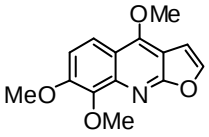
8 R = Me
9 R = isopentenyl
10 R = geranyl



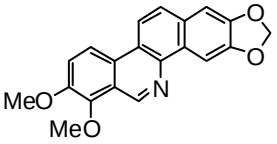
11



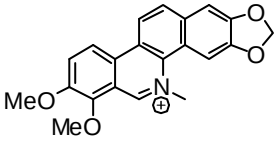
12



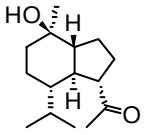
13



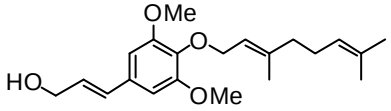
14



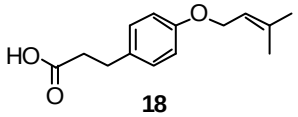
15



16



17



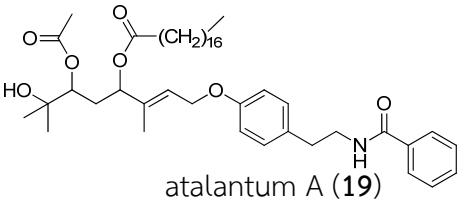
18

จากการทดสอบฤทธิ์ทางชีวภาพพบว่า สาร **2** แสดงฤทธิ์ต้านเซลล์มะเร็ง KB และ NCI-H187 และเซลล์ปรกติ Vero cells ด้วยค่า IC_{50} เท่ากับ 32.2, 5.8 และ 17.6 μg/mL สารคูมาริน **3** แสดงค่า IC_{50} อยู่ในช่วง 7.4 ถึง 31.0 μg/mL กับเซลล์ KB, NCI-H187 และ MCF-7 แต่สาร **4** ไม่แสดงฤทธิ์ เมื่อเปรียบเทียบโครงสร้างของสารเหล่านี้ จะเห็นได้ว่าหมู่ prenylalkoxy น่าจะมีผลต่อการแสดงฤทธิ์ สาร **11** แสดงฤทธิ์ที่แรงต่อเซลล์ NCI-H187 (IC_{50} = 0.8 μg/mL) ซึ่งใกล้เคียงกับสารมาตรฐาน ellipticine แต่อย่างไรก็ตามสารนี้ก็เป็นพิษต่อเซลล์ปรกติ สาร **5** และ **13** แสดงฤทธิ์ต่อเซลล์ MCF-7 ด้วยค่า IC_{50} เท่ากับ 23.4 และ 8.7 μg/mL

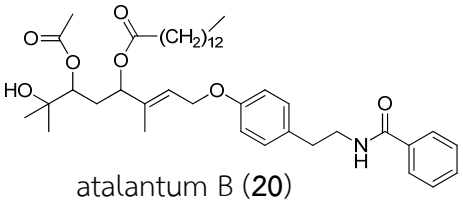
ตามลำดับ แต่ไม่เป็นพิษต่อเซลล์ปรกติ จากข้อมูลทั้งหมดนี้จะเห็นได้ว่าสาร **5** และ **13** จากว่านดีงูเห่า สามารถเป็นสารต้นแบบในการพัฒนาเป็นสารต้านมะเร็งได้

เปลือกผลมะนาวผี

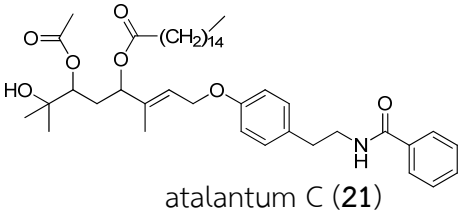
เมื่อนำสารที่แยกได้จากเปลือกของผลมะนาวผีทั้งหมด ไปหาโครงสร้างทางเคมีโดยวิธีทางสเปกโตรสโกปีเช่น IR, MS, 1D และ 2D พร้อมทั้งคุณสมบัติทางกายภาพอื่นๆ พบว่าสารทั้งหมดมีโครงสร้างดังแสดงตามภาพข้างล่าง และสารที่ได้เป็นสารใหม่ 7 สาร และสารที่ทราบโครงสร้างแล้ว 5 สาร ดังนี้ atalantums A-G (**19**-**25**) *N*-{2-[4-(4,6,7-trihydroxy-3,7-dimethyl-2-octen-1-yl)oxy]phenyl}ethylbenzamide (**26**) *N*-{2-[4-(4-acetoxy-6,7-dihydroxy-3,7-dimethyl-2-octen-1-yl)oxy]phenyl}ethylbenzamide (**27**), *N*-{2-[4-(6-acetoxy-4,7-dihydroxy-3,7-dimethyl-2-octen-1-yl)oxy]phenyl}ethylbenzamide (**28**), severine palmitate (**29**) และ severine acetate (**30**) เมื่อนำสารทั้งหมดไปทดสอบฤทธิ์ทางชีวภาพในการฆ่าเซลล์มะเร็งท่อน้ำดี สามารถแสดงฤทธิ์ทางชีวภาพได้ดังตาราง



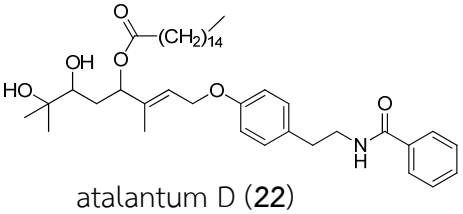
atalantum A (**19**)



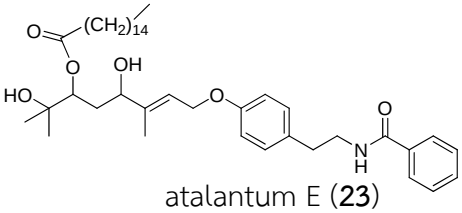
atalantum B (**20**)



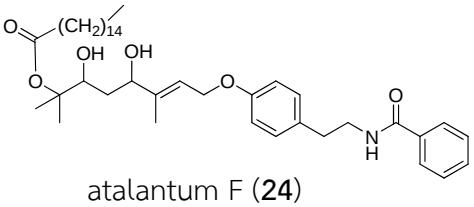
atalantum C (**21**)



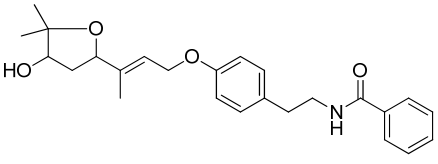
atalantum D (**22**)

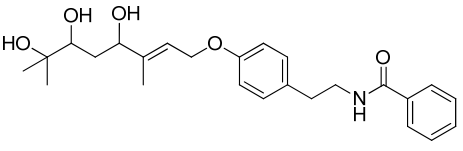


atalantum E (**23**)

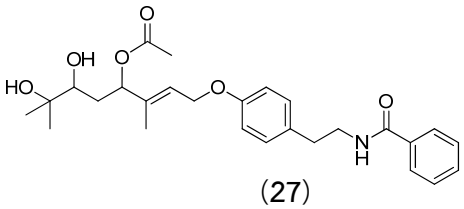


atalantum F (**24**)

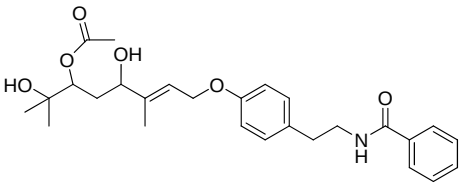




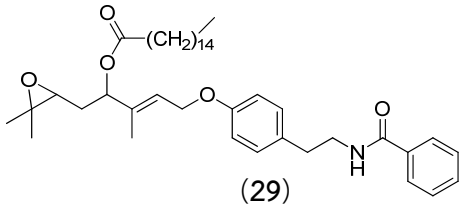
atalantum G (**25**)



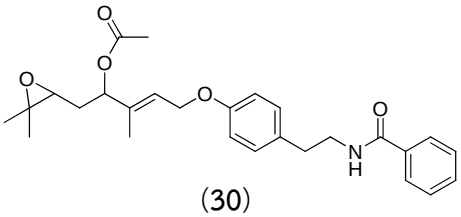
(**26**)



(**27**)



(**28**)



(**29**)

(**30**)

ตารางที่ 1 ฤทธิ์ต้านเซลล์มะเร็งท่อน้ำดีของสารจากเปลือกผลมะนาวผี (μM)*

compound	KKU-M214	KKU-M213	KKU-M156
19	3.06±0.51	34.77±1.40	22.02±1.55
20	24.00±1.21	2.36±0.20	24.47±1.98
21	12.36±1.44	29.05±1.48	24.02±0.46
22	27.43±0.27	5.63±0.22	2.80±0.22
23	8.44±0.47	23.47±1.01	1.97±0.73
24	20.52±0.17	16.14±0.84	31.49±1.08
25	7.37±1.29	12.21±1.07	21.51±0.46
26	11.11±1.03	25.84±5.00	26.76±0.11
27	44.48±1.26	28.79±1.64	49.72±0.80
29	14.92±0.68	2.71±0.23	20.98±0.54
30	31.49±7.22	11.14±1.02	29.10±2.52
Ellipticine	—	6.58±1.74	9.34±1.66
5-Fluorouracil	3.76±0.16	—	—

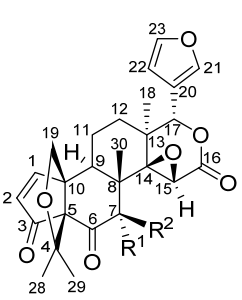
*Data shown are from triplicate experiments

จากการทดสอบฤทธิ์ต้านมะเร็งท่อน้ำดีชนิดเซลล์ KKU-M213 KKU-M214 และ KKU-M156 ด้วยวิธี sulforhodamine B (SRB) assay พบว่าสาร **19** แสดงฤทธิ์ที่ดี (IC_{50} เท่ากับ 3.06 μM) ใกล้เคียงสารมาตรฐาน 5-fuorouracil สาร **20** แสดงฤทธิ์กับเซลล์ KKU-M213 ด้วยค่า IC_{50} เท่ากับ 2.36 μM ซึ่งต่ำกว่าสารมาตรฐาน ellipticine แต่สำหรับสาร **21** ซึ่งมีสายโซ่ไฮโดรคาร์บอนยาวกว่า แสดงฤทธิ์ที่ไม่ดี นั่นอาจเนื่องมาจากสภาพขั้วของโมเลกุลอาจมีผลต่อฤทธิ์ต้านเซลล์มะเร็ง

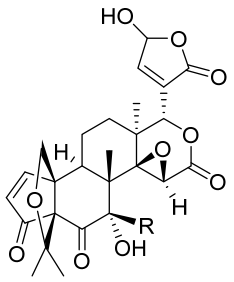
เมื่อเปรียบเทียบสารที่เป็น diol (**22**-**24**) สาร **22** แสดงฤทธิ์ที่ดีต่อเซลล์ KKU-M213 และ KKU-M156 (IC_{50} = 5.63 และ 2.80 μM) ขณะที่สาร **23** แสดงฤทธิ์ต่อเซลล์ KKU-M214 และ KKU-M156 (IC_{50} = 8.44 และ 1.97 μM ตามลำดับ แต่สาร **24** แสดงฤทธิ์ที่ไม่ดี นั่นอาจเนื่องมาจาก ตำแหน่งของหมู่ palmitoyloxy มีความสำคัญ จากการเปรียบเทียบฤทธิ์ระหว่างสาร **19** และ **22** กับเซลล์ KKU-M214 จะเห็นว่าหมู่ acetoxy ที่ตำแหน่ง 6 จะช่วยให้ฤทธิ์ดีขึ้นประมาณ 9 เท่า แต่ในทางตรงข้าม ฤทธิ์กับเซลล์ KKU-M213 และ KKU-M156 นั้น พบว่าสาร **22** แสดงฤทธิ์ที่แรงประมาณ 7 และ 11 เท่ากว่าสาร **19** ในส่วนของสาร **23** แสดงค่า IC_{50} เท่ากับ of 1.97 μM กับเซลล์ KKU-M156 ซึ่งแรงกว่าสาร **22** ประมาณ 1.42 เท่า นั่นอาจจะอธิบายได้ว่า diol **22** และ **23** จะจำเพาะเจาะจงกับเซลล์ KKU-M156 ขณะที่สาร **24** แสดงฤทธิ์ที่อ่อนมาก เมื่อพิจารณาจากโครงสร้างอาจอธิบายได้ว่า ตำแหน่งของหมู่อัลกอฮอล์จำเป็นต่อการแสดงฤทธิ์ เมื่อเปรียบเทียบระหว่างสาร **29** และ **30** สาร **29** แสดงฤทธิ์ที่แรงกว่า อาจเป็นผลมาจากหมู่ palmitoyloxy ที่ C-4 มีอิทธิพลต่อ เซลล์ KKU-M213 มากกว่าหมู่ acetoxy

ต้นมะนาวผี

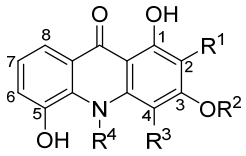
จากการแยกต้นมะนาวผี พบว่าได้สารบริสุทธิ์ 16 สาร ได้แก่ limonophyllines A-C (**31**, **34** และ **35**), ซึ่งเป็นสารใหม่ นอกจากนี้ยังได้สารที่ทราบโครงสร้างแล้วจำนวน 13 สารได้แก่ known compounds, 7-hydroxycycloatalantin (**32**), cycloepiatalantin (**33**), N-methylataphylline (**36**), atalaphylline (**37**), citrusinine II (**38**), citrusinion I (**39**), glycosparvarine (**40**), citruscridone (**41**), buxifoliadine C (**42**), atalaphyllinine (**43**), N-methylatalaphyllinine (**44**), N-methylcycloatalaphylline A (**45**) และ buxifoliadine E (**46**) ดังแสดงในภาพข้างล่าง



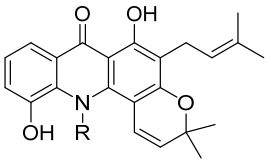
31 $R^1 = OCH_3$, $R^2 = OH$
32 $R^1 = R^2 = OH$
33 $R^1 = OH$, $R^2 = H$



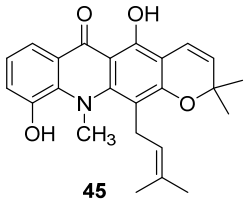
34 R = H
35 R = OH



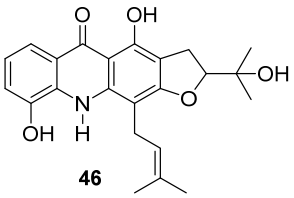
36 R^1 = prenyl, R^2 = H, R^3 = prenyl, $R^4 = CH_3$
37 R^1 = prenyl, R^2 = H, R^3 = prenyl, R^4 = H
38 R^1 = H, R^2 = H, $R^3 = OCH_3$, $R^4 = CH_3$
39 R^1 = H, $R^2 = CH_3$, $R^3 = OCH_3$, $R^4 = CH_3$
40 $R^1 = OCH_3$, R^2 = H, R^3 = H, $R^4 = CH_3$
41 $R^1 = OCH_3$, R^2 = H , $R^3 = OCH_3$, $R^4 = CH_3$
42 R^1 = prenyl, $R^2 = CH_3$, R^3 = H, R^4 = H



43 R = H
44 R = CH_3



45



46

รูปที่ 1 สารที่แยกได้จากต้นมะนาวผี

ตารางที่ 2 ฤทธิ์ต้านเซลล์มะเร็งของสารที่แยกได้จากต้นมะนาวผี (IC_{50}, μg/ml)

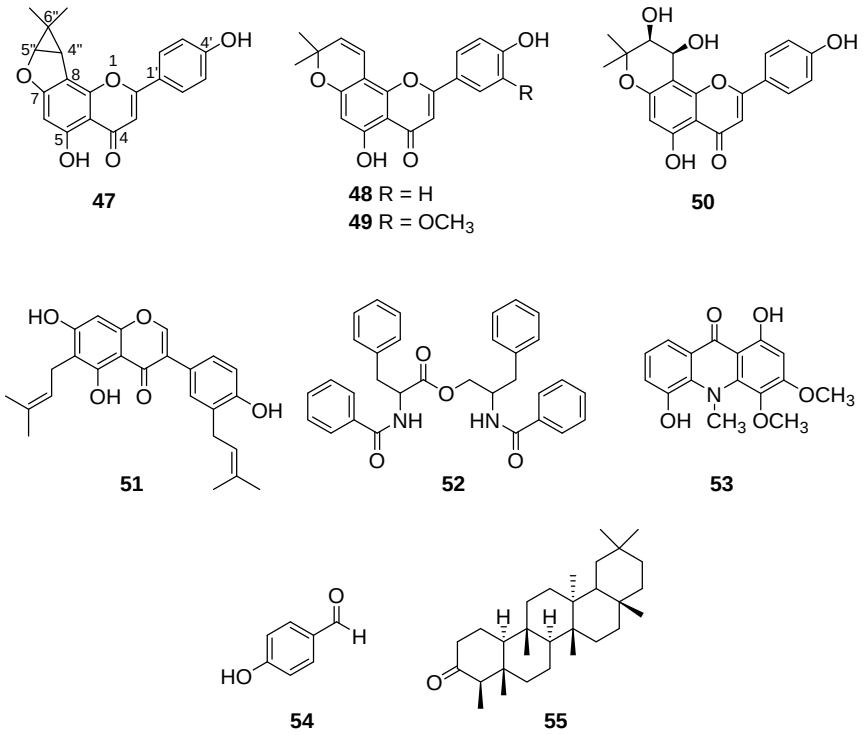
compound	KKU-M156		HepG2	
	IC_{50} (μg/ml)	Emax (%)	IC_{50} (μg/ml)	Emax (%)
31	139 ± 45.8	87.7 ± 19.8	72.1 ± 20.0	54.1 ± 19.1
34	52.3 ± 16.1	100	17.2 ± 8.9	42 ± 7.3
35	83.6 ± 29.1	100	2.1 ± 1.1	24.2 ± 2.6
36	35.9 ± 18.7	100	42.1 ± 16.0	100
37	3.7 ± 1.1	70.3 ± 5.0	29.0 ± 13.1	68.4 ± 11.8
38	9.9 ± 3.2	91.8 ± 8.4	47.6 ± 15.0	100
39	11.8 ± 4.9	100	26.9 ± 6.8	88.8 ± 7.6
40	15.6 ± 1.8	84.1 ± 3.0	64.6 ± 21.7	100
41	60.1 ± 22.2	100	103.6 ± 51.8	100
42	4.1 ± 3.4	100	8.4 ± 4.2	100
44	3.39 ± 0.24	100	1.43 ± 0.69	100
46	3.8 ± 0.72	98.4 ± 5.6	2.0 ± 0.4	89.4 ± 3.7
the others	inactive	-	inactive	-
Cisplatin	3.36 ± 1.44	74.5 ± 14.2	0.66 ± 0.21	66.8 ± 3.7

*****Data shown are from triplicate experiments

จากการทดสอบฤทธิ์ต้านมะเร็งท่อน้ำดี (KKU-M156) และมะเร็งตับ พบว่าสาร acridone alkaloid **44** แสดงฤทธิ์ฆ่าเซลล์มะเร็งทั้งสองได้ดีที่สุด (IC_{50} เท่ากับ 3.39 และ 1.43 μg/ml) สาร **36** แสดงฤทธิ์กับเซลล์ KKU-M156 ที่ IC_{50} เท่ากับ 35.9 μg/ml ซึ่งเสมือนว่าโครงสร้างของไพแรนน่าจะมีส่วนสำคัญในการแสดงฤทธิ์ จากค่า IC_{50} ของสาร **38** และ **39** กับเซลล์ KKU-M156 แสดงว่าหมู่เมทอกซีที่ตำแหน่ง 3 จำเป็นกับความสามารถในการออกฤทธิ์ แต่หากหมู่เมทอกซีอยู่ในตำแหน่ง 4 (สาร **40** และ **41**) เสมือนจะลดความสามารถลง เมื่อเปรียบเทียบระหว่างสาร **36** และ **37** พบว่าสาร **37** แสดงฤทธิ์ที่ดีกว่าถึง 9.7 ซึ่งเสมือนว่าหมู่ N-CH_3 จะลดฤทธิ์ในการฆ่าเซลล์ KKU-M156 ในกรณีของเซลล์มะเร็งตับ (HepG2) เมื่อเปรียบเทียบระหว่างสาร **37** และ **46** พบว่าหมู่ฟิวแรนเสมือนมีความสำคัญในการออกฤทธิ์

ใบมะนาวผี

ในส่วนของใบมะนาวผี พบว่าได้สารทั้งสิ้น 9 สาร เป็นสารใหม่ 1 สารได้แก่ atalantraflavone (**47**) และที่เหลือเป็นสารที่ทราบโครงสร้างแล้ว 8 สาร ได้แก่ atalantoflavone (**48**), racemoflavone (**49**), 5,4′-dihydroxy-(3″,4″-dihydro-3″,4″-dihydroxy)-2″,2″-dimethylpyrano-(5″,6″:7,8)-flavone (**50**), lupalbigenin (**51**), anabellamide (**52**), citrusinine I (**53**), *p*-hydroxybenzaldehyde (**54**) และ frideline (**55**) ดังแสดง



รูปที่ 2 สารที่แยกได้จากใบมะนาวผี

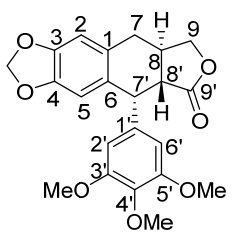
ตารางที่ 3 ฤทธิ์ต้านเอนไซม์อะซิติลโคลีนเอสเทอร์เลส และฤทธิ์ต้านอนุมูลอิสระ

compound	AChE inhibitory action at 100 μM (% inhibition)	Antioxidant activity (ABTS scavenging)
48	Inactive	13.99 ± 1.33
49	Inactive	32.30 ± 0.57
50	25.03 ± 4.17	14.18 ± 0.78
51	79.21 ± 4.79	47.64 ± 0.85
52	6.84 ± 2.67	5.26 ± 0.83
53	30.41 ± 1.24	90.68 ± 0.97
54	12.67 ± 4.63	3.86 ± 0.41
55	25.34 ± 4.35	4.72 ± 0.89
Tacrine	54.74 ± 3.42	-
Trolox (80 μM)	-	64.66 ± 0.62

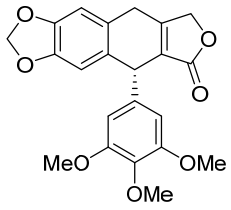
การทดสอบฤทธิ์ต้านเอนไซม์ acetylcholine esterase และ ฤทธิ์ต้านอนุมูลอิสระด้วยวิธี modified Ellman's method และ the ABTS scavenging assay ตามลำดับ พบว่าไอโซฟลาโวนอยด์ 21 แสดงฤทธิ์ที่ดีที่สุด (79.21% inhibition) แต่แสดงฤทธิ์ต้านอนุมูลอิสระปานกลาง เมื่อเปรียบเทียบระหว่าง ฟลาโวนอยด์ (2-4) และไอโซฟลาโวนอยด์ (5) พบว่าไอโซฟลาโวนอยด์แสดงฤทธิ์ที่ดีกว่า เมื่อเปรียบเทียบโครงสร้างของสารกลุ่มฟลาโวนอยด์ พบว่าหมู่ phenolic และ hydroxyl มีความสำคัญต่อการแสดงฤทธิ์

เมล็ดโพกระดิ่ง

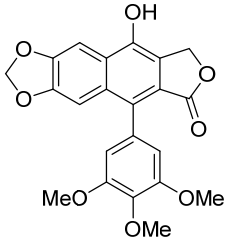
จากการแยกสกัดเมล็ดโพกระดิ่ง พบว่าได้สารทั้งหมด 11 สาร โดยสารเหล่านี้เป็นสารที่ทราบโครงสร้างแล้วทั้งสิ้น ไม่พบสารใหม่เลย มีชื่อดังนี้ (-)-deoxypodophyllotoxin (**56**), β-apopicropodophyllin (**57**), dehydropodophyllotoxin (**58**), deoxypodorhizone (**59**), 5′-methoxyyatein (**60**), podorhizol (**61**), bursehernin (**62**), (-)-maculatin (**63**), hernanol (**64**), (+)-epimagnolin (**65**) และ (+)-epiaschantin (**66**)



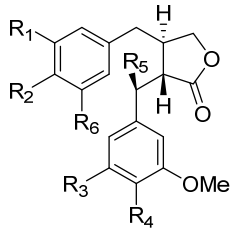
56

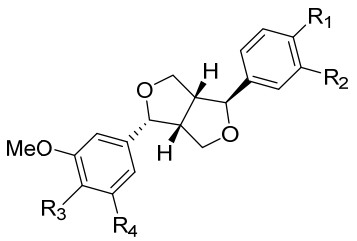


57



58

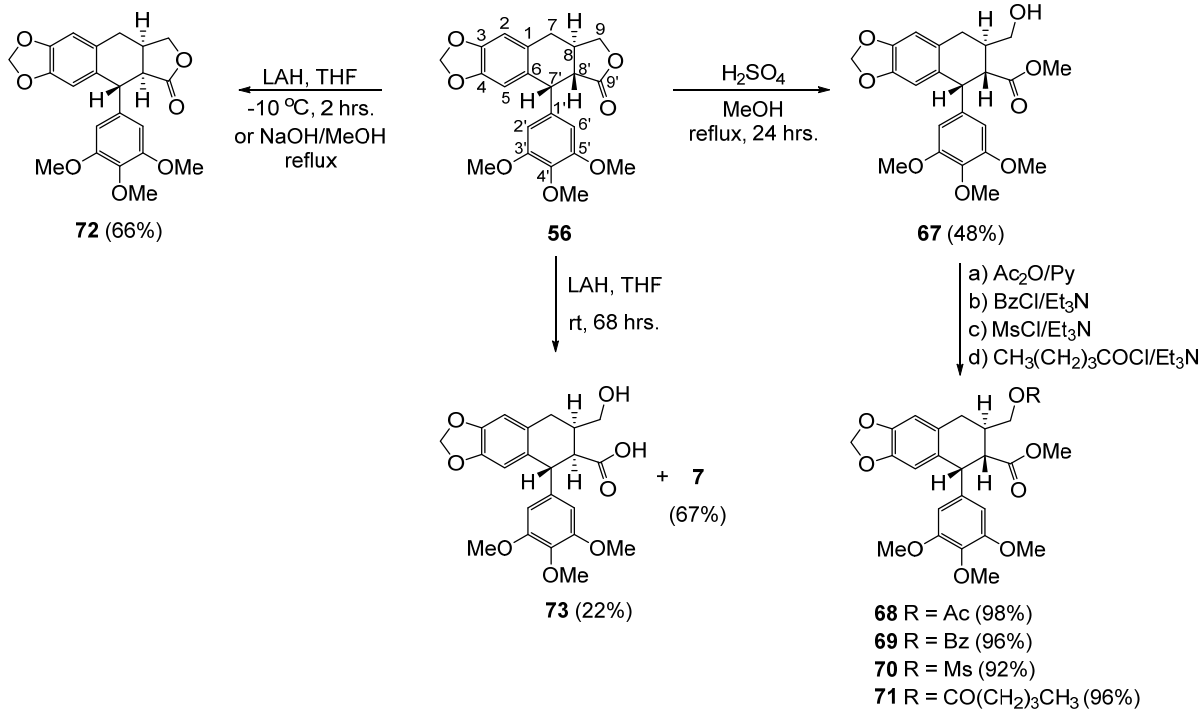




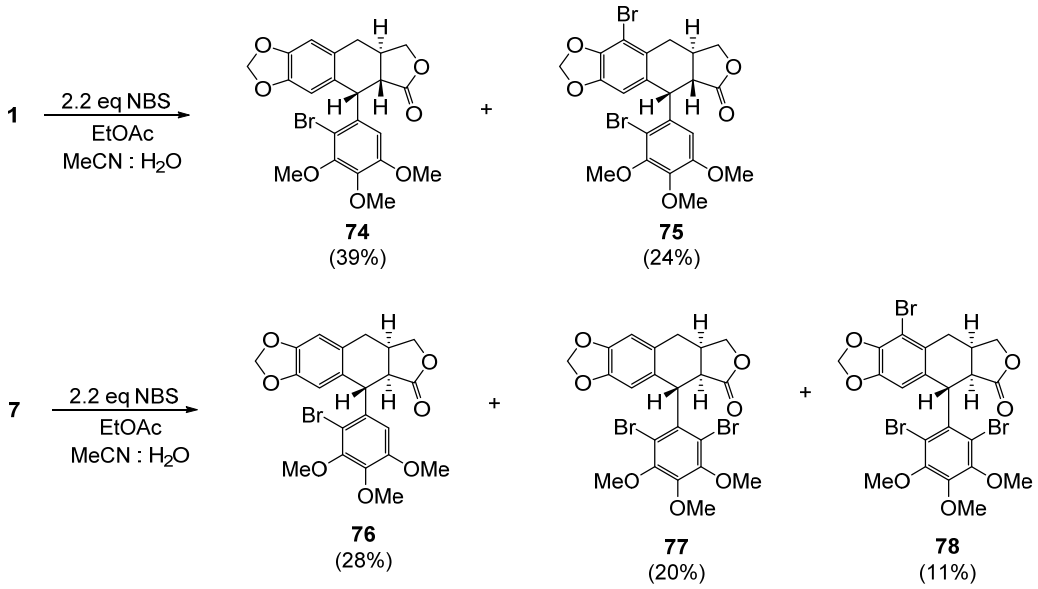
65 $R_1 = R_2 = R_3 = R_4 = OMe$
66 $R_1, R_2 = OCH_2O$, $R_3 = R_4 = OMe$

59 $R_1, R_2 = OCH_2O$, $R_3 = R_4 = OMe$, $R_5 = R_6 = H$
60 $R_1, R_2 = OCH_2O$, $R_3 = R_4 = OMe$, $R_5 = H$, $R_6 = OMe$
61 $R_1, R_2 = OCH_2O$, $R_3 = R_4 = OMe$, $R_5 = OH$, $R_6 = H$
62 $R_1, R_2 = OCH_2O$, $R_3 = H$, $R_4 = OMe$, $R_5 = R_6 = H$
63 $R_1 = R_2 = OMe$, $R_3 = H$, $R_4 = OMe$, $R_5 = R_6 = H$
64 $R_1 = OMe$, $R_2 = OH$, $R_3 = R_4 = OMe$, $R_5 = R_6 = H$

รูปที่ 3 สารที่แยกได้จากเมล็ดโพกระดิ่ง



รูปที่ 4 อนุพันธ์ลิกแนนที่สังเคราะห์ได้



รูปที่ 4 อนุพันธ์ลิกแนนที่สังเคราะห์ได้ (ต่อ)

ตารางที่ 4 ฤทธิ์ต้านเซลล์มะเร็งท่อน้ำดี (KKU-100) และมะเร็งตับ (HepG2) ของสารจากเมล็ดโพกระดิ่ง

compound	KKU—M156		HepG2	
	IC_{50} (μM)	Emax (%)	IC_{50} (μM)	Emax (%)
Crude EtOAc	8.8*	93	1.9*	62
Crude MeOH	3.6*	73	5.1*	82
56	34.6	83	8.1	84
57	15.4	99	1.7	84
58	29.7	98	4.1	74
59	5.2	96	4.5	68
61	40.1	90	16.3	83
62	24.0	81	10.6	85
63	5.4	59	5.2	78
65	16.5	77	18.2	89
66	12.5	82	12.5	71
Cisplatin	3.3	100	5.7	100

เมื่อนำสารจากเมล็ดโพกระดิ่งไปทดสอบฤทธิ์ต้านเซลล์มะเร็งท่อน้ำดีและมะเร็งตับ พบว่าสาร **59** และ **63** แสดงฤทธิ์ที่ดีมาก (IC_{50} เท่ากับ 5.2 และ 5.4 µM) เมื่อเปรียบเทียบระหว่างสาร **59** และ **61** พบว่าหมู่

ไฮดรอกซิลที่ตำแหน่ง C-7′ จะลดความสามารถในการออกฤทธิ์ เมื่อเปรียบเทียบระหว่างสาร **62** และ **63** พบว่าหมู่ OMe ที่ตำแหน่ง 3 และ 4 สำคัญต่อฤทธิ์ทางชีวภาพ

ในส่วนของฤทธิ์ต้านมะเร็งตับ สาร **57**, **58**, **59** และ **63** แสดงฤทธิ์ที่ดีมาก จากข้อมูลของสาร **56** และ **57** พบว่าสาร 57 แสดงฤทธิ์ที่ดีกว่า อาจหมายถึงว่าหมู่ α,β-unsaturated lactone สำคัญต่อการออกฤทธิ์

ตารางที่ 5 ฤทธิ์ต้านเซลล์มะเร็งท่อน้ำดี (KKU-100) และมะเร็งตับ (HepG2) ของอนุพันธ์ลิกแนน

compound	KKU-100	HepG2
56	25.78 ± 1.78	8.10 ± 2.00
67	1.63 ± 0.79	6.73 ± 1.37
68	2.01 ± 0.74	1.94 ± 1.12
69	14.96 ± 7.85	3.55 ± 1.44
70	0.84 ± 0.47	16.12 ± 4.32
71	4.47 ± 1.94	41.39 ± 10.49
72	9.04 ± 5.70	1.50 ± 0.53
73	34.58 ± 10.80	14.65 ± 7.20
74	1.84 ± 0.54	1.90 ± 0.77
75	0.75 ± 0.52	0.46 ± 0.19
76	4.82 ± 2.09	5.86 ± 1.88
77	22.6 ± 203	14.92 ± 7.37
78	1.01 ± 0.39	0.42 ± 0.29
ellipticine	25.21 ± 0.20	
cisplatin		2.2 ± 0.70

สรุปผลการทดลอง

จากการสกัดแยกรากว่านดีงูเห่า พบสารใหม่ที่เป็นอนุพันธ์คูมาริน และสารที่เป็นอนุพันธ์ dihydro chelerythrine-cadinane และยังพบสารที่ทราบโครงสร้างแล้วอีก 16 สาร จากการทดสอบฤทธิ์ทางชีวภาพ พบว่าสาร **12** แสดงฤทธิ์ฆ่าเชื้อมาลาเรียที่ดี และไม่เป็นพิษต่อเซลล์ปรกติ สาร **13** แสดงฤทธิ์ฆ่าเซลล์มะเร็ง MCF-7 ด้วยค่า IC_{50} เท่ากับ 8.7 μg/mL และไม่เป็นพิษต่อเซลล์ปรกติ ซึ่งผลการทดลองที่ได้ เป็นข้อมูลที่น่าสนใจ และอาจนำไปพัฒนาต่อยอดได้

จากการสกัดเปลือกมะนาวผีพบว่าได้สารประเภท tyramine โดยเป็นสารใหม่ 7 สาร ได้แก่ atalantums A—G และสารที่ทราบโครงสร้างแล้ว 5 สาร นำสารทั้งหมดไปทดสอบฤทธิ์ต้านมะเร็งท่อน้ำดีชนิด KKU-M214, KKU-M213 และ KKU-M156 พบว่าสาร **23** แสดงฤทธิ์ที่ดีกับเซลล์ KKU-M156 ด้วยค่า IC_{50} เท่ากับ 1.97 $\pm$ 0.73 μM ซึ่งดีกว่าสารมาตรฐาน ellipticine สาร **19** แสดงฤทธิ์ต่อเซลล์ KKU-M214 ด้วยค่า IC_{50} เท่ากับ 3.06 $\pm$ 0.51 μM สำหรับเซลล์ KKU-M213 นั้น สาร **20**, **22** และ **29** แสดงฤทธิ์ที่ดี โดยมีค่า IC_{50} เท่ากับ 2.36 $\pm$ 0.20, 5.63 $\pm$ 0.22 และ 2.71 $\pm$ 0.23 μM ตามลำดับ สาร **19**, **23** และ **25** แสดงฤทธิ์กับเซลล์ M214 ด้วยค่า IC_{50} เท่ากับ 3.06 $\pm$ 0.51, 8.44 $\pm$ 0.47 และ 7.37 $\pm$ 1.29 ตามลำดับ

จากการแยกสารจากต้นมะนาวผี และใบมะนาวผี ได้สารทั้งสิ้น 25 สาร เป็นสารใหม่ 4 สาร (เป็นไลโมนอยด์ 3 สาร ได้แก่ limonophyllines A-C และเป็นฟลาโวนอยด์ 1 สาร นอกจากนี้ยังพบสารประเภท acridone alkaloids 12 สาร เป็นสารประเภทฟลาโวนอยด์ 4 สาร นอกนั้นเป็นสารอื่นๆ ในส่วนของเมล็ดโพกระดิ่งนั้น สารที่แยกได้เป็นสารประเภทลิกแนนทั้งสิ้น จำนวน 11 สาร แต่เป็นสารที่ทราบโครงสร้างแล้วทั้งหมด

จากการทดสอบฤทธิ์ทางชีวภาพพบว่า ลิกแนนจากเมล็ดโพกระดิ่ง แสดงฤทธิ์ต้านเซลล์มะเร็งท่อน้ำดีชนิด KKU-M156 และเซลล์มะเร็งตับ HepG2 ดีที่สุด มีค่า IC_{50} ประมาณ 1.7 ถึง 5.2 μM (Emax 96%) สาร **42** และ **44** จากต้นมะนาวผีแสดงฤทธิ์ฆ่าเซลล์มะเร็งท่อน้ำดี KKU-M156 และเซลล์มะเร็งตับ HepG2 ด้วยค่า IC_{50} ในช่วง 1.43 ถึง 8.4 μg/ml ในส่วนของการทดสอบฤทธิ์การยับยั้งเอนไซม์ acetylcholine esterase พบว่า lupalbigenin แสดงความสามารถในการยับยั้ง 79% และมีค่าสูงประมาณ 1.4 เท่าเมื่อเทียบกับสารมาตรฐาน tacrine ในส่วนของ citrusinine I แสดงฤทธิ์ต้านออกซิเดชันเท่ากับ 90.68% เมื่อทดสอบด้วยวิธี ABTS assay.

เอกสารอ้างอิง

Basa, S.C. *Phytochemistry* 1975, 14, 835–836.

Braga, P.A.C., Dos Santos, D.A.P., Da Silva, M.F.D.G.F., Vieira, P.C., Fernandes, J.B., Houghton, P. J., Fang, R. *In vitro* cytotoxicity activity on several cancer cell lines of acridone alkaloids and *N*-phenylethyl-benzamide derivatives from *Swinglea glutinosa* (Bl.) Merr. *Nat. Prod. Res.* 2007, 21(1), 47—55.

Bunyapraphatsara, N. Traditional Herb; Prachachon Printing: Thailand, 1999, Vol 3, p 568.

Cerqueira, C.N., Santos, D.A.P., Malaquias, K.S., Lima, M.M.C., Silva, M.F.G.F., Vieira, J.B.F.P.C. Noval *N*-benzoyl tyramine from *Swinglea glutinosa* (Rutaceae). *Quim. Nova* 2012, 35(11), 2181—2185.

Dryer, D.L., Rigod, J.F., Basa, S.C., Mahanty, P., Das, D.P. Chemotaxonomy of the Rutaceae-XII. *Tetrahedron* 1980, 36(6), 827—829.

Franke, K., Porzel, A., Masaoud, M., Adam, G., Schmidt, J.,. Furanocoumarins from *Dorstenia gigas*. Phytochemistry 2001, 56, 611—621.

Gao, Y., Liu, Y., Wang, Z.G., Zhang, H.L. Chemical constituents of *Heracleum dissectum* and their cytotoxic activity. Phytochem. Lett. 2014, 10, 276—280.

Genovese, S., Epifano, F., Curini, M., Dudra-Jastrzebska, M., Luszczki, J.J., Prenyloxyphenylpropanoids as a novel class of anticonvulsive agents. Bioorg. Med. Chem. Lett. 2009, 19, 5419-5422.

Ghosh, P., Ghosh, M. K., Thakur, S., Datta, J. D., Akihisa, T., Tamura, T., Kimura, Y. Dihydroxy acidissiminol and acidissiminol epoxide, two tyramine derivatives from *Limonia acidissima*. *Phytochemistry* 1994, 37(3), 757—760.

Ghosh, P., Sil, P., Das, S., Thakur, S., Kokke, W.C.M.C., Akihisa, T., Shimizu, N., Tamura, T., Matsumoto, T. Tyramine derivatives from the fruit of *Limonia acidissima*. *J. Nat. Prod.* 1991, 54, 1389—1393.

Heinke, R., Franke, K., Porzel, A., Wessjohann, L.A., Ali, N.A.A., Schmidt, J., Furanocoumarins from *Dorstenia foetida*. Phytochemistry 2011, 72, 929—934.

Hu, J., Shi, X., Chen, J., Mao, X., Zhu, L., Yu, L., Shi, J., Alkaloids from *Toddalia asiatica* and their cytotoxic, antimicrobial and antifungal activities. Food Chem. 2014, 148, 437—444.

Ishii, H., Kobayashi, J., Ishikawa, T., Toddalenone: a new coumarin from *Toddalia asiatica* (*T. aculeata*) structure establishment based on the chemical conversion of limettin into toddalenone. Chem. Pharm. Bull. 1983, 31, 3330—3333.

Li, W., Zhou, W., Shim, S. H., Kim, Y. H., Chemical constituents of *Zanthoxylum schinifolium* (Rutaceae). Biochem. Syst. Ecol. 2014, 55, 60–65.

Lukas, B., Schmiderer, C., Novak, J. Essential oil diversity of European *Origanum vulgare* L. (Lamiaceae). Phytochemistry, 2015, 119, 32–40.

Lv, H.N., Wang, S., Zeng, K.W., Li, J., Guo, X.Y., Ferreira, D., Zjawiony, J.K., Tu, P.F., Jiang, Y. Anti-inflammatory coumarin and benzocoumarin derivatives from *Murraya alata*. J. Nat. Prod. 2015, 78, 279–285.

Panda, H. Handbook on Medicinal Herbs with Uses. Asia Pacific Business Press Inc: 2004, p 166–167.

Phatchana, R., Yenjai, C., Cytotoxic coumarins from *Toddalia asiatica*. Planta med. 2014, 80, 719–722.

Vazquez, R., Riveiro, M.E., Vermeulen, M., Mondillo, C., Coombes, P.H., Crouch, N.R., Ismail, F., Mulholland, D.A., Baldi, A., Shayo, C., Davio, C., Toddaculin, a natural coumarin from *Toddalia asiatica*, induces differentiation and apoptosis in U-937 leukemic cells. Phytomedicine 2012, 19, 737–746.

Wang, F., Xu, Y., Liu, J.K., New geranyloxycoumarins from *Toddalia asiatica*. J. Asian Nat. Prod. Res. 2009, 11, 752–756.

Zou, H.L., Li, H.Y., Liu, B.L., Zhou, G.X., A new cytotoxic benzophenanthridine isoquinoline alkaloid from the fruits of *Macleaya cordata*. J. Asian Nat. Prod. Res. 2015, 17, 856–860.

ภาคผนวก

ผลงานตีพิมพ์

1. Sombatsri, A., Thummanant, Y., Sribuhom, T., Wongphakham, P., Senawong T., Yenjai C. Atalantums H-K from the peels of *Atalantia monophylla* and their cytotoxicity Natural Product Research, 2019, DOI:10.1080/14786419.2019.1576042

2. Posri, P., Suthiwong, J., Takomthong, P., Wongsa, C., Chuenban, C., Boonyarat, C., Yenjai, C*. A new flavonoid from the leaves of *Atalantia monophylla* (L.) DC*,* Natural Product Research, 2018, DOI:10.1080/14786419.2018.1457667
3. Suthiwong J, Wandee J, Pitchuanchom S, Sojikul P, Kukongviriyapan V, Yenjai C*. Cytotoxicity against cholangiocarcinoma and HepG2 cell lines of lignan derivatives from *Hernandia nymphaeifolia*. Medicinal Chemistry Research, 2018, 27, 2042-2049.
4. Sombatsri, A., Thummanant, Y., Sribuhom, T., Boonmak, J., Youngme, S., Phusrisom, S., Kukongviriyapan, V., Yenjai, C*. New limonophyllines A-C from the stem of *Atalantia monophylla* and cytotoxicity against cholangiocarcinoma and HepG2 cell lines, Archives of Pharmacal Research, 2018, 41, 431-437.
5. Suthiwong, J., Boonloh, K., Kukongviriyapan, V., Yenjai, C*. Cytotoxicity against Cholangiocarcinoma and HepG2 Cell Lines of Lignans from *Hernandia nymphaeifolia*, Natural Product Communication, 2018, 13 (1), 61-63.
6. Sukieum, S., Sang-aroon W., Yenjai C*. Coumarins and alkaloids from the roots of *Toddalia asiatica,* Natural Product Research, 2018, 32(8), 944-952.
7. Sribuhom, T., Boueroy, P., Hahnvajanawong, C., Phatchana, R., Yenjai, C*. Benzoyltyramine alkaloids atalantums A-G from the peels of *Atalantia monophylla* and their cytotoxicity against cholangiocarcinoma cell lines, Journal of Natural Products, 2017, 80, 403–408.
8. Phatchana R., Thongsri Y., Yenjai C*. Canangalias C-H, juvenile hormone III analogues from the roots of *Cananga latifolia.* Fitoterapia, 2016, 114, 45-50.
9. Hirunwong, C., Sukieum, S., Phatchana, R., Yenjai C*. Cytotoxic and antimalarial constituents from the roots of *Toddalia asiatica.* Phytochemistry Letters, 2016, 17, 242-245.

NATURAL PRODUCT RESEARCH
https://doi.org/10.1080/14786419.2019.1576042

Taylor & Francis
Taylor & Francis Group

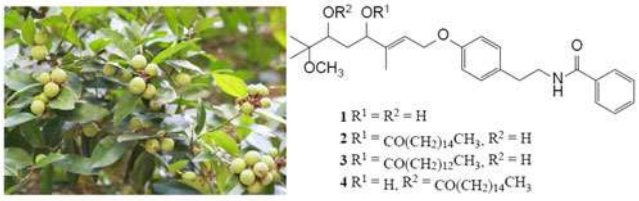
Atalantums H-K from the peels of *Atalantia monophylla* and their cytotoxicity

Aonnicha Sombatsri[a], Yutthapong Thummanant[a], Thurdpong Sribuhom[a], Paweena Wongphakham[b], Thanaset Senawong[b] and Chavi Yenjai[a]

[a]Natural Products Research Unit, Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Khon Kaen University, Khon Kaen, Thailand; [b]Natural Products Research Unit, Department of Biochemistry, Faculty of Science, Khon Kaen University, Khon Kaen, Thailand

ABSTRACT

Four new benzoyltyramines, atalantums H-K (**1–4**) and seven known compounds were isolated from the peels of *Atalantia monophylla*. All compounds were tested for cytotoxicity against HeLa, HCT116 and MCF-7 cell lines, as well as normal cells (Vero cells). Compound **5** showed cytotoxicity against HeLa, HCT116 and MCF-7 cell lines with IC_{50} values ranging from 16-25 μg/mL but was inactive against Vero cells. Compound **6** also showed interesting results as compound **5** with IC_{50} values ranging from 15-18 μg/mL and an IC_{50} value of 80.20 μg/mL against Vero cells. This means compounds **5** and **6** can be used as lead compounds for anticancer agents.



Four new atalantums H-K (**1-4**) were isolated from the peels of *Atalantia monophylla*.

ARTICLE HISTORY
Received 7 December 2018
Accepted 26 January 2019

KEYWORDS
Atalantia monophylla; atalantum; benzoyltyramine

1. Introduction

Atalantia monophylla is a plant in the family Rutaceae. It is a small woody climber which has a brown stem full of thorns. The fruits and leaves look and smell similar to lime, although the fruits are not edible. It can be found in South India, Sri Lanka, East Bengal, Ceylon, and Southeast Asia including Thailand, where it is called "Ma Nao Phee" (Bunyapraphatsara 1999; Panda 2004). Various parts of this plant have been used in traditional medicine for several purposes. For instance, this plant is used as an

CONTACT Chavi Yenjai chayen@kku.ac.th Natural Products Research Unit, Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Khon Kaen University, Khon Kaen, 40002, Thailand

Supplemental data for this article can be accessed at https://doi.org/10.1080/14786419.2019.1576042.

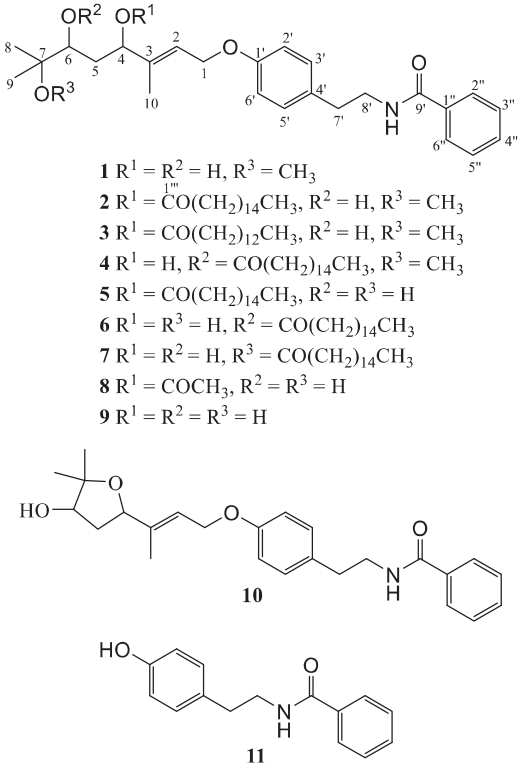


Figure 1. Structures of compounds **1–11**.

antispasmodic, and is also used to cure paralysis, hemiplegia, and chronic rheumatism (Basa 1975). Oil from the fruits can be used to treat rheumatism and respiratory diseases (Basa 1975). Pathogenic fungi and itching can also be treated using the oil from its leaves (Panda 2004). There are many groups of compounds that have been isolated from *A. monophylla* comprising limonoids, acridone alkaloids, furoquinoline alkaloids (Kumar et al. 2010), coumarins, flavonoids (Posri et al. 2018) and benzoyltyramines (Govindachari et al. 1970; Sribuhom et al. 2017). In this study, eleven *N*-benzoyltyramine derivatives including four new compounds (**1 – 4**) and seven known compounds (**5 – 11**), were isolated. All isolated compounds were evaluated for cytotoxicity against HeLa (cervical cancer), HCT116 (colon cancer), MCF-7 (breast cancer), and Vero cell lines (Kumnerdkhonkaen et al. 2018).

2. Results and discussion

2.1. Chemistry

Phytochemical investigation of the MeOH extract of the peels of *A. monophylla* led to the isolation of four new benzoyltyramines, atalantums H-K (**1–4**) (Figure 1). Seven known compounds including atalantums D-G (**5–7, 10**) (Sribuhom et al. 2017), *N*-{2-[4-(4-acetoxy-6,7-dihydroxy-3,7-dimethyl-2-octen-1-yl)oxy]phenyl}-ethyl benzamide (**8**) (Cerqueira et al. 2012), *N*-{2-[4-(4,6,7-trihydroxy-3,7-dimethyl-2-octen-1-yl)oxy]phenyl}ethyl benzamide (**9**) (Ghosh et al. 1994) and *N*-benzoyltyramine (**11**) (Cerqueira et al.

2012) were also discovered. The specific rotations of **1–10** showed $[\alpha]_D^{25}$ ± 0 which confirmed the racemic mixture of all compounds.

Compound **1** was found as a pale yellow oil, and its molecular formula was determined as $C_{26}H_{35}O_5N$ by HRESIMS data which showed a molecular ion at *m/z* $[M + Na]^+$ 464.2405 (calcd. 464.2413). The IR spectrum showed absorption bands of hydroxyl and carbonyl groups at 3341 and 1736 cm^{-1}, respectively. Five aromatic protons at δ 7.66 (2 H, d, $J = 8.0$ Hz), δ 7.38 (2 H, t, $J = 8.0$ Hz) and δ 7.46, (1 H, t, $J = 8.0$ Hz) were assigned as H-2″/H-6″, H-3″/H-5″ and H-4″, respectively (Table S1). Two doublet signals at δ 7.12 (2 H, d, $J = 8.0$ Hz, H-3′/H-5′) and δ 6.84 (2 H, d, $J = 8.0$ Hz, H-2′/H-6′) indicated a para-substituted benzene. In the HMBC experiment, correlations between H-2″/H-6″ and amide carbonyl at δ 167.2 (C-9′) indicated it contained a benzoyl moiety (Figure S1). Two triplet signals ($J = 8.0$ Hz) at δ 2.84 (H-7′) and 3.65 (H-8′) correlated to carbon at δ 34.1 and δ 40.6, respectively, in the HMQC spectrum. Correlation between H-8′ and amide carbonyl (δ 167.2) indicated the connection of this ethyl group and the benzamide moiety. The ^1H and ^{13}C NMR spectra showed signals at δ_H/δ_C 3.20/48.6, which were assigned to a methoxy group (Table S2). From the ^{13}C NMR and DEPT spectra, it was found that the remaining ten carbons signals contained three methyl, two methylene, two methine, two olefinic and one quaternary carbons. The oxygenated methylene signal at δ_H/δ_C 4.54/64.0 was assigned as H-1 and coupled to the olefinic proton H-2 (δ 5.76, t, $J = 8.0$ Hz). Correlations between H-1 and C-2 (120.3), C-3 (141.3) and C-1′ (156.8) were observed in the HMBC experiment. The methyl group at δ_H/δ_C 1.72/11.8 (CH_3-10) showed long range coupling with C-2 (120.3), C-3 (141.3) and C-4 (76.5) in this experiment. In the HMQC spectrum, the proton signals at δ 4.27 and δ 3.64 connected with carbons at δ 76.5 and δ 76.9, respectively, confirming the presence of dihydroxy group at C-4 and C-6 positions. Terminal dimethyl group CH_3-8 (δ_C 19.6) and CH_3-9 (δ_C 18.7) correlated with C-7 (76.5) in the HMBC spectrum. In addition, the correlation between the methoxy proton and C-7 was observed in the HMBC experiment. Therefore, compound **1**, atalantum H, was characterized as *rac-N*-{2-[4-(4,6-dihydroxy-7-methoxy-3,7-dimethyl-2-octen-1-yl)oxy]phenyl}ethylbenzamide (Figure 1).

Compound **2** showed a molecular ion at *m/z* 702.4700 $[M + Na]^+$ (calcd. 702.4710) indicative of the molecular formula $C_{42}H_{65}O_6N$ and corresponding to 11 indices of hydrogen deficiency. The ^1H and ^{13}C NMR spectra showed the same patterns as those of **1**, except for the presence of a palmitoyloxy moiety instead of a hydroxyl group at the C-4 position. The ^{13}C NMR spectrum also displayed an additional ester-type carbonyl carbon at δ 172.9 (C-1‴). In the HMBC spectrum, cross-peaks between H-4 and C-1‴ were observed. The rest of the proton and carbon signals were consistent with the hydrocarbon $[(CH_2)_{14}CH_3]$ chain of palmitic acid. Therefore, compound **2**, atalantum I, was characterized as *rac-N*-{2-[4-(4-palmitoyloxy-6-hydroxy-7-methoxy-3,7-dimethyl-2-octen-1-yl)oxy]phenyl}ethylbenzamide (Figure 1).

The HRESIMS data of compound **3** indicated a molecular formula of $C_{40}H_{61}O_6N$ (*m/z* 674.4396 $[M + Na]^+$) (calcd. 674.4397). The ^1H and ^{13}C NMR spectra displayed the same patterns as those of **2**, except for resonances reminiscent of a myristoyloxy instead of a palmitoyloxy moiety. Thus, the structure of compound **3**, atalantum J, was identified as *rac-N*-{2-[4-(4-myristoyloxy-6-hydroxy-7-methoxy-3,7-dimethyl-2-octen-1-yl)oxy]phenyl}ethyl benzamide.

The HRESIMS data indicated a molecular formula of $C_{42}H_{65}O_6N$ (*m/z* 702.4703 $[M+Na]^+$) (calcd. 702.4710) for compound **4**. The ^1H and ^{13}C NMR spectra displayed the same patterns as those of **2**, except for the cross-peaks in the HMBC spectrum. Correlation between H-6 and C-1‴ (δ 173.7) was evident in this spectrum. Thus, the structure of compound **4**, atalantum K, was identified as *rac-N*-{2-[4-(6-palmitoyloxy-4-hydroxy-7-methoxy-3,7-dimethyl-2-octen-1-yl)oxy]phenyl}ethylbenzamide.

2.2. Biological activity

All isolated compounds were evaluated for cytotoxicity against HeLa (cervical cancer), HCT116 (colon cancer), MCF-7 (breast cancer) and Vero cells (normal cells) by MTT assay. The results at 72 hr. showed compound **5** exhibited cytotoxicity against HeLa, HCT116 and MCF-7 cell lines with IC_{50} values ranging from 16 to 25 μg/mL and displayed an IC_{50} value of more than 100 μg/mL against normal cells (Table S3). This indicates this compound can be used as a lead compound for an anti-cancer agent. Comparing between **5** and **2**, compound **2** showed inactivity against these cell lines. The results indicate that the hydroxyl group may play an important role for cytotoxicity. This evidence can be observed in the cases of compounds **6** and **4**. The IC_{50} values of **6** were 15-18 μg/mL against HeLa, HCT116 and MCF-7 cell lines and showed an IC_{50} value of 80.20 μg/mL against Vero cells. These data confirmed that the hydroxyl group may play an important role for cytotoxicity. In the case of cytotoxicity against Vero cells of compounds **6** and **7**, compound **7** showed stronger cytotoxicity with an IC_{50} value of 25.20 μg/mL. These results mean the palmitoyloxy group at the C-7 position selected to Vero cells. Comparing between compounds **9** and **10**, compound **10** displayed stronger cytotoxicity against all cell lines than **9**. This evidence shows that the tetrahydrofuran moiety was necessary for the activity.

3. Experimental

3.1. General experimental procedures

The NMR spectra were recorded on a Varian Mercury plus spectrometer operating at 400 MHz (^1H) and at 100 MHz (^{13}C). IR spectra were recorded as thin films, using a Perkin Elmer Spectrum One FT-IR spectrophotometer. Mass spectra were determined on a Micromass Q-TOF 2 hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer with a Z-spray ES source (Micromass, Manchester, UK). UV spectra were measured on an Agilent 8453 UV-Visible spectrophotometer. Melting points were determined on a SANYO Gallenkamp melting point apparatus and were uncorrected. Thin layer chromatography (TLC) was carried out on MERCK silica gel 60 F254 TLC aluminum sheet. Column chromatography was done with silica gel 0.063-0.200 mm or less than 0.063 mm and RP-18 column chromatography was also used. Preparative layer chromatography (PLC) was carried out on glass supported silica gel plates using silica gel 60 PF254 for preparative layer chromatography. All solvents were routinely distilled prior to use.

3.2. Plant material

The peels of *A. monophylla* were collected in June 2016 from Phuwieng District, Khon Kaen Province, Thailand. The plant was identified by Prof. Dr. Pranom Chantaranothai, Faculty of Science, Khon Kaen University, Thailand. A botanically identified voucher specimen (KKU022015) was deposited at Faculty of Science, Khon Kaen University.

3.3. Extraction and isolation

Air-dried and finely powdered peels (2 kg) of *A. monophylla* were sequentially extracted at room temperature for three days with hexane (3 × 12 L), EtOAc (3 × 5 L) and MeOH (3 × 5 L). The extracts were evaporated *in vacuo* to obtain three dry extracts, crude hexane (121 g), EtOAc (270 g), and MeOH (220 g). The crude MeOH extract (220 g) was subjected to CC, eluted with a gradient system of hexane, hexane-EtOAc and EtOAc-MeOH. On the basis of their TLC characteristic, the fractions which contained the same major compounds were combined to give eight fractions, F1-F8. Fraction F3 was purified by silica gel column chromatography and eluted with pure CH_2Cl_2 to get three subfractions, F3.1–F3.3. Subfraction F3.2 was purified by FCC using 20% EtOAc:hexane as eluent to afford two subfractions. Fraction F4 was purified by silica gel CC and eluted with 30% EtOAc:hexane to get three subfractions, F4.1-F4.3. Subfraction F4.2 was subjected to a column of Sephadex LH-20 using MeOH as eluent and gave three subfractions, F4.2.1-F4.2.3. Subfraction F4.2.2 was rechromatographed on FCC, by silica gel reverse phase (RP-18) eluted with 10% H_2O:MeOH to afford **1** (12.3 mg, 0.00059%), **2** (18.4 mg, 0.00088%) and **3** (60.0 mg, 0.00285%). Fraction F5 was purified by silica gel CC and eluted with 1% MeOH:CH_2Cl_2 to give four subfractions, F5.1-F5.4. Subfraction F5.1 was subjected to a column of Sephadex LH-20, using MeOH as eluent and gave **9** (18.5 mg, 0.00088%). Gel filtration (Sephadex LH-20) was carried out on subfraction F5.2, eluting with MeOH afforded **11** (12.8 mg, 0.00060%). Subfraction F5.3 was rechromatographed on FCC by silica gel reverse phase (RP-18) eluted with 10% H_2O:MeOH to afford **5** (14.6 mg, 0.00069%), **6** (13.5 mg, 0.00064%) and **7** (15.8 mg, 0.00075%). Fraction F6 was purified by silica gel CC and eluted with 1% MeOH:CH_2Cl_2 to give three subfractions, F6.1-F6.3. Subfraction F6.3 was subjected to a column of Sephadex LH-20 using MeOH as eluent and gave three subfractions, F6.3.1-F6.3.3. Subfraction F6.3.2 was purified by silica gel FCC and eluted with 1% MeOH:CH_2Cl_2 to obtain **10** (12.2 mg, 0.00058%) and three subfractions, F6.3.2.1-F6.3.2.3. Both subfractions F6.3.2.1 and F6.3.2.2 were further purified by FCC (3% MeOH:CH_2Cl_2) and gave **4** (3.0 mg, 0.00014%) and **8** (17.5 mg, 0.00083%), respectively.

3.4. Spectroscopic data of compounds

Atalantum H (**1**): Colorless oil; UV ($CHCl_3$) λ_{max} (log ε) 242 (3.81), 277 (3.35) nm; IR (neat) ν_{max} 3341, 2926, 1736, 1641, 1511, 1237, 1073, 772 cm^{-1}; HRESIMS *m/z* 464.2405 $[M + Na]^+$ (calcd. 464.2413); ^1H and ^{13}C NMR spectroscopic data, see Tables S1 and S2.

Atalantum I (**2**): Colorless oil; UV ($CHCl_3$) λ_{max} (log ε) 242 (3.71), 276 (3.24) nm; IR (neat) ν_{max} 3344, 2924, 1736, 1641, 1511, 1221, 772 cm^{-1}; HRESIMS *m/z* 702.4700 $[M + Na]^+$ (calcd. 702.4710); ^1H and ^{13}C NMR spectroscopic data, see Tables S1 and S2.

Atalantum J (**3**): Colorless oil; UV ($CHCl_3$) λ_{max} (log ε) 242 (4.05), 277 (3.61) nm; IR (neat) ν_{max} 3341, 2923, 2853, 1731, 1642, 1511, 1231, 1075, 772 cm^{-1}; HRESIMS *m/z* 674.4396 $[M + Na]^+$ (calcd. 674.4397); ^1H and ^{13}C NMR spectroscopic data, see Tables S1 and S2.

Atalantum K (**4**): Colorless oil; UV ($CHCl_3$) λ_{max} (log ε) 242 (3.92), 277 (3.46) nm; IR (neat) ν_{max} 3344, 2924, 2853, 1732, 1642, 1511, 1237, 772 cm^{-1}; HRESIMS *m/z* 702.4703 $[M + Na]^+$ (calcd. 702.4710); ^1H and ^{13}C NMR spectroscopic data, see Tables S1 and S2.

3.5. Cytotoxic activity assay

Cytotoxic effect on cancer cells was evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells (8×10^3 cells/well) were seeded onto 96-well plates and incubated for 24 hours to adhere. Human cervical cancer (HeLa) cell line was obtained from Dr. C. Pientong (Khon Kaen University, Khon Kaen, Thailand). Human breast adenocarcinoma (MCF-7) and human colon cancer (HCT116) cell lines were kindly provided by Dr. O. Tetsu (University of California, San Francisco, U.S.A.). A non-cancer (Vero) cell line was kindly provided by Dr. S. Barusrux (Khon Kaen University, Khon Kaen, Thailand), respectively. All cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) (Gibco-BRL, USA) and incubated at 37 °C in a humidified atmosphere of 5% CO_2. For preliminary testing, cells were exposed to the selected compounds at a concentration of 100 μg/mL for 24, 48 and 72 hours. The compounds that caused cell viability less than 50% were further evaluated for their half maximal inhibitory concentration (IC_{50}) values. To evaluate IC_{50} values, cells were exposed to increasing concentrations (10 - 100 μg/mL) of selected compounds for 24, 48 and 72 hours. Control groups were treated with a solvent (a mixture of DMSO and ethanol; 1:1). After incubation for the indicated times, the medium was replaced with 110 μL of fresh medium containing MTT (0.5 mg/mL in PBS) (Sigma Chemical Co., St Louis, MO, USA) and incubated for 2 h. Formazan formed after conversion of MTT was dissolved in DMSO. The absorbance of formazan was measured with a microplate reader (Bio-Rad Laboratories, USA) at the wavelength of 550 nm using 655 nm as a reference wavelength. Each assay was replicated four times. The percentage of viable cells which corresponds to the production of formazan was calculated as previously described (Kumnerdkhonkaen et al. 2018):

$$\%\ \text{Cell viability} = [\text{Sample } (A_{550} - A_{655})/\text{Control } (A_{550} - A_{655})] \times 100$$

4. Conclusion

Chemical investigation of the methanol extract of the peels of *Atalantia monophylla* led to the isolation of four new benzoyltyramines, atalantums H-K (**1**–**4**) and seven known compounds. Cytotoxicity against HeLa, HCT116 and MCF-7 cell lines, as well as normal cells (Vero cells) was evaluated using MTT assay. The results showed that **5** exhibited cytotoxicity against HeLa, HCT116 and MCF-7 cell lines with IC_{50} values ranging from 16-25 μg/mL but was inactive against Vero cells. In addition, **6** also showed IC_{50} values ranging from 15-18 μg/mL and an IC_{50} value of 80.20 μg/mL against Vero

cells. From all data, it means the hydroxyl group may play an important role for cytotoxicity.

Acknowledgements

We thank the Science Achievement Scholarship of Thailand (SAST) for the support to A. Sombatsri. We acknowledge the Thai Research Fund (RSA5980022) and Khon Kaen University for financial support. The Center of Excellence for Innovation in Chemistry (PERCH-CIC), Office of the Higher Education Commission, Ministry of Education is gratefully acknowledged.

Disclosure statement

The authors declare no conflict of interest.

Funding

This work was partially supported by the Science Achievement Scholarship of Thailand [SAST] for A. Sombatsri and Thailand Research Fund and Khon Kaen University [RSA5980022].

References

Basa SC. 1975. Atalaphyllinine, a new acridone base from *Atalantia monophylla*. Phytochemistry. 14(3):835–836.

Bunyapraphatsara N. 1999. Traditional herb, Vol. 3. Thailand: Prachachon Printing, pp. 568.

Cerqueira CDN, Santos DAPD, Malaquias KDS, Lima MMDC, Silva MFDGFD, Fernandes JB, Vieira PC. 2012. Novels *N*-benzoyltyramines of *Swinglea glutinosa* (Rutaceae). Quím Nova. 35(11): 2181–2185.

Ghosh P, Ghosh MK, Thakur S, Datta JD, Akihisa T, Tamura T, Kimura Y. 1994. Dihydroxy acidissiminol and acidissiminol epoxide, Two tyramine derivatives from *Limonia acidissima*. Phytochemistry. 37(3):757–760.

Govindachari TR, Viswanathan N, Pai BR, Ramachandran VN, Subramaniam PS. 1970. Alkaloids of *Atalantia monyphylla*. Tetrahedron. 26(12):2905–2910.

Kumar TSSPNSS, Krupadanam GLD, Kumar KA. 2010. 5-Hydroxydictamnine, a new alkaloid from *Atalantia monophylla*. Nat Prod Res. 24(16):1514–1517.

Kumnerdkhonkaen P, Saenglee S, Asgar MA, Senawong G, Khongsukwiwat K, Senawong T. 2018. Antiproliferative activities and phenolic acid content of water and ethanolic extracts of the powdered formula of *Houttuynia cordata* Thunb. fermented broth and *Phyllanthus emblica* Linn. Fruit. BMC Complem Altern Med. 18:130. https://doi.org/10.1186/s12906-018-2185-x.

Panda H. 2004. *Handbook on Medicinal Herbs with Uses*. Delhi: Asia Pacific Business Press Inc, pp. 166–167.

Posri P, Suthiwong J, Takomthong P, Wongsa C, Chuenban C, Boonyarat C, Yenjai C. 2018. A new flavonoid from the leaves of *Atalantia monophylla* (L.) DC. *Nat Prod Res*. 1: 1–7. DOI: 10.1080/14786419.2018.1457667.

Sribuhom T, Boueroy P, Hahnvajanawong C, Phatchana R, Yenjai C. 2017. Benzoyltyramine alkaloids atalantums A-G from the peels of *Atalantia monophylla* and their cytotoxicity against cholangiocarcinoma cell lines. J Nat Prod. 80(2):403–408.

NATURAL PRODUCT RESEARCH, 2018
https://doi.org/10.1080/14786419.2018.1457667

Taylor & Francis
Taylor & Francis Group

A new flavonoid from the leaves of *Atalantia monophylla* (L.) DC

Priyapan Posri[a], Jittra Suthiwong[a], Pitchayakarn Takomthong[b], Chatchawan Wongsa[a], Chindawadee Chuenban[a], Chantana Boonyarat[b] and Chavi Yenjai[a]

[a]Natural Products Research Unit, Faculty of Science, Department of Chemistry and Center of Excellence for Innovation in Chemistry, Khon Kaen University, Khon Kaen, Thailand; [b]Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, Thailand

ABSTRACT

A new flavonoid, atalantraflavone (**1**) as well as eight known compounds including atalantoflavone (**2**), racemoflavone (**3**), 5,4′-dihydroxy-(3″,4″-dihydro-3″,4″-dihydroxy)-2″,2″-dimethylpyrano-(5″,6″:7,8)-flavone (**4**), lupalbigenin (**5**), anabellamide (**6**), citrusinine I (**7**), *p*-hydroxybenzaldehyde (**8**), and frideline (**9**), were isolated from the leaves of *Atalantia monophylla* (L.) DC. Focusing on Alzheimer's disease, acetylcholine esterase (AChE) inhibition and antioxidant activity were evaluated using the modified Ellman's method and the ABTS scavenging assay, respectively. It was found that isoflavonoid **5**, lupalbigenin, showed 79% inhibition to AChE and was 1.4-fold stronger than the tacrine standard. In addition, acridone **7**, citrusinine I, displayed 90.68% antioxidant activity.

ARTICLE HISTORY
Received 15 January 2018
Accepted 21 March 2018

KEYWORDS
Atalanta monophylla; flavonoid; acetylcholine esterase; antioxidant

1. Introduction

Atalanta monophylla (L.) DC belongs to the family Rutaceae and known in Thai as 'Ma Nao Phee'. It has been used to treat chronic rheumatism, paralysis, hemiplegia, and as an anti-spasmodic (Basa 1975). Oil from the leaves is used to treat some pathogenic fungi and itching (Panda 2004). It has been reported that the leaves of this plant contain triterpenoids, steroids, and flavonoids (Talapatra et al. 1970; Saraswathy et al. 1998). Limonoids, acridone alkaloids,

CONTACT Chavi Yenjai chayen@kku.ac.th
Supplemental data for this article can be accessed at https://doi.org/10.1080/14786419.2018.1457667.

furoquinoline alkaloids (Kumar et al. 2010), coumarins, and benzoyltyramines have been isolated from this plant (Govindachari et al. 1970; Sribuhom et al. 2017).

Alzheimer's disease, a neurodegenerative disease, affects millions of elderly people (Khoobi et al. 2015). This disease causes loss of language skills, attention, depression and memory. It is believed that a low level of acetylcholine (ACh), deposition of β–amyloid plaques, formation of neurofibrillary tangles containing tau protein, and oxidative stress may cause this disease (Lan et al. 2014). The ester bond in acetylcholine can be cleaved by acetylcholinesterase (AChE) enzyme. The inhibition of AChE results in a rising ACh level in the brain (Anand and Singh 2013). Thus, an AChE inhibitor is one of the strategies that is used to treat Alzheimer's disease. Some agents such as tacrine, serine, donepezil, rivastigamine, and galantamine are used to treat Alzheimer's patients. However these agents show side effects such as diarrhea, nausea and vomiting (Rogers et al. 1998). Thus, AChE inhibitors, especially from natural sources, are still necessary. Nowadays, many studies have reported the role of free radical formation and oxidative cell damage in the pathogenesis of Alzheimer's disease. It has been found that oxidative stress plays a key role in initiating the aggregation of Ab and tau protein hyperphosphorylation, involved in the early stage of the pathologic cascade. Thus, antioxidants have also been an important target for Alzheimer's disease treatment (Tan et al. 2003).

In continuation of our work on bioactive constituents from Thai herbal plants, the chemical constituents of the leaves of *A. monophylla* were isolated and evaluated for their acetylcholine esterase inhibition, and antioxidant activities (Sribuhom et al. 2016).

2. Results and discussion

Nine isolated compounds were obtained from the leaves of *A. monophylla* using chromatographic methods. Among these, a new flavonoid (**1**) and eight known compounds including atalantoflavone (**2**) (Kapche et al. 2017), racemoflavone (**3**) (Banerji et al. 1988), 5,4′-dihydroxy-(3″,4″-dihydro-3″,4″-dihydroxy)-2″,2″-dimethylpyrano-(5″,6″:7,8)-flavone (**4**) (Kassem et al. 2000), lupalbigenin (**5**) (Tedasen et al. 2016), anabellamide (**6**) (Nwodo et al. 2014), citrusinine I (**7**) (Shan et al. 2013), *p*-hydroxybenzaldehyde (**8**), and frideline (**9**) were identified (Figure 1). All compounds were evaluated for acetylcholine esterase inhibition and antioxidant activity.

Atalantraflavone (**1**) was obtained as a yellow solid and displayed a molecular formula at *m/z* 337.1075 $[M + H]^+$ indicating a molecular formula of $C_{20}H_{16}O_5$. The IR spectrum showed the absorption bands of a carbonyl group at 1657 cm^{-1}. The ^1H and ^{13}C NMR spectra indicated the characteristics of flavonoid skeleton by showing 4-chromone moiety and a 1,4-disubstituted benzene group. The ^1H NMR spectrum shows two doublet signals ($J = 8.0$ Hz) at δ 7.68 (H-2′ and 6′) and at δ 6.85 (H-3′ and 5′) which indicates 1,4-disubstituted benzene (Table S1). The ^{13}C NMR spectrum displayed two overlapping resonances accounting for two carbons each at δ 128.2 (C-2′ and 6′) and at δ 116.0 (C-3′ and 5′). The HMBC spectrum showed the correlations of H-2′ and H-6′ with C-2 (δ 164.3) which indicated the connection of aryl group at C-2 of chromone moiety. Two singlet signals of chromone moiety at δ 6.43 and 6.16 were assigned as H-3 and H-6, respectively, which indicated 5,7,8-trisubstituted chromone (Banerji et al. 1988). Correlation between H-3 with C-1′ (δ 122.0) was observed in the HMBC spectrum confirmed the structure of flavonoid skeleton. These two protons, H-3 and H-6, showed correlations with C-10 (δ 104.8) in the HMBC spectrum (Figure S1). In the chromone

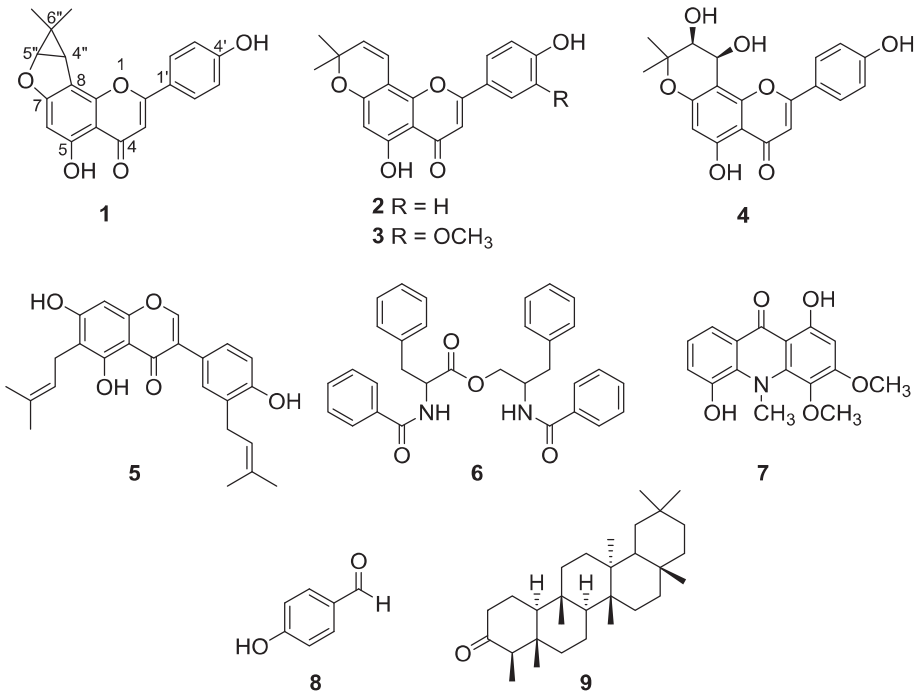


Figure 1. Chemical structures of isolated compounds **1–9**.

moiety, three oxygenated aromatic carbons were observed at δ 161.2 (C-5), δ 168.0 (C-7) and δ 153.0 (C-9). The doublet signal at δ 4.46 (J = 5.6 Hz) was assigned to an oxygenated methine proton H-5″, and correlated with carbon at δ 73.1 in the HMQC spectrum. Another doublet signal (J = 5.6 Hz) at δ 2.57 belongs to H-4′′ and is located on a methine carbon at δ 28.7. The small coupling constant, J = 5.6 Hz, confirms the *cis*-orientation of these two protons. In addition, the ^{1}H-^{1}H COSY spectrum shows cross peaks between H-5″ and H-4″. These two protons correlate with C-7 (δ 168.0) in the HMBC spectrum, which indicates the dihydrofuran moiety. Two methyl groups display signals at δ_H/δ_C 0.66/12.6 and δ_H/δ_C 1.07/22.6. In the HMBC spectrum, these two methyl groups correlate with C-6″ (δ 15), C-4″ (δ 28.7), and C-5″ (δ 73.1), indicating the cyclopropane moiety. The NOE experiment shows the correlations between H-4″ (and H-5″) and methyl proton at CH_3-7″ (δ_H/δ_C 1.07/22.6), which indicate the same face for these groups. Thus, the structure of this compound was identified as 5,4′-dihydroxy-6″,6″-dimethyl-4″,5″-dihydrocyclopropa[4″,5″] furano[2″,3″:7,8] flavone which was named atalantraflavone.

Acetylcholine esterase inhibition and antioxidant activity were evaluated using the modified Ellman's method and the ABTS scavenging assay, respectively. The results are shown in Table S2. Among the tested compounds, isoflavonoid derivative **5**, lupalbigenin, shows the strongest inhibition to AChE (79.21% inhibition) which is about 1.4-fold stronger than the tacrine standard. However, this compound displays moderate antioxidant activity at 47.64%. Comparing between flavonoid derivatives (**2–4**) and isoflavonoid **5**, it may be suggested that the isoflavonoid structure may play an important role in AChE inhibition. These results convinced that the effect of more phenolic groups in A and B ring of isoflavonoid **5** may be enhance the activity. These data are similar to the results of Salah which stated that

isoflavonoid showed higher activity than flavonoid derivatives (Salah et al. 2017). In case of flavonoids **2** and **4**, **4** showed a bit more active than **2**, it may be due to dihydroxyl groups in pyrone ring of flavonoid **4** effect to AChE inhibitory. These results showed convincingly that phenolic or hydroxyl groups play an important role for acetylcholine esterase inhibition. In addition, our obtained data are in agreement with the finding of Xie's data (Xie et al. 2014). Acridone **7** exhibits the strongest antioxidant activity (90.68%) but displays weak AChE inhibition (30.41%). These information implies that isoflavonoid **5** seem to be useful as a lead compound for the development of AChE inhibitors.

3. Experimental

3.1. General experimental procedures

Melting points were determined on a SANYO Gallenkamp (Leicester, UK) melting point apparatus and are uncorrected. UV spectra were measured on an Agilent 8453 UV-Visible spectrophotometer (Waldbronn, Germany). IR spectra were recorded as KBr disks or thin films, using a Perkin Elmer Spectrum One FT-IR spectrophotometer (Shelton, CT, USA). The NMR spectra were recorded on a Varian Mercury plus spectrometer (California, USA) operating at 400 MHz (^{1}H) and at 100 MHz (^{13}C). Mass spectra were determined on a Micromass Q-TOF 2 hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer with a Z-spray ES source (Micromass, Manchester, UK). Thin layer chromatography (TLC) was carried out on MERCK silica gel 60 F_{254} TLC aluminium sheets. Column chromatography was done with silica gel 0.063–0.200 mm or less than 0.063 mm (Darmstadt, Germany). Preparative thin layer chromatography (PLC) was carried out on glass supported silica gel plates using silica gel 60 PF_{254} for preparative layer chromatography (Darmstadt, Germany). All solvents were routinely distilled prior to use.

3.2. Plant material

The leaves of *A. monophylla* were collected in June 2016 from Phuwieng District, Khon Kaen Province, Thailand. The plant was identified by Dr. Pranom Chantaranothai, Faculty of Science, Khon Kaen University, Thailand where a voucher specimen (KKU022015) was deposited.

3.3. Extraction and isolation

The air-dried leaves (9.0 kg) of *A. monophylla* were ground and extracted with hexane (2 × 20 L), EtOAc (2 × 20 L), and MeOH (2 × 20 L) at room temperature. After evaporation, crude hexane (126 g), EtOAc (174 g), and MeOH (685 g) extracts were obtained. The EtOAc extract was separated by flash column chromatography (FCC) and using gradients of hexane, EtOAc and MeOH as an elution system. On the basis of the TLC pattern, nine fractions, F1 to F9, were collected. Fraction F3 was separated by silica gel FCC, and a gradient of EtOAc:hexane was used as an eluent to give three subfractions, F3.1–F3.3. The further purification of F3.2 by FCC and elution with EtOAc:hexane afforded **9** (25.7 mg, 0.0029%). Fraction 5 was subjected to silica gel FCC, and a gradient system of MeOH:CH_2Cl_2 yielded two subfractions, F5.1 and F5.2. Purification of F5.1 by silica gel FCC and using 100% CH_2Cl_2 as eluent obtained **3** (12.8 mg, 0.0014%). Compound **2** was obtained from the purification of subfraction F5.2 by

silica gel FCC, and 100% CH_2Cl_2 was used as eluent. The purification of fraction F7 using silica gel FCC (100% CH_2Cl_2) yielded four subfractions, F7.1–F7.4. Subfraction F7.2 was separated by gel filtration (Sephadex LH20) and MeOH as eluent to obtain **7** (18.4 mg, 0.0020%) and **6** (20.2 mg, 0.0022%). Further purification of subfraction F7.3 by preparative TLC (100% CH_2Cl_2) yielded **1** (1.5 mg, 0.00002%), **5** (4.3 mg, 0.00005%) and **8** (2.0 mg, 0.00002%). Fraction F9 was purified by FCC (5% MeOH:CH_2Cl_2) to yield **4** (7.6 mg, 0.00008%).

3.4. Spectroscopic data of compounds

3.4.1. *Atalantraflavone* (1)

a yellow solid; mp 235–238 °C; $[\alpha]_D^{24} = +86.1$ (*c* 0.1 MeOH); UV (MeOH) λ_{max} (log ε) 214 (4.10), 277 (3.95), 304 (3.86) nm; IR (thin film) ν_{max} cm^{-1}: 3596, 2924, 1657, 1589, 1448, 1251, 770; ^1H NMR (400 MHz, $CDCl_3$) δ 7.68 (d, *J* = 8.0 Hz, H-2′ and H-6′), 6.85, (d, *J* = 8.0 Hz, H-3′ and H-5′), 6.43 (s, H-3), 6.16 (s, H-6), 4.46 (d, *J* = 5.6 Hz, H-5″), 2.57 (d, *J* = 5.6 Hz, H-4″), 1.07 (CH_3-7″), 0.66 (CH_3-8″); ^{13}C NMR (100 MHz, $CDCl_3$) δ 164.3 (C-2), 103.2 (C-3), 182.6 (C-4), 161.2 (C-5), 93.9 (C-6), 168.0 (C-7), 105.9 (C-8), 153.0 (C-9), 104.8 (C-10), 122.0 (C-1′), 128.2 (C-2′ and C-6′), 116.0 (C- C-3′ and C-5′), 160.9 (C-4′), 28.7 (C-4″), 73.1 (C-5″), 15.0 (C-6″), 22.6 (C-7″), 12.6 (C-8″); HRESIMS *m/z* 337.1075 $[M + H]^+$ (calcd 337.1076).

3.5. Bioassay

3.5.1. In vitro *cholinesterase activity assay*

Acetylcholinesterase (AChE) activity was evaluated using the modified Ellman's spectrophotometric method. Tacrine was used as a reference standard. The assay was performed in a 96-well plate by adding 25 µL of 1 mM acetylthiocholine iodide as substrate in the assay, 125 µL of 1 mM 5, 5′-dithiobis-(2-nitrobenzoic acid) (DTNB), 25 µL of 0.1 M phosphate buffer pH 7.4, 25 µL of testing substance in various concentrations and 50 µL of 0.2 Units/ml AChE from an electric eel (type VI-S). The absorbance changes at 405 nm were detected every 30 s over a period of 5 min with a microplate reader. The enzyme activity and the percentage inhibition were determined (Ellman et al. 1961). The specific activity of enzyme AChE was calculated as follows:

$$\text{Specific activity (Rs)} = \frac{\left[\Delta\text{OD} \times \text{Volume of assay (mL)}\right]}{\text{Molar absorptivity of DTNB} \times \text{mg of protien}}$$

where: Rs is the rate of enzyme activity in µmole of ATCI hydrolyzed/minute/mg of protein; Δ OD is the change in absorbance per minute = Slope; The molar absorptivity of DTNB, which is 13,600 M^{-1} cm^{-1}; The percentage inhibition for each test compound was then calculated using the following equation:

$$\%\text{inhibition} = \left[(\text{Rs enzyme} - \text{Rs sample})/(\text{Rs enzyme} - \text{Rs control})\right] \times 100\%$$

3.5.2. In vitro *antioxidant activity assays*

The radical scavenging activity of the test compounds was measured by the ABTS method. The ABTS was dissolved in water to obtain a 7 mM concentration of ABTS stock solution. ABTS radical cation ($ABTS^{\cdot+}$) was generated by adding 2.45 mM potassium persulfate to the

ABTS stock solution and keeping it in the dark at room temperature for 12–16 h. The $ABTS^{\bullet+}$ solution was diluted with ethanol to give an absorbance of 0.70 ± 0.02 at 734 nm. Ten ml of the test compounds were allowed to react with 990 µl of $ABTS^{\bullet+}$ solution. The absorbance was measured 15 min after initial mixing. Trolox was used as a standard (Miller and Rice-Evans 1997). The percentage inhibition of the samples was calculated by the following equation: %Inhibition = $(1 - A/A_0) \times 100$, where A_0 is the absorbance at 734 nm of the negative control (Ethanol 99%), and A is the absorbance at 734 nm of the assay mixture with sample. All determinations were carried out at least three times, and in triplicate.

4. Conclusion

Chemical investigation of the leaves of *A. monophylla* led to the isolation of a new flavonoid named atalantraflavone and eight known compounds. All compounds including four flavonoids (**1–4**), a isoflavonoid (**5**), a dipeptide (**6**), an acridone (**7**), a benzaldehyde derivative (**8**), and a triterpene (**9**) were isolated. The isolated compounds were measured for acetylcholine esterase inhibition and antioxidant activity. It was found that lupalbigenin (**5**) showed 79% inhibition to AChE and was 1.4-fold stronger than the tacrine standard under the modified Ellman's spectrophotometric method. Citrusinine I (**7**) displayed 90.68% antioxidant activity under ABTS assay.

Acknowledgements

We thank the Thailand Research Fund and Khon Kaen University (RSA5980022) for financial support. We are indebted to The Center of Excellence for Innovation in Chemistry (PERCH-CIC), Office of the Higher Education Commission, Ministry of Education, Thailand.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was partially supported by the Royal Golden Jubilee Scholarship [grant number PHD/0007/2558] for P. Phosri and Thailand Research Fund and Khon Kaen University [grant number RSA5980022].

References

Anand P, Singh B. 2013. A review on cholinesterase inhibitors for Alzheimer's disease. Arch Pharm Res. 36:375–399.

Banerji A, Luthria DL, Prabhu BR. 1988. Prenylated compounds from *Atalantia racemosa*: isolation and synthesis of two pyranoflavones. Phytochemistry. 27:3637–3640.

Basa SC. 1975. Atalaphyllinine, a new acridone base from *Atalantia monophylla*. Phytochemistry. 14:835–836.

Ellman GL, Courtney KD, AndresV Feather-Stone RM. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 7:88–95.

Govindachari TR, Viswanathan N, Pai BR, Ramachandran VN, Subramaniam PS. 1970. Alkaloids of *Atalantia monyphylla*. Tetrahedron. 26:2905–2910.

Kapche DWFG, Lekane NM, Kulabas SS, Ipek H, Tok TT, Nagdjui BT, Demirtas I, Tumer TB. 2017. Aryl benzofuran derivatives from the stem bark of *Calpocalyx dinklagei* attenuate inflammation. Phytochemistry. 141:70–79.

Kassem M, Mosharrafa SA, Saleha NAM, Abdel-Wahab SM. 2000. Two new flavonoids from *Retama raetam*. Fitoterapia. 71:649–654.

Khoobi M, Ghanoni F, Nadri H, Moradi A, Hamedani MP, Moghadam FH, Emami S, Vosooghi M, et al. 2015. New tetracyclic tacrine analogs containing pyrano[2,3-c]pyrazole: Efficient synthesis, biological assessment and docking simulation study. Eur J Med Chem. 89:296–303.

Kumar TSSPNSS, Krupadanam GLD, Kumar KA. 2010. 5-Hydroxydictamnine, a new alkaloid from *Atalantia monophylla*. Nat Pro Res. 24:1514–1517.

Lan JS, Xie SS, Li SY, Pan LF, Wang XB, Kong LY. 2014. Design, synthesis and evaluation of novel tacrine-(β-carboline) hybrids as multifunctional agents for the treatment of Alzheimer's disease. Bioorg Med Chem. 22:6089–6104.

Miller NJ, Rice-Evans CA. 1997. Factors influencing the antioxidant activity determined by the ABTSdþ radical cation assay. Free Radic Res. 26:195–199.

Nwodo NJ, Okoye FBC, Lai D, Debbab A, Brun R, Proksch P. 2014. Two trypanocidal dipeptides from the roots of *Zapoteca portoricensis* (Fabaceae). Molecules. 19:5470–5477.

Panda H. 2004. Handbook on medicinal herbs with uses. India: Asia Pacific Business Press Inc: p. 166–167.

Rogers SL, Farlow MR, Doody RS, Mohs R, Friedhoff LT. 1998. A 24-week, double-blind, placebo-controlled trial of donepezil in patients with Alzheimer's disease. Neurology. 50:136–145.

Salah NM, Souleman AMA, Shaker KH, El-Hawary S, El-Shahid ZA, El-Hady FKA. 2017. Acetylcholinesterase, alpha-glucosidase and tyrosinase inhibitors from Egyptian Propolis. Inter J Pharmacog Phytochem Res. 9:528–536.

Saraswathy A, Balakrishna K, Rao RB, Allirani T, Patra A, Pichai R. 1998. Carpachromene from *Atalantia monophylla*. Fitoterapia. 69:463–464.

Shan F, Yin YQ, Huang F, Huang YC, Guo LB, Wu YF. 2013. A novel acridone alkaloid from *Atalantia buxifolia*. Nat Pro Res. 27:1956–1959.

Sribuhom T, Saraphon C, Decharchoochart P, Boonyarat C, Yenjai C. 2016. Acetylcholine esterase inhibition and cytotoxicity of flavonoids and chalcones from *Derris indica*. ScienceAsia. 42:247–251.

Sribuhom T, Boueroy P, Hahnvajanawong C, Phatchana R, Yenjai C. 2017. Benzoyltyramine alkaloids atalantums A-G from the peels of *Atalantia monophylla* and their cytotoxicity against cholangiocarcinoma cell lines. J Nat Prod. 80:403–408.

Talapatra SK, Bhattacharya S, Talapatra B. 1970. Terpenoid and related compounds. VI. Terpenoid and coumarin constituents of *Atalantia monophylla*. J Indian Chem Soc. 47:600–604.

Tan DX, Manchester LC, Sainz R, Mayo JC, Alvares FL. 2003. Antioxidant strategies in protection against neurodegenerative disorders. Expert Opin Ther Pat. 13:1515–1543.

Tedasen A, Sukrong S, Sritularak B, Srisawat T, Graidist P. 2016. 5,7,4′-Trihydroxy-6,8-diprenylisoflavone and lupalbigenin, active components of Derris scandens, induce cell death on breast cancer cell lines. Biomed Pharmacother. 81:235–241.

Xie Y, Yang W, Chen X, Xiao J. 2014. Inhibition of flavonoids on acetylcholine esterase: binding and structure–activity relationship. Food Funct. 5:2582–2589.

Medicinal Chemistry Research (2018) 27:2042–2049
https://doi.org/10.1007/s00044-018-2214-9

MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Cytotoxicity against cholangiocarcinoma and HepG2 cell lines of lignan derivatives from *Hernandia nymphaeifolia*

Jittra Suthiwong[1] · Jaroon Wandee[2] · Siripit Pitchuanchom[3] · Punchapat Sojikul[4] · Veerapol Kukongviriyapan[2] · Chavi Yenjai[1]

Received: 6 April 2018 / Accepted: 28 June 2018 / Published online: 10 July 2018

Abstract

Twelve lignan derivatives were synthesized from deoxypodophyllotoxin isolated from *Hernandia nymphaeifolia*. Cytotoxicity evaluation against cholangiocarcinoma, KKU-100, and HepG2 cell lines showed that compounds **3**, **9**, **10**, and **13** exhibited stronger cytotoxicity than the starting material, **1**, with IC_{50} ranging from 0.42 to 2.01 μM. Compound **10** displayed interesting activity by showing IC_{50} values of 0.75 and 0.46 μM against KKU-100 and HepG2 cell lines, respectively. From these observation, **10** seems to be useful as a lead compound for the development of anticancer agents.

Keywords *Hernandia nymphaeifolia* · Deoxypodophyllotoxin · KKU-100 · HepG2

Introduction

Hernandia nymphaeifolia is a coastal tree that grows throughout the tropical and subtropical areas. It is found in the southern part of Thailand and called Pho Kra Ding. Its seed is used as a cathartic (Kan 1970). This plant mainly contains lignans and aporphine alkaloids (Chen et al. 1996; Chao et al. 2002; Suthiwong et al. 2018). Several compounds from this plant have shown antiplatelet aggregation and cytotoxicity (Chen et al. 2000). It has been reported that some compounds exhibit anticancer activity against murine P388 lymphocytic leukemia and human cancer, KB16, A549, and HT-29 cell lines (Chen et al. 1997). Many lignans from medicinal plants have shown anti-oxidant, anti-estrogenic, anti-mitotic, and anti-viral activities (Pettit et al. 2004). Because of this pharmacological information, we are interested in the structural modification of a major lignan, deoxypodophyllotoxin, from the seeds of *H. nymphaeifolia* for cancer therapy, especially for cholangiocarcinoma. Deoxypodophyllotoxin has shown cytotoxicity against several cancer cell lines such as KB16, A549, HT-29, Colo205, k562, LNCaP, and PC-3 (Bogucki and Charlton 1995; Wickramaratne et al. 1995; Lim et al. 1999; Jiang et al. 2007). In addition, this compound inhibits 12-O-tetradecanoylphorbol 13-acetate-induced ornithine decarboxylase in cultured mouse epidermal cells (Chang et al. 2000).

In Thailand, cholangiocarcinoma is the cancer of most interest, due to its being one of the major health problems, especially in the northeastern area (Sripa and Pairojkul 2008). It is believed that infection by the liver fluke, *Opisthorchis viverrini*, is the cause of the disease (McGlynn et al. 2006). In this country, this cancer has been found in men more than women because of the high prevalence of liver fluke infections. There is no effective chemotherapy treatment with advanced cholangiocarcinoma patients (Sampson et al. 1997). Surgical resection is the best treatment and is a potentially curative therapy for this cancer. Thus, effective therapeutic agents from natural sources are still needed. It has been reported that KKU-100 was the

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00044-018-2214-9) contains supplementary material, which is available to authorized users.

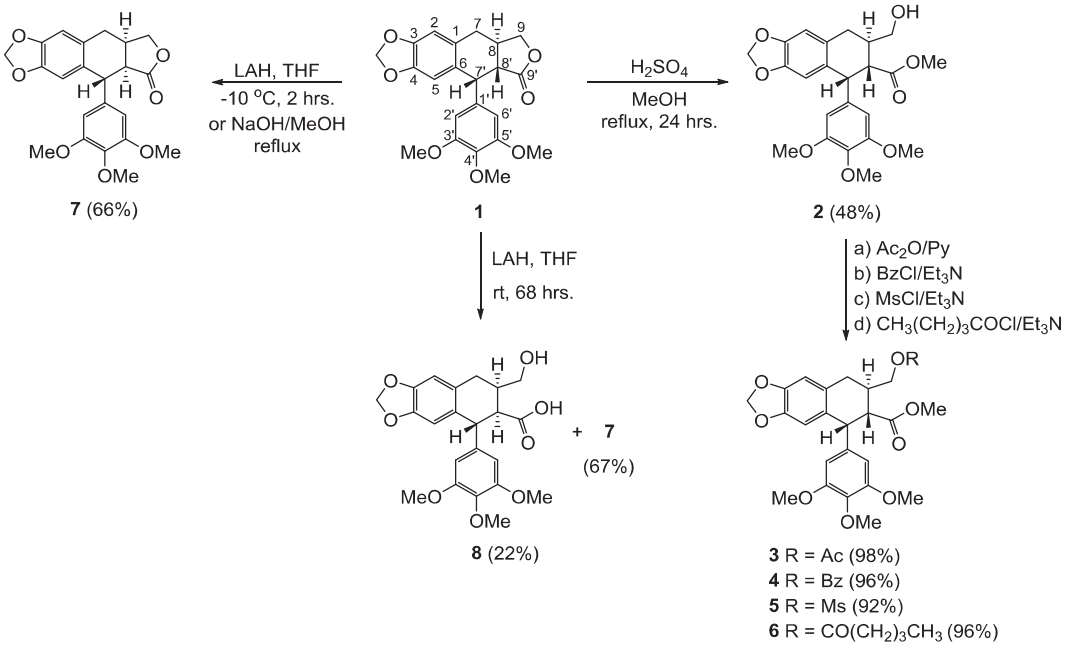
✉ Chavi Yenjai
chayen@kku.ac.th

[1] Department of Chemistry, Faculty of Science, Natural Products Research Unit, The Center of Excellence for Innovation in Chemistry, Khon Kaen University, Khon Kaen 40002, Thailand

[2] Department of Pharmacology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

[3] Department of Chemistry, Faculty of Science, Natural Products Research Unit, The Center of Excellence for Innovation in Chemistry, Mahasarakham University, Mahasarakham 44150, Thailand

[4] Department of Biotechnology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand



Scheme 1 Derivatives of deoxypodophyllotoxin

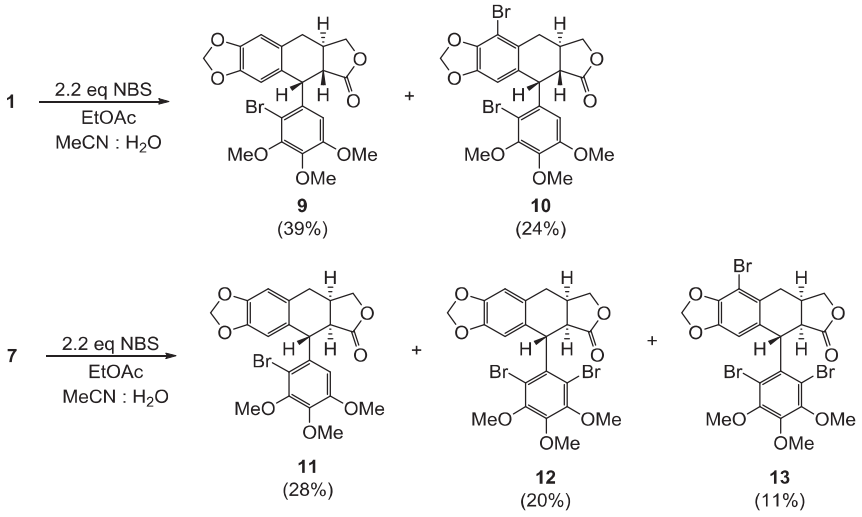
least sensitive cell line which showed the highest IC_{50} value of 25.21 μM to ellipticine, thus this cell line was selected for the study (Songsiang et al. 2010).

Results and discussion

Chemistry

Deoxypodophyllotoxin (**1**), a lignan derivative, was isolated using a chromatographic method from the EtOAc extract of the seed of *H. nymphaeifolia* with a good yield (Suthiwong et al. 2018). In our cytotoxicity screening, this compound showed cytotoxicity against cholangiocarcinoma (KKU-M100) and HepG2 cell lines, with IC_{50} values of 25.78 μM and 8.10 μM, respectively. To explore the structure-cytotoxicity relationship, a series of deoxypodophyllotoxin derivatives have been synthesized and evaluated for cytotoxicity to find novel compounds for cholangiocarcinoma and HepG2 treatment. Twelve lignan derivatives were successfully synthesized using simple organic reactions. Treatment of **1** with conc. sulfuric acid in methanol at room temperature yields methyl ester **2** (48%) (Scheme 1). The stereochemistry at C-8′ is still the same as the starting material by showing a coupling constant of *cis* configuration, $J_{7'/8'} = 5.7$ Hz, while *trans* configuration showed $J_{7'/8'} = 6.4$ Hz. (Gordaliza et al. 1997). The ^{1}H NMR spectrum of **2** showed a singlet signal at δ 3.53, indicating the presence of a methyl ester group. Alcohol **2** was further treated with acetic anhydride in pyridine affording **3** (98%). Lignan derivatives **4**, **5**, and **6** were synthesized by treatment of **2** with benzoyl chloride, methanesulfonyl chloride and valeroyl chloride, respectively, in the presence of Et_3N (Scheme 1). Compounds **3–6** showed the coupling constant of the *cis* configuration as $J_{7'/8'} = 5.7$–5.9 Hz. After treatment of **1** with LAH in THF at −10 °C, deoxypicropodophyllotoxin (**7**) was obtained with 66% yield, and no reduced product was obtained. This result indicates that LAH acts as a base and abstracted acidic proton at the *alpha* position leads to the epimerization of the starting material. The ^{1}H NMR spectrum displayed a double of doublet signal of H-8′ at δ 3.34 ($J = 9.6$, 3.0 Hz) where **1** showed a multiplet signal at δ 2.73. In addition, compound **7** showed a doublet signal of H-7′ at δ 4.37 ($J = 3.0$ Hz) where **1** exhibited at δ 4.60. Further treatment of **1** with LAH at room temperature for 68 h, interestingly, carboxylic acid derivative **8** (22%) and epimerized product **7** (67%) were observed. The ^{13}C NMR spectrum of **8** displayed signals at δ 31.4 (C-7) and δ 63.8 (C-9), where **1** showed at δ 33.3 (C-7) and δ 72.2 (C-9). Bromination of **1** with NBS in the presence of aqueous acetonitrile at room temperature yields monobromolignan **9** (39%) and dibromolignan **10** (24%) (Scheme 2). The effect of high electron density at C-2′ (and C-6′) in **1** led to bromination at the C-2′ position. The ^{13}C NMR data of **9** at C-2′ (C-Br) showed a signal at δ_C 114.6 where **1** showed at δ_C 108.5 and the proton signal of H-6′ changed from δ_H 6.34 to δ_H 6.12 (Feliciano et al. 1993). It was found that the addition of the second bromine atom at C-2 in **10** may be due to steric hindrance at C-6′. The HMBC spectrum of dibromolignan **10** showed

Scheme 2 Bromolignan derivatives of deoxypodophyllotoxin



correlations between H-7 (δ_{7a} 3.31 and δ_{7b} 2.56) and C-2 (δ 103.2). The reaction of **7** with NBS in the same condition gave monobromolignan **11** (28%), dibromolignan **12** (20%) and tribromolignan **13** (11%). The ^{13}C NMR signal of **11** at C-2′ changed from δ_C 105.3 to δ_C 110.9 where the signal of H-6′ changed from δ_H 6.33 to δ_H 6.31. In the case of **7**, the bromination of the second bromine atom occurring at C-6′ may be due to this compound being less steric than **10**. The spectroscopic data of dibromolignan **12** exhibited the containing of bromine atoms at C-2′ and C-6′ positions, which were different from **10**. The ^{1}H and ^{13}C NMR of **12** showed signals at δ_H/δ_C 6.69/108.7 (H-2) and δ_H/δ_C 6.06/106.5 (H-5), which correlated with C-3 (δ 146.1) and C-4 (δ 146.9) in the HMBC experiment. In the case of tribromolignan **13**, it showed only one aromatic proton at δ_H/δ_C 6.04/105.8 of the H-5 position. Correlations between H-5 and C-3 (δ 144.8), C-4 (δ 146.6), C-1 (δ 127.3) and C7′ (δ 45.1) were observed in the HMBC spectrum. In this spectrum, the correlations between H-7 and C-2 (δ 103.0), C-6 (δ 131.7) were also observed in the HMBC spectrum.

Table 1 Cytotoxicity of all compounds (IC_{50}, μM)*

Compound	KKU-100	HepG2
1	25.78 ± 1.78	8.10 ± 2.00
2	1.63 ± 0.79	6.73 ± 1.37
3	2.01 ± 0.74	1.94 ± 1.12
4	14.96 ± 7.85	3.55 ± 1.44
5	0.84 ± 0.47	16.12 ± 4.32
6	4.47 ± 1.94	41.39 ± 10.49
7	9.04 ± 5.70	1.50 ± 0.53
8	34.58 ± 10.80	14.65 ± 7.20
9	1.84 ± 0.54	1.90 ± 0.77
10	0.75 ± 0.52	0.46 ± 0.19
11	4.82 ± 2.09	5.86 ± 1.88
12	22.6 ± 203	14.92 ± 7.37
13	1.01 ± 0.39	0.42 ± 0.29
Ellipticine	25.21 ± 0.20	
Cisplatin		2.2 ± 0.70

*Data shown are from triplicate experiments

Biological activity

Natural lignan from *H. nymphaeifolia*, deoxypodophyllotoxin, was used as the starting material and 12 derivatives were synthesized. All compounds were evaluated for cytotoxicity against cholangiocarcinoma cells (KKU-M100, poorly-differentiated adenocarcinoma) and HepG2 cell lines (Tusskorn et al. 2013). KKU-100 cell is a poorly differentiated adenocarcinoma and is the least sensitive among cholangiocarcinoma cells. Most lignan derivatives displayed stronger cytotoxicity against two cell lines, KKU-100 and HepG2 cells, except compounds **8** and **12** (Table 1). In cases of KKU-100 cells, methyl ester derivatives (**2–6**) showed cytotoxicity with IC_{50} values ranging from 0.84 to 4.47 μM, except compound **4** (IC_{50} was 14.96 μM), which is about 5–30 fold stronger than the starting material. The results show convincingly that polarity may play an important role in cytotoxicity. Among the ester derivatives, **3** exhibited strong cytotoxicity against both cells with IC_{50} values of 2.01 and 1.94 μM, respectively. Compound **7** displayed cytotoxicity with IC_{50} values of 9.04 and 1.5 μM, respectively, which were 2.8 and 5.4 fold stronger than the starting material. These results suggest that the stereochemistry which led to the molecular form may affect the activity. Comparing between compounds **2** and **8**, carboxylic acid, which is more polar than methyl ester, exhibited weaker cytotoxicity (IC_{50} = 34.58 and 14.65 μM). These results confirm that the polarity of a compound may play an important role in activity. In cases of bromide derivatives, they showed strong cytotoxicity,

with the exception of compound **12**, against KKU-100 and HepG2 cell lines, with IC_{50} values ranging from 0.42 to 5.86 μM. Comparing between compounds **9** and **10**, the presence of a bromine atom at the C-2 position increased cytotoxicity. In cases of compounds **12** and **13**, the bromine atom at the C-2 position also improved the cytotoxicity against the two cell lines. Compound **13** exhibited the activity with IC_{50} values of 1.01 and 0.42 μM to KKU-100 and HepG2 cells, respectively. These results confirm that the bromine atom at the C-2 position can enhance the cytotoxicity. Among all derivatives, **10** is the most active compound, showing IC_{50} values of 0.75 and 0.46 μM against KKU-100 and HepG2 cell lines, respectively.

Conclusion

Lignan derivatives were synthesized by using deoxypodophyllotoxin as the starting material. All compounds were evaluated for cytotoxicity against cholangiocarcinoma cells, KKU-100, and hepatoma carcinoma cells, HepG2. Compounds **3**, **9**, **10**, and **13** exhibited cytotoxicity against the two cell lines. It is believed that polarity of these compounds may play an important role in cytotoxicity. Among bromide derivatives (**9–13**), it was found that the bromine atom at the C-2 position was favorable for cytotoxicity. Compound **10** displayed highly potent activity against the two cell lines.

Experimental part

General experimental procedures

All melting points were determined on a SANYO Gallenkamp (UK) melting point apparatus. Optical rotations were identified using a JASCO P–1020 digital polarimeter. UV spectra were recorded using an Agilent 8453 UV-Visible spectrophotometer (Germany). IR spectra were taken as thin films using a Perkin Elmer Spectrum One FT-IR spectrophotometer (UK) ^{1}H NMR spectra were determined with a Varian Mercury plus spectrometer (UK) operating at 400 MHz (^{1}H NMR) and at 100 MHz (^{13}C NMR). Mass spectra were recorded on a Micromass Q-TOF 2 hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer with a Z-spray ES source (Micromass, UK). Silica gel 60 (100–200 mesh, Merck) was employed for column chromatography. Preparative TLC was carried out using silica gel 60 GF254. TLC was examined on silica gel 60 F_{254} (Merck) precoated aluminum sheets. Spot on TLC were visualized under UV light and by spraying with acidic anisaldehyde solution followed by heating. All solvents were distilled throughout the separation process.

Extraction and isolation of deoxypodophyllotoxin (1)

Air-dried seed (300 g) of *H. nymphaeifolia* were ground and successively extracted at room temperature with EtOAc (3 × 1.5 L), and MeOH (3 × 1.5 L). After filtration and evaporation, the crude EtOAc (79 g) and MeOH (23 g) extracts were obtained. The crude EtOAc extract was separated by silica gel flash column chromatography (FCC) and eluted with a gradient system of hexane and EtOAc to obtain 4 fractions, F1-F4. Fraction F1 (30 g) was identified as the natural oil. Fraction F2 was purified by silica gel FCC, and 5% EtOAc-hexane was used as an eluent to obtain two subfractions, F2.1 and F2.2. Further purification of subfraction F2.2 by CC and elution with 100% CH_2Cl_2 afforded **1** (321 mg, 0.11%) which was used as the starting material.

Deoxypodophyllotoxin (1)

White solid; mp 166–168 °C, $[\alpha]^{23}_D$ −54° (*c* 0.1 $CHCl_3$); FT-IR (film) ν_{max} cm^{-1}: 2923, 1763, 1588, 1504, 1479, 1459, 1421, 1377, 1336, 1270, 1219, 1121, 1033, 997, 929; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.66 (1H, s, H-2), 6.52 (1H, s, H-5), 6.34 (2H, br s, H-2′,6′), 5.95 (1H, s, –OCH_2O–), 5.92 (1H, s, –OCH_2O–), 4.60 (1H, br s, H-7′), 4.45 (1H, dd, J = 8.3, 5.4 Hz, H-9), 3.92 (1H, t, J = 9.0 Hz, H-9), 3.80 (3H, s, 4′–OCH_3), 3.75 (6H, s, 3′,5′–OCH_3), 3.07 (1H, br d, J = 11.3 Hz, H-7a), 2.73 (3H, m, H-7b, 8, 8′); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.0 (C-9′), 152.7 (C-3′, 5′), 147.2 (C-3), 146.9 (C-4), 137.3 (C-4′), 136.4 (C-1′), 130.8 (C-6), 128.4 (C-1), 110.6 (C-5), 108.6 (C-2), 108.5 (C-2′, 6′), 101.3 (–OCH_2O–), 72.2 (C-9), 60.9 (4′-OCH_3), 56.4 (3′, 5′-OCH_3), 47.6 (C-8′), 43.9 (C-7′), 33.3 (C-7), 32.9 (C-8); HRESI-MS *m/z* 421.1277 $[M + Na]^+$ (calcd. for $C_{22}H_{22}O_7$ +Na, 421.1263).

Preparation of lignan derivatives

Delactonization

A solution of compound **1** (172.4 mg, 0.4337 mmol) in MeOH (3 mL) was treated with conc. H_2SO_4 (0.2 mL) at 100 °C for about 24 h. Water was added and the mixture was extracted with EtOAc (2×50 mL). The organic layer was combined, washed with water, brine and dried with anhydrous Na_2SO_4. Evaporation of solvent gave a crude oil, which was purified by PLC (50 % EtOAc:hexane) to give a semi-solid of compound **2** (89.1 mg, 48%).

Methyl deoxypodophyllate (2) White solid; mp 154–157 °C, $[\alpha]^{24}_D$ −95° (*c* 0.1 $CHCl_3$); FT-IR (film) ν_{max} cm^{-1}: 3498, 2936, 1734, 1588, 1503, 1483, 1459, 1419, 1328, 1224,

1123, 1035, 1005, 925, 862, 749; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.58 (1H, s, H-2), 6.34 (1H, s, H-5), 6.09 (2H, s, H-2′,6′), 5.83 (2H, br s, –OCH_2O–), 4.32 (1H, d, J = 5.7 Hz, H-7′), 3.74 (3H, s, 4′-OMe), 3.70 (6H, s, 3′ and 5′-OMe), 3.64 (1H, dd, J = 10.9, 3.8 Hz, H-9a), 3.54 (1H, dd overlap, J = 10.9, 5.4 Hz, H-9), 3.53 (3H, s, 9′-OMe), 3.06–2.89 (2H, m, H-8′, 7a), 2.65 (1H, dd, J = 17.1, 10.8 Hz, H-7b), 2.35 (1H, m, H-8); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.9 (C-9′), 152.7 (C-3′,5′), 146.6 (C-4), 146.2 (C-3), 138.0 (C-4′), 137.1 (C-1′), 130.0 (C-6), 128.5 (C-1), 109.2 (C-5), 108.0 (C-2), 106.8 (C-2′ and 6′), 100.8 (-OCH_2O-), 65.4 (C-9), 60.8 (4′-OMe), 56.1 (3′ and 5′-OMe), 51.5 (9′-OMe), 48.3 (C-8′), 47.8 (C-7′), 32.9 (C-8), 32.1 (C-7); HRESI-MS *m/z* 453.1521 $[M + Na]^+$ (calcd. for $C_{23}H_{26}O_8$+Na, 453.1525).

Acetylation

To a solution of compound **2** (15.3 mg, 0.0355 mmol) in pyridine (1 mL) was added dropwise Ac_2O (excess) at 0 °C, and the reaction mixture was stirred at room temperature for 2 h. The entire reaction mixture was poured into the cold water and extracted with EtOAc (2×20 mL). The organic layers were combined, washed with water, saturated NaCl, dried over anhydrous Na_2SO_4 and evaporated. After recrystallization, compound **3** was obtained as a colorless solid (16.5 mg, 98%).

Methyl acetoxydeoxypodophyllate (3) Viscous oil; $[\alpha]_D^{25}$ −75° (*c* 0.1 $CHCl_3$); FT-IR (film) ν_{max} cm^{-1}: 2930, 1738, 1588, 1503, 1484, 1460, 1230, 1127, 1037; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.62 (1H, s, H-2), 6.40 (1H, s, H-5), 6.12 (2H, s, H-2′ and 6′), 5.89 (1H, s, –OCH_2O–), 5.88 (1H, s, –OCH_2O–), 4.37 (1H, d, J = 5.8 Hz, H-7′), 4.21 (1H, dd, J = 11.0, 3.2 Hz, H-9a), 4.13 (1H, dd, J = 11.0, 5.8 Hz, H-9b), 3.79 (3H, s, 4′-OMe), 3.74 (6H, s, 3′ and 6′-OMe), 3.54 (3H, s, 9′-OMe), 3.00 (1H, dd, J = 11.0, 5.8 Hz, H-8′), 2.98 (1H, dd, J = 16.9, 5.8 Hz, H-7a) 2.70 (1H, dd, J = 16.9, 10.8 Hz, H-7b), 2.62–2.50 (1H, m, H-8), 2.05 (3H, s, H-2″); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.6 (C-9′), 171.2 (C-1″), 152.9 (C-3′ and 5′), 146.8 (C-4), 146.4 (C-3), 137.8 (C-4′), 137.3 (C-1′), 130.0 (C-6), 128.0 (C-1), 109.4 (C-5), 107.9 (C-2), 106.9 (C-2′ and 6′), 101.0 (-OCH_2O-), 66.8 (C-9), 60.9 (4′-OMe), 56.3 (3′ and 5′-OMe), 51.5 (9′-OMe), 47.73 (C-7′), 47.69 (C-8′), 31.9 (C-7), 30.1 (C-8), 21.0 (C-2″); HRESI-MS *m/z* 495.1628 $[M + Na]^+$ (calcd. for $C_{25}H_{28}O_9$+Na, 495.1631).

General procedure to prepare 4–6

To a solution of compound **2** (14.6 mg, 0.034 mmol) in Et_3N (1 mL) was added dropwise BzCl (excess) at 0 °C and the reaction mixture was stirred at room temperature for 30 min. The entire reaction mixture was poured into cold water and extracted with EtOAc (2×20 mL). The organic layer were combined, washed with water, saturated NaCl, dried over anhydrous Na_2SO_4 and evaporated to give a crude oil, which was purified by PLC (20 % EtOAc:hexane) to give **4** (17.4 mg, 96%).

The reaction of **2** with MsCl or valerory chloride was examined in the same procedure as described above and then **5** (92%) or **6** (96%) were obtained, respectively.

Methyl benzyloxydeoxypodophyllate (4)

White solid; mp 171–173 °C; $[\alpha]_D^{25}$ −86° (*c* 0.1 $CHCl_3$); FT-IR (film) ν_{max} cm^{-1}: 2934, 1718, 1587, 1503, 1483, 1454, 1271, 1222, 1123, 1036, 1005, 938, 749, 710; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 (2H, d, J = 7.9 Hz, H-3″), 7.56 (1H, t, J = 7.6 Hz, H-5″), 7.44 (2H, t, J = 7.6 Hz, H-4″), 6.63 (1H, s, H-2), 6.43 (1H, s, H-5), 6.16 (2H, s, H-2′ and 6′), 5.90 (1H, s, –OCH_2O–), 5.88 (1H, s, –OCH_2O–), 4.48 (1H, dd, J = 11.1, 3.1 Hz, H-9a), 4.41 (1H, d, J = 5.7 Hz, H-7′), 4.39 (1H, m overlap, H-9b) 3.81 (3H, s, 4′-OMe), 3.76 (6H, s, 3′,6′-OMe), 3.55 (3H, s, 9′-OMe), 3.14 (1H, dd, J = 11.5, 5.7 Hz, H-8′), 3.09 (1H, dd, J = 16.9, 5.8 Hz, H-7a) 2.84 (1H, dd, J = 16.9, 10.8 Hz, H-7b), 2.77–2.66 (1H, m, H-8); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.6 (C-9′), 166.6 (C-1″), 152.9 (C-3′,5′), 146.9 (C-4), 146.5 (C-3), 137.8 (C-4′), 137.4 (C-1′), 133.2 (C-5″), 130.2 (C-6), 130.0 (C-2″), 129.7 (C-3″), 128.6 (C-4″), 128.1 (C-1), 109.4 (C-5), 108.0 (C-2), 106.9 (C-2′ and 6′), 101.0 (−OCH_2O−), 67.1 (C-9), 61.0 (4′-OMe), 56.3 (C-3′ and 5′-OMe), 51.6 (9′-OMe), 47.9 (C-8′), 47.8 (C-7′), 32.1 (C-8), 30.4 (C-7); HRESI-MS *m/z* 557.1789 $[M+Na]^+$ (calcd. for $C_{30}H_{30}O_9$ +Na, 557.1788).

Methyl mesyloxydeoxypodophyllate (5)

White solid; mp 175–177 °C; $[\alpha]_D^{25}$ −62° (*c* 0.1 $CHCl_3$); FT-IR (film) ν_{max} cm^{-1}: 2935, 1733, 1588, 1503, 1483, 1460, 1420, 1352, 1331, 1223, 1172, 1124, 1035, 926, 752; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.63 (1H, s, H-2), 6.39 (1H, s, H-4), 6.10 (2H, s, H-2′ and 6′), 5.90 (2H, s, −OCH_2O−), 4.42 (1H, d, J = 5.9 Hz, H-7′), 4.40–4.32 (2H, m, H-9), 3.80 (3H, s, 4′-OMe), 3.74 (6H, s, 3′ and 5′-OMe), 3.61 (3H, s, 9′-OMe), 3.10–3.00 (2H, m, H-7a and 8′), 2.98 (3H, s, SO_2Me), 2.85 (1H, dd, J = 17.1, 11.2 Hz, H-7b), 2.68–2.56 (1H, m, H-8); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.3 (C-9′), 153.0 (C-3′ and 5′), 147.0 (C-4), 146.6 (C-3), 137.4 (C-1′ and 4′), 129.7 (C-6), 127.5 (C-1), 109.3 (C-5), 107.9 (C-2), 106.9 (C-2′ and 6′), 101.1(−OCH_2O−), 72.2 (C-9), 61.0 (4′-OMe), 56.3 (3′ and 5′-OMe), 51.7 (9′-OMe), 47.6 (C-8′), 46.8 (C-7′), 37.2 (SO_2Me), 31.6 (C-8), 30.4 (C-7); HRESI-MS *m/z* 531.1283 $[M + Na]^+$ (calcd. for $C_{24}H_{28}O_{10}$+Na, 531.1301).

Methyl valeroyloxydeoxypodophyllate (6)

White solid; mp 168–169 °C; $[\alpha]_D^{25}$ −52° (*c* 0.1 $CHCl_3$); FT-IR (film) ν_{max} cm^{-1}: 2954, 1732, 1587, 1503, 1483, 1459, 1419, 1390, 1330, 1222, 1162, 1124, 1035, 1007, 937, 752; ^1H NMR (400 MHz, $CDCl_3$) δ 6.62 (1H, s, H-2), 6.40 (1H, s, H-5), 6.12 (2H, s, H-2′ and 6′), 5.89 (1H, s, –OCH_2O–), 5.88 (1H, s, –OCH_2O–), 4.37 (1H, d, $J = 5.7$ Hz, H-7′), 4.20 (1H, dd, $J = 11.2$, 3.0 Hz, H-9a), 4.15 (1H, dd, $J = 11.0$, 5.6 Hz, H-9b), 3.80 (3H, s, 4′-OMe), 3.74 (6H, s, 3′ and 5′-OMe), 3.54 (3H, s, 9′-OMe), 3.05–2.95 (2H, m, H-7a and 8′), 2.71 (1H, dd, $J = 16.9$, 10.8 Hz, H-7b), 2.61–2.51 (1H, m, H-8), 2.31 (2H, t, $J = 7.6$ Hz, H-2″), 1.59 (2H, m, H-3″), 1.33 (2H, m, H-4″), 0.91 (3H, t, $J = 7.3$ Hz, H-5″); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.9 (C-1″), 172.6 (C-9′), 152.9 (C-3′,5′), 146.8 (C-4), 146.4 (C-3), 137.8 (C-4′), 137.3 (C-1′), 130.0 (C-6), 128.1 (C-1), 109.4 (C-5), 108.0 (C-2), 106.9 (C-2′ and 6′), 101.0 (−OCH_2O−), 66.4 (C-9), 61.0 (4′-OMe), 56.3 (3′ and 5′-OMe), 51.5 (9′-OMe), 47.7 (C-8′), 47.7 (C-7′), 34.1 (C-2″), 32.0 (C-8), 30.2 (C-7), 27.2 (C-3″), 22.4 (C-4″), 13.8 (C-5″); HRESI-MS *m/z* 537.2101 $[M + Na]^+$ (calcd. for $C_{28}H_{34}O_9$+Na, 537.2101).

Epimerization

A solution of compound **1** (46.2 mg, 0.1159 mmol) in THF at −10 °C was treated with LAH (4.5 mmol, 1 M solution in THF) under argon. The reaction mixture was stirred at −10 °C for 2 h. The reaction mixture was quenched with 10% HCl followed by extraction of the aqueous layer with EtOAc (2×20 mL). The combined organic layers were washed with water and brine, dried with Na_2SO_4, and concentrated in vacuo. Chromatographic separation on silica gel with EtOAc-hexane (2:3) as the eluent afforded the epimerized product **7** (30.5 mg, 66%).

Deoxypicropodophyllotoxin (7)

White solid; mp 159–161 °C, $[\alpha]_D^{24}$ +61° (*c* 0.1 $CHCl_3$); FT-IR (film) ν_{max} cm^{-1}: 2924, 1761, 1589, 1505, 1478, 1459, 1423, 1325, 1245, 1180, 1121, 1033, 1005, 926, 791; ^1H NMR (400 MHz, $CDCl_3$) δ 6.66 (1H, s, H-2), 6.58 (1H, s, H-5), 6.33 (2H, s, H-2′, 6′), 5.95 (1H, br s, –OCH_2O–), 5.92 (1H, br s, –OCH_2O–), 4.45 (1H, dd, $J = 9.2$, 7.4 Hz, H-9a), 4.37 (1H, d, $J = 3.0$ Hz, H-7′), 3.97 (1H, dd, $J = 9.2$, 3.2 Hz, H-9b), 3.82 (3H, s, 4′-OMe), 3.78 (6H, s, 5′ and 3′-OMe), 3.34 (1H, dd, $J = 9.6$, 3.0 Hz, H-8′), 3.06–2.96 (1H, m, H-8), 2.86 (1H, dd, $J = 15.4$, 6.4 Hz, H-7a), 2.48 (1H, dd, $J = 15.4$, 5.5 Hz, H-7b); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.5 (C-9′), 153.5 (C-3′,5′), 147.0 (C-3), 146.9 (C-4), 138.4 (C-1′), 130.6 (C-6), 128.4 (C-1), 110.0 (C-5), 108.9 (C-2), 105.3 (C-2′), 105.2 (C-6′), 101.1 (−OCH_2O−), 72.9 (C-9), 61.0 (4′-OMe), 56.4 (3′,5′-OMe), 46.5 (C-8′), 45.5 (C-7′), 33.2 (C-8), 32.2 (C-7); HRESI-MS *m/z* 421.1261 $[M + Na]^+$ (calcd. for $C_{22}H_{22}O_7$+Na, 421.1263).

Hydrolysation of 1

A solution of compound **1** (89.7 mg, 0.2251 mmol) in THF at −10 °C was treated with LAH (4.5 mmol, 1 M solution in THF) under argon. The reaction mixture was stirred at −10 °C for 2 h. and then warmed to room temperature until the disappearance of the starting material (68 h) which was determined by TLC. The reaction mixture was quenched with 10% HCl followed by extraction of the aqueous layer with EtOAc (2×20 mL). The combined organic layers were washed with water and brine, dried with Na_2SO_4, and concentrated in vacuo. Chromatography on silica gel with EtOAc-hexanes (2:3) as the eluent afforded products **8** (20.5 mg, 22%) and **7** (60.3 mg, 67%).

Deoxypicropodophyllic acid (8)

White solid; mp 162–165 °C; $[\alpha]_D^{25}$+21° (*c* 0.1 CH_3OH); FT-IR (film) ν_{max} cm^{-1}: 3402, 2918, 1762, 1589, 1504, 1482, 1461, 1424, 1328, 1228, 1128, 1007, 982; ^1H NMR (400 MHz, Methanol-d4) δ 6.62 (1H, s, H-2), 6.38 (2H, s, H-2′ and 6′), 6.30 (1H, s, H-5), 5.86 (1H, s, –OCH_2O–), 5.85 (1H, s, –OCH_2O–), 4.36 (1H, d, $J = 6.1$ Hz, H-7′), 3.75 (6H, s, 3′ and 5′-OMe), 3.73 (3H, s, 4′-OMe), 3.70 (1H, dd, $J = 10.9$, 5.9 Hz, H-9a), 3.55 (1H, dd, $J = 10.9$, 8.3 Hz, H-9b), 3.06 (1H, dd, $J = 6.1$, 3.5 Hz, H-8′), 2.95 (1H, dd, $J = 16.6$, 5.5 Hz, H-7a), 2.84 (1H, dd, $J = 16.6$, 7.8 Hz, H-7b), 2.40–2.31 (1H, m, H-8); ^{13}C NMR (100 MHz, CD_3OD) δ 176.9 (C-9′), 154.3 (C-3′ and 5′), 147.8 (C-4), 147.5 (C-3), 143.4 (C-1′), 137.7 (C-4′), 131.0 (C-6), 129.9 (C-1), 110.4 (C-5), 109.3 (C-2), 107.7 (C-2′ and 6′), 102.0 (-OCH_2O-), 63.8 (C-9), 61.1 (4′-OMe), 56.6 (3′ and 5′-OMe), 50.6 (C-8′), 47.5 (C-7′), 37.0 (C-8), 31.4 (C-7); HRESI-MS *m/z* 439.1372 $[M + Na]^+$ (calcd. for $C_{22}H_{24}O_7$+Na, 439.1369).

Addition with N-Bromosuccinamide

To a solution of **1** (33 mg, 0.0828 mmol) in EtOAc (2 mL) was added dropwise the solution of NBS (2.2 eq.) in MeCN: H_2O (2 mL) at 0 °C and this was stirred at 0 °C for 1 h and kept stirring at room temperature for 1 h. The entire reaction mixture was poured into cold water and extracted with EtOAc (2×20 mL). The organic layers were combined, washed with water, saturated NaCl, dried over anhydrous Na_2SO_4 and evaporated to give solid products. After purification by PLC, lignan derivatives **9** (15.5 mg, 39%) and **10** (10.9 mg, 24%) were obtained.

The reaction of **7** (29.9 mg) with NBS was examined in the same procedure as described above and then **11**

(10.2 mg, 28%), **12** (8.4 mg, 20%) and **13** (11.3 mg, 24%) were obtained.

2′-Bromodeoxypodophyllotoxin (9)

White solid; mp 221–223 °C; $[\alpha]_D^{25}$ −93° (*c* 0.1 $CHCl_3$); FT-IR (film) ν_{max} cm^{-1}: 2927, 1779, 1563, 1480, 1389, 1334, 1313, 1283, 1224, 1197, 1164, 1103, 1037, 999, 944, 928, 872, 754; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.63 (1H, s, H-2), 6.38 (1H, s, H-5), 6.12 (1H, br s, H-6′), 5.90 (2H, s, –OCH_2O–), 5.28 (1H, d, J = 5.9 Hz, H-7′), 4.51 (1H, dd, J = 8.6, 6.7 Hz, H-9a), 3.93 (1H, dd, J = 10.2, 8.6 Hz, H-9b), 3.91 (3H, s, 3′-OMe), 3.86 (3H, s, 4′-OMe), 3.63 (3H, s, 5′-OMe), 3.08 (1H, dd, J = 15.5, 4.7 Hz, H-7a), 3.03–2.89 (1H, m, H-8), 2.83–2.73 (2H, m, H-7b and 8′); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.4 (C-9′), 152.3 (C-5′), 151.0 (C-3′), 147.1 (C-4), 147.0 (C-3), 142.7 (C-4′), 136.6 (C-1′), 131.3 (C-6), 127.6 (C-1), 114.6 (C-2′), 110.8 (C-6′), 110.4 (C-5), 108.5 (C-2), 101.3 (–OCH_2O–), 71.7 (C-9), 61.2 (4′-OMe), 61.1 (3′-OMe), 56.5 (5′-OMe), 46.8 (C-8′), 41.5 (C-7′), 34.4 (C-8), 32.9 (C-7); HRESI-MS *m/z* 499.0385, 501.0365 $[M + Na]^+$ (calcd. for $C_{22}H_{21}BrO_7$+Na, 499.0368, 501.0348).

2,2′-Dibromodeoxypodophyllotoxin (10)

White solid; mp 225–227 °C; $[\alpha]_D^{25}$ −84° (*c* 0.1 $CHCl_3$); FT-IR (film) ν_{max} cm^{-1}: 2936, 1778, 1563, 1465, 1390, 1334, 1313, 1223, 1198, 1167, 1104, 1040, 1000, 932, 837, 752; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.39 (1H, s, H-5), 6.10 (1H, br s, H-6′), 6.01 (1H, s, –OCH_2O–), 5.99 (1H, s, –OCH_2O–), 5.29 (1H, br d, J = 6.4 Hz, H-7′), 4.56 (1H, dd, J = 8.5, 6.8 Hz, H-9a), 3.98 (1H, dd, J = 10.5, 8.5 Hz, H-9b), 3.91 (3H, s, 5′-OMe), 3.86 (3H, s, 4′-OMe), 3.66 (3H, s, 3′-OMe), 3.31 (1H, dd, J = 16.4, 5.0 Hz, H-7a), 3.00–2.86 (1H, m, H-8), 2.75 (1H, dd, J = 14.3, 6.4 Hz, H-8′), 2.56 (1H, dd, J = 16.4, 11.6 Hz, H-7b); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.0 (C-9′), 152.4 (C-3′), 151.2 (C-5′), 146.9 (C-4), 145.8 (C-3), 142.9 (C-4′), 135.9 (C-1′), 133.5 (C-6), 127.2 (C-1), 144.7 (C-2′), 110.9 (C-6′), 109.8 (C-5), 103.2 (C-2), 101.7 (–OCH_2O–), 71.7(C-9), 61.2 (4′-OMe), 61.1 (5′-OMe), 56.7 (3′-OMe), 46.4 (C-8′), 42.0 (C-7′), 34.3 (C-8), 32.5 (C-7); HRESI-MS *m/z* 576.9457, 578.9458, 580.9421 $[M + Na]^+$ (calcd. for $C_{22}H_2Br_2O_7$ +Na, 576.9473, 578.9453, 580.9433).

2′-Bromodeoxypicropodophyllotoxin (11)

Viscous oil; $[\alpha]_D^{24}$+34° (*c* 0.1 $CHCl_3$); FT-IR (film) ν_{max} cm^{-1}: 2928, 1768, 1566, 1481, 1390, 1328, 1256, 1161, 1104, 1036, 933, 801, 756; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.67 (1H, s, H-2), 6.40 (1H, s, H-5), 6.31 (1H, s, H-6′), 5.93 (1H, br s, –OCH_2O–), 5.90 (1H, br s, –OCH_2O–), 4.65 (1H, d, J = 3.8 Hz, H-7′), 4.43 (1H, dd, J = 9.2, 6.3 Hz, H-9a), 4.08 (1H, dd, J = 9.2, 2.0 Hz, H-9b), 3.92 (3H, s, 5′-OMe), 3.89 (3H, s, 4′-OMe), 3.70 (3H, s, 3′-OMe), 3.35 (1H, dd, J = 8.8, 3.8 Hz, H-8′), 2.97 (1H, dd, J = 15.4, 6.5 Hz, H-7a), 2.92–2.82 (1H, m, H-8), 2.60 (1H, dd, J = 15.4, 7.8 Hz, H-7b); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.7 (C-9′), 152.7 (C-3′), 151.6 (C-5′), 147.0 (C-4), 146.9 (C-3), 142.3 (C-4′), 137.1 (C-1′), 129.8 (C-6), 128.4 (C-1), 110.9 (C-2′), 110.1 (C-6′), 109.2 (C-5), 108.5 (C-2), 101.1 (–OCH_2O–), 72.8 (C-9), 61.20 (5′-OMe), 61.16 (4′-OMe), 56.3 (3′-OMe), 45.1 (C-8′), 45.0 (C-7′), 33.3 (C-8), 32.2 (C-7); HRESI-MS *m/z* 499.0376, 501.0353 $[M + Na]^+$ (calcd. for $C_{22}H_{21}BrO_7$ +Na, 499.0368, 501.0348).

2′,6′-Dibromodeoxypicropodophyllotoxin (12)

White solid; mp 220–223 °C $[\alpha]_D^{24}$+23° (*c* 0.1 $CHCl_3$); FT-IR (film) ν_{max} cm^{-1}: 2925, 1772, 1503, 1481, 1464, 1407, 1388, 1334, 1242, 1215, 1159, 1126, 1088, 1038, 1009, 929, 837, 754; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.69 (1H, s, H-2), 6.06 (1H, s, H-5), 5.91 (1H, br s, –OCH_2O–), 5.87 (1H, br s, –OCH_2O–), 5.16 (1H, d, J = 6.4 Hz, H-7′), 4.51 (1H, dd, J = 9.3, 5.6 Hz, H-9a), 4.26 (1H, d, J = 9.3 Hz, H-9b), 3.98 (3H, s, 4′-OMe), 3.96 (3H, s, 5′-OMe), 3.89 (3H, s, 3′-OMe), 3.43 (1H, t, J = 7.3 Hz, H-8′), 2.90–2.80 (2H, m, H-7a, 8), 2.62 (1H, t, J = 14.5 Hz, H-7b); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.6 (C-9′), 151.6 (C-3′), 151.1 (C-5′), 146.9 (C-4), 146.9 (C-4′), 146.1 (C-3), 136.3 (C-1′), 129.8 (C-6), 128.2 (C-1), 119.5 (C-2′), 112.7 (C-6′), 108.7 (C-2), 106.5 (C-5), 101.0 (–OCH_2O–), 71.9 (C-9), 61.4 (4′-OMe), 61.2 (5′-OMe), 61.2 (3′-OMe), 46.0 (C-8′), 44.6 (C-7′), 35.9 (C-8), 32.8 (C-7); HRESI-MS *m/z* 576.9452, 578.9453, 580.9416 $[M + Na]^+$ (calcd. for $C_{22}H_{20}Br_2O_7$ +Na, 576.9473, 578.9453, 580.9433).

2,2′,6′-Tribromodeoxypicropodophyllotoxin (13)

White solid; mp 233–235 °C $[\alpha]_D^{24}$+53° (*c* 0.1 $CHCl_3$); FT-IR (film) ν_{max} cm^{-1}: 2924, 1774, 1607, 1499, 1465, 1406, 1388, 1316, 1238, 1160, 1086, 1041, 1009, 930, 837, 754; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.04 (1H, s, H-5), 5.99 (1H, br s, –OCH_2O–), 5.97 (1H, br s, –OCH_2O–), 5.16 (1H, d, J = 6.5 Hz,, H-7′), 4.54 (1H, dd, J = 9.4, 5.9 Hz, H-9a), 4.32 (1H, d, J = 9.4 Hz, H-9b), 3.98 (3H, s, 4′-OMe), 3.96 (3H, s, 3′-OMe), 3.89 (3H, s, 5′-OMe), 3.46–3.37 (2H, m, H-7a, 8′), 2.87–2.78 (1H, m, H-8), 2.42 (1H, dd, J = 15.1, 13.0 Hz, H-7b); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.2 (C-9′), 151.6 (C-3′), 151.1 (C-5′), 147.0 (C-4′), 146.6 (C-4), 144.8 (C-3), 135.8 (C-1′), 131.7 (C-6), 127.3 (C-1), 119.5 (C-2′), 114.8 (C-6′), 105.8 (C-5), 103.0 (C-2), 101.4 (-OCH_2O-), 71.9 (C-9), 61.4 (4′-OMe), 61.3 (3′-OMe), 61.2 (5′-OMe), 45.9 (C-8′), 45.1 (C-7′), 35.5 (C-8), 30.6 (C-7); HRESI-MS *m/z* 654.8581, 656.8575, 658.8549, 660.8506

$[M + Na]^+$ (calcd. for $C_{22}H_{19}Br_3O_7$+Na, 654.8518, 656.8558, 658.8538, 660.8518).

Cell culture

The human cholangiocarcinoma (CCA) cell lines; KKU-M100 cells were routinely cultured in Ham's F12, supplemented with 10% fetal bovine serum, 12.5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, pH 7.3), 100 U/ml penicillin G and 100 µg/ml gentamicin. Human hepatoma cell line, HepG2 cells, from the American Type Culture Collection (ATCC HB 8065), were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (1%), MEM nonessential amino acids (Gibco), 12.5 mM HEPES, pH 7.3, 100 U/ml penicillin and 100 µg/ml gentamicin. Cultured cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. The cells were subcultured every 2–3 days before cultured cell confluence using 0.25% trypsin–EDTA, and medium was changed after an overnight incubation.

Cytotoxicity assay

KKU-M100 and HepG2 cells were seeded onto 96-well plates at a density of 7.5×10^3 and 1.5×10^4 cells/well, respectively. After an overnight incubation, cultured media were changed to serum-free media. Test compounds were dissolved in DMSO and diluted with medium to various concentrations before use. The compounds were added into cultured cells and incubated for 24 h. The cytotoxicity was assessed by the sulphorhodamine B (SRB) assay as previously described (Tusskorn et al. 2013). In brief, cultured cells were fixed with 15% trichloroacetic acid and stained with 0.4% SRB. The protein-bound dye was solubilized with 10 mM Tris-base solution for determination of the absorbance at 540 nm with a microplate reader. The cytotoxicity was calculated as percent absorbance of controls. The IC_{50} value was calculated by a non-linear curve-fitting program.

Acknowledgements The Post-Doctoral Training Program from Research Affairs and Graduate School, Khon Kaen University for Jittra Suthiwong is acknowledged (Grant no. 590012). This work was supported by the Thailand Research Fund (RSA5980022), Khon Kaen University and The Center of Excellence for Innovation in Chemistry (PERCH-CIC), Office of the Higher Education Commission, Ministry of Education.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Bogucki DE, Charlton JL (1995) An asymmetric synthesis of (-)-deoxypodophyllotoxin. J Org Chem 60:588–593

Chang LC, Song LL, Park EJ, Luyengi L, Lee KJ, Farnsworth NR, Pezzuto JM, Kinghorn AD (2000) Bioactive constituents of *Thuja occidentalis*. J Nat Prod 63:1235–1238

Chao YY, Su W, Jan CR, Ko YC, Chen JJ, Cheng JS, Liu CP, Lo YK, Chou KJ, Lee KC, Chen WC, Chen IS (2002) Novel action of lignans isolated from *Hernandia nymphaeifolia* on Ca^{2+} signaling in human neutrophils. Arch Toxicol 75:695–702

Chen IS, Chen JJ, Duh CY, Tsai IL (1997) Cytotoxic lignans from Formosan *Hernandia nymphaeifolia*. Phytochemistry 45:991–996

Chen JJ, Chang YL, Teng CM, Chen IS (2000) Anti-platelet aggregation alkaloids and lignans from *Hernandia nymphaeifolia*. Planta Med 66:251–256

Chen JJ, Tsai IL, Chen IS (1996) New oxoaporphine alkaloids from *Hernandia nymphaeifolia*. J Nat Prod 59:156–158

Feliciano AS, Medarde M, Lamamie de Clairaca RP, López JL, Puebla P, Gravalos DG, Lázaro PR, Garcia de Quesada T (1993) Synthesis and biological activity of bromolignans and cyclolignans. Arch Pharm 326:421–426

Gordaliza M, Castro A, Miguel del Corral J, López-Vázquez L, Garcia PA, Feliciano AS, Garcia-Grávalos D, Broughton H (1997) Preparation and cytotoxicity of podophyllotoxin derivatives lacking the lactone ring. Tetrahedron 53:15743–15760

Jiang RW, Zhou JR, Hon PM, Li SL, Zhou Y, Li LL, Ye WC, Xu HX, Shaw PC, But PPH (2007) Lignans from *Dysosma wersipellis* with inhibitory effects on prostate cancer cell lines. J Nat Prod 70:283–286

Kan WS (1970) Manual of Medicinal Plants in Taiwan. Vol. 1. National Research Insititute of Chinese Medicine, Taiwan, p 178–179

Lim YH, Leem MJ, Shin DH, Chang HB, Hong SW, Moon EY, Lee DK, Yoon SJ, Woo WS (1999) Cytotoxic constituents from the roots of *Anthriscus sylvestris*. Arch Pharm Res 22:208–212

McGlynn KA, Tarone RE, El-Serag HB (2006) A comparison of trends in the incidence of hepatocellular carcinoma and intrahepatic cholangiocarcinoma in the United States. Cancer Epidemiol Biomark Prev 15:1198–1203

Pettit GR, Meng Y, Gearing RP, Herald DL, Pettit RK, Doubek DL, Chapuis JC, Tackett LP (2004) Antineoplastic Agents. 522. *Hernandia peltata* (Malaysia) and *Hernandia nymphaeifolia* (Republic of Maldives). J Nat Prod 67:214–220

Sampson LK, Vickers SM, Ying W, Phillips JO (1997) Tamoxifen-mediated growth inhibition of human cholangiocarcinoma. Cancer Res 57:1743–1749

Songsiang U, Pitchuanchom S, Boonyarat C, Hahnvajanawong C, Yenjai C (2010) Cytotoxicity against cholangiocarcinoma cell lines of zerumbone derivatives. Eur J Med Chem45:3794–3802

Sripa B, Pairojkul C (2008) Cholangiocarcinoma: lessons from Thailand. Curr Opin Gastroenterol 24:349–356

Suthiwong J, Boonloh K, Kukongviriyapan V, Yenjai C (2018) Cytotoxicity against cholangiocarcinoma and HepG2 cell lines of lignans from *Hernandia nymphaeifolia*. Nat Prod Commun 13:61–63

Tusskorn O, Prawan A, Senggunprai L, Kukongviriyapan U, Kukongviriyapan V (2013) Phenethyl isothiocyanate induces apoptosis of cholangiocarcinoma cells through interruption of glutathione and mitochondrial pathway. Naunyn Schmiede Arch Pharmacol 386:1009–1016

Wickramaratne DB, Mar W, Chai H, Castlllo J, Farnsworth NR, Soejarto DD, Cordell GA, Pezzuto IM, Kinghorn AD (1995) Cytotoxic constituents of *Bursera permollis*. Planta Med 61:80–81

Arch. Pharm. Res. (2018) 41:431–437
https://doi.org/10.1007/s12272-018-1021-7

Online ISSN 1976-3786
Print ISSN 0253-6269

Archives of Pharmacal Research
www.springer.com/12272

CrossMark

RESEARCH ARTICLE

New limonophyllines A-C from the stem of *Atalantia monophylla* and cytotoxicity against cholangiocarcinoma and HepG2 cell lines

Aonnicha Sombatsri[1] · Yutthapong Thummanant[1] · Thurdpong Sribuhom[1] · Jaursup Boonmak[2] · Sujittra Youngme[2] · Suphanthip Phusrisom[3] · Veerapol Kukongviriyapan[3] · Chavi Yenjai[1]

Received: 31 October 2017 / Accepted: 6 March 2018 / Published online: 15 March 2018

Abstract Three new limonoids, limonophyllines A-C (**1**, **4** and **5**), along with two known limonoids (**2** and **3**) and 11 acridone alkaloids (**6-16**) were isolated from the stems of *Atalantia monophylla*. All isolates were evaluated against cholangiocarcinoma, KKU-M156, and HepG2 cancer cell lines. Compounds **12**, **14** and **16** displayed cytotoxicity against KKU-M156 cell line with IC_{50} ranging from 3.39 to 4.1 μg/mL while cytotoxicity against HepG2 cell line with IC_{50} ranging from 1.43 to 8.4 μg/mL. The structures of all isolated compounds were established by spectroscopic methods including 1D and 2D NMR, IR and mass spectrometry.

Keywords *Atalantia monophylla* · Limonophylline · Acridones · Cholangiocarcinoma · HepG2

Dedication In remembrance of His Majesty King Bhumibol Adulyadej (1927–2016).

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12272-018-1021-7) contains supplementary material, which is available to authorized users.

✉ Chavi Yenjai
chayen@kku.ac.th

[1] Natural Products Research Unit, Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand

[2] Materials Chemistry Research Center, Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand

[3] Department of Pharmacology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

Introduction

As part of our ongoing research on cytotoxicity against cholangiocarcinoma (KKU-M156) and hepatoma (HepG2) cell lines from natural sources (Suthiwong et al. 2014; Decharchoochart et al. 2014; Saraphon et al. 2017; Sribuhom et al. 2017). *A. monophylla*, known in Thai as "Ma Nao Phee", belongs to the family Rutaceae (Bunyapraphatsara 1999). It can be found over the Indian subcontinent and Southeast Asia. In Thailand, this medicinal plant has been used to treat chronic rheumatism, paralysis, hemiplegia, and as an antispasmodic, while the oil from the fruits has been used to treat respiratory diseases and rheumatism (Basa 1975; Panda 2004). It was reported that the roots of this plant contain limonoids and acridone alkaloids while its leaves contain steroids, triterpenoids, and flavonoids (Govindachari et al. 1970; Talapatra et al. 1970; Saraswathy et al. 1998). We report herein the isolation of three new limonoids, two known limonoids, and eleven acridone alkaloids from the stems of *A. monophylla* and cytotoxicity evaluation of isolated compounds.

Materials and methods

General experimental procedures

IR spectra were obtained using a Bruker Tenser 27 spectrophotometer. The UV spectra were measured using a JASCO J-810 apparatus. Optical rotations were obtained using a JASCO P-1020 digital polarimeter. The NMR spectra were recorded on a Varian Mercury plus spectrometer operating at 400 MHz (^{1}H) and at 100 MHz (^{13}C). Mass spectra were determined on a Micromass Q-TOF 2 hybrid quadrupole time-of-flight (Q-TOF) mass

spectrometer with a Z-spray ES source. Thin layer chromatography (TLC) was carried out on Merck silica gel 60 F_{254} TLC aluminium sheet. Column chromatography was done with silica gel 0.063–0.200 mm or less than 0.063 mm. Preparative layer chromatography (PLC) was carried out on glass supported silica gel plates using silica gel 60 PF_{254} for preparative layer chromatography. All solvents were routinely distilled prior to use.

Plant material

The stems of *A. monophylla* were collected in June 2016 from Phuwieng District, Khon Kaen Province, Thailand. The plant was identified by Dr. Pranom Chantaranothai, Faculty of Science, Khon Kaen University, Thailand where a voucher specimen (KKU022015) was deposited.

Extraction and isolation

Air-dried and finely powdered stems (6 kg) of *A. monophylla* were sequentially extracted at room temperature for 3 days with hexane (2 × 20 L), EtOAc (2 × 20 L) and MeOH (2 × 20 L). The extracts were evaporated in vacuo to obtain three dry extracts, crude hexane (12 g), EtOAc (80 g), and crude MeOH (100 g). The crude EtOAc extract was subjected to silica gel flash column chromatography (FCC) and subsequently eluted with a gradient of three solvents (hexane, EtOAc and MeOH) by gradually increasing the polarity of the elution solvents system. The eluents were collected and monitored by thin layer chromatography (TLC), resulting in 8 groups of eluting fractions which were designated as F1 to F8. Fraction F2 was purified by silica gel column chromatography and eluted with an isocratic system of 70% CH_2Cl_2:hexane to yield three subfractions, F2.1–F2.3. Subfraction F2.3 was purified by FCC using 80% CH_2Cl_2:hexane as developing solvent to yield **6** (199.8 mg, 0.0033%) and **14** (60.6 mg, 0.001%). Fraction F3 was purified by silica gel FCC using pure CH_2Cl_2 as eluent to give five subfractions, F3.1–F3.5. Subfractions F3.2 and F3.5 were purified by FCC using 20% EtOAc:hexane as eluent to afford **15** (47.9 g, 0.0008%) and **7** (167.4 mg, 0.0028%), respectively. Purification of F5 by FCC using pure CH_2Cl_2 as eluting solvent gave three subfractions, F5.1–F5.3. Fraction F5.3 was purified by silica gel FCC and eluted with a gradient system of EtOAc:hexane to give three subfractions, F5.3.1–F5.3.3. Subfraction F5.3.1 was recrystallized to obtain **13** (19.9 mg, 0.0003%). Subfractions F5.3.2 and F5.3.3 were subjected to column of Sephadex LH-20 using MeOH as eluent and yielded **9** (7.9 mg, 0.0001%) and **16** (24.1 mg, 0.0004%), respectively. Fraction F7 was purified by FCC (pure CH_2Cl_2) to give two subfractions F7.1 and F7.2. Recrystallization of solid in F7.2 yielded **1** (165.0 mg, 0.0027%). Subfraction F7.2 was further purified by FCC (pure CH_2Cl_2) and gave three subfractions, F7.2.1–F7.2.3. Recrystallization of solid in F7.2.1 yielded **12** (21.7 mg, 0.0004%). Subfraction F7.2.2 was subjected to FCC (1% MeOH:CH_2Cl_2) to obtain **8** (66.0 mg, 0.0011%) and **10** (19.3 mg, 0.0003%). Further purification of F7.2.3 with gel filtration (Sephadex LH-20) and eluting with MeOH afforded **5** (42.8 mg, 0.0007%) and **11** (16.2 mg, 0.0003%). Purification of F8 by FCC and elution with a gradient system of EtOAc:hexane gave two subfractions, F8.1 and F8.2. Further purification of F8.1 by gel filtration (Sephadex LH-20) gave **2** (63.6 mg, 0.0011%). Gel filtration (Sephadex LH-20) was carried out on subfractio F8.2, eluting with MeOH, and subsequent crystallization afforded **3** (10.1 mg, 0.00017%) and **4** (9.8 mg, 0.00016%).

Bioassay

Cell culture

Culture media, Ham F12, Dulbecco's modified Eagle's medium (DMEM) and MEM nonessential amino acids were purchased from Gibco BRL (Grand Island, NY, USA). *N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and sulphorhodamine B were from Sigma Chemical (St. Louis, MO, USA).

The human cholangiocarcinoma (CCA) cell line, KKU-M156, was routinely cultured in Ham's F12, supplemented with 10% fetal bovine serum, 12.5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES, pH 7.3), 100 U/mL penicillin G and 100 μg/ml gentamicin. For the human hepatoma cell line, HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (1%), MEM nonessential amino acids (Gibco), 12.5 mM HEPES, pH 7.3, 100 U/mL penicillin and 100 μg/mL gentamicin. Cultured cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. The cells were sub-cultured every 2 days with 0.25% trypsin–EDTA, and the medium was changed after an overnight incubation.

Cytotoxicity assay

KKU-M156 and HepG2 cells were seeded onto 96-well plates at densities of 7.5×10^3 and 1.5×10^4 cells/well, respectively. After an overnight incubation, cultured media were changed to the serum-free media. Test compounds were dissolved in DMSO and diluted with medium to various concentrations (1–200 μg/mL). The compounds were added into cultured cells and incubated for 24 h. The cytotoxicity was assessed by the sulphorhodamine B (SRB) assay as previously described (Tusskorn et al. 2013). In

brief, cultured cells were fixed with 10% trichloroacetic acid and stained with 0.4% SRB. The protein-bound dye was solubilized with 10 mM Tris-base solution for determination of the absorbance at 540 nm with a microplate reader. The cytotoxicity was calculated as percentage absorbance of controls. The IC_{50} value was calculated by a non-linear curve-fitting program from triplicate assay of two experiments.

Results and discussion

Chemical investigation of the stems of *A. monophylla* using chromatographic methods led to the isolation of 16 compounds, including five limonoids (**1–5**) and eleven acridone alkaloids (**6–16**) (Fig. 1). Among all isolated compounds, three new limonoids named limonophyllines **A-C** (**1, 4** and **5**), were discovered. In cases of known compounds, 7-hydroxycycloatalantin (**2**) (Bennett et al. 1994), cycloepiatalantin (**3**) (Bennett et al. 1994), *N*-methylatalaphylline (**6**) (Shan et al. 2013), atalaphylline (**7**) (Chukaew et al. 2008), citrusinine II (**8**) (Braga et al. 2007), citrusinion I (**9**) (Braga et al. 2007), glycosparvarine (**10**) (Chansriniyom et al. 2009), citruscridone (**11**) (Phetkul et al. 2013), buxifoliadine C (**12**) (Wu and Chen 2000), atalaphyllinine (**13**) (Kawaii et al. 1999), *N*-methylatalaphyllinine (**14**) (Auzi et al. 1996), *N*-methylcycloatalaphylline A (**15**) (Chukaew et al. 2008), and buxifoliadine E (**16**) (Chukaew et al. 2008) were isolated.

Compound **1** was found as a white solid and its molecular formula was determined as $C_{27}H_{30}O_9$ by a quasi-molecular ion peak at *m/z* 521.2023 $[M + Na]^+$ in the HRESIMS. The IR spectrum showed the absorption bands of ketone and α,β-unsaturated ketone groups at 1729 and 1688 cm^{-1}, respectively. The ^1H NMR spectrum showed two doublet signals (J = 5.6 Hz) at δ 7.95 (H-1) and δ 6.19 (H-2). The small coupling constant (J = 5.6 Hz) indicated that this moiety was a five-membered ring. The ^{13}C NMR spectrum confirmed the presence of α,β-unsaturated ketone moiety at δ 171.8 (C-1), δ 130.4 (C-2) and δ 199.7 (C-3).

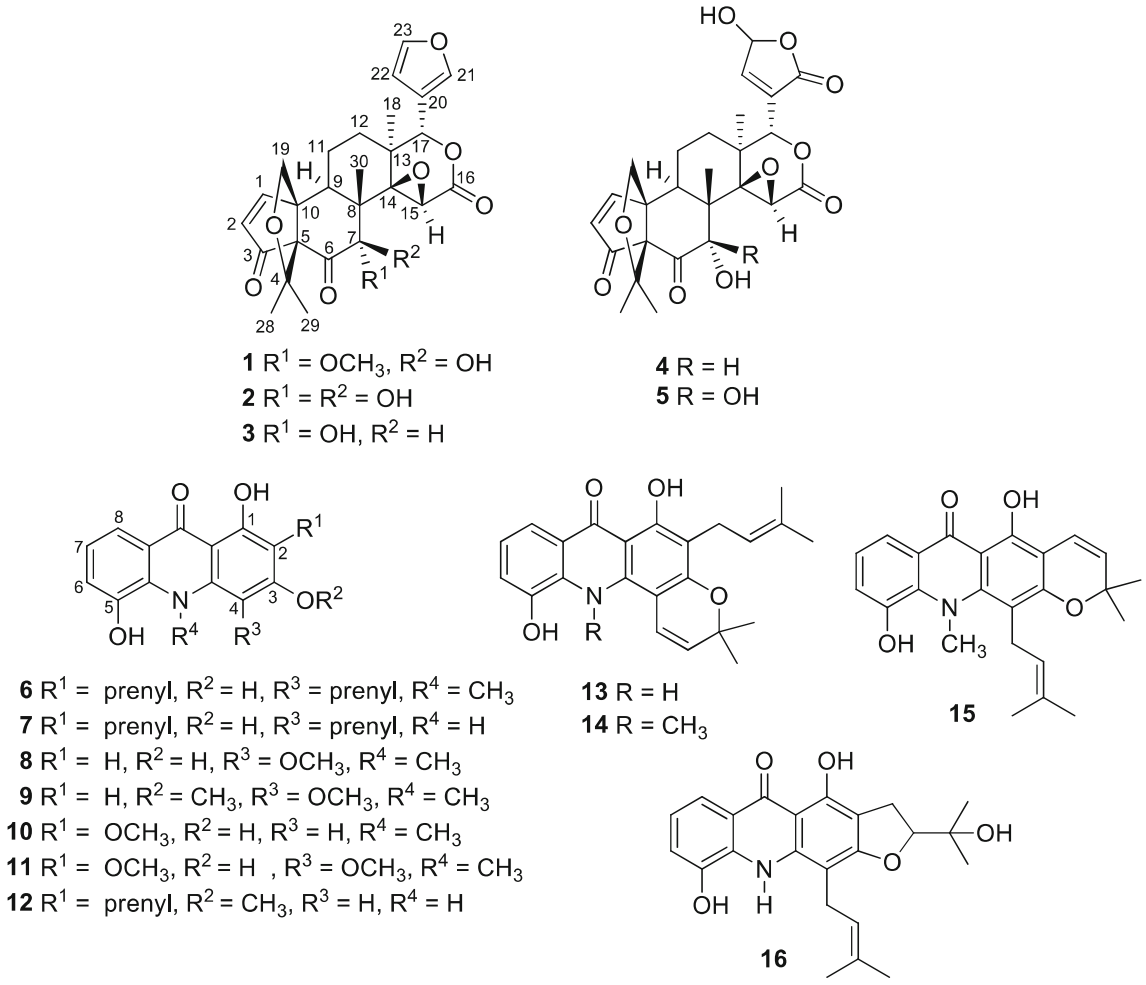


Fig. 1 Chemical structures of compounds **1–16**

The oxygenated methylene protons at δ 4.05 (J = 10 Hz, H-19a) and δ 3.89 (J = 10 Hz, H-19b) correlated with carbon at δ 70.6 (C-19) in the HMQC spectrum, in addition, they correlated with C-1 (δ 171.8) in the HMBC spectrum (Fig. 2). The ^{1}H-^{1}H COSY spectrum showed correlations for the H-9/H-11/H-12 system. A doublet of doublet signal at δ 2.98 (J = 13.0, 6.6 Hz) was assigned as methine proton H-9. The large coupling constant (J = 13.0 Hz) suggested the axial orientation of this proton. Correlations of H-9 and C-1 (δ 171.8), C-8 (δ 47.9), C-11 (δ 18.0), and C-19 (δ 70.6) were observed in the HMBC spectrum. In this spectrum, the methyl proton, CH_3-30, correlated with C-7 (δ 99.0), C-8 (δ 47.9), C-9 (δ 36.7), and C-14 (δ 70.2). The ^{13}C NMR signal of this methyl group was shielded (δ 15.2) by the epoxide oxygen at C-14,15 position which indicated the same face orientation of these groups. Epoxide lactone moiety of ring D displayed the signals of carbons at δ 70.2 (C-14), δ 57.3 (C-15) and δ 170.2 (C-16). The singlet signal at δ 5.60 was assigned to H-17 and correlated with carbon at δ 79.1 in the HMQC spectrum. A furan moiety showed the characteristic signals at δ_H/δ_C 7.46/144.1 (H-21), δ_H/δ_C 7.47/142.5 (H-23) and δ_H/δ_C 6.38/110.8 (H-22). Correlations of H-17 with C-13 (δ 39.8), C-14 (δ 70.2), C-18 (δ 18.3), C-20 (δ 121.6), C-21 (δ 144.1) and C-22 (δ 110.8) were observed in the HMBC experiment. The NOESY experiment showed cross-peaks between H-19a (δ 4.05) and CH_3-29 (δ 25.5), which indicated the same face of these two groups. In addition, correlations between CH_3-30 and H-17β, and between H-19b (δ 3.89) and H-1, and between H-9 and CH_3-18 and H-11α were observed in this experiment. The methoxy proton showed signals at δ_H/δ_C 2.88/47.9 and correlated with C-7 (δ 99.0) in the HMBC spectrum. The methoxy proton and H-15 were located on the same face which was confirmed by the NOE experiment. In addition, X-ray diffraction data confirmed the structure and stereochemistry of this compound as shown in Fig. 3. From all data, this limonoid was named limonophylline A.

Compound **4**, a white solid, displayed a molecular ion at m/z 523.1579 $[M + Na]^+$ indicating a molecular formula, $C_{26}H_{28}O_{10}$. The ^{1}H and ^{13}C NMR spectra displayed the

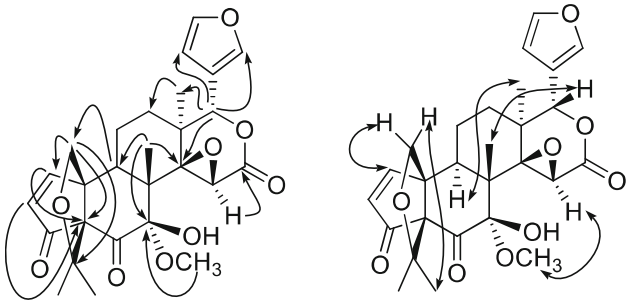


Fig. 2 Key HMBC and NOESY of compound **1**

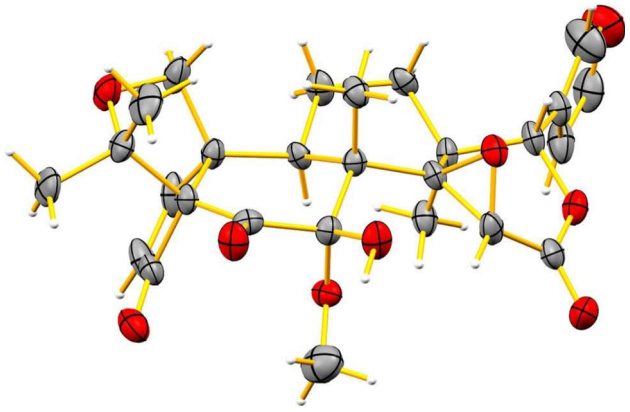


Fig. 3 X-ray crystal structure of limonophylline A

same patterns as those of **1**, except for the absence of furan and methoxy moieties. The ^{1}H NMR showed a singlet signal at δ 7.37 (H-22), which correlated with a carbon at δ 152.9 in the HMQC spectrum. This proton showed cross-peaks with lactone carbonyl carbon at δ 171.4 (C-21) and at δ 99.5 (C-23) in the HMBC experiment. This information indicated that **3** contained a γ-hydroxy butylrolactone moiety which was an oxidized form of furan. The ^{1}H NMR showed a singlet signal at δ 3.21 which was assigned as H-7 and located on C-7 (δ 77.4). The NOE experiment indicated the cofacial nature of H-7 and CH_3-30. Thus, the structure of compound **4**, limonophylline B, was defined as shown in Fig. 1.

Compound **5** was found as a white solid and displayed a molecular ion at m/z 539.1524 $[M + Na]^+$ indicating a molecular formula, $C_{26}H_{28}O_{11}$. The ^{1}H and ^{13}C NMR spectra displayed the same patterns as those of **4**, except for the absence of proton H-7. The ^{13}C NMR spectrum of **5** displayed a signal of C-7 at δ 99.4 while **4** showed at δ 77.4, which indicated the geminal diol group in this position. Comparing between **4** and **5**, the ^{1}H NMR signal of H-15 (δ 4.59) in **5** had a greater downfield shift than in **4** (δ 3.81) due to the effect of the geminal diol at C-7. The carbon signals at δ 134.2 (C-20), 171.8 (C-21), δ 152.5 (C-22), and δ 99.4 (C-23) indicated the γ-hydroxy butylrolactone group. Thus, the structure of compound **5**, limonophylline C, was defined as shown in Fig. 1.

All isolated compounds were evaluated for cytotoxicity against KKU-M156 and HepG2 cell lines using a sulforhodamine B (SRB) assay and the results are shown in Table 2. Acridone **14** showed strong cytotoxicity with IC_{50} values of 3.39 and 1.43 μg/mL against KKU-M156 and HepG2 cell lines, respectively, and showed Emax (maximum efficacy; maximal cell killing effect) at 100%. It is interesting to note that this compound was selective to HepG2 cells. Compound **6** showed cytotoxicity against KKU-M156 with an IC_{50} value of 35.9 μg/ml and Emax was 100%. These results showed convincingly that the

Table 1 ^{1}H and ^{13}C NMR spectral data of compounds **1**, **4**, and **5** ($CDCl_3$, δ in ppm)

Position	**1** ($CDCl_3$ + CD_3OD)		**4** (CD_3OD)		**5** (CD_3OD)	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	171.8	7.95 d (5.6)	171.4	7.97 d (8.0)	172.3	7.99 d (8.0)
2	130.4	6.19 d (5.6)	130.7	6.20 d (8.0)	130.6	6.17 d (8.0)
3	199.7		202.8		200.1	
4	87.0		86.6		87.1	
5	73.0		73.4		73.4	
6	198.7		202.9		198.8	
7	99.0		77.4	3.21 s	99.4	
8	47.9		44.5		48.2	
9	36.7	2.98 dd (13.0, 6.6)	34.2	2.95 dd (12.0, 4.0)	36.9	2.91dd, (12.0, 8.0)
10	61.7		62.4		62.0	
11	18.0	2.10 m	17.2	2.18 m	18.0	2.11 m
		1.84 m		1.97 m		1.90 m
12	26.8	1.76 m	26.4	1.90 m	26.4	1.83 m
		1.52 t (10.8)		1.49 t (16.0)		1.42 t (12.0)
13	39.8		40.2		40.9	
14	70.2		70.3		70.3	
15	57.3	4.64 s	58.1	3.81 s	57.5	4.59 s
16	170.2		168.8		169.5	
17	79.1	5.60 s	77.1	5.47 s	76.9	5.42 s
18	18.3	1.17 s	17.6	1.20 s	17.6	1.15 s
19	70.6	4.05 d (10.0)	70.9	4.05 d (12.0)	70.8	7.33 s
		3.89 d (10.0)		3.89 d (12.0)		3.87 d (12.0)
20	121.6		133.9		134.2	
21	144.1	7.46 s	171.4		171.8	
22	110.8	6.38 s	152.9	7.37 s	152.5	7.33 s
23	142.5	7.47 br d (4.0)	99.5	6.20 d (8.0)	99.4	6.17 d (8.0)
28	28.9	1.20 s	29.2	1.17 s	29.0	1.15 s
29	25.5	1.32 s	25.5	1.34 s	25.5	1.28 s
30	15.2	1.18 s	16.1	1.11 s	15.4	1.16 s
OCH_3	47.9	2.88 s				

pyran ring was necessary to cytotoxicity. In the case of HepG2 cells, compound **6** displayed weak activity with an IC_{50} value of 42.1 μg/ml. Compound **12** exhibited cytotoxicity against KKU-M156 and HepG2 cell lines with IC_{50} values of 4.1 and 8.4 μg/ml (E_{max} = 100%). Comparing between compounds **8** and **9**, cytotoxicity toward KKU-M156 cells seem nearly equal to each other ($IC_{50} \cong$ 10–11) but cytotoxicity of **9** against HepG2 cells was about 2 times more active than **8**. These results indicate that the methoxy group at the C-3 position is essential for cytotoxicity. In cases of **10** and **11**, the methoxy at C-4 position seems to dramatically decrease cytotoxicity. Compounds **16** and **7** displayed cytotoxicity against KKU-M156 cell line with IC_{50} values of 3.8 μg/mL (E_{max} 98%) and 3.7 μg/ml (E_{max} = 70%), respectively. On the other hand, cytotoxicity against HepG2 cell line was different, by showing IC_{50} values of 2.0 and 29.0 μg/mL for **16** and **7,** respectively. The results show the furan moiety may play an important role in cytotoxicity against HepG2 cells. In the cases of **6** and **7**, **7** showed 9.7-fold stronger cytotoxicity than **6** against KKU-M156 cells. The results indicate the *N*-CH_3 group led to a reduction in potency. Unfortunately, isolated limonoids showed weak cytotoxicity against KKU-M156 and HepG2 cell lines. Limonophylline C (**5**) showed strong cytotoxicity against HepG2 cells with an IC_{50} value of 2.1 μg/mL but the E_{max} was 24.2% which is not suitable to develop as a cytotoxic agent.

Table 2 Cytotoxicity of isolated compounds (IC_{50}, μg/ml)

Compound	KKU-M156		HepG2	
	IC_{50} (μg/mL)	E_{max} (%)	IC_{50} (μg/mL)	E_{max} (%)
1	139 ±45.8	87.7 ± 19.8	72.1 ± 20.0	54.1 ± 19.1
4	52.3 ± 16.1	100	17.2 ± 8.9	42 ± 7.3
5	83.6 ± 29.1	100	2.1 ± 1.1	24.2 ± 2.6
6	35.9 ± 18.7	100	42.1 ± 16.0	100
7	3.7 ± 1.1	70.3 ± .0	29.0 ± 13.1	68.4 ± 11.8
8	9.9 ± 3.2	91.8 ± 8.4	47.6 ± 15.0	100
9	11.8 ± 4.9	100	26.9 ± 6.8	88.8 ± .6
10	15.6 ± 1.8	84.1 ± 3.0	64.6 ± .7	100
11	60.1 ± 22.2	100	103.6 ± .8	100
12	4.1 ± 3.4	100	8.4 ± .2	100
14	3.39 ± 0.24	100	1.43 ± 0.69	100
16	3.8 ± 0.72	98.4 ± 5.6	2.0 ± 0.4	89.4 ± 3.7
the others	Inactive	–	Inactive	–
Cisplatin	3.36 ± 1.44	74.5 ± 14.2	0.66 ± 0.21	66.8 ± 3.7

*Data shown are from triplicate experiments

Conclusion

Sixteen compounds including three new limonoids, limonophyllines A-C (**1**, **4**, and **5**), two known limonoids, and 11 acridone alkaloids (**6-16**) were isolated from the stems of *Atalantia monophylla.* Cytotoxicity evaluation against KKU-M156 and HepG2 cell lines demonstrated that acridone alkaloids showed strong to moderate cytotoxicity while limonoids showed weak activity. Compounds **12** and **14** exhibited cytotoxicity against KKU-M156 and HepG2 cell lines with IC_{50} ranging from 1.43 to 8.4 μg/mL and showed 100% maximum efficacy. These substances are likely to be useful as lead compounds for the development of anticancer agents.

Spectroscopic data

Limonophylline A (**1**): white solid; mp. 173-175°C; $[\alpha]_D^{24}$ -40 (*c* 1.00, CH_2Cl_2); UV (MeOH) λ_{max} (log ε) 247 (3.14), 320 (2.17) nm; IR (Neat) ν_{max} 3395, 2950, 1729, 1688, 1259, 1059, 808 cm^{-1}; ^1H ($CDCl_3$, 400 MHz) and ^{13}C NMR data ($CDCl_3$, 100 MHz), see Tables 1 and 2; HRESIMS *m/z* 521.2023 (calcd. for $C_{27}H_{30}O_9$ + Na, 521.1788).

Limonophylline B (**2**): white solid; mp. 241-243°C; $[\alpha]_D^{26}$ +1.3 (*c* 1.00, CH_2Cl_2); UV (MeOH) λ_{max} (log ε) 217 (3.78) nm; IR (Neat) ν_{max} 3420, 1731, 1385, 1263, 1022, 772 cm^{-1}; ^1H ($CDCl_3$, 400 MHz) and ^{13}C NMR data ($CDCl_3$, 100 MHz), see Tables 1 and 2; HRESIMS *m/z* 523.1579 (calcd. for $C_{26}H_{28}O_{10}$ + Na, 523.1580).

Limonophylline C (**3**): white solid; $[\alpha]_D^{25}$ -4.2 (*c* 1.00, CH_2Cl_2); UV (MeOH) λ_{max} (log ε) 204 (4.03) nm; IR (Neat) ν_{max} 3409, 2946, 1730, 1262, 1014, 929, 811 cm^{-1}; ^1H ($CDCl_3$, 400 MHz) and ^{13}C NMR data ($CDCl_3$, 100 MHz), see Tables 1 and 2; HRESIMS *m/z* 539.1524 (calcd. for $C_{26}H_{28}O_{11}$ + Na, 539.1529).

Acknowledgements We thank the Science Achievement Scholarship of Thailand (SAST) for the support to A. Sombatsri. We acknowledge the Thai Research Fund (RSA5980022) and Khon Kaen University for financial support. The Center of Excellence for Innovation in Chemistry (PERCH-CIC), Office of the Higher Education Commission, Ministry of Education is gratefully acknowledged.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to report.

References

Auzi AA, Hartley TG, Waigh RD, Waterman PG (1996) Acridone alkaloids from *Bosistoa transversa*. Phytochemistry 42:235–238

Basa SC (1975) Atalaphyllinine, a new acridone base from *Atalantia monophylla*. Phytochemistry 14:835–836

Bennett RD, Hasegawa S, Wong RY (1994) Limonoids from *Atalantia zeylanica*. Phytochemistry 36:163–166

Braga PAC, Dos Santos DAP, Da Silva MFDGF, Vieira PC, Fernandes JB, Houghton PJ, Fang R (2007) In vitro cytotoxicity activity on several cancer cell lines of acridone alkaloids and *N*-phenylethyl-benzamide derivatives from Swinglea glutinosa (Bl.) Merr. Nat Prod Res 21:47–55

Bunyapraphatsara N (1999) Traditional Herb. Prachachon Print 3:568

Chansriniyom C, Ruangrungsi N, Lipipun V, Kumamoto T, Ishikawa T (2009) Isolation of acridone alkaloids and *N*-[(4-monoterpenyloxy)phenylethyl]-substituted sulfur-containing propanamide derivatives from *Glycosmis parva* and their anti-herpes simplex virus activity. Chem Pharm Bul 57:1246–1250

Chukaew A, Ponglimanont C, Karalai C, Tewtrakul S (2008) Potential anti-allergic acridone alkaloids from the roots of *Atalantia monophylla*. Phytochemistry 69:2616–2620

Decharchoochart P, Suthiwong J, Samatiwat P, Kukongviriyapan V, Yenjai C (2014) Cytotoxicity of compounds from the fruits of *Derris indica* against cholangiocarcinoma and HepG2 cell lines. J Nat Med 68:730–736

Govindachari TR, Viswanathan N, Pai BR, Ramachandran VN, Subramaniam PS (1970) Alkaloids of *Atalantia monyphylla*. Tetrahedron 26:2905–2910

Kawaii S, Tomono Y, Katase E, Ogawa K, Yano M, Takemura Y, Juichi M, Ito C, Furukawa H (1999) Acridones as inducers of HL-60 cell differentiation. Leukemia Res 23:263–269

Panda H (2004) Handbook on medicinal herbs with uses. Asia Pacific Business Press Inc, Delhi, pp 166–167

Phetkul U, Wanlaso N, Mahabusarakam W, Phongpaichit S, Carroll AR (2013) New acridone from the wood of *Citrus reticulata* Blanco. Nat Prod Res 27:1922–1926

Saraphon C, Boonloh K, Kukongviriyapan V, Yenjai C (2017) Cytotoxic flavonoids from the fruits of *Derris indica*. J Asian Nat Prod. https://doi.org/10.1080/10286020.2017.1317750

Saraswathy A, Balakrishna K, Rao RB, Allirani T, Patra A, Pichai R (1998) Carpachromene from *Atalantia monophylla*. Fitoterapia 69:463–464

Shan F, Yin YQ, Huang F, Huang YC, Guo LB, Wu YF (2013) A novel acridone alkaloid from *Atalantia buxifolia*. Nat Prod Res 27:1956–1959

Sribuhom T, Boueroy P, Hahnvajanawong C, Phatchana R, Yenjai C (2017) Benzoyltyramine alkaloids atalantums A-G from the peels of *Atalantia monophylla* and their cytotoxicity against cholangiocarcinoma cell lines. J Nat Prod 80:403–408

Suthiwong J, Pitchuanchom S, Wattanawongdon W, Hahnvajanawong C, Yenjai C (2014) Terpenoids from the root bark of *Pterolobium macropterum*. J Nat Prod 77:2432–2437

Talapatra SK, Bhattacharya S, Talapatra B (1970) Terpenoid and related compounds. VI. Terpenoid and coumarin constituents of *Atalantia monophylla*. J Indian Chem Soc 47:600–604

Tusskorn O, Prawan A, Senggunprai L, Kukongviriyapan U, Kukongviriyapan V (2013) Phenethyl isothiocyanate induces apoptosis of cholangiocarcinoma cells through interruption of glutathione and mitochondrial pathway. Naunyn Schmiedebergs Arch Pharmacol 386:1009–1016

Wu TS, Chen CM (2000) Acridone alkaloids from the root bark of *Severinia buxifolia* in Hainan. Chem Pharm Bull 48:85–90

NPC Natural Product Communications

2018
Vol. 13
No. 1
61 - 63

Cytotoxicity against Cholangiocarcinoma and HepG2 Cell Lines of Lignans from *Hernandia nymphaeifolia*

Jittra Suthiwong[a], Kampeebhorn Boonloh[b], Veerapol Kukongviriyapan[b] and Chavi Yenjai[a]*

[a]*Natural Products Research Unit*, *Center of Excellence for Innovation in Chemistry*, *Department of Chemistry*, *Faculty of Science*, *Khon Kaen University*, *Khon Kaen 40002*, *Thailand*
[b]*Department of Pharmacology*, *Faculty of Medicine*, *Khon Kaen University*, *Khon Kaen 40002*, *Thailand*

chayen@kku.ac.th

In remembrance of His Majesty King Bhumibol Adulyadej (1927-2016)

Received: September 27th, 2017; Accepted: November 22nd, 2017

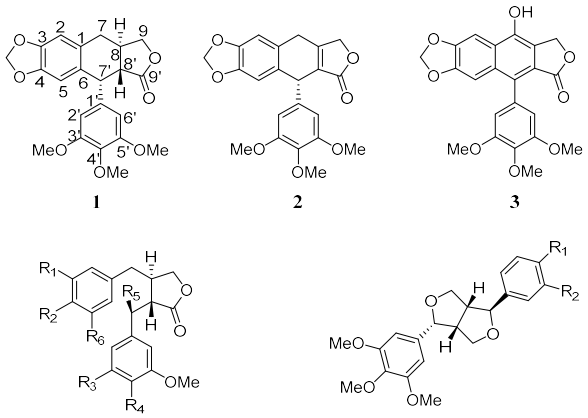
Eleven lignans (**1-11**) were isolated from the seed of *Hernandia nymphaeifolia*. Most of the lignans exhibited strong to moderate cytotoxicity against cholangiocarcinoma KKU–M156 and HepG2 cell lines. Compounds **4** and **8** showed cytotoxicity against the KKU–M156 cell line with IC_{50} values of 5.2 μM (Emax 96%) and 5.4 (Emax 59%) μM, respectively. In the cases of cytotoxicity against the HepG2 cell line, compounds **2**, **3**, **4**, and **8** showed cytotoxicity with IC_{50} values of 1.7 μM (Emax 84%), 4.1 μM (Emax 74%), 4.5 μM (Emax 68%), and 5.2 μM (Emax 78%), respectively.

Keywords: *Hernandia nymphaeifolia*, Cholangiocarcinoma, KKU–M156, HepG2, Lignan.

Hernandia nymphaeifolia belongs to the Family Hernandiaceae and is an evergreen tree that grows along seashores [1]. It is widely distributed in tropical countries such as the Maldives, Japan, Taiwan and Thailand. The chemical investigation of the stem of this plant led to the isolation of lignans and aporphine alkaloids [2-3]. Some lignans such as *epi*-aschantin, *epi*-magnolin, *epi*-yangambin, deoxypodophyllotoxin and yatin showed strong *anti*-platelet aggregation activity [4]. In addition, deoxypodophyllotoxin and yatin displayed cytotoxicity against several cancer cell lines [2,5-11]. It was found that deoxypodophyllotoxin inhibits 12-*O*-tetradecanoylphorbol 13-acetate-induced ornithine decarboxylase in cultured mouse epidermal cells [12-14].

In the continuation of our research on the cytotoxicity of natural compounds, we are interested in the seed of *H. nymphaeifolia* which contains cytotoxic lignans. In this work, the cytotoxicity against cholangiocarcinoma cells (KKU–M156) and hepatoma (HepG2) were evaluated. Cholangiocarcinoma is the most interesting cancer in the northeast of Thailand because it is one of the major health problems in this area [15]. This disease occurs from the liver fluke, *Opisthorchis viverrini,* infection [16]. The search for active compounds from natural sources is an attractive way to find effective anticancer agents.

Crude EtOAc and methanol extracts from the seed of *H. nymphaeifolia* were separated by column chromatography and preparative TLC, leading to eleven lignans, (-)-deoxy-podophyllotoxin (**1**) [17], *β*-apopicropodophyllin (**2**) [18], dehydropodophyllotoxin (**3**) [19], deoxypodorhizone (**4**) [20], 5′-methoxyyatein (**5**) [21], podorhizol (**6**) [22], bursehernin (**7**) [23], (-)-maculatin (**8**) [24], hernanol (**9**) [25], (+)-epimagnolin (**10**) [25], (+)-epiaschantin (**11**) [25]. The structure of all the isolated compounds were elucidated by spectroscopic methods and by comparison with those previously reported in the literature. The isolated compounds were tested for their cytotoxicity in both KKU–M156 and HepG2 cells using sulphorhodamine B (SRB) assay. Deoxypodorhizone (**4**) and (-)-maculatin (**8**) showed



4 $R_1, R_2 = OCH_2O$, $R_3 = R_4 = OMe$, $R_5 = R_6 = H$
5 $R_1, R_2 = OCH_2O$, $R_3 = R_4 = OMe$, $R_5 = H$, $R_6 = OMe$
6 $R_1, R_2 = OCH_2O$, $R_3 = R_4 = OMe$, $R_5 = OH$, $R_6 = H$
7 $R_1, R_2 = OCH_2O$, $R_3 = H$, $R_4 = OMe$, $R_5 = R_6 = H$
8 $R_1 = R_2 = OMe$, $R_3 = H$, $R_4 = OMe$, $R_5 = R_6 = H$
9 $R_1 = OMe$, $R_2 = OH$, $R_3 = R_4 = OMe$, $R_5 = R_6 = H$
10 $R_1 = R_2 = OMe$
11 $R_1, R_2 = OCH_2O$

Figure 1: Structure of isolated compounds.

cytotoxicity against the KKU–M156 cell lines, with IC_{50} values of 5.2 and 5.4 μM, respectively. Comparing compounds **4** and **6**, they showed cytotoxicity against KKU–M156 with IC_{50} values of 5.2 and 40.1 μM, respectively. It is suggested that the hydroxyl group at C-7′ is dramatically detrimental to the activity. In the cases of compounds **7** and **8**, they displayed cytotoxic activity with IC_{50} values of 24.0 and 5.4 μM, respectively. These results seem to indicate that dimethoxy groups at C-3 and C-4 may be important for cytotoxicity.

In the case of the HepG2 cell line, compounds **2**, **3**, **4** and **8** showed strong cytotoxicity with IC_{50} values of 1.7, 4.1, 4.5, and 5.2 μM, respectively. Comparing between **1** and **2**, **2** showed stronger cytotoxicity, about 4.8 fold, against the HepG2 cell line. This may

Table 1: Cytotoxicity of isolated compounds.

Compds	KKU-M156		HepG2	
	IC_{50} (μM)	Emax (%)	IC_{50} (μM)	Emax (%)
Crude EtOAc	8.8*	93	1.9*	62
Crude MeOH	3.6*	73	5.1*	82
1	34.6	83	8.1	84
2	15.4	99	1.7	84
3	29.7	98	4.1	74
4	5.2	96	4.5	68
6	40.1	90	16.3	83
7	24.0	81	10.6	85
8	5.4	59	5.2	78
10	16.5	77	18.2	89
11	12.5	82	12.5	71
Cisplatin	3.3	100	5.7	100

*IC_{50} expressed as μg/mL; Emax was percent of maximal cancer cell killing effect.

be due to the α,β-unsaturated lactone moiety necessary for the activity. Comparing cytotoxicity between **4** and **7**, **4** showed 2.4 fold stronger activity than **7**. These results suggest the methoxy group at the C-3 position may play an important role for the activity. Cytotoxicity measurements of **7** and **8** showed IC_{50} values of 10.6 and 5.2 μM.

These results indicate that the dimethoxy groups at C-3 and C-4 may enhance the activity, similarly to the results for the KKU–M156 cell line. From all the data, compounds **4** and **2** are interesting as lead compounds for drug development, since they show strong cytotoxicity and also exhibit high values of Emax (maximum efficacy) at 96% and 84%, respectively.

In conclusion, chemical investigation of the seed of *Hernandia nymphaeifolia* led to the isolation of eleven lignans (**1-11**). Cytotoxicity evaluation of isolated compounds against cholangiocarcinoma, KKU–M156, and HepG2 cell lines were examined. It was found that compounds **4** and **8** showed strong cytotoxicity against the KKU–M156 cell line with IC_{50} values of 5.2 μM (Emax 96%) and 5.4 μM (Emax 59%), respectively. In the cases of the HepG2 cell line, compounds **2**, **3**, **4**, and **8** showed cytotoxicity with IC_{50} values ranging from 1.7 to 5.2 μM.

Experimental

General experimental procedures: All melting points were determined on a SANYO Gallenkamp (Leicester, UK) melting point apparatus. Optical rotations were identified using a JASCO P-1020 digital polarimeter. UV spectra were recorded using an Agilent 8453 UV-Visible spectrophotometer (Waldbronn, Germany). IR spectra were taken from thin films using a Perkin Elmer Spectrum One FT-IR spectrophotometer (Shelton, CT, USA). NMR spectra were determined with a Varian Mercury plus spectrometer (California, USA) operating at 400 MHz (^{1}H) and at 100 MHz (^{13}C). Mass spectra were recorded on a Micromass Q-TOF 2 hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer with a Z-spray ES source (Micromass, Manchester, UK). Silica gel 60 (100-200 mesh, Merck, Darmstadt, Germany) was employed for column chromatography. Preparative TLC was carried out using silica gel PF_{254} (Merck, Darmstadt, Germany). TLC was examined on silica gel 60 F_{254} (Merck, Darmstadt, Germany) precoated aluminum sheets. Spots on TLC were visualized under UV light and by spraying with acidic anisaldehyde solution followed by heating. All solvents were distilled throughout the separation process.

Plant materials: The seed of *H. nymphaeifolia* was collected in May 2013 from Krabi province in Thailand. Voucher specimens were deposited at the Herbarium of the Department of Chemistry, Faculty of Science, Khon Kaen University.

Extraction and isolation: Extraction and isolation: Air dried seed (300 g) of *H. nymphaeifolia* were ground into a powder and then extracted successively with EtOAc (1.5 L x 3) and MeOH (1.5 L x 3) at room temperature. The filtrates were combined and the solvents were evaporated *in vacuo* to yield crude EtOAc (79 g) and MeOH (23 g), respectively. The crude EtOAc extract (79 g) was subjected to flash column chromatography (FCC) and eluted with a gradient system of hexane:EtOAc and EtOAc:MeOH. The fractions which contained the same major compounds were combined to give four fractions, EF_1-EF_4. Fraction EF_3 was chromatographed by Sephadex LH-20 (MeOH) to remove pigments, and the residue was subjected to silica gel FCC eluted with CH_2Cl_2 to obtain two subfractions $EF_{3.2.1}$ and $EF_{3.2.2}$. Subfraction $EF_{3.2.2}$ was purified by silica gel FCC and eluted with a gradient of 20% EtOAc-hexane to give **1** and two subfractions, $EF_{3.2.2.1}$ and $EF_{3.2.2.2}$. Further purification of these two subfractions by preparative thin layer chromatography (PLC), and developing with 30% EtOAc-hexane, afforded **11** and **4**, respectively. Fraction EF_4 was subjected to gel filtration over Sephadex LH-20 (MeOH) to afford **3** and two subfractions, $EF_{4.1}$ and $EF_{4.2}$. Subfracttion $EF_{4.2}$ was further purified by silica gel FCC and eluted with a gradient of 5% EtOAc-hexane to obtain two subfractions, $EF_{4.2.1}$ and $EF_{4.2.2}$, which were purified by PLC (1% MeOH-CH_2Cl_2) to afford compounds **10** and **8** from $EF_{4.2.1}$ and gave compounds **6** and **2** from $EF_{4.2.2}$.

The crude MeOH extract (23 g) was subjected to flash column chromatography (FCC) and eluted with a gradient system of hexane:EtOAc and EtOAc:MeOH. The fractions which contained the same major compounds were combined to give four fractions, MF_1-MF_4. Fraction MF_2 was purified by silica gel FCC, and 20 % EtOAc-hexane was used as an eluent to give three subfractions, $MF_{2.1}$-$MF_{2.3}$. Further purification of $MF_{2.2}$ and $MF_{2.3}$ by PLC and developing with CH_2Cl_2 afforded **5** and **7**, respectively. Fraction MF_3 was purified by silica gel FCC (CH_2Cl_2 as eluent) to give subfractions $MF_{3.1}$ and $MF_{3.2}$. Further purification of $MF_{3.1}$ with PLC (50% acetone-hexane) afforded **9**.

Cell cultures: The human cholangiocarcinoma (CCA) cell line, KKU–M156 cells, was routinely cultured in Ham's F12, supplemented with 10% fetal bovine serum, 12.5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, pH 7.3), 100 U/mL penicillin G and 100 μg/mL gentamicin. The human hepatoma cell line, HepG2 cells, was grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (1%), MEM nonessential amino acids (Gibco), 12.5 mM HEPES, pH 7.3, 100 U/mL penicillin and 100 μg/mL gentamicin. Cultured cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. The cells were subcultured every 2 days with 0.25% trypsin–EDTA, and the medium was changed after an overnight incubation.

Cytotoxicity assay: KKU–M156 and HepG2 cells were seeded onto 96 well-plates at densities of $7.5x10^3$ and $1.5x10^4$ cells/well, respectively. After an overnight incubation, cultured media were changed to serum-free media. Test compounds, dissolved in DMSO and diluted with medium to various concentrations (1-200 μg/mL), were added into cultured cells and incubated for 24 h. The cytotoxicity was assessed by the sulphorhodamine B (SRB) assay as previously described [26]. In brief, cultured cells were fixed with 10% trichloroacetic acid and stained with 0.4% SRB. The protein-bound dye was solubilized with 10 mM Tris-base solution for determination of the absorbance at 540 nm with a microplate reader. The cytotoxicity was calculated as percent absorbance of controls. The IC_{50} value was calculated by a non-linear curve-fitting program from triplicate assay of two experiments.

Supplementary data: ^{1}H and ^{13}C NMR spectra of all compounds (**1**–**11**).

Acknowledgements - The authors thank the Royal Golden Jubilee Scholarship [PHD/0020/2556], and also thank the Thailand Research Fund [Grant No. RSA5980022] and Khon Kaen University for financial support. The Center of Excellence for Innovation in Chemistry (PERCH-CIC), Office of the Higher Education Commission, Ministry of Education, Thailand, is gratefully acknowledged.

References

[1] Yang DM, Lu SY. (**1996**) Flora of Taiwan, Hernandiaceae, vol. 2. Editorial Committee of the Flora of Taiwan, 2nd ed. Taipei, Taiwan: p 500–501.
[2] Chen JJ, Tsai IL, Chen IS. (**1996**) New oxoaporphine alkaloids from *Hernandia nymphaeifolia*. *Journal of Natural Products*, ***59***, 156–158.
[3] Chen IS, Chen JJ, Duh CY, Tsai IL. (**1997**) Cytotoxic lignans from Formosan *Hernandia nymphaeifolia*. *Phytochemistry*, ***45***, 991–996.
[4] Chen JJ, Chang YL, Teng CM, Chen TS. (**2000**) Anti-platelet aggregation alkaloids and lignans from *Hernandia nymphaeifolia*. *Planta Medica*, ***66***, 251–256.
[5] Fang JM, Lee CK, Cheng YS. (**1992**) Lignans from leaves of *Juniperus chinensis*. *Phytochemistry*, ***31***, 3659–3661.
[6] Wickramaratne DB, Mar E, Chai H, Castillo JJ, Farnsworth NR, Soejarto DD, Cordell GA, Pezzuto JM, Kinghom AD. (**1995**) Cytotoxic constituents of *Bursera permollis*. *Planta Medica*, ***61***, 80–81.
[7] Ikeda R, Nagao T, Okabe H, Nakano Y, Matsunaga H, Katano M, Mori M. (**1998**) Antiproliferative constituents in umbelliferae plants. III. Constituents in the root and the ground part of *Anthriscus sylvestris* Hoffm. *Chemical & Pharmaceutical Bulletin*, ***46***, 871–874.
[8] Lim YH, Leem MJ, Shin DH, Chang HB, Hong SW, Moon EY, Lee DK, Yoon SJ, Woo WS. (**1999**) Cytotoxic constituents from the roots of *Anthriscus sylvestris*. *Archives of Pharmacal Research*, ***22***, 208–212.
[9] Jiang RW, Zhou JR, Hon PM, Li SL, Zhou Y, Li LL, Ye WC, Xu HX, Shaw PC, But P PH. (**2007**) Lignans from *Dysosma versipellis* with inhibitory effects on prostate cancer cell lines. *Journal of Natural Products*, ***70***, 283–286.
[10] Yong Y, Shin SY, Lee YH, Lim Y. (**2009**) Antitumor activity of deoxypodophyllotoxin isolated from *Anthriscus sylvestris*: Induction of G2/M cell cycle arrest and caspase-dependent apoptosis. *Bioorganic & Medicinal Chemistry Letters*, ***19***, 4367–4371.
[11] Wu M, Jiang Z, Duan H, Sun L, Zhang S, Chen M, Wang Y, Gao Q, Song Y, Zhu X, Zhang L. (**2013**) Deoxypodophyllotoxin triggers neocrotosis in human non-small cell lung cancer NCI-H460 cells. *Biomedicine & Pharmacotherapy*, ***67***, 701–706.
[12] Chang LC, Song LL, Park EJ, Luyengi L, Lee KJ, Farnsworth NR, Pezzuto JM, Kinghorn AD. (**2000**) Bioactive constituents of *Thuja occidentalis*. *Journal of Natural Products*, ***63***, 1235–1238.
[13] Ito C, Itoigawa M, Ogata M, Mou XY, Tokuda H, Nishino H, Furakawa H. (**2001**) Lignans as anti-tumor-promoter from the seeds of *Hernandia ovigera*. *Planta Medica*, ***67***, 166–168.
[14] Gu JQ, Park EJ, Totura S, Riswan S, Fong HHS, Pezzuto JM, Kinghorn AD. (**2002**) Constituents of the twigs of *Hernandia ovigera* that inhibit the transformation of JB6 murine epidermal cells. *Journal of Natural Products*, ***65***, 1065–1068.
[15] Sripa B, Pairojkul C. (**2008**) Cholangiocarcinoma: lessons from Thailand. *Current Opinion in Gastroenterology*, ***24***, 349–356.
[16] McGlynn, KA, Tarone RE, El-Serag HB. (**2006**) A comparison of trends in the incidence of hepatocellular carcinoma and intrahepatic cholangiocarcinoma in the United States. *Cancer Epidemiology Biomarkers & Prevention*, ***15***, 1198–1203.
[17] Dickson RA, Houghton PJ, Hylands PJ. (**2007**) Antibacterial and antioxidant cassane diterpenoids from *Caesalpinia benthamiana*. *Phytochemistry*, ***68***, 1436–1441.
[18] Lane AL, Kubanek J. (**2006**) Structure–activity relationship of chemical defenses from the freshwater plant *Micranthemum umbrosum*. *Phytochemistry*, ***67***, 1224–1231.
[19] Kofod H, Jorgensen C. (**1954**) Dehydropodophyllotoxin, a new compound isolated from *Podophyllum peltatum*. *Acta Chemica Scandinavica*, ***8***, 1296–1297.
[20] Kuo YC, Kuo, YH, Lin YL, Tsai WJ. (**2006**) Yatein from *Chamaecyparis obtusa* suppresses herpes simplex virus type 1 replication in HeLa cells by interruption the immediate-early gene expression. *Antiviral Research*, ***70***, 112–120.
[21] Richomme P, Bruneton J, Cabalion P, Debray MM. (**1984**) Study of Hernandiaceae. IX. Lignans from two Melanesian *Hernandia*. *Journal of Natural Products*, ***47***, 879–881.
[22] San Feliciano A, Medarde M, Lopez JL, Puebla P, Miguel del Corral JM, Barrero AF. (**1989**) Lignans from *Juniperus thurifera*. *Phytochemistry*, ***28***, 2863–2866.
[23] Gonzalez AG, Estevez-Reyes R, Mato C, Estevez-Braun AM. (**1990**) Three lignans from *Bupleurum salicifolium*. *Phytochemistry*, ***29***, 1981–1983.
[24] Takaoka D, Imooka M, Hiroi M. (**1977**) Studies of lignoids in Lauraceae. III. A new lignan from the heart wood of *Cinnamomum Camphora* Siep. *Bulletin of the Chemical Society of Japan*, ***50***, 2821–2822.
[25] Pettit GR, Meng Y, Gearing RP, Herald DL, Pettit RK, Doubek DL, Chapuis JC, Tackett LP. (**2004**) Antineoplastic agents. 522. *Hernandia peltata* (Malaysia) and *Hernandia nymphaeifolia* (Republic of Maldives). *Journal of Natural Products*, ***67***, 214–220.
[26] Tusskorn O, Prawan A, Senggunprai L, Kukongviriyapan U, Kukongviriyapan V. (**2013**) Phenethyl isothiocyanate induces apoptosis of cholangiocarcinoma cells through interruption of glutathione and mitochondrial pathway. *Naunyn-Schmiedeberg's Archives of Pharmacology*, ***386***, 1009–1016.

NATURAL PRODUCT RESEARCH, 2018
VOL. 32, NO. 8, 944–952
https://doi.org/10.1080/14786419.2017.1374264

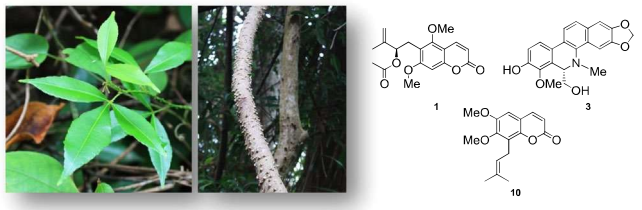
Coumarins and alkaloids from the roots of *Toddalia asiatica*

Sanwat Sukieum[a], Wichien Sang-aroon[b] and Chavi Yenjai[a]

[a]Natural Products Research Unit, Faculty of Science, Department of Chemistry and Center of Excellence for Innovation in Chemistry, Khon Kaen University, Khon Kaen, Thailand; [b]Faculty of Engineering, Department of Chemistry, Rajamangala University of Technology Isan, Khon Kaen, Thailand

ABSTRACT

The EtOAc and MeOH extracts of the roots of *Toddalia asiatica* Lam. were investigated for the roots' chemical constituents. Two new compounds including 2′*R*-acetoxytoddanol (**1**) and 8*S*-10-*O*-demethylbocconoline (**3**) as well as 15 known compounds were isolated. Compound **10** showed strong cytotoxicity against KB cells with an IC_{50} value of 2.60 µg/mL, which is nearly equal to the ellipticine standard, but showed no activity against Vero cells. Alkaloid **3** displayed weak cytotoxicity against the KB cell line with an IC_{50} value of 21.69 µg/mL.



ARTICLE HISTORY
Received 5 June 2017
Accepted 12 August 2017

KEYWORDS
Toddalia asiatica; 2′*R*-Acetoxytoddanol; quinolone alkaloid; phenanthridine

1. Introduction

Toddalia asiatica (Linn.) Lam., a member of the Rutaceae family, is a medicinal plant widely distributed in humid tropical areas such as east Africa, south Asia, southeast Asia and China. It is a liana, with woody and thorny stems, and climbs on trees reaching a height of 10 m. This plant has been used to treat chronic lumbago, scelalgia, colds, stomachache and injuries from falls. The root bark of this plant shows some therapeutic activities such as to treat diarrhoea, gonorrhoea, cough, influenza and for toothache (Jain et al. 2006; Hu et al. 2014). Many compounds from this plant showed cytotoxicity against a leukaemic cell line (U-937) (Vázquez et al. 2012). The leaves are used to cure lung diseases and for curing bowel complaints (Jain et al. 2006). The EtOAc extract of the leaves has shown significant antidiabetic and antioxidant effects in STZ-induced diabetic rats (Stephen Irudayaraj et al. 2012). The isolation of volatile oils (Saxena and Sharma 1999), coumarins (Karunai Raj et al. 2012),

CONTACT Chavi Yenjai chayen@kku.ac.th
Supplemental data for this article can be accessed at https://doi.org/10.1080/14786419.2017.1374264.

phenanthridine alkaloids (Hu et al. 2014), quinolone alkaloid (Duraipandiyan and Ignacimuthu 2009) and amide from this plant (Tsai et al. 1997; Hu et al. 2015) has been reported. The ethanol extract from the roots of this plant contain alkaloid derivatives and shows cytotoxic, antimicrobial and antifungal properties (Hu et al. 2014). It was found that nitidine, phenanthridine alkaloid, inhibited human lymphoblastoid cell killing by HIV-1 (Rashid et al. 1995). The aim of the present study was to explore the chemical constituents from *T. asiatica* and to evaluate the cytotoxicity against KB, NCI-H187 and Vero cell lines of all isolated compounds. It was expected that some compounds from this plant would exhibit interesting results against KB and NCI-H187 cell lines.

2. Results and discussion

Chemical investigation of the EtOAc and MeOH extracts of the roots of *T. asiatica* led to the isolation of 17 compounds, including a new coumarin, 2′*R*-acetoxytoddanol (**1**), a new alkaloid, 8*S*-10-*O*-demethylbocconoline (**3**) (Figure 1), and 15 known compounds. All known compounds including 11 coumarins, (+) toddanol (**2**) (Tsai et al. 1996), 8-hydroxybergapten (**4**) (Thompson et al. 1978), phellopterin (**5**) (Hirunwong et al. 2016), 10-hydroxy-8,8-dimethyl-2H,8H-pyrano[3,2-g]chromen-2-one (**6**), toddanone (**7**) (Sharma et al. 1981), toddalolactone methyl ether (**8**) (Phatchana and Yenjai 2014), toddalenone (**9**) (You et al. 2014), *O*-methylcedrelopsin (**10**) (Mulholland et al. 2002), toddaculin (**11**), coumurrayin (**12**) (Hirunwong et al. 2016) and toddacoumaquinone (**13**) (Lin et al. 2014), together with 4 alkaloid derivatives, chelerythrine (**14**) (Ishii et al. 1983), oxynorchelerythrine (**15**) (Chung et al. 2013), arnottin II (**16**) (Ishikawa et al. 1995) and *p*-coumaroyltyramine (**17**) (Sun et al. 2015) (Figure 1).

Compound **1** showed the molecular formula as $C_{18}H_{20}O_6$ by ^{13}C NMR and HRESIMS data (*m/z* 355.1161 $[M + Na]^+$). The IR spectrum showed absorption bands of a conjugated carbonyl group at 1736 and 1612 cm^{-1}. The characteristic of α,β-unsaturated lactone in a coumarin moiety was observed at δ 6.25 (J = 9.6 Hz, H-3) and δ 7.85 (J = 9.6 Hz, H-4). A singlet signal at δ_H/δ_C 6.60/95.4 was assigned as H-8 (Table S1). Two doublet of doublet signals at δ 3.04 (J = 13.6, 8.4 Hz) and δ 2.96 (J = 13.6, 6.4 Hz) were assigned as H-1a′ and H-1b′, and these protons correlated with H-2′ (δ 5.52, J = 8.4, 6.4 Hz) in the ^1H-^1H COSY spectrum. In the HMQC spectrum, a correlation between H-2′ and oxygenated carbon (δ 75.9) was observed. The olefinic methylene proton H-4′ displayed at δ 4.84 and δ 4.82, and connected to carbon at δ 112.4 in the HMQC experiment. The allylic methyl group, CH_3-5′, showed long length correlation to the olefinic proton in the COSY spectrum. The ^1H NMR spectrum exhibited an acetoxy group at δ 1.95 and showed correlation with a carbonyl carbon at δ 170.0 in the HMBC experiment (Figure S1). In this experiment, correlations between H-1′ and C-5, C-6, C-7, and C-2′ confirmed the connection of the side chain at the C-6 position. The CH_3-5′ showed correlations with C-2′, C-3′, and C-4′ in the HMBC spectrum. The specific rotation of this compound was $[\alpha]_D^{24}$ = + 90.5 (*c* 0.1, $CHCl_3$) which was the same sign as (+) toddanol; $[\alpha]_D^{24}$ + 51.0 (*c* 2.09, $CHCl_3$) (Tsai et al. 1996). Thus, compound **1** was identified as 6-(2′-acetoxy-3′-methyl-3′-butenyl)-5,7-dimethoxycoumarin and was named 2′*R*-acetoxytoddanol.

Compound **3** was assigned the molecular formula $C_{21}H_{19}NO_5$ by HRESIMS analysis (*m/z* 388.1160 $[M + Na]^+$). The ^1H NMR spectrum showed two pairs of doublets and two singlet signals in the aromatic region. Two singlet signals at δ 7.57 and 7.03 were assigned as H-1 and H-4, respectively. The ^1H–^1H COSY spectrum showed correlations between H-5 (δ 7.41,

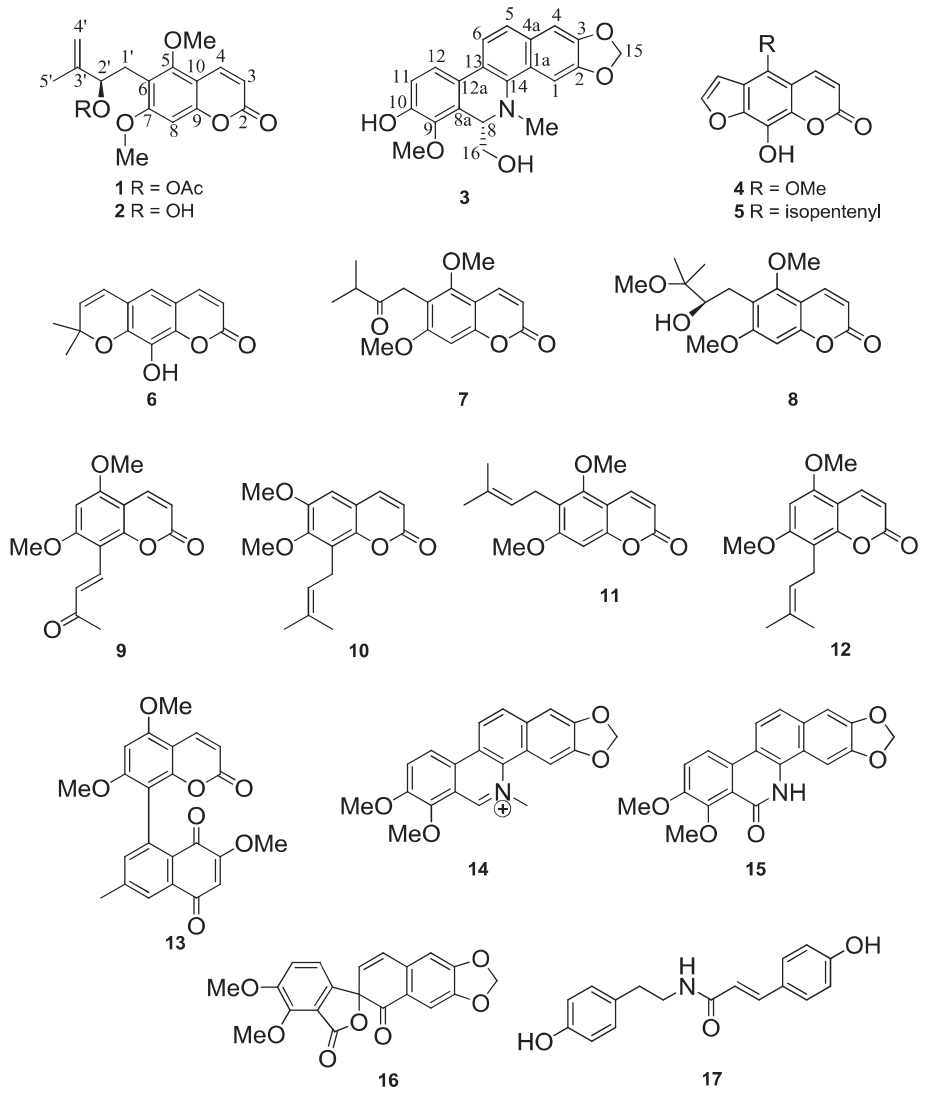


Figure 1. Chemical structures of compounds **1–17**.

d, J = 8.8 Hz) and H-6 (δ 7.60, d, J = 8.8 Hz), as well as between H-11 (δ 6.87, d, J = 8.4 Hz) and H-12 (δ 7.39, d, J = 8.4 Hz) (Table S1). The methylenedioxy proton displayed a singlet at δ 5.98, and correlated with C-2 (δ 148.3) and C-3 (δ 147.4) in the HMBC experiment (Figure S1). In this spectrum, H-1 correlated with C-3 (δ 147.4), C-4a (δ 130.8), and C-14 (δ 137.3), as well as H-4 correlated with C-1a (δ 126.9), C-2 (δ 148.3) and C-5 (δ 124.2). Correlations between H-11 and C-9 (δ 145.1), C-12a (δ 124.2) were also observed. The methoxy proton showed a correlation with C-9 (δ 145.1). An *N*-methyl group showed signals at δ_H/δ_C 2.65/42.5 which correlated with C-8 (δ 59.6) and C-14 (δ 137.3) in the HMBC experiment. The methine proton H-8 displayed a doublet of doublet signal at δ 4.52 (J = 10.8, 4.8 Hz) while methylene H-16 showed a doublet of doublet signal at δ 3.40 (J = 10.8, 4.8 Hz) and a triple signal at δ 3.04 (J = 10.8 Hz). The correlation between H-8 and C-9 (δ 145.1) confirmed that the methoxy group was located at the C-9 position. In addition, this compound showed the specific

rotation as $[\alpha]_D^{24}$=−13.3 (*c* 0.1, $CHCl_3$). The CD spectrum of this compound showed a negative value at 236 nm (Fig. S16). The ECD of 8*S*-configuration isomer also showed the same pattern as this compound (Figure S17). Thus, the structure of **3** was determined as 8*S*-10-*O*-demethylbocconoline.

Cytotoxicity against KB and NCI-H187 cell lines was evaluated using Resazurin Microplate Assay (REMA). Among all isolated compounds, **10** showed the most activity and was selective against KB cells with an IC_{50} value of 2.60 µg/mL, which was nearly equal to the ellipticine standard and showed no activity against NCI-H187 cells (Table 1). The results suggest that the positions of methoxy and prenyl groups are necessary for cytotoxicity. Alkaloids **3** and **15** displayed cytotoxicity against the KB cell line with IC_{50} values of 21.69 and 43.77 µg/mL, respectively. In the case of cytotoxicity against NCI-H187 cell line, compounds **5–7** and **15** exhibited weak cytotoxic activity with IC_{50} ranging from 21 to 35 µg/mL.

3. Experimental

3.1. General experimental procedures

Melting points were determined on a SANYO Gallenkamp (Leicester, UK) melting point apparatus and are uncorrected. UV spectra were measured on an Agilent 8453 UV-Visible spectrophotometer (Waldbronn, Germany). IR spectra were recorded as KBr disks or thin films, using Perkin Elmer Spectrum One FT-IR spectrophotometer (Shelton, CT, USA). The NMR spectra were recorded on a Varian Mercury plus spectrometer (California, USA) operating at 400 MHz (^1H) and at 100 MHz (^{13}C). Mass spectra were determined on a Micromass Q-TOF 2 hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer with a Z-spray ES source (Micromass, Manchester, UK). Thin layer chromatography (TLC) was carried out on MERCK silica gel 60 F_{254} TLC aluminium sheets. Column chromatography was done with silica gel 0.063–0.200 mm or less than 0.063 mm (Darmstadt, Germany). Preparative thin layer chromatography (PLC) was carried out on glass-supported silica gel plates using silica gel 60 PF_{254} for preparative layer chromatography (Darmstadt, Germany). All solvents were routinely distilled prior to use.

3.2. Plant material

The roots of *T. asiatica* were collected in June 2014 from Khon Kaen Province and the plant was identified by Dr Pranom Chantaranothai, Faculty of Science, Khon Kaen University. A

Table 1. Cytotoxicity of all compounds (IC_{50}, µg/mL).*

Compound	KB	NCI-H187	Vero cells
2	IA	IA	47.53 ± 4.69
3	21.69 ± 2.32	IA	IA
5	18.80 ± 1.47	21.20 ± 2.78	IA
6	IA	34.87 ± 4.10	7.36 ± 1.85
7	IA	34.63 ± 3.97	IA
10	2.60 ± 0.12	IA	IA
15	43.77 ± 3.11	24.84 ± 2.51	IA
The others	IA	IA	IA
Ellipticine	3.27	4.01	7.59
Doxorubicin	1.19	0.21	–

Note: IA = Inactive at >50 µg/mL.
*Data shown are from triplicate experiments.

voucher specimen (KKU0042011) was deposited at the herbarium of the Department of Chemistry, Faculty of Science, Khon Kaen University, Thailand.

3.3. Extraction and isolation

Air-dried and finely powdered roots (7 kg) of *T. asiatica* was sequentially extracted at room temperature for three days with hexane (2 × 10 L), EtOAc (2 × 10 L), and MeOH (2 × 10 L). The extracts were evaporated *in vacuo* to obtain three dry extracts, crude hexane (147 g), EtOAc (89 g), and crude MeOH (121 g). The crude EtOAc extract (62 g) was subjected to silica gel flash column chromatography (FCC), eluted with a gradient system of hexane, hexane:CH_2Cl_2, hexane:EtOAc, CH_2Cl_2:EtOAc and EtOAc:MeOH. On the basis of their thin layer chromatography (TLC) characteristics, the fractions which contained the same major compounds were combined to give 14 fractions, F_1–F_{14}. Fraction F_6 (0.58 g) was purified by silica gel flash column chromatography (FCC) and eluted with a gradient system of hexane:EtOAc to give eight fractions, $F_{6.1}$–$F_{6.8}$. Subfraction $F_{6.4}$ was purified by FCC (hexane:EtOAc) followed by preparative thin layer chromatography (PLC) of $F_{6.4.2}$ (hexane:EtOAc, 90:10) and gave toddaculin (**11**, 32.1 mg).

Fraction F_8 (3.12 g) was purified by FCC and eluted with a gradient system of hexane:EtOAc to give eleven fractions, $F_{8.1}$–$F_{8.11}$. Subfraction $F_{8.3}$ was purified by PLC (hexane:EtOAc, 70:30) and afforded coumurrayin (**12**, 55.4 mg). Subfraction $F_{8.5}$ was purified by FCC (EtOAc:CH_2Cl_2:hexane, 20:20:60) to give seven fractions, $F_{8.5.1}$–$F_{8.5.7}$. Subfraction $F_{8.5.3}$ was purified by PLC (EtOAc:CH_2Cl_2:hexane, 20:20:60) to afford a yellow amorphous solid of *o*-methylcedrelopsin (**10**, 7.0 mg). Subfraction $F_{8.5.5}$ was rechromatographed on FCC, by silica gel reverse phase (RP-18) and eluted with an isocratic system (H_2O:MeOH, 30:70) to afford a white solid of **1** (9.5 mg).

Fraction F_9 (3.22 g) was purified by silica gel FCC and eluted with a gradient system (EtOAc:hexane, 15:85) to give 10 fractions, $F_{9.1}$–$F_{9.10}$. Subfraction $F_{9.6}$ was subjected to Sephadex LH-20 CC and eluted with an isocratic system of MeOH to give four fractions, $F_{9.6.1}$–$F_{9.6.4}$. Subfraction $F_{9.6.2}$ was rechromatographed on CC and eluted with a gradient system (EtOAc:hexane, 30:70) to give a pale yellow solid of toddanone **7** (35.5 mg). Subfraction $F_{9.7}$ was isolated by Sephadex LH-20 CC and eluted with an isocratic system (MeOH:CH_2Cl_2, 90:10) to give a brown solid of prangenidin (**5**, 8.2 mg). Subfraction $F_{9.8}$ was isolated by Sephadex LH-20 CC and eluted with an isocratic system (MeOH:CH_2Cl_2, 90:10) to give a brown solid of **6** (8.2 mg). Subfraction $F_{9.9}$ was purified by PLC (EtOAc:CH_2Cl_2:hexane, 40:10:50) to afford a brown amorphous solid of (+)-toddanol (**2**, 34.6 mg).

Fraction F_{10} (3.62 g) was subjected to silica gel FCC and eluted with a gradient system (EtOAc:CH_2Cl_2:hexane, 25:25:50) to give eight fractions, $F_{10.1}$–$F_{10.8}$. Subfraction $F_{10.2}$ was purified by Sephadex LH-20 CC and eluted with an isocratic system (MeOH:CH_2Cl_2, 80:20) to afford a dark yellow solid of 8-hydroxybergapten (**4**, 12.5 mg).

Fraction F_{11} (4.39 g) was purified by silica gel CC and eluted with a gradient system (EtOAc:CH_2Cl_2:hexane, 10:10:80) to give nine fractions, $F_{11.1}$–$F_{11.9}$. Subfraction $F_{11.4}$ was isolated and eluted by CC with a gradient system (EtOAc:CH_2Cl_2:hexane, 20:20:60) to afford a yellow solid of arnottin II (**16**, 16.9 mg). Subfraction $F_{11.6}$ was purified by CC and eluted with a gradient system (EtOAc:CH_2Cl_2:hexane, 30:30:40) to give a pale yellow solid of toddalenone (**9**, 8.8 mg). Subfraction $F_{11.7}$ was purified by PLC, eluted with an isocratic system (EtOAc:CH_2Cl_2, 10:90) to afford a white solid of chelerythrine (**14**, 6.5 mg).

The crude MeOH extract (200 g) was subjected to silica gel flash column chromatography (FCC) and eluted with a gradient system of hexane, hexane:CH_2Cl_2, hexane:EtOAc, CH_2Cl_2:EtOAc and EtOAc:MeOH. On the basis of their thin layer chromatography (TLC) characteristics, the fractions which contained the same major compounds were combined to give 26 fractions, MF_1–MF_{26}. Fraction F_{12} (1.59 g) was purified by RP-18 CC, isolated and eluted with a gradient system (MeOH:H_2O, 60:40) and further purified by PLC with an isocratic system (MeOH:CH_2Cl_2, 2:98) to give a brown solid of **3** (8.5 mg). Fraction MF_{14} (2.21 g) was purified by RP-18 CC with an isocratic system (MeOH:H_2O, 50:50) to give a yellow oil of **8** (4.3 mg).

Fraction MF_{15} (2.30 g) was purified by FCC over silica gel, isolated and eluted with a gradient system (EtOAc:CH_2Cl_2:hexane, 30:30:40) to give seven fractions, $F_{15.1}$–$F_{15.7}$. Subfraction $F_{15.4}$ was purified by CC, eluted with an isocratic system (EtOAc:CH_2Cl_2, 20:80) to give orange crystals of toddacoumaquinone (**13**, 6.6 mg). Subfraction $F_{15.6}$ was purified by FCC, eluted with a gradient system (EtOAc:CH_2Cl_2, 20:80) to give 10 fractions, $F_{15.6.1}$–$F_{15.6.10}$, and also a yellow solid of *trans-N-p*-coumaroyl tyramine (**17**, 22.4 mg). Subfraction $F_{15.6.6}$ was purified by Sephadex LH-20 CC, eluted with an isocratic system (MeOH:CH_2Cl_2, 95:5) to give a yellow amorphous powder of oxynorchelerythrine (**15**, 3.0 mg).

3.4. Spectroscopic data of compounds

2′*R*-Acetoxytoddanol (**1**): a white solid; mp 93–95 °C; $[\alpha]_D^{24}$=+ 90.5 (*c* 0.1 $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 206 (4.27), 224 (4.01), 246 (3.53), 256 (3.37), 327 (3.68) nm; IR (thin film) ν_{max} cm^{-1}: 3081, 2923, 2850, 1736, 1612, 1458, 1376, 1239, 1201, 1131, 1100, 1020, 957, 911, 825, 772; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.85 (d, *J* = 9.6 Hz, H-4), 6.60, (s, H-8), 6.25 (d, *J* = 9.6 Hz, H-3), 5.52 (dd, *J* = 8.4, 6.4, H-2′), 4.84 (s, H-4′a), 4.82 (s, H-4′b), 3.89 (s, OCH_3-5), 3.87 (s, OCH_3-7), 3.04 (dd, *J* = 13.6, 8.4, H-1′), 2.96 (dd, *J* = 13.6, 6.4, H-1′), 1.95 (s, $CO\underline{C}H_3$), 1.81 (s, H-5′); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.0 ($\underline{C}OCH_3$), 161.9 (C-5), 161.0 (C-2), 156.3 (C-7), 155.2 (C-9), 143.2 (C-3′), 138.8 (C-4), 116.1 (C-6), 112.5 (C-3), 112.4 (C-4′), 107.0 (C-10), 95.4 (C-8), 75.9 (C-2′), 63.2 (OCH_3-7), 56.1 (OCH_3-5), 27.6 (C-1′), 21.1 ($CO\underline{C}H_3$), 18.1 (C-5′); HRESIMS *m/z* 355.1161 $[M + Na]^+$ (calcd 355.1158).

8*S*-10-*O*-Demethylbocconoline (**3**) was obtained as a white solid, mp 244–245 °C; $[\alpha]_D^{24}$ =−13.3, (*c* 0.1, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 211 (4.02), 229 (4.15), 284 (4.24), 322 (3.72), 336 (3.56), 351 (3.18) nm; IR (thin film) ν_{max} cm^{-1}: 3412, 3190, 2950, 2923, 2893, 1600, 1508, 1458, 1398, 1354, 1294, 1237, 1189, 1106, 1028, 989, 941, 873, 841, 802; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.60 (d, *J* = 8.8, H-7), 7.57 (s, H-1), 7.41 (d, *J* = 8.8, H-6), 7.39 (d, *J* = 8.4, H-12), 7.03 (s, H-4), 6.87 (d, *J* = 8.4, H-12), 5.98 (s, H-15), 4.52 (dd, *J* = 10.8, 4.8, H-8), 3.85 (s, OCH_3-9), 3.40 (dd, *J* = 10.8, 4.8, H-16a), 3.04, (t, *J* = 10.8, H-16b), 2.65 (s, NCH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 149.3 (C-10), 148.3 (C-2), 147.4 (C-3), 145.1 (C-9), 137.3 (C-14), 130.8 (C-4a), 126.9 (C-1a), 125.3 (C-8a), 124.2 (C-5), 124.2 (C-12a), 123.6 (C-13), 119.7 (C-6), 119.4 (C-12), 116.2 (C-11), 104.5 (C-4), 101.0 (C-15), 99.7 (C-1), 61.8 (C-16), 61.1 (OCH_3-9), 59.6 (C-8), 42.5 (NCH_3); HRESIMS *m/z* 388.1160 $[M + Na]^+$ (calcd 388.1161).

3.5. Bioassay

Cytotoxicity assay against human epidermoid carcinoma of oral cavity (KB, ATCC CCL-17), and human small cell lung cancer (NCI-H187, ATCC CRL-5804) cell lines were performed

employing Resazurin Microplate Assay (REMA) (Sigma-Aldrich, Dye content 75%) (O'Brien et al. 2000). In brief, cells at a logarithmic growth phase are harvested and diluted to 7×10^4 cells/mL for KB and 9×10^4 cells/mL for NCI-H187, in fresh medium. Successively, 5 µL of test sample diluted in a 5% DMSO, and 45 µL of cell suspension are added to 384-well plates, incubated at 37 °C in 5% CO_2 incubator. After the incubation period (3 days for KB and 5 days for NCI-H187), 12.5 µL of 62.5 µg/mL resazurin solution is added to each well, and the plates are then incubated at 37 °C for 4 h. The fluorescence signal is measured using SpectraMax M5 multi-detection microplate reader (Molecular Devices, USA) at the excitation and emission wavelengths of 530 and 590 nm. Per cent inhibition of cell growth is calculated by the following equation: % Inhibition = $[1 - (FU_T/FU_C)] \times 100$, where FU_T and FU_C are the mean fluorescent units from treated and untreated conditions, respectively. Dose response curves are plotted from six concentration of twofold serially diluted test compounds and the sample concentrations that inhibit cell growth by 50% (IC_{50}) can be derived using the SOFTMax Pro software (Molecular Devices, USA). Ellipticine (Fluka, purity ≥ 99%) and doxorubicin (Fluka, purity ≥ 98%) were included as the reference substances.

3.6. ECD

The electronic structure of the molecule was computed by the single-point calculation of the LDSA/3-21G, optimised structure using TDHF/3-21G in chloroform. The calculation was performed by Gaussian 03 W package. The Gaussum 2.1.4 was utilised to generate the CD spectrum.

4. Conclusion

A new coumarin, 2'*R*-acetoxytoddanol (**1**), and a new alkaloid, 8*S*-10-*O*-demethyl bocconoline (**3**), along with 15 known compounds were isolated from the root of *T. asiatica*. *O*-Methylcedrelopsin showed strong cytotoxicity against KB cells with an IC_{50} value of 2.60 µg/mL, which is nearly equal to the ellipticine standard, but showed no activity against Vero cells. New alkaloids **3** displayed weak cytotoxicity against the KB cell line with an IC_{50} value of 21.69 µg/mL.

Acknowledgements

We thank the Thailand Research Fund and Khon Kaen University (RSA5980022) for financial support. We are indebted to The Center of Excellence for Innovation in Chemistry (PERCH-CIC), Office of the Higher Education Commission, Ministry of Education, Thailand. The Bioassay Laboratory of the National Center for Genetic Engineering and Biotechnology, Pathumthani, Thailand is also gratefully acknowledged for biological activity assays.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was partially supported by Faculty of Science, Khon Kaen University for Sanwat Sukieum and Thailand Research Fund and Khon Kaen University [grant number RSA5980022].

Dedication

In remembrance of His Majesty King Bhumibol Adulyadej (1927–2016).

References

Chung CY, Hwang TL, Kuo LM, Kuo WL, Cheng MJ, Wu YH, Sung PJ, Chung MI, Chen JJ. 2013. New Benzo[c]phenanthridine and Benzenoid derivatives, and other constituents from zanthoxylum ailanthoides: effects on neutrophil pro-inflammatory responses. Int J Mol Sci. 14:22395–22408.

Duraipandiyan V, Ignacimuthu S. 2009. Antibacterial and antifungal activity of Flindersine isolated from the traditional medicinal plant, *Toddalia asiatica* (L.) Lam. J Ethnopharm. 123:494–498.

Hirunwong C, Sukieum S, Phatchana R, Yenjai C. 2016. Cytotoxic and antimalarial constituents from the roots of *Toddalia asiatica*. Phytochem Lett. 17:242–246.

Hu J, Shi X, Chen J, Mao X, Zhu L, Yu L, Shi J. 2014. Alkaloids from *Toddalia asiatica* and their cytotoxic, antimicrobial and antifungal activities. Food Chem. 148:437–444.

Hu J, Shi X, Mao X, Chen J, Li H. 2015. Amides from the roots of *Toddalia asiatica*. Chem Nat Compd. 51:726–729.

Ishii H, Kobayashi J, Ishikawa T. 1983. Toddalenone: a new coumarin from *Toddalia asiatica* (*T. aculeata*) structure establishment based on the chemical conversion of limettin into toddalenone. Chem Pharm Bull. 31:3330–3333.

Ishikawa T, Murota M, Watanabe T, Harayama T, Ishii H. 1995. Arnottin II, a unique spiro compound composed of a 3, 4-dehydro-1-tetralone and a phthalide skeleton: is it biosynthetically related to a benzo[c]phenanthridine alkaloid? Tetrahedron Lett. 36(24):4269–4272.

Jain SC, Pandey MK, Upadhyay RK, Kumar R, Hundal G, Hundal MS. 2006. Alkaloids from *Toddalia aculeata*. Phytochemistry. 67:1005–1010.

Karunai Raj M, Balachandran C, Duraipandiyan V, Agastian P, Ignacimuthu S. 2012. Antimicrobial activity of ulopterol isolated from *Toddalia asiatica* (L.) Lam.: a traditional medicinal plant. Journal of Ethnopharmacology. 140:161–165.

Lin TT, Huang YY, Tang GH, Cheng ZB, Liu X, Luo HB, Yin S. 2014. Prenylated coumarins: Natural phosphodiesterase-4 inhibitors from *Toddalia asiatica*. J Nat Prod. 77:955–962.

Mulholland DA, Kotsos M, Mahomed HA, Koorbanally NA, Randrianarivelojosia M, van Ufford LQ, van den Berg AJJ. 2002. Coumarins from *Cedrelopsis grevei* (Ptaeroxylaceae). Phytochemistry. 61:919–922.

O'Brien JO, Wilson I, Orton T, Pognan F. 2000. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur J Biochem. 267:5421–5426.

Phatchana R, Yenjai C. 2014. Cytotoxic coumarins from *Toddalia asiatica*. Planta med. 80:719–722.

Rashid MA, Gustafson KR, Kashman Y, Cardellina JH, McMahon JB, Boyd MR. 1995. Anti-HIV alkaloids from *Toddalia asiatica*. Nat Prod Lett. 6:153–156.

Saxena VK, Sharma RN. 1999. Antimicrobial activity of the essential oil of *Toddalia asiatica*. Fitoterapia. 70:64–66.

Sharma PN, Shoeb A, Kapil RS, Popli SP. 1981. Toddanol and toddanone, two coumarins from *Toddalia asiatica*. Phytochemistry. 20:335–336.

Stephen Irudayaraj SS, Sunil C, Duraipandiyan V, Ignacimuthu S. 2012. Antidiabetic and antioxidant activities of *Toddalia asiatica* (L.) Lam. leaves in Streptozotocin induced diabetic rats. J Ethnopharm. 143:515–523.

Sun J, Huo HX, Zhang J, Huang Z, Zheng J, Zhang Q, Zhao YF, Li J, Tu PF. 2015. Phenylpropanoid amides from the roots of *Solanum melongena* L. (Solanaceae). Biochem Syst Ecol. 58:265–269.

Thompson HJ, Sharma SK, Brown SA. 1978. O-methyltransferases of furanocoumarin biosynthesis. Arch Biochem Biophys. 188:272–281.

Tsai IL, Chang RG, Fang SC, Ishikawa T, Chen IS. 1996. Chemical constituents from the root bark of Formosan *Toddalia asiatica*. Chinese Pharm J (Taipei). 48(1):63–75.

Tsai IL, Fang SC, Ishikawa T, Chang CT, Chen IS. 1997. N-cyclohexyl amides and a dimeric coumarin from formosan *Toddalia asiatica*. Phytochemistry. 44:1383–1386.

Vázquez R, Riveiro ME, Vermeulen M, Mondillo C, Coombes PH, Crouch NR, Ismail F, Mulholland DA, Baldi A, Shayo C, et al. 2012. Toddaculin, a natural coumarin from *Toddalia asiatica*, induces differentiation and apoptosis in U-937 leukemic cells. Phytomedicine. 19:737–746.

You CX, Yang K, Wang CF, Zhang WJ, Wang Y, Han J, Fan L, Du SS, Geng ZF, Deng ZW. 2014. Cytotoxic compounds isolated from Murraya tetramera. Molecules 19:13225–13234.

JOURNAL OF NATURAL PRODUCTS

Article

pubs.acs.org/jnp

Benzoyltyramine Alkaloids Atalantums A–G from the Peels of *Atalantia monophylla* and Their Cytotoxicity against Cholangiocarcinoma Cell Lines

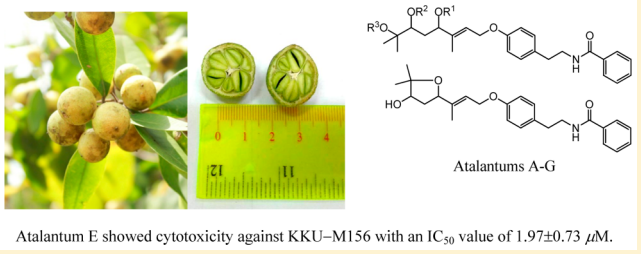
Thurdpong Sribuhom,[†] Parichart Boueroy,[‡] Chariya Hahnvajanawong,[‡] Ratchanee Phatchana,[§] and Chavi Yenjai*[,†]

[†]Natural Products Research Unit, Center of Excellence for Innovation in Chemistry, Department of Chemistry, Faculty of Science, and [‡]Department of Microbiology, Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

[§]Department of Chemistry, Faculty of Engineering, Rajamangala University of Technology Isan, Khon Kaen Campus, Khon Kaen 40000, Thailand

Supporting Information

ABSTRACT: Seven new benzoyltyramines, atalantums A–G (**1**–**7**), and five known compounds were isolated from the peels of *Atalantia monophylla*. All compounds were examined for cytotoxicity against the cholangiocarcinoma cell lines KKU-M214, KKU-M213, and KKU-M156. Compound **5** exhibited the strongest cytotoxicity against KKU-M156 cells, with an IC_{50} value of 1.97 ± 0.73 μM, an approximately 4.7-fold higher activity than that of the ellipticine standard. Compound **1** displayed strong cytotoxicity against KKU-M214 cells, with an IC_{50} value of 3.06 ± 0.51 μM, nearly equal to that of the 5-fluorouracil standard. In the case of the KKU-M213 cell line, compounds **2**, **4**, and **11** exhibited stronger cytotoxicity than the ellipticine standard, with IC_{50} values of 2.36 ± 0.20, 5.63 ± 0.22, and 2.71 ± 0.23 μM, respectively. Compounds **1**, **5**, and **7** displayed cytotoxicity against KKU-M214 cells, with IC_{50} values of 3.06 ± 0.51, 8.44 ± 0.47, and 7.37 ± 1.29 μM, respectively.



Atalantum E showed cytotoxicity against KKU–M156 with an IC_{50} value of 1.97±0.73 μM.

Cholangiocarcinoma (CCA) is one of the cancers that is usually found in the northeast of Thailand.[1] It is believed that liver fluke infection and hepatolithiasis lead to this disease. *Opisthorchis viverrini*, the endemic liver fluke in northeast Thailand, is related to the high incidence of bile duct cancer.[2] There is a poor prognosis for this tumor, so the best treatment is surgical resection, which is a potential curative therapy for CCA.[3] In the case of unresectable malignancy patients, chemotherapy has been used to control the disease and improve the patients' survival rates.[4] However, there is no effective agent for treating CCA. Thus, medicinal plants are interesting sources for effective and potent compounds. In continuing research on bioactive substances from natural sources,[5] *Atalantia monophylla* may be a source of anticancer agents against cholangiocarcinoma cell lines, including moderately differentiated adenocarcinoma, KKU-M214 and KKU-M156, and adenosquamous carcinoma, KKU-M213.

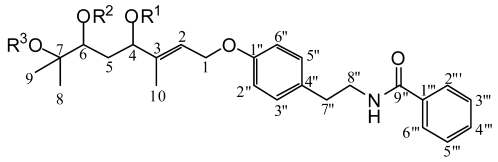
A. monophylla (DC.) Corrêa (Rutaceae), known in Thai as "Ma Nao Phee", is an evergreen shrub that grows up to 6 m with a brown bark and thorny branches. This plant is distributed over the Indian subcontinent and Southeast Asia and can be found in the northeastern and southern parts of Thailand.[6,7] This medicinal plant has been used to treat chronic rheumatism, paralysis, and hemiplegia and as an antispasmodic.[8] The essential oil from the fruit has been used for rheumatism and to cure respiratory disease, while the oil from the leaves is used to treat some pathogenic fungi and itching.[6] The isolation of limonoids and acridone alkaloids from the roots of this plant has been reported.[9] The leaves of this plant contain triterpenoids, steroids, and flavonoids.[10,11] In this study, the seven new *N*-benzoyltyramine derivatives, atalantums A–G (**1**–**7**), along with five known compounds are reported.

RESULTS AND DISCUSSION

The compounds from the peels of *A. monophylla* were extracted, isolated, and characterized to obtain seven new tyramine derivatives, atalantums A–G (**1**–**7**). Five known compounds, namely, *N*-{2-[4-(4,6,7-trihydroxy-3,7-dimethyl-2-octen-1-yl)oxy]phenyl}ethylbenzamide (**8**)[12] *N*-{2-[4-(4-acetoxy-6,7-dihydroxy-3,7-dimethyl-2-octen-1-yl)oxy]phenyl}-ethylbenzamide (**9**),[13] *N*-{2-[4-(6-acetoxy-4,7-dihydroxy-3,7-dimethyl-2-octen-1-yl)oxy]phenyl}ethylbenzamide (**10**),[13] severine palmitate (**11**),[14,15] and severine acetate (**12**),[16] were also isolated and identified (Figure 1). All compounds were optically inactive, $[\alpha]^{25}_{D}$ ±0.[14,17] Their racemic nature was confirmed by electronic circular dichroism (ECD) data.

Received: October 5, 2016
Published: January 31, 2017

DOI: 10.1021/acs.jnatprod.6b00908
J. Nat. Prod. 2017, 80, 403–408



1 R^1 = C(=O)(CH2)14CH3, R^2 = Ac, R^3 = H

2 R^1 = C(=O)(CH2)12CH3, R^2 = Ac, R^3 = H

3 R^1 = C(=O)(CH2)16CH3, R^2 = Ac, R^3 = H

4 R^1 = C(=O)(CH2)14CH3, R^2 = R^3 = H

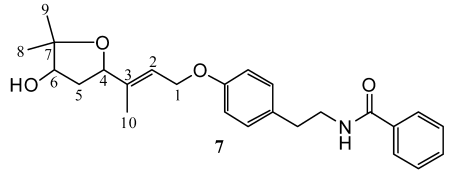
5 R^1 = R^3 = H, R^2 = C(=O)(CH2)14CH3

6 R^1 = R^2 = H, R^3 = C(=O)(CH2)14CH3

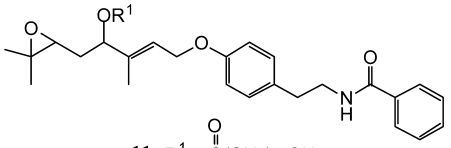
8 R^1 = R^2 = R^3 = H

9 R^1 = Ac, R^2 = R^3 = H

10 R^1 = R^3 = H, R^2 = Ac



7



11 R^1 = C(=O)(CH2)14CH3

12 R^1 = C(=O)CH3

Figure 1. Structures of compounds **1**−**12**.

Compound **1** showed a molecular ion at *m*/*z* 730.4659 $[M + Na]^+$ indicative of the molecular formula $C_{43}H_{65}O_7N$ and corresponds to 12 indices of hydrogen deficiency. The ^{13}C NMR and DEPT spectra showed 43 carbon signals, including five methyl, 18 methylene, 12 methine (two aliphatic, an olefinic, and nine aromatic), three quaternary (one olefinic, two aromatic), two oxygenated tertiary, and three ester/amide-type carbonyl carbons. The ^1H NMR data showed five aromatic protons at δ 7.69 (2H, d, J = 7.6 Hz, H-2‴ and H-6‴), 7.47 (1H, t, J = 7.6 Hz, H-4‴), and 7.39 (2H, t, J = 7.6 Hz, H-3‴ and H-5‴) (Table 1). In the ^{13}C NMR spectrum, the signal at δ 167.6 was assigned to an amide carbonyl (C-9″) (Table 2). In the HMBC spectrum, cross-peaks of H-2‴ and H-6‴ with C-9″ (δ 167.6) and C-4‴ (131.5) were observed (Figure 2). A broad triplet at δ 6.28 was assigned to an NH proton. The $^1H-^1H$ COSY spectrum showed the connection of two methylene groups at δ 3.66 (2H, q, J = 6.4 Hz, H-8″) and δ 2.85 (2H, t, J = 6.4 Hz, H-7″). Cross-peaks between H-8″ (δ 3.66) and C-9″ (δ 167.6), C-4″ (δ 131.2), and C-7″ (δ 34.9) were evident in the HMBC spectrum. A pair of two-proton doublets at δ 7.13 (2H, d, J = 8.0 Hz, H-3″ and H-5″) and δ 6.85 (2H, d, J = 8.0 Hz, H-2″ and H-6″) in the ^1H NMR spectrum indicated the presence of a 1,4-disubstituted benzene moiety. Cross-peaks between H-3″ and C-7″ (δ 34.9) in the HMBC spectrum indicated the connection of the aromatic and *N*-ethylbenzamide moieties. The HMBC spectrum also showed interactions of H-3″ with C-1″ (δ 157.4) and C-2″ (δ 115.2). All of these data confirmed that this molecule contains a benzoyltyramine moiety.

The remaining signals in the ^1H and ^{13}C NMR spectra showed the presence of a geranyl structure. The ^{13}C NMR spectrum also displayed two ester-type carbonyl carbons at δ 172.9 (C-1′) and δ 171.0 ($O\underline{C}OCH_3$). The methylene protons at δ_H 4.55 (H_a-1) correlated with the carbons at C-2 (δ 124.9), C-3 (δ 136.6), and C-1″ (δ 157.4) in the HMBC experiment. The olefinic proton H-2 ($\delta_{H/C}$ 5.74/124.9) displayed HMBC cross-peaks with C-4 (δ 76.0) and C-10 (δ 12.6). The ^{13}C NMR spectrum showed three aliphatic oxygenated carbons at δ 76.0 (C-4), δ 76.4 (C-6), and δ 72.4 (C-7). HMBC cross-peaks were observed between CH_3-8/CH_3-9 and C-7 and C-6. The $^1H-^1H$ COSY spectrum showed correlations for the H-4/H-5/H-6 system. The HMBC spectrum displayed correlations between H-4 and C-1′, as well as between H-6 and the ester carbonyl carbon at δ 171.0 ($O\underline{C}OCH_3$). In this spectrum, the acetoxy proton at δ_H 2.07 also correlated with an ester carbonyl carbon ($O\underline{C}OCH_3$). The rest of the proton and carbon signals were reminiscent of the hydrocarbon $[(CH_2)_{14}CH_3]$ chain of palmitic acid at C-1′ (δ 172.9). Therefore, compound **1**, atalantum A, was characterized as *rac-N*-{2-[4-(4-palmitoyloxy-6-acetoxy-7-hydroxy-3,7-dimethyl-2-octen-1-yl)oxy]phenyl}-ethylbenzamide (Figure 1).

Compound **2**, a white solid, displayed a molecular ion at *m*/*z* 702.4391 $[M + Na]^+$, indicating a molecular formula of $C_{41}H_{61}O_7N$, which corresponds to 12 indices of hydrogen deficiency. The ^1H and ^{13}C NMR spectra displayed the same patterns as those of **1**, except for the presence of a myristoyloxy moiety instead of a palmitoyloxy moiety. Thus, the structure of compound **2**, atalantum B, was defined as *rac-N*-{2-[4-(4-myristoyloxy-6-acetoxy-7-hydroxy-3,7-dimethyl-2-octen-1-yl)-oxy]phenyl}ethylbenzamide.

The HRESIMS data indicated a molecular formula of $C_{45}H_{69}O_7N$ (*m*/*z* 758.4972 $[M + Na]^+$) for compound **3**. The ^1H and ^{13}C NMR spectra displayed the same patterns as those of **1**, except for resonances reminiscent of a stearoyloxy instead of a palmitoyloxy moiety. Thus, the structure of compound **3**, atalantum C, was identified as *rac-N*-{2-[4-(4-stearoyloxy-6-acetoxy-7-hydroxy-3,7-dimethyl-2-octen-1-yl)-oxy]phenyl}ethylbenzamide.

The HRESIMS data of compound **4** indicated a molecular formula of $C_{41}H_{63}O_6N$ (*m*/*z* 688.4556 $[M + Na]^+$). The ^1H and ^{13}C NMR spectroscopic data showed the presence of benzoyltyramine and palmitoyloxy moieties. The signals at δ 5.38 (1H, t, J = 7.0 Hz) and δ 3.27 (1H, d, J = 10.0 Hz) were assigned to H-4 and H-6, respectively. These two protons correlated with the oxygenated methine carbons at δ 76.8 (C-4) and 75.2 (C-6) in the HMQC experiment. Correlations of H-4 and C-2 (δ 124.1), C-5 (δ 34.4), C-10 (δ 12.3), and C-1′ (δ 172.8) were observed in the HMBC spectrum. These data confirmed the C-4 location of the palmitoyloxy moiety. The $^1H-^1H$ COSY spectrum showed correlation for the H-4/H-5/H-6 system. The two methyl groups at δ_H 1.13 (CH_3-8) and δ_H 1.13 (CH_3-9) showed correlations with C-6 (75.2) and C-7 (δ 72.5) in the HMBC experiment. From these data, the vicinal dihydroxy groups in this molecule are confirmed. Thus, the structure of compound **4**, atalantum D, was defined as *rac-N*-{2-[4-(4-palmitoyloxy-6,7-dihydroxy-3,7-dimethyl-2-octen-1-yl)oxy]phenyl}ethylbenzamide.

DOI: 10.1021/acs.jnatprod.6b00908
J. Nat. Prod. 2017, 80, 403−408

Table 1. ^{1}H NMR Spectroscopic Data of Compounds 1−7 ($CDCl_3$, δ in ppm)

position	1	2	3	4	5	6	7
1	4.55, d (5.6)	4.56, d (5.6)	4.56, d (6.0)	4.58, d (6.0)	4.54, d (6.0)	4.55, d (6.0)	4.56, d (5.5)
2	5.74, t (5.6)	5.74, t (5.6)	5.75, t (6.0)	5.82, t (6.0)	5.72, t (6.0)	5.77, t (6.0)	5.81, t (5.5)
4	5.18, t (8.0)	5.19, t (8.0)	5.20, t (8.0)	5.38, t (7.0)	4.18, t (6.8)	3.93, d (10.0)	4.57, m
5	2.01, m	2.00, m	2.01, m	1.81, m	2.00, m	1.83, m	2.05, m
	1.90, m	1.91, m	1.90, m	1.69, m	1.87, m	1.64, m	
6	4.69, d (9.2)	4.68, d (9.2)	4.69, d (8.8)	3.27, d (10.0)	4.76, t (6.0)	4.97, d (7.6)	4.00, t (4.4)
8	1.13, s	1.13, s	1.13, s	1.13, s	1.19, s	1.22, s	1.27, s
9	1.13, s	1.13, s	1.13, s	1.13, s	1.20, s	1.23, s	1.24, s
10	1.72, s	1.72, s	1.72, s	1.73, s	1.71, s	1.74, s	1.69, s
2′	2.27, t (7.6)	2.23, t (7.6)	2.28, t (7.6)	2.86, t (7.4)	2.33, t (7.4)	2.38, t (7.2)	
3′	1.59, m	1.59, m	1.60, m	1.59, m	1.62, m	1.65, m	
CH_2 (4′-13′, 15′, 18′)	1.24, m	1.24, m	1.25, m	1.25, m	1.24, m	1.25, m	
CH_3 (14′ or 16′ or 18′)	0.87, t (7.2)	0.88, t (6.0)	0.87, t (6.2)	0.87, t (6.6)	0.87, t (6.6)	0.87, t (6.6)	
$OCOCH_3$	2.07, s	2.08, s	2.08, s				
2″, 6″	6.85, d (8.0)	6.85, d (8.0)	6.86, d (8.4)	6.84, d (8.4)	6.84, d (8.0)	6.86, d (8.4)	6.86, d (8.4)
3″, 5″	7.13, d (8.0)	7.13, d (8.0)	7.14, d (8.4)	7.13, d (8.4)	7.12, d (8.0)	7.14, d (8.4)	7.14, d (8.4)
7″	2.85, t (6.4)	2.85, t (6.4)	2.86, t (6.8)	2.86, t (6.8)	2.85, t (6.4)	2.87, t (6.8)	2.87, t (6.4)
8″	3.66, q (6.4)	3.66, q (6.4)	3.67, q (6.8)	3.67, q (6.8)	3.66, q (6.4)	3.68, q (6.8)	3.68, q (6.4)
2‴, 6‴	7.69, d (7.6)	7.69, d (7.6)	7.69, d (7.6)	7.69, d (7.6)	7.68, d (7.6)	7.68, d (7.6)	7.68, d (7.2)
3‴, 5‴	7.39, t (7.6)	7.39, t (7.6)	7.40, t (7.6)	7.40, t (7.6)	7.39, t (7.6)	7.40, t (7.6)	7.40, t (7.2)
4‴	7.47, t (7.6)	7.47, t (7.6)	7.48, t (7.6)	7.48, t (7.6)	7.47, t (7.6)	7.48, t (7.6)	7.48, t (7.2)
NH	6.28, br t (6.0)	6.22, br t (6.4)	6.21, br t (6.0)	6.27, br t (6.0)	6.28, br t (6.2)	6.14, br t (6.0)	6.13, br t (6.8)

Table 2. ^{13}C NMR Spectroscopic Data of Compounds 1−7 ($CDCl_3$, δ in ppm)

position	1	2	3	4	5	6	7
1	64.3	64.3	64.4	64.0	64.6	64.7	64.8
2	124.9	124.9	124.9	124.1	121.7	120.7	120.9
3	136.6	136.6	136.6	137.2	141.5	141.8	140.2
4	76.0	76.0	76.0	76.8	74.2	72.5	80.7
5	32.8	32.9	32.9	34.4	35.4	35.9	39.4
6	76.4	76.5	76.5	75.2	77.3	72.2	78.4
7	72.4	72.5	72.5	72.5	72.3	77.3	83.4
8	26.0	26.1	26.1	25.8	26.2	26.4	28.0
9	25.3	25.3	25.3	23.4	25.9	25.9	21.7
10	12.6	12.6	12.6	12.3	12.4	13.1	12.7
1′	172.9	172.9	172.9	172.8	174.0	174.9	
2′	34.6	34.6	34.7	34.4	34.7	34.6	
3′	25.0	25.7	25.1	24.8	25.1	25.3	
12′, 14′, 17′	22.8	22.8	22.8	22.5	22.8	22.8	
4′-13′, 15′, 18′	29.3−29.8	29.3−29.8	29.3−29.8	29.0−29.5	29.3−29.8	29.4−29.8	
14′ or 16′ or 18′	14.2	14.3	14.3	13.9	14.2	14.3	
$OCO\underline{C}H_3$	21.2	21.3	21.2				
$O\underline{C}OCH_3$	171.0	171.0	171.0				
1″	157.4	157.4	157.4	157.4	157.5	157.6	157.7
2″, 6″	115.2	115.2	115.3	114.9	115.1	115.3	115.8
3″, 5″	129.9	129.9	129.9	129.6	129.9	129.9	129.9
4″	131.2	131.2	131.3	131.1	131.2	131.2	131.0
7″	34.9	34.9	35.0	34.7	34.9	35.0	35.0
8″	41.4	41.4	41.4	41.1	41.4	41.4	41.4
9″	167.6	167.6	167.6	167.4	167.6	167.6	167.6
1‴	134.8	134.8	134.9	134.5	134.8	134.8	134.9
2‴, 6‴	127.0	127.0	127.0	126.6	126.9	126.9	126.9
3‴, 5‴	128.6	128.6	128.7	128.4	128.7	128.7	128.7
4‴	131.5	131.5	131.5	131.2	131.7	131.5	131.5

The HRESIMS data of compound **5** indicated a molecular formula of $C_{41}H_{63}O_6N$. The ^{1}H NMR spectrum was similar to that of **4**, except for the chemical shifts of H-4, H-6, CH_3-8, and CH_3-9. The two one-proton triplets at δ 4.76 (1H, J = 6.0 Hz) and 4.18 (1H, J = 6.8 Hz) were determined as H-6 and H-4, respectively, and two three-proton singlets at δ 1.19 (CH_3-8) and δ 1.20 (CH_3-9) were evident. The ^{13}C NMR spectrum displayed oxygenated carbons at δ 74.2 (C-4), δ 77.3 (C-6), and δ 72.3 (C-7). Correlations between H-4 and C-2 (δ 121.7) and between H-6 and C-4 (δ 74.2) and the ester-type carbonyl

DOI: 10.1021/acs.jnatprod.6b00908
J. Nat. Prod. 2017, 80, 403−408

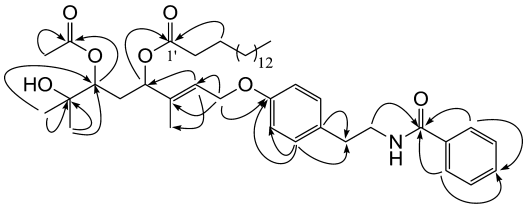


Figure 2. HMBC correlations of compound **1**.

carbon C-1′ (δ 174.0) were observed. This confirmed that the palmitoyloxy moiety is located at C-6. Therefore, the structure of compound **5**, atalantum E, was defined as *rac-N*-{2-[4-(6-palmitoyloxy-4,7-dihydroxy-3,7-dimethyl-2-octen-1-yl)oxy]-phenyl}ethylbenzamide.

The HRESIMS data of compound **6** indicated a molecular formula of $C_{41}H_{63}O_6N$. The ^{1}H NMR spectrum was similar to that of **5**, except for the chemical shifts of H-4 and H-6 at δ 3.93 and 4.97, respectively. The ^{1}H−^{1}H COSY spectrum showed correlation for the H-4/H-5/H-6 system. In the ^{13}C NMR spectrum, oxygenated carbons resonated at δ 72.5 (C-4), 72.2 (C-6), and 77.3 (C-7). The HMBC spectrum showed cross-peaks between H-4 and C-2 and C-10 and between H-6 and CH_3-8 and CH_3-9. No HMBC interaction was evident between the oxymethine proton and the ester-type carbonyl C-1′ (δ 174.9). The ^{1}H and ^{13}C NMR spectra also displayed the palmitoyloxy moiety located at C-7. Thus, the structure of compound **6**, atalantum F, was defined as *rac-N*-{2-[4-(7-palmitoyloxy-4,6-dihydroxy-3,7-dimethyl-2-octen-1-yl)oxy]-phenyl}ethylbenzamide.

It should be noted that compound **5** may be obtained from an intramolecular *trans*-esterification of **4** during the isolation process, and **6** may similarly be derived from **5**. However, **5** showed interesting results by revealing the strongest cytotoxicity against cholangiocarcinoma cell lines (Table 3).

Table 3. Cytotoxicity of Isolated Compounds (μM)[a]

compound	KKU-M214	KKU-M213	KKU-M156
1	3.06 ± 0.51	34.77 ± 1.40	22.02 ± 1.55
2	24.00 ± 1.21	2.36 ± 0.20	24.47 ± 1.98
3	12.36 ± 1.44	29.05 ± 1.48	24.02 ± 0.46
4	27.43 ± 0.27	5.63 ± 0.22	2.80 ± 0.22
5	8.44 ± 0.47	23.47 ± 1.01	1.97 ± 0.73
6	20.52 ± 0.17	16.14 ± 0.84	31.49 ± 1.08
7	7.37 ± 1.29	12.21 ± 1.07	21.51 ± 0.46
8	11.11 ± 1.03	25.84 ± 5.00	26.76 ± 0.11
9	44.48 ± 1.26	28.79 ± 1.64	49.72 ± 0.80
11	14.92 ± 0.68	2.71 ± 0.23	20.98 ± 0.54
12	31.49 ± 7.22	11.14 ± 1.02	29.10 ± 2.52
ellipticine		6.58 ± 1.74	9.34 ± 1.66
5-fluorouracil	3.76 ± 0.16		

[a]Data shown are from triplicate experiments.

The HRESIMS data of compound **7** indicated a molecular formula of $C_{25}H_{31}O_4N$ and corresponded to 11 indices of hydrogen deficiency. The ^{1}H NMR data showed the absence of a palmitoyloxy, myristoyloxy, or stearoyloxy moiety, but the ^{1}H and ^{13}C NMR spectra displayed resonances reminiscent of a benzoyltyramine moiety. The ^{13}C NMR spectrum showed an additional 10 carbons representing a geranyl unit. The ^{13}C NMR and DEPT spectra exhibited oxygenated methine carbons at δ 80.7 (C-4) and 78.4 (C-6), while there was an oxygenated tertiary carbon at δ 83.4 (C-7). A correlation between H-4 ($\delta_{H/C}$ 4.57/80.7) and C-2 (δ 120.9) was observed in the HMBC spectrum, and correlations between CH_3-8/CH_3-9 and C-6 and C-7 were evident. The NOESY spectrum showed a correlation between H-4 and CH_3-8/CH_3-9. These data show the presence of a tetrahydrofuran moiety. Thus, the structure of **7**, atalantum G, was defined as rac-*N*-{2-[4-(6-hydroxy-4,7-oxolane-3,7,7-trimethyl-2-octen-1-yl)oxy]phenyl}-ethylbenzamide.

The cytotoxicity against KKU-M214, KKU-M213, and KKU-M156 cell lines was evaluated using a sulforhodamine B (SRB) assay. Compound **1** showed cytotoxicity against KKU-M214 (IC_{50} value of 3.06 μM) that is nearly equal to that of the standard drug, 5-fuorouracil (Table 3). Compound **2** exhibited stronger cytotoxicity toward KKU-M213 cells than the standard ellipticine with an IC_{50} value of 2.36 μM. In contrast, **3**, which contains the longest hydrocarbon chain, showed only weak cytotoxicity (IC_{50} = 12 to 29 μM). These finding revealed that the polarity of the compound is related to the cytotoxicity. Among the diols **4**−**6**, compound **4** displayed strong cytotoxicity toward the KKU-M213 and KKU-M156 (IC_{50} = 5.63 and 2.80 μM) cell lines and **5** showed strong cytotoxicity against the KKU-M214 and KKU-M156 (IC_{50} = 8.44 and 1.97 μM, respectively) cell lines, but **6** exhibited only weak activity (IC_{50} = 16.14 to 31.49 μM for the three cell lines). These results indicate that the position of the palmitoyloxy group plays an important role. Comparing **1** and **4**, it is believed that the acetoxy group at C-6 resulted in a dramatic improvement, approximately 9-fold, in cytotoxicity toward the KKU-M214 cell line. On the other hand, the 6,7-dihydroxy derivative **4** showed approximately 7- and 11-fold stronger cytotoxicity than the 6-acetoxy derivative (compound **1**) against the KKU-M213 and KKU-M156 cell lines, respectively. Compound **5** exhibited the strongest cytotoxicity against the KKU-M156 cell line, with an IC_{50} value of 1.97 μM, which is approximately 1.42-fold stronger than that of **4**. It should be noted that diols **4** and **5** were selective for KKU-M156 cells, while diol **6** showed only weak activity. These results seem to indicate that the position of the hydroxy group may be critical for the cytotoxicity. Comparing **11** and **12**, **11** displayed strong cytotoxicity against the KKU-M213 cell line, with an IC_{50} value of 2.71 μM. It is suggested that the palmitoyloxy group at C-4 was more effective against KKU-M213 cells than the acetoxy group. Compounds **2**, **4**, and **11** showed stronger cytotoxicity against KKU-M213 cells than the standard drug ellipticine, with IC_{50} values of 2.36, 5.63, and 2.71 μM, respectively. Against the KKU-M156 cell line, **4** and **5** also displayed stronger cytotoxicity than the standard drug, with IC_{50} values of 2.80 and 1.97 μM, respectively. These results provide interesting information that should be useful for cholangiocarcinoma research.

In conclusion, seven new tyramines, atalantums A−G (**1**−**7**), along with five known tyramines were isolated from the peels of *A. monophylla*. Compounds **1**, **5**, and **7** showed cytotoxicity against the KKU-M214 cell line, with IC_{50} values of 3.06, 8.44, and 7.37 μM, respectively. Compounds **2**, **4**, and **11** exhibited strong cytotoxicity against KKU-M213 cells, with IC_{50} values of 2.36, 5.63, and 2.71 μM, higher than that of the ellipticine standard. In addition, both **4** and **5** displayed stronger cytotoxicity than ellipticine against KKU-M156 cells, with IC_{50} values of 2.80 and 1.97 μM, respectively.

DOI: 10.1021/acs.jnatprod.6b00908
J. Nat. Prod. 2017, 80, 403−408

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were measured using a SANYO Gallenkamp (UK) melting point apparatus. A JASCO P-1020 digital polarimeter was used to measure optical rotations. The UV spectra were recorded on an Agilent 8453 UV−visible spectrophotometer (Germany). IR spectra were obtained using a Perkin−Elmer Spectrum One FT-IR spectrophotometer (UK). The NMR spectra were recorded on a Varian Mercury Plus spectrometer (UK) operating at 400 MHz (^{1}H) and at 100 MHz (^{13}C). The chemical shifts δ_H 7.26 and δ_C 77.2 using $CDCl_3$ as a residual solvent were used as references. A Micromass Q-TOF 2 hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer (Micromass, UK) was used to measure the mass spectra. Silica gel column chromatography was carried out over silica gel 60 (100−200 mesh, Merck). Preparative liquid chromatography (PLC) was conducted on silica gel 60 F_{254} (Merck). UV light at 254 and 365 nm was used to detect compounds, and acidic anisaldehyde solution was used as spraying agent. The organic solvents were distilled before use in the separation process.

Plant Material. The fruits of *Atalantia monophylla* (voucher specimen KKU022015) were collected locally from Khon Kaen Province, Thailand, in August 2015. The plant was characterized by Prof. Dr. Pranom Chantaranothai, Faculty of Science, Khon Kaen University.

Extraction and Isolation. The air-dried peels (2.0 kg) of *A. monophylla* were ground and extracted with hexanes (3 × 12 L), EtOAc (3 × 5 L), and MeOH (3 × 5 L) at room temperature. After evaporation, crude hexanes (120 g), EtOAc (150 g), and MeOH (250 g) extracts were produced. Silica gel flash column chromatography (FCC) was used to separate the hexanes extract using an elution gradient of hexanes and EtOAc. On the basis of the TLC pattern, nine fractions, HF1 to HF9, were collected. Fraction HF6 was separated by silica gel FCC, and MeOH/CH_2Cl_2 (1:99) was used as an eluent to give three subfractions, HF6.1−HF6.3. The further purification of HF6.2 by FCC and elution with EtOAc/hexanes (5:95) afforded **11** (50.5 mg, 0.0025%). Five subfractions, HF7.1−HF7.5, were obtained from the separation of fraction HF7 over silica gel FCC (acetone/hexanes, 5:95). Subfraction HF7.4 was further purified by PLC (25:75 acetone/hexanes) to obtain **12** (37.3 mg, 0.0019%). Subfraction HF7.5 was purified by FCC (1:99 MeOH/CH_2Cl_2) to give five subfractions, HF7.5.1−HF7.5.5. Subfraction HF7.5.3 was separated using reversed-phase CC (1:1 H_2O/MeOH) to afford **1** (9.3 mg, 0.00046%), **2** (5.9 mg, 0.0003%), and **3** (4.2 mg, 0.00021%). The purification of subfraction HF7.5.4 by FCC (1:99 MeOH/CH_2Cl_2) afforded three subfractions, HF7.5.4.1−HF7.5.4.3, and the further purification of subfraction HF7.5.4.2 by PLC (70:29:1 hexanes/actone/MeOH) afforded **5** (15.2 mg, 0.00076%) and **7** (4.8 mg, 0.00024%). Subfraction HF7.5.4.3 was purified by PLC (70:29:1 hexanes/actone/MeOH) to yield **4** (11.1 mg, 0.00055%) and **6** (4.5 mg, 0.0002%). The purification of fraction HF8 by FCC and elution with MeOH/CH_2Cl_2 (2:98) followed by PLC (65:34:1 hexanes/acetone/MeOH) gave **9** (6.2 mg, 0.0003%).

The crude EtOAc extract was separated over silica gel FCC (hexanes/EtOAc), affording eight subfractions, EF1−EF8. Subfraction EF6 was subjected to silica gel FCC (acetone/hexanes) to yield five subfractions, EF6.1−EF6.5. Purification of EF6.2 by reversed-phase CC (2:3 H_2O/MeOH) yielded **10** (3.2 mg, 0.00016%), and the purification of EF6.3 by PLC (3:7 acetone/hexanes) gave **8** (10.3 mg, 0.0005%).

Cytotoxicity Assay. Cells ((1−2) × 10^4 cells/well) were seeded in 96-well plates at 37 °C and incubated for 24 h. Then, the cells were treated with 0.1% DMSO (as solvent-control cells) and the compounds by adding 10 μL/well of each concentration in triplicate to obtain a final concentration of 0.025−20 μg/well at 37 °C. After incubation for 1 h (starting cells) and 72 h, the determination of cell growth was examined using the SRB assay.[18] The percent cell viability was calculated as [(OD treated cells on day 3 − OD starting cells)/(OD control on day 3 − OD starting cells)] × 100. The IC_{50} (50% growth inhibitory concentration) values of the compounds on the CCA cell lines were calculated from the dose−response curves. The IC_{50} values were calculated through computation using CalcuSyn software.

Atalantum A (1): white, crystalline solid (MeOH); mp 94−97 °C; $[\alpha]^{25}_D$ 0 (*c* 0.1, $CHCl_3$); UV (CH_3OH) λ_{max} (log ε) 226 (4.23), 283 (3.17) nm; IR (neat) ν_{max} 3351, 2924, 2853, 1736, 1644, 1540, 1511, 1239, 1176 cm^{-1}; ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data ($CDCl_3$), see Tables 1 and 2; HRESIMS *m*/*z* 730.4659 [M + Na]$^+$ (calcd for $C_{43}H_{65}O_7NNa$, 730.4659).

Atalantum B (2): white, crystalline solid (MeOH); mp 77−80 °C; $[\alpha]^{25}_D$ 0 (*c* 0.1, $CHCl_3$); UV (CH_3OH) λ_{max} (log ε) 226 (4.32), 283 (3.24) nm; IR (neat) ν_{max} 3355, 2924, 2853, 1735, 1645, 1539, 1511, 1239, 1176 cm^{-1}; ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data ($CDCl_3$), see Tables 1 and 2; HRESIMS *m*/*z* 702.4391 [M + Na]$^+$ (calcd for $C_{41}H_{61}O_7NNa$, 702.4346).

Atalantum C (3): white, crystalline solid (MeOH); mp 67−70 °C; $[\alpha]^{25}_D$ 0 (*c* 0.1, $CHCl_3$); UV (CH_3OH) λ_{max} (log ε) 226 (4.34), 282 (3.29) nm; IR (neat) ν_{max} 3365, 2924, 2853, 1736, 1645, 1539, 1511, 1239, 1176 cm^{-1}; ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data ($CDCl_3$), see Tables 1 and 2; HRESIMS *m*/*z* 758.4972 [M + Na]$^+$ (calcd for $C_{45}H_{69}O_7NNa$, 758.4972).

Atalantum D (4): pale yellow oil; $[\alpha]^{25}_D$ 0 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 226 (4.35), 283 (3.31) nm; IR (neat) ν_{max} 3351, 2924, 2854, 1727, 1642, 1541, 1512, 1235, 1177 cm^{-1}; ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data ($CDCl_3$), see Tables 1 and 2;HRESIMS *m*/*z* 688.4556 [M + Na]$^+$ (calcd for $C_{41}H_{63}O_6NNa$, 688.4553).

Atalantum E (5): pale yellow oil; $[\alpha]^{25}_D$ 0 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 226 (4.22), 283 (3.02) nm; IR (neat) ν_{max} 3348, 2924, 2853, 1730, 1642, 1540, 1511, 1237, 1176 cm^{-1}; ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data ($CDCl_3$), see Tables 1 and 2; HRESIMS *m*/*z* 688.4555 [M + Na]$^+$ (calcd for $C_{41}H_{63}O_6NNa$, 688.4553).

Atalantum F (6): amorphous gum; $[\alpha]^{25}_D$ 0 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 226 (4.32), 284 (3.36) nm; IR (neat) ν_{max} 3348, 2923, 2853, 1731, 1642, 1541, 1511, 1239, 1177 cm^{-1}; ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data ($CDCl_3$), see Tables 1 and 2; HRESIMS *m*/*z* 688.4558 [M + Na]$^+$ (calcd for $C_{41}H_{63}O_6NNa$, 688.4553).

Atalantum G (7): pale yellow oil; $[\alpha]^{25}_D$ 0 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 226 (3.96), 283 (2.92) nm; IR (neat) ν_{max} 3333, 2925, 2856, 1641, 1542, 1512, 1237, 1178 cm^{-1}; ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data ($CDCl_3$), see Tables 1 and 2; HRESIMS *m*/*z* 432.2147 [M + Na]$^+$ (calcd for $C_{25}H_{31}O_4NNa$, 432.2151).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.6b00908.

^{1}H, ^{13}C, and 2D NMR and mass spectra for **1**−**7** (PDF)

AUTHOR INFORMATION

Corresponding Author

*Tel: +66-4320-2222-41, ext. 12243. Fax: +66-4320-2373. E-mail: chayen@kku.ac.th (C. Yenjai).

ORCID

Chavi Yenjai: 0000-0002-5803-0312

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank the Development and Promotion of Science and Technology Talents Project (DPST) for the support for T.S. We also thank the Thailand Research Fund (Grant No. RSA5980022) and Khon Kaen University for financial support.

DOI: 10.1021/acs.jnatprod.6b00908
J. Nat. Prod. 2017, 80, 403−408

The Center of Excellence for Innovation in Chemistry (PERCH−CIC), Office of the Higher Education Commission, Ministry of Education, Thailand, is gratefully acknowledged.

DEDICATION

In remembrance of His Majesty King Bhumibol Adulyadej (1927−2016).

REFERENCES

(1) Sripa, B.; Pairojkul, C. *Curr. Opin. Gastroenterol.* **2008**, *24*, 349−356.
(2) Loilome, W.; Yongvanit, P.; Wongkham, C.; Tepsiri, N.; Sripa, B.; Sithithaworn, P.; Hanai, S.; Miwa, M. *Mol. Carcinog.* **2006**, *45* (5), 279−287.
(3) McGlynn, K. A.; Tarone, R. E.; El-Serag, H. B. *Cancer Epidemiol., Biomarkers Prev.* **2006**, *15*, 1198−1203.
(4) Sampson, L. K.; Vickers, S. M.; Ying, W.; Phillips, J. O. *Cancer Res.* **1997**, *57*, 1743−1749.
(5) Suthiwong, J.; Pitchuanchom, S.; Wattanawongdon, W.; Hahnvajanawong, C.; Yenjai, C. *J. Nat. Prod.* **2014**, *77*, 2432−2437.
(6) Panda, H. *Handbook on Medicinal Herbs with Uses*; Asia Pacific Business Press Inc, 2004; pp 166−167.
(7) Bunyapraphatsara, N. *Traditional Herb*; Prachachon Printing: Thailand, 1999; Vol 3, p 568.
(8) Basa, S. C. *Phytochemistry* **1975**, *14*, 835−836.
(9) Govindachari, T. R.; Viswanathan, N.; Pai, B. R.; Ramachandran, V. N.; Subramaniam, P. S. *Tetrahedron* **1970**, *26*, 2905−2910.
(10) Talapatra, S. K.; Bhattacharya, S.; Talapatra, B. *J. Indian Chem. Soc.* **1970**, *47* (6), 600−604.
(11) Saraswathy, A.; Balakrishna, K.; Rao, R. B.; Allirani, T.; Patra, A.; Pichai, R. *Fitoterapia* **1998**, *69* (5), 463−464.
(12) Ghosh, P.; Ghosh, M. K.; Thakur, S.; Datta, J. D.; Akihisa, T.; Tamura, T.; Kimura, Y. *Phytochemistry* **1994**, *37* (3), 757−760.
(13) Cerqueira, C. N.; Santos, D. A. P.; Malaquias, K. S.; Lima, M. M. C.; Silva, M. F. G. F.; Vieira, J. B. F.P. C. *Quim. Nova* **2012**, *35* (11), 2181−2185.
(14) Dryer, D. L.; Rigod, J. F.; Basa, S. C.; Mahanty, P.; Das, D. P. *Tetrahedron* **1980**, *36* (6), 827−829.
(15) Ghosh, P.; Sil, P.; Das, S.; Thakur, S.; Kokke, W. C. M. C.; Akihisa, T.; Shimizu, N.; Tamura, T.; Matsumoto, T. *J. Nat. Prod.* **1991**, *54*, 1389−1393.
(16) Braga, P. A. C.; Dos Santos, D. A. P.; Da Silva, M. F. D. G. F.; Vieira, P. C.; Fernandes, J. B.; Houghton, P. J.; Fang, R. *Nat. Prod. Res.* **2007**, *21* (1), 47−55.
(17) Ghosh, P.; Bandyopadhyay, A. K.; Thakur, S.; Akihisa, T.; Shimizu, N.; Tamura, T.; Matsumoto, T. *J. Nat. Prod.* **1989**, *52*, 1323−1326.
(18) Skehan, P.; Storeng, R.; Scdiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. *J. Natl. Cancer Inst.* **1990**, *82*, 1107−1112.

DOI: 10.1021/acs.jnatprod.6b00908
J. Nat. Prod. 2017, 80, 403−408

Fitoterapia 114 (2016) 45–50

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Canangalias C-H, juvenile hormone III analogues from the roots of *Cananga latifolia*

Ratchanee Phatchana [a], Yordhathai Thongsri [b], Chavi Yenjai [a,*]

[a] Natural Products Research Unit, Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand
[b] Cellular and Molecular Immunology Research Unit, Department of Medical Technology, Faculty of Allied Health Science, Naresuan University, Phitsanulok 65000, Thailand

ARTICLE INFO

Article history:
Received 15 July 2016
Received in revised form 22 August 2016
Accepted 25 August 2016
Available online 26 August 2016

Keywords:
Cananga latifolia
Annonaceae
Canangalias
Juvenile hormone III
Sesquiterpenes

ABSTRACT

Chemical investigation of the roots of *Cananga latifolia* led to the isolation and purification of thirteen juvenile hormone III analogues. Six new analogues, canangalias C-H (**1**–**6**) and a new natural product, (2*E*,6*E*,10*R*)-10-acetoxy-11-hydroxy-3,7,11-trimethyldodeca-2,6-dienoic acid methyl ester (**7**), were isolated. In addition, six known juvenile hormone III analogues were isolated. Their structures were established by spectroscopic methods including 1D and 2D NMR, IR and mass spectrometry.

1. Introduction

Cananga latifolia Finet & Gagnep. (Annonaceae) is commonly used as a traditional medicine in South-East Asia, such as in Thailand, Myanmar, Laos, Vietnam and Cambodia [1]. The roots are used for curing infectious diseases in early childhood [2]. The stem and stem barks are used as an antipyretic [3], and for nasal polyposis [4], dizziness and fever [5]. The seeds are used as antirheumatism, antimalarial and antidiarrhoeal treatments [6]. It was reported that flavonoids, flavonoid glycosides, fatty acids, alkaloids and juvenile hormone III analogues were isolated from *C. latifolia* [7,8]. In our previous study, we reported two new juvenile hormone III analogues, a new phenylpropanoid derivative and ten known compounds from the stem of this plant, and all isolates were evaluated for antifungal activity against *Pythium insidiosum* [9]. It was found that three juvenile hormone III analogues showed strong activity against this fungus. *P. insidiosum*, which causes pythiosis disease, is found in tropical and subtropical areas [9,10]. In the past this disease has been found in cats, dogs, horses, and calves, but nowadays occurrence in humans has also been found [11]. Because its cell wall does not containing ergosterol like a true fungus, the existing antifungal agents are not effective [12]. Therefore, the search for potent compounds from natural sources is still important. As part of our ongoing research on antifungal activity against *P. insidiosum*, the bioactive metabolites from the roots of *C. latifolia* have been studied. In this work, we report the isolation of 13 juvenile hormone III analogues, including six new juvenile hormone III analogues (**1**–**6**), a new natural product juvenile hormone III derivative (**7**), along with six known compounds (**8**–**13**). Herein, we describe the isolation and structural elucidation of the six new compounds and bioactivity results.

2. Experimental

2.1. General experimental procedures

IR spectra were obtained using a Bruker Tenser 27 spectrophotometer. The CD and UV spectra were measured using a JASCO J-810 apparatus. Optical rotations were obtained using a JASCO P-1020 digital polarimeter. The NMR spectra were recorded on a Varian Mercury plus spectrometer operating at 400 MHz (^{1}H) and at 100 MHz (^{13}C). Mass spectra were determined on a Micromass Q-TOF 2 hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer with a *Z*-spray ES source. Thin layer chromatography (TLC) was carried out on Merck silica gel 60 F_{254} TLC aluminium sheets. Column chromatography was done with silica gel 0.063–0.200 mm or less than 0.063 mm. Preparative layer chromatography (PLC) was carried out on glass supported silica gel plates using silica gel 60 PF_{254} for preparative layer chromatography. All solvents were routinely distilled prior to use.

2.2. Plant material

The roots of *C. latifolia* were collected in October 2013 from Phuwieng District, Khon Kaen Province, Thailand. The plant was identified by Dr. Pranom Chantaranothai, Faculty of Science, Khon Kaen University, Thailand where a voucher specimen (KKU012013) was deposited.

* Corresponding author.
E-mail address: chayen@kku.ac.th (C. Yenjai).

http://dx.doi.org/10.1016/j.fitote.2016.08.018
0367-326X/

2.3. Extraction and isolation

Air-dried and finely powdered roots (3.1 kg) of *C. latifolia* were sequentially extracted at room temperature for three days with hexane (2 × 12 L), EtOAc (2 × 10 L), and MeOH (2 × 10 L). The extracts were evaporated *in vacuo* to obtain three dry extracts, crude hexane (20.1 g), EtOAc (28.9 g), and crude MeOH (124.2 g). The crude EtOAc extract was subjected to silica gel flash column chromatography (FCC) and subsequently eluted with a gradient of three solvents (hexane, EtOAc and MeOH) by gradually increasing the polarity of the elution solvents system. The eluents were collected and monitored by thin layer chromatography (TLC) resulting in 7 groups of eluting fractions which were designated as F_1 to F_7. Fraction F_1 was purified by silica gel column chromatography and eluted with an isocratic system of 5% EtOAc:CH_2Cl_2 to yield three subfractions, $F_{1.1}$–$F_{1.3}$. Subfraction $F_{1.2}$ was purified by PLC using 10% EtOAc:hexane as developing solvent to yield **9** (10.2 mg, 0.0003%). Fraction F_2 was purified by silica gel FCC using 5% EtOAc:hexane as eluent to give three subfractions, $F_{2.1}$-$F_{2.3}$. Subfraction $F_{2.1}$ was purified by PLC and 10% EtOAc:hexane was used as developer to afford **10** (2.3 g, 0.0740%). Purification of subfraction $F_{2.2}$ by PLC using 15% EtOAc:hexane as developing solvent yielded **11** (13.5 mg, 0.0004%). Fraction F_3 was purified by silica gel FCC and eluted with a gradient system of EtOAc:hexane to give eight subfractions, $F_{3.1}$–$F_{3.8}$. Subfraction $F_{3.3}$ was subjected to a column of Sephadex LH-20, using MeOH as eluent and then by PLC (40% EtOAc:hexane) to give **13** (15.1 mg, 0.0005%). Further purification of subfraction $F_{3.5}$ with gel filtration (Sephadex LH-20) and eluting with MeOH gave three subfractions, $F_{3.5.1}$–$F_{3.5.3}$. Subfraction $F_{3.5.1}$ was purified by PLC and 50% EtOAc:hexane was used as developing solvent to afford **12** (23.7 mg, 0.0008%). Subfractions $F_{3.7}$ and $F_{3.8}$ were subjected to a column of Sephadex LH-20, using MeOH as eluent and then by PLC (40% EtOAc:hexane) to give **7** (22.3 mg, 0.0007%) and **8** (7.8 g, 0.25%), respectively. Fraction F_5 was purified by silica gel FCC and eluted with a gradient system of EtOAc:hexane to give five subfractions, $F_{5.1}$–$F_{5.5}$. Further purification of subfraction $F_{5.2}$ by gel filtration (Sephadex LH-20), eluted with MeOH, gave three subfractions, $F_{5.2.1}$–$F_{5.2.3}$. Subfraction $F_{5.2.2}$ was purified by silica gel CC, eluted with an isocratic system of 20% EtOAc:hexane and then by PLC (1% MeOH:CH_2Cl_2) to give **1** (21.5 mg, 0.0007%). Subfractions $F_{5.3}$ and $F_{5.4}$ were subjected to a column of Sephadex LH-20, using MeOH as eluent and then purified by silica gel CC, eluted with an isocratic system of 20% EtOAc:hexane to give **5** (10.4 mg, 0.0003%) and **6** (9.8 mg, 0.0003%), respectively. Fraction F_6 was purified by silica gel FCC and eluted with a gradient system of EtOAc:hexane to give four subfractions, $F_{6.1}$–$F_{6.4}$. Further purification of subfraction $F_{6.4}$ with gel filtration (Sephadex LH-20) and eluting with MeOH gave three subfractions, $F_{6.4.1}$–$F_{6.4.3}$. Subfraction $F_{6.4.1}$ was further purified by silica gel CC, eluted with an isocratic system of 15% EtOAc:CH_2Cl_2 to give four subfractions, $F_{6.4.1.1}$–$F_{6.4.1.4}$. Subfractions $F_{6.4.1.2}$ and $F_{6.4.1.4}$ were purified by PLC using 10%MeOH:CH_2Cl_2 as eluent to give **3** (12.5 mg, 0.0004%) and **4** (11.4 mg, 0.0004%). Fraction F_7 was purified by silica gel FCC and eluted with a gradient system of EtOAc:hexane to give four subfractions, $F_{7.1}$–$F_{7.4}$. Subfraction $F_{7.2}$ was purified on a column of Sephadex LH-20, using MeOH as eluent and then by silica gel FCC, eluted with an isocratic system of 20% EtOAc:hexane to give **2** (14.2 mg, 0.0005%).

2.4. Antifungal activity; disk diffusion assay

All purified compounds were dissolved in CH_2Cl_2 to final volumes of 100 μL. Then 20 μL of the tested compounds were impregnated on sterilized discs (6.0 mm) (Whatman, England) and placed on the Sabouraud Dextrose Agar (SDA) plate (Oxoid, UK) which had been inoculated with an agar block of *P. insidiosum* (1 × 1 cm). Plates were kept at room temperature for 2 h in the laminar flow cabinet, then inverted and incubated at 25 °C for 3, 6 and 9 days. Terbinafine (20 mg/100 μL; 20 μL/disk) (Sigma-Aldrich, USA) and a disk with CH_2Cl_2 only were used as control discs. Inhibition of the mycelial growth of *P. insidiosum* compared with the control was observed and reported as positive antifungal activity.

3. Results and discussion

Purification and isolation of the EtOAc extract of air dried roots of *C. latifolia* was accomplished by repeated column chromatography and Sephadex LH-20 followed by PLC, to obtain six new compounds, canangalias C-H (**1**–**6**), a new natural product compound, (2*E*,6*E*,10*R*)-10-acetoxy-11-hydroxy-3,7,11-trimethyldodeca-2,6-dienoic acid methyl ester (**7**) [13], together with six known compounds, (2*E*,6*E*,10*R*)-10,11-dihydroxy-3,7,11-trimethyldodeca-2,6-dienoic acid methyl ester (**8**) [14], (2*E*,6*E*)-3,7,11-trimethyldodeca-2,6,10-trienoic acid methyl ester (**9**) [15], (2*E*,6*E*,10*R*)-10,11-epoxy-3,7,11-trimethyldodeca-2,6-dienoic acid methyl ester (**10**) [16], (2*E*,6*E*,10*R*)-10-hydroxy-3,7,11-trimethyldodeca-2,6,11-trienoic acid methyl ester (**11**) [8], (2*E*,6*E*)-11-hydroxy-3,7,11-trimethyldodeca-10-one-2,6-dienoic acid methyl ester (**12**) and (2*E*)-5-(3′-hydroxy-2′2′-dimethyl-6′-methylenecyclohexyl)-3-methyl-2-pentenoic acid methyl ester (**13**) [9] (Fig. 1). All isolated compounds were juvenile hormone III derivatives, which can be found in some plants [17,18]. The structure of all compounds were identified on the basis of spectroscopic data, including IR, 1D, 2D NMR experiment (COSY, HMQC and HMBC) and by comparison with those previously reported in the literature for the known compounds.

Compound **1** was determined as $C_{19}H_{32}O_6$ by a quasi-molecular ion peak at *m/z* 379.2098 $[M + Na]^+$ in the HRESIMS. The IR spectrum showed the absorption bands at 3431 and 1719 cm^{-1} ascribable to hydroxyl and α,β-unsaturated ester groups. The ^1H NMR spectrum showed a methyl ester group at δ_H 3.68 (Table 1). These protons correlated with carbon at δ 167.3 (C-1) in the HMBC spectrum (Fig. 2). The ^{13}C NMR spectrum showed two carbonyl carbons at δ_C 167.3 (C-1) and 175.8 (C-1′) (Table 2). Two olefinic protons at δ_H 5.68 (1H, s, H-2) and 5.06 (1H, br s, H-6) correlated with carbons at δ_C 115.4 (C-2) and δ_C 124.0 (C-6), respectively, in the HMQC spectrum. In the HMBC experiment, H-2 correlated with carbons at δ 18.8 (C-15) and 40.6 (C-4) and H-6 correlated with carbons C-5, C-8 and C-14 in the HMBC spectrum. The methyl groups at CH_3-12 and CH_3-13 showed singlet signals at δ 1.19 and 1.20 and correlated with C-10 and C-11 in the HMBC spectrum. The methine proton H-10 (4.85, dd, J = 10.5, 2.3 Hz) showed correlation with an oxygenated carbon at δ 80.9 in the HMQC experiment. This proton displayed correlations with carbons at δ 35.9 (C-8) and δ 175.8 (C-1′) in the HMBC spectrum which indicated the connection of the other ester group at this position. In this spectrum, the methine proton (q, J = 6.9 Hz, H-2′) at $\delta_{H/C}$4.31/66.8 correlated with carbons at δ_C 175.8 (C-1′) and 20.6 (C-3′). The COSY spectrum also displayed the correlation between methyl proton at δ 1.56 (d, J = 6.9 Hz, CH_3-3′) with H-2′. These data indicated the presence of an α-hydroxy ester moiety which was attached at the C-10 position of the methyl farnesoate part. The absolute stereochemistry at C-10 was assumed as 10*R* due to the specific rotation of this compound being $[\alpha]^{28}_D$ + 1.4 (*c* 0.55) which has the same sign as the major component in this plant, (2*E*,6*E*,10*R*)-10,11-dihydroxy-3,7,11-trimethyldodeca-2,6-dienoic acid methyl ester ($[\alpha]_D$ + 17.4; [8,9]). Thus, this compound was (2*E*,6*E*,10*R*)-10-lactoxy-11-hydroxy-3,7,11-trimethyldodeca-2,6-dienoic acid methyl ester, which was named canangalia C.

Compound **2** was assigned as $C_{20}H_{32}O_7$ from its a quasi-molecular ion peak at *m/z* 407.2048 $[M + Na]^+$ in the HRESIMS. The IR spectrum showed broad absorption bands at 3464 and 1715 cm^{-1} which indicated the presence of a carboxylic acid group. The ^{13}C NMR spectrum showed three carbonyl carbons at δ_C 167.4 (C-1), 172.3 (C-1′) and 176.3 (C-4′) (Table 2). The ^1H and ^{13}C NMR spectral data of **2** are similar to those of compound **1** except that the hydroxyl group at C-10 was acylated by a succinic acid. The ^1H NMR spectrum showed multiplet signals of methylene protons H-2′

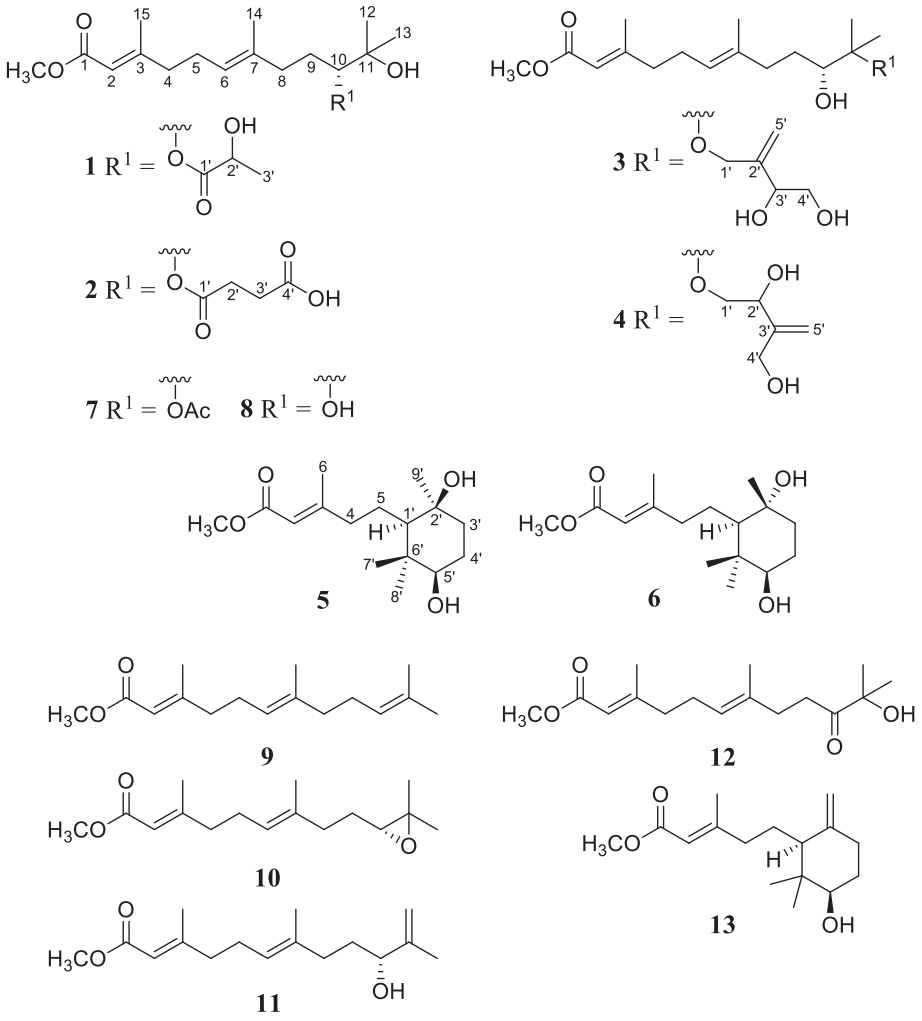


Fig. 1. The structures of isolated compounds (**1**−**13**).

and H-3′ at δ 2.63 and 2.69 (Table 1). The ^{13}C NMR and DEPT spectra also exhibited two methylene carbons at δ 29.1 (C-2′) and 29.3 (C-3′). Correlations of H-2′ and H-3′ with C-1′ and C-4′ were observed in the HMBC spectrum. The correlation of the H-10 with carbonyl carbon C-1′ confirmed the linkage of a succinic moiety at the C-10 position. From the above evidence, compound **2** was determined as (2*E*,6*E*,10*R*)-11-hydroxy-10-succinoxy-3,7,11-trimethyldodeca-2,6-dienoic acid methyl ester, which was named canangalia D.

Table 1
^{1}H NMR (400 MHz, $CDCl_3$) spectral data of compounds **1**–**6** (*J* in Hz).

Position	1	2	3	4	5	6
2	5.68, s	5.67, s	5.66, s	5.66, s	5.69, s	5.69, s
4	2.17, m	2.16, m	2.17, m	2.18, m	2.14, m	H_a 2.37, H_b 2.17, m
5	2.17, m	2.16, m	2.17, m	2.18, m	1.60, m	H_a 1.75, H_b 1.49, m
6	5.06, br s	5.06, br s	5.13, br s	5.14, br s	2.18, s	2.17, s
8	1.97, m	1.94, m	H_a 2.26, H_b 2.04, m	H_a 1.47, H_b 1.41, m		
9	1.69, m	1.65, m	H_a 1.48, H_b 1.40, m	H_a 2.26, H_b 2.05, m		
10	4.85, dd (10.5, 2.3)	4.84, dd (10.3, 2.4)	3.52, br d (10.4)	3.48, m		
12	1.19, s	1.18, s	1.15, s	1.13, s		
13	1.20, s	1.18, s	1.18, s	1.15, s		
14	1.58, s	1.57, s	1.60, s	1.61, s		
15	2.15, s	2.14, s	2.15, s	2.15, s		
1′			H_a 4.08, d (10.5) H_b 3.90, d (10.5)	3.48, m	0.84, t (4.4)	1.13, t (4.4)
2′	4.31, q (6.9)	2.63, m		4.41, dd (7.6, 3.2)		
3′	1.56, d (6.9)	2.69, m	4.35, t (5.0)		H_a 1.64, H_b 1.47, m	H_a 1.60, H_b 1.49, m
4′			3.69, d (5.0)	4.20, s	H_a 1.79, H_b 1.54, m	H_a 1.75, H_b 1.49, m
5′			H_a 5.18, s H_b 5.20, s	H_a 5.17, s H_b 5.19, s	3.24, dd (11.7, 4.0)	3.32, m
6′						
7′					0.92, s	0.78, s
8′					0.98, s	1.02, s
9′					1.17, s	1.18, s
OCH_3	3.68, s	3.68, s	3.68, s	3.68, s	3.68, s	3.68, s

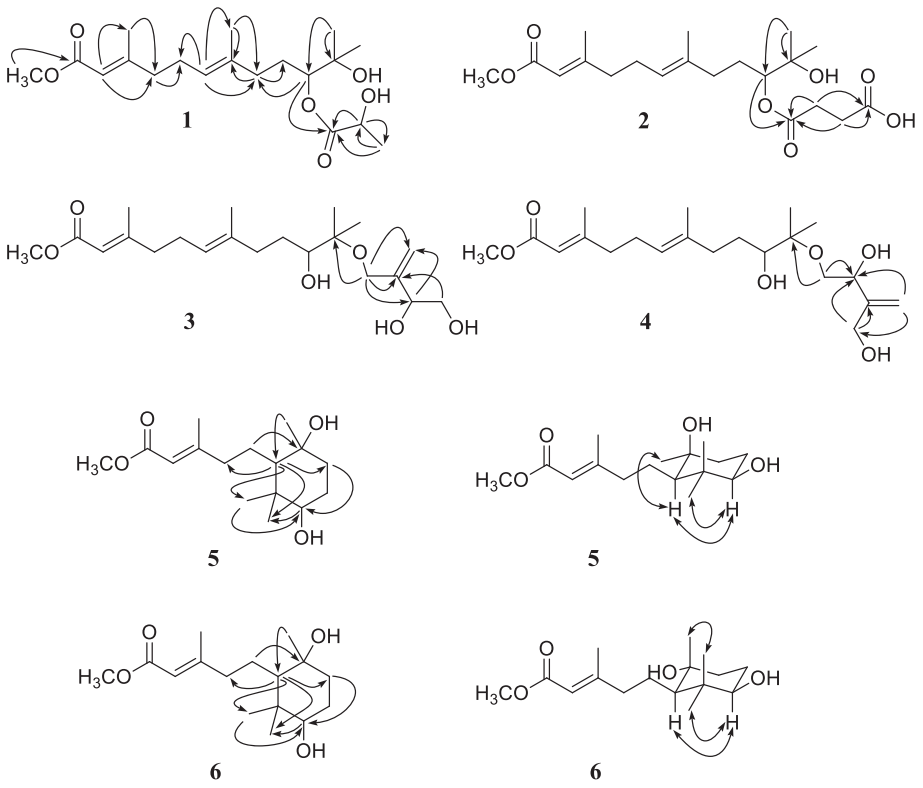


Fig. 2. The HMBC and NOESY correlations of compounds **1–6**.

Compound **3** was concluded to be $C_{21}H_{36}O_6$ by a quasi-molecular ion peak at *m/z* 407.2428 $[M + Na]^+$ in the HRESIMS. The ^1H and ^{13}C spectra showed the same pattern as compounds **1** and **2** by showing a 3,7,11-trimethyldodeca-2,6-dienoic acid methyl ester moiety. The remaining ^{13}C NMR signals displayed five carbons, including two oxygenated aliphatic methylenes, an olefinic methylene, an oxygenated aliphatic methine, and a quaternary olefinic carbon. The ^1H NMR spectrum displayed two doublet signals ($J = 10.5$ Hz) at δ 4.08 and 3.90 (H-1′) and correlated with a carbon at δ 62.7 (C-1′) in the HMQC spectrum (Tables 1 and 2). In the HMBC experiment, these protons showed long range correlations with carbons at δ 145.6 (C-2′), 74.4 (C-3′), and 115.4 (C-5′), in addition, a correlation between H-1′ (δ 3.90) and C-11 (δ 78.6) was observed, which indicated the connection of an isoprene unit at this position. The triplet signal at δ 4.35 (1H, $J = 5.0$ Hz, H-3′) correlated with a carbon at δ 74.4 in the HMQC spectrum. This proton correlated with a terminal olefinic carbon at δ 115.4 (C-5′) in the HMBC spectrum. The oxygenated methylene proton H-4′ showed a signal at $\delta_{H/C}$ 3.69/66.4 and correlated with carbons at δ 145.6 (C-2′) and 74.4 (C-3′) in the HMBC experiment. The COSY spectrum showed the correlation of H-3′/H-4′ which confirmed the structure of this moiety. Therefore, compound **3** was identified as (2*E*,6*E*,10*R*)-11-(3′,4′-dihydroxy-2′-methylenebutoxy)-10-hydroxy-3,7,11-trimethyldodeca -2.6-dienoic acid methyl ester which was named canangalia E.

Table 2
^{13}C NMR (100 MHz, $CDCl_3$) spectral data of compounds **1–6**.

Position	1	2	3	4	5	6
1	167.3 C	167.4 C	167.3 C	167.3 C	167.2 C	167.3 C
2	115.4 CH	115.4 CH	115.3 CH	115.3 CH	115.1 CH	114.9 CH
3	159.7 C	159.9 C	160.0 C	159.9 C	160.1 C	160.9 C
4	40.6 CH_2	40.7 CH_2	40.8 CH_2	40.8 CH_2	44.4 CH_2	43.8 CH_2
5	25.8 CH_2	25.8 CH_2	25.8 CH_2	25.8 CH_2	39.1 CH_2	41.1 CH_2
6	124.0 CH	123.7 CH	123.4 CH	123.4 CH	19.0 CH_3	19.0 CH_3
7	134.7 C	135.0 C	135.9 C	135.9 C		
8	35.9 CH_2	35.9 CH_2	36.7 CH_2	36.7 CH_2		
9	27.7 CH_2	28.1 CH_2	29.7 CH_2	29.6 CH_2		
10	80.9 CH	79.9 CH	75.5 CH	75.7 CH		
11	72.4 C	72.8 C	78.6 C	78.3 C		
12	24.8 CH_3	24.3 CH_3	20.1 CH_3	19.8 CH_3		
13	26.4 CH_3	26.3 CH_3	21.5 CH_3	21.5 CH_3		
14	15.9 CH_3	15.9 CH_3	16.0 CH_3	16.0 CH_3		
15	18.8 CH_3	18.8 CH_3	18.8 CH_3	18.8 CH_3		
1′	175.8 C	172.3 C	62.7 CH_2	65.2 CH_2	53.1 CH	55.4 CH
2′	66.8 CH	29.1 CH_2	145.6 C	73.1 CH	72.3 C	73.6 C
3′	20.6 CH_3	29.3 CH_2	74.4 CH	147.4 C	23.9 CH_2	24.1 CH_2
4′		176.3 C	66.4 CH_2	64.3 CH_2	26.8 CH_2	28.9 CH_2
5′			115.4 CH_2	113.7 CH_2	78.4 CH	78.1 CH
6′					40.3 C	40.3 C
7′					14.6 CH_3	14.8 CH_3
8′					26.9 CH_3	28.0 CH_3
9′					30.4 CH_3	23.2 CH_3
OCH_3	50.8 CH_3	50.8 CH_3	50.8 CH_3	50.8 CH_3	50.8 CH_3	50.8 CH_3

Compound **4** was assigned the molecular formula $C_{21}H_{36}O_6$ from its quasi-molecular ion peak at *m/z* 407.2405 $[M + Na]^+$ in the HRESIMS. The ^1H and ^{13}C NMR spectral data of **4** are similar to those of compound **3** except for the pattern of an isoprene unit at C-11 (Tables 1 and 2). The COSY spectrum showed the correlation between H-1′ (δ 3.48, 2H, m) and H-2′ (δ 4.41, 1H, dd, $J = 7.6$, 3.2 Hz) and these protons connected to carbons at δ 65.2 (C-1′) and 73.1 (C-2′), respectively, in the HMQC spectrum. The ^1H NMR spectrum showed olefinic methylene signals at δ 5.17 and 5.19 (s each, H-5′) which correlated with carbon at δ 113.7 (C-5′) in the HMQC spectrum. These methylene protons showed long range correlations with carbons at δ 73.1 (C-2′), 147.4 (C-3′) and 64.3 (C-4′) in the HMBC experiment (Fig.2). The signal at $\delta_{H/C}$ 4.20/64.3 (H-4′) correlated with carbons at δ 73.1 (C-2′), 147.4 (C-3′), and 113.7 (C-5′) in the HMBC spectrum. The connection of an isoprene unit at the C-11 position was determined from the long range correlation between H-1′ (δ 3.48) and C-11 (δ 78.3) in the HMBC spectrum. From these data, compound **4** was identified as (2*E*,6*E*,10*R*)-11-(2′,4′-dihydroxy-3′-methylenebutoxy)-10-hydroxy-3,7,11-trimethyldodeca-2,6-dienoic acid methyl ester which was named canangalia F.

Compound **5** was assigned as $C_{16}H_{28}O_4$ from its quasi-molecular ion peak at *m/z* 307.1856 $[M + Na]^+$ in the HRESIMS. In the HMBC spectrum, correlations between methylene proton at δ 1.60 (H-5) with carbons at C-4 (δ 44.4), C-1′ (δ 53.1) and C-2′ (δ 72.3) were observed. The triplet signal at δ 0.84 ($J = 4.4$ Hz) was assigned as H-1′ and connected to the carbon at δ 53.1 in the HMQC spectrum. This proton showed long range correlations with C-4 (δ 44.4), C-6′ (δ 40.3), C-3′ (δ 23.9), C-7′ (δ 14.6), and C-8′ (δ 26.9) in the HMBC experiment (Fig. 2). The ^{1}H-^{1}H COSY spectrum showed correlations in the aliphatic proton, representing H-4/H-5/H-1′ and H-3′/H-4′/H-5′. Two methyl protons at CH_3-7′ ($\delta_{H/C}$ 0.92/14.6) and CH_3-8′ ($\delta_{H/C}$ 0.98/26.9) showed correlations with C-1′, C-6′ and C-5′ in the HMBC spectrum. The signal at $\delta_{H/C}$ 1.17/30.4 was assigned as CH_3-9′, and this proton showed correlations with C-5, C-1′, and C-2′ in the HMBC spectrum. The NOESY experiment showed correlations between H-1′/H-5′/CH_3-8′/CH_3-9′, which indicated that these protons are cofacial. The results indicate that H-1′ and H-5′ are located in an α-axial orientation and CH_3-9′ was in an α-equatorial orientation (Fig. 2). From all data, compound **5** was determined as (*rel*-1′*R*,2′*S*,5′*R*,2*E*)-5-(2′,5′-dihydroxy-2′,6′,6′-trimethylcyclohexyl)-3-methyl-2-pentenoic acid methyl ester which was named canangalia G.

Compound **6** was assigned as $C_{16}H_{28}O_4$ from its quasi-molecular ion peak at *m/z* 307.1886 $[M + Na]^+$ in the HRESIMS. The ^{1}H NMR spectrum of this compound was similar to that of **5**, except for the chemical shift of the methine proton, H-1′ (δ_H 1.13, t, $J = 4.4$ Hz) which appeared at a slightly lower field than **5**. In addition, the ^{13}C NMR signal of CH_3-9′ in **5** showed a signal at δ 30.4 but in **6** showed at δ 23.2. Consequently, the NOESY experiment showed correlations between H-1′/H-5′/CH_3-8′, indicating that these protons are located in an α- orientation. This means that CH_3-7′ is located in an β-axial orientation. The NOESY experiment displayed the correlation between CH_3-7′ and CH_3-9′, which indicated the β-axial orientation of CH_3-9′. From all the data, compound **6** was determined as (*rel*-1′*R*,2′*R*,5′*R*,2*E*,)-5-(2′,5′-dihydroxy-2′,6′,6′-trimethylcyclohexyl)-3-methyl-2-pentenoic acid methyl ester which was named canangalia H.

Biogenetics of cyclic derivatives **5** and **6** were produced from linear methyl farnesoate. Epoxide derivative **10** was the key starting material. Electrophilic addition of a double bond at C-6 with carbocation at C-11 generated carbocation at C-7. Further hydroxylation at carbocation led to epimers **5** and **6**. All isolated compounds were juvenile hormone III derivatives which are rare in higher plants. Juvenile hormone III is an important hormone for development from insect larvae to insect adults. It is believed that higher plants produce juvenile hormone III analogues to defend themselves from insects [19].

The isolated compounds were evaluated for antifungal activity against *P. insidiosum*. From our previous study, compounds **8**, **10** and **11** exhibited potent antifungal activity with inhibition zones of 24, 16 and 14 mm, respectively. Unfortunately, the other compounds showed no activity. It should be noted that compounds **1**–**7** were ester or ether derivatives of **8**, but only **8** exhibited strong activity. These results suggest dihydroxyl groups at C-10 and C-11 may play an important role in this activity.

Canangalia C (**1**): colorless oil; $[\alpha]^{28}_D$ + 1.4 (*c* 0.55, MeOH); UV (MeOH) λ_{max} (log ε) 218 (4.23) nm; IR (Neat) ν_{max} 3431, 2931, 1719, 1648, 1223, 1146 cm^{-1}; ^{1}H ($CDCl_3$, 400 MHz) and ^{13}C NMR data ($CDCl_3$, 100 MHz), see Tables 1 and 2; HRESIMS *m/z* 379.2098 $[M + Na]^+$(calcd. for $C_{19}H_{32}O_6$ + Na, 379.2097).

Canangalia D (**2**): colorless oil; $[\alpha]^{28}_D$ + 5.6 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε) 218 (4.23) nm; IR (Neat) ν_{max} 3464, 2936, 1715, 1648, 1225, 1153 cm^{-1}; ^{1}H ($CDCl_3$, 400 MHz) and ^{13}C NMR data ($CDCl_3$, 100 MHz), see Tables 1 and 2; HRESITOFMS *m/z* 407.2048 $[M + Na]^+$(calcd. for $C_{20}H_{32}O_7$ + Na, 407.2046).

Canangalia E (**3**): colorless oil; $[\alpha]^{28}_D$ + 9.3 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.29), 217 (4.30) nm; IR (Neat) ν_{max} 3392, 2929, 1717, 1648, 1224, 1147 cm^{-1}; ^{1}H ($CDCl_3$, 400 MHz) and ^{13}C NMR data ($CDCl_3$, 100 MHz), see Tables 1 and 2; HRESIMS *m/z* 407.2428 $[M + Na]^+$(calcd. for $C_{21}H_{36}O_6$ + Na, 407.2410).

Canangalia F (**4**): colorless oil; $[\alpha]^{28}_D$ + 10 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.21), 217 (4.22) nm; IR (Neat) ν_{max} 3387, 2928, 1717, 1648, 1225, 1147 cm^{-1}; ^{1}H ($CDCl_3$, 400 MHz) and ^{13}C NMR data ($CDCl_3$, 100 MHz), see Tables 1 and 2; HRESIMS *m/z* 407.2405 $[M + Na]^+$(calcd. for $C_{19}H_{32}O_6$ + Na, 407.2410).

Canangalia G (**5**): colorless oil; $[\alpha]^{28}_D$ + 15.5 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε) 220 (4.21) nm; IR (Neat) ν_{max} 3428, 2943, 1700, 1645, 1226, 1150 cm^{-1}; ^{1}H ($CDCl_3$, 400 MHz) and ^{13}C NMR data ($CDCl_3$, 100 MHz), see Tables 1 and 2; HRESIMS *m/z* 307.1856 $[M + Na]^+$(calcd. for $C_{16}H_{28}O_4$ + Na, 307.1885).

Canangalia H (**6**) colorless oil; $[\alpha]^{28}_D$ − 2.0 (*c* 1.4, MeOH); UV (MeOH) λ_{max} (log ε) 220 (4.21) nm; IR (Neat) ν_{max} 3430, 2942, 1717, 1646, 1227, 1150 cm^{-1}; ^{1}H ($CDCl_3$, 400 MHz) and ^{13}C NMR data ($CDCl_3$, 100 MHz), see Tables 1 and 2; HRESIMS *m/z* 307.1886 $[M + Na]^+$(calcd. for $C_{16}H_{28}O_4$ + Na, 307.1885).

4. Conclusion

Six new juvenile hormone III analogues named canangalias C-H (**1**–**6**) and a new natural product juvenile hormone III, (2*E*,6*E*,10*R*)-10-acetoxy-11-hydroxy-3,7,11-trimethyldodeca-2,6-dienoic acid methyl ester (**7**), together with six known juvenile hormone III analogues (**8**–**13**) were isolated from the roots of *C. latifolia*. Their structures were defined by 1D, 2D NMR (COSY, NOESY, HMQC and HMBC).

Conflict of interest

The authors have no conflict of interest to report.

Acknowledgments

We acknowledge financial support from Rajamangala University of Technology Isan, Khon Kaen campus. The Thai Research Fund (RSA5980022) and The Center of Excellence for Innovation in Chemistry (PERCH-CIC), Office of the Higher Education Commission, Ministry of Education are gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fitote.2016.08.018.

References

[1] W. Nanakorn, Queen Sirikit Botanic Garden, O.S, 5, Printing House, Bangkok, 1998 73.
[2] W. Chuakul, P. Saralamp, A. Boonpleng, Medicinal plants used in the Kutchum District, Yasothon Province, Thailand, Thai J. Phytopharm. 9 (2002) 22–49.
[3] W. Chuakul, A. Boonpleng, Survey on medicinal plants in Ubon Ratchathani Province, Thailand, Thai J. Phytopharm. 11 (2004) 33–54.
[4] W. Chuakul, N. Soonthornchareonnon, S. Sappakun, Medicinal plants used in Kungkrabaen Royal Development Study Center, Chanthaburi province, Thai J. Phytopharm. 13 (2006) 27–42.
[5] G. Gyatso, Dictionary of Plants Used in Cambodia, Dy Phon Pauline, 2000.
[6] S. Hout, A. Chea, S.S. Bun, R. Elias, M. Gasquet, P. Timon-David, G. Balansard, N. Azas, Screening of selected indigenous plants of Cambodia for antiplasmodial activity, J. Ethnopharmacol. 107 (2006) 12–18.
[7] Y.Y. Siv, R.R. Paris, Flavonoids from Cambodian plants belonging to genera *Cananga*, *Colona*, *Grewia*, *Leea*, and *Melastoma*, Plantes Med. Phytother. 6 (1972) 299–305.
[8] H. Yang, H.S. Kim, E.J. Jeong, P. Khiev, Y.W. Chin, S.H. Sung, Plant-derived juvenile hormone III analogues and other sesquiterpenes from the stem bark of *Cananga latifolia*, Phytochemistry 94 (2013) 277–283.
[9] R. Phatchana, Y. Thongsri, R. Somwaeng, K. Piboonpol, C. Yenjai, Canangalias A and B from the stem bark of *Cananga latifolia*, Phytochem. Lett. 13 (2015) 147–151.
[10] T. Sribuhom, U. Sriphana, Y. Thongsri, C. Yenjai, Chemical constituents from the stems of *Alyxia schlechteri*, Phytochem. Lett. 11 (2015) 80–84.
[11] A.W. De Cock, L. Mendoza, A.A. Padhye, L. Ajello, L. Kaufman, *Pythium insidiosum* sp. nov., the etiologic agent of pythiosis, J. Clin. Microbiol. 25 (1987) 344–349.
[12] L. Mendoza, Pythium insidiosum, in: W.G. Merz, R.J. Hay (Eds.), Topley and Wilson's Microbiology and Microbial Infections, Medical Mycology, 10th ed.ASM Press, London, UK 2005, pp. 617–630.

[13] K. Mori, H. Mori, Synthesis of compounds with juvenile hormone activity. 25. Synthesis of both the enantiomers of juvenile hormone III, Tetrahedron 43 (1987) 4097–4106.
[14] H. Jacobs, F. Ramadayal, S. Mclean, M. Perpickdumont, F. Puzzuoli, W.F. Reynolds, Constituents of *Hortia regia*: 6,7-dimethoxycoumarin, rutaecarpine, skimmianine, and (+)-methyl (*E*, *E*)-10,11-dihydroxy-3,7,11-trimethyl-2,6-dodecadienoate, J. Nat. Prod. 50 (1987) 507–509.
[15] P.E.A. Teal, D. Jones, G. Jones, B. Torto, V. Nyasembe, C. Borgemeister, et al., Identification of methyl farnesoate from the hemolymph of insects, J. Nat. Prod. 77 (2014) 402–405.
[16] J.B. Rodriguez, E.G. Gros, A convenient method for the preparation of (±) juvenile hormone-III, Naturforsch. B 45 (1990) 93–95.
[17] Y.C. Toong, D.A. Schooley, F.C. Baker, Isolation of insect juvenile hormone-III from a plant, Nature 333 (1988) 170–171.
[18] J.C. Bede, W.G. Goodman, S.S. Tobe, Quantification of juvenile hormone III in the sedge *Cyperus iria* L.: comparison of HPLC and radioimmunoassay, Phytochem. Anal. 11 (2000) 21–28.
[19] R.K. Chaitanya, P. Sridevi, B. Senthikumaran, A. Dutta-Gupta, Effect of juvenile hormone analog, methoprene on H-fibroin regulation during the last instar larval development of *Corcyra cephalonica*, Gen.Comp. Endocrinol. 181 (2012) 10–17.

Phytochemistry Letters 17 (2016) 242–246

Contents lists available at ScienceDirect

Phytochemistry Letters

journal homepage: www.elsevier.com/locate/phytol

Short communication

Cytotoxic and antimalarial constituents from the roots of *Toddalia asiatica*

Chayanis Hirunwong[a], Sanwat Sukieum[a], Ratchanee Phatchana[b], Chavi Yenjai[a,*]

[a] Natural Products Research Unit, Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand
[b] Department of Chemistry, Faculty of Engineering, Rajamangala University of Technology Isan, Khon Kaen Campus, Khon Kaen 40000, Thailand

ARTICLE INFO

Article history:
Received 31 May 2016
Received in revised form 13 July 2016
Accepted 8 August 2016
Available online xxx

Keywords:
Toddalia asiatica
Rutaceae
Coumarin
Terpenealkaloid

ABSTRACT

A rare isoprene coumarin, toddayanin (**1**), and a new dihydrochelerythrine-cadinane derivative, toddayanis (**2**), along with 16 known compounds were isolated from the root of *Toddalia asiatica* Lam. Compound **12** showed strong antimalarial activity against *Plasmodium falciparum* with an IC_{50} value of 5.4 μg/mL and was inactive against normal, Vero cells. Compound **13** showed cytotoxicity against the MCF-7 cell line with an IC_{50} value of 8.7 μg/mL and was inactive against Vero cells. Alkaloid **11** displayed cytotoxicity against KB, NCI-H187, MCF-7 and Vero cells lines with IC_{50} values ranging from 0.8 to 11.6 μg/mL.

1. Introduction

In the continuation of our research investigation of bioactive compounds from natural sources, we have intensively evaluated biological activities such as cytotoxicity, antimalarial, antifungal, and antituberculosis properties of isolated compounds from Thai medicinal plants (Thongthoom et al., 2011; Suthiwong et al., 2014). In the present work, the chemical constituents from the roots of *Toddalia asiatica* (Linn.) Lam. were studied. Nine coumarins, six alkaloids, two cinnamic derivatives, and a sesquiterpene were isolated and biological activities of all isolates were evaluated. Cytotoxicity against KB, MCF-7, and NCI-H187 cell lines were investigated. In addition, antimalarial activity against *Plasmodium falciparum* was also evaluated.

T. asiatica (Linn.) Lam. (Rutaceae), a Thai medicinal plant, is a woody liana with a corky, thorny stem which climbs on trees. The root bark of this plant is used for curing diarrhea, gonorrhea, cough, influenza and for toothache (Jain et al., 2006; Hu et al., 2014). The fresh leaves are used to treat lung diseases and for curing bowel complaints (Jain et al., 2006). The fruits are believed to be a treatment for malaria and cough (Karunai et al., 2012). An ointment of the roots and unripe fruits has been used to treat rheumatism (Lakshmi et al., 2002). There are many reports on the chemical constituents and biological activities of this plant such as prenylated and geranylated coumarins, triterpenes, phenanthridine alkaloids, and also volatile oils (Saxena and Sharma, 1999). Biological activities of compounds from this plant have been investigated including anticancer against the U-937 cell line (Vazquez et al., 2012), antidiabetic, antioxidant (Irudayaraj et al., 2012) and antibacterial activities (Karunai et al., 2012).

2. Results and discussion

The extraction and purification of the hexane extract from the root of *T. asiatica* led to the isolation of 18 compounds. Two of them, a rare coumarin named toddayanin (**1**) and a terpenealkaloid named toddayanis (**2**) were new compounds. Sixteen known compounds including artanin (**3**) (Wang et al., 2009), coumurrayin (**4**) (Lv et al., 2015), toddaculine (**5**) (Vazquez et al., 2012), toddanol (**6**) (Vazquez et al., 2012), toddalolactone (**7**) (Phatchana and Yenjai, 2014), isopimpinellin (**8**) (Gao et al., 2014), phellopterin (**9**) (Heinke et al., 2011), 5-methoxy-8-geranyloxypsoralen (**10**) (Franke et al., 2001), 8-methoxydihydrochelerythrine (**11**) (Zou et al., 2015), 8-methoxynorchelerythrine (**12**) (Hu et al., 2014), skimmiamine (**13**) (Li et al., 2014), norchelerythrine (**14**) (Phatchana and Yenjai, 2014), chelerythrine (**15**) (Ishii et al., 1983), oplopanone (**16**) (Lukas et al., 2015), nelumol (**17**) (Phatchana and

* Corresponding author.
E-mail address: chayen@kku.ac.th (C. Yenjai).

http://dx.doi.org/10.1016/j.phytol.2016.08.008
1874-3900/

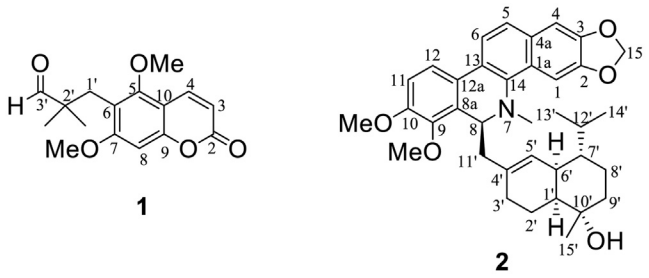


Fig. 1. The structure of new compounds **1** and **2**.

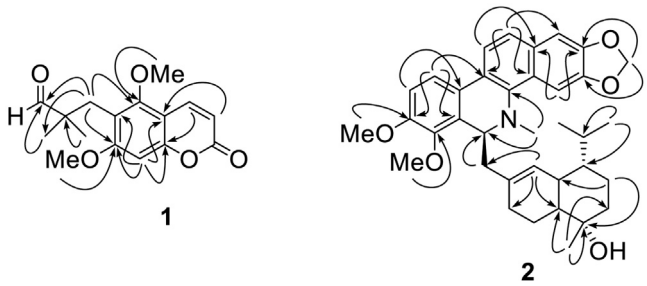


Fig. 2. Key HMBC correlations of compounds **1** and **2**.

Yenjai, 2014), and *p*-isopentenoxybenzenepropanoic acid (**18**) (Genovese et al., 2009) were also isolated (Fig. 1).

Compound **1** was found as a pale yellow oil, and its molecular formula was determined as $C_{16}H_{18}O_5$ by ^{13}C NMR and HRESIMS data. The IR spectrum showed absorption bands of carbonyl groups at 1729 and 1609 cm^{-1}. The ^1H NMR spectrum showed a singlet signal at δ 9.49 indicating an aldehydic proton (H-3′) (Table 1). This proton correlated with carbon at δ 204.9 in the HMQC spectrum. Two doublet signals at δ 7.83 and 6.25 (J = 9.6 Hz) were assigned as H-4 and H-3, respectively. The signal at δ_H/δ_C 6.60/95.5 was assigned as H-8. In the HMBC spectrum, a correlation between H-4 and C-9 (δ 155.2) was observed (Fig. 2). The methoxy protons at δ_H/δ_C 3.79/62.8 showed the correlation with C-5 (δ 156.3) and at δ_H/δ_C 3.82/55.7 displayed a correlation with C-7 (δ 161.4) in the HMBC experiment. In this experiment, H-8 displayed correlations with C-6 (δ 116.2), C-7 (δ 161.4), C-9 (δ 155.2) and C-10 (δ 107.0). The signal at δ 2.84 (2H, s) was assigned as H-1′ and correlated with carbon at δ 30.9 in the HMQC experiment. This methylene proton showed correlations with C-5 (δ 156.3), C-7 (δ 161.4), C-3′ (δ 204.9) and C-4′/5′ (δ 21.5) in the HMBC spectrum. Two methyl groups (CH_3-4′ and CH_3-5′) displayed the signal at δ_H/δ_C 1.03/21.5 and correlated with C-1′ (δ 30.9), C-2′ (δ 47.1), and C-3′ (δ 204.9) in the HMBC experiment. All these data confirmed an irregular isoprene-coumarin, **1,** which was identified as 6-(2′,2′-dimethyl-3′-propanal)-5,7-dimethoxycoumarin and was named toddayanin.

Table 1
^1H and ^{13}C NMR spectral data of compounds **1** and **2** (*d* in ppm).

position	**1** ($CDCl_3$)		**2** ($CDCl_3$)	
	d_C, type	d_H (J in Hz)	d_C, type	d_H (J in Hz)
1			101.5 CH	7.60 s
1a			127.5C	
2	160.9C		147.3C	
3	112.7 CH	6.25 d (9.6)	147.7C	
4	138.7 CH	7.83 d (9.6)	104.1 CH	7.08 s
4a			130.9C	
5	156.3C		123.3 CH	7.46 d (8.8)
6	116.2C		119.7 CH	7.72 d (8.8)
7	161.4C			
8	95.5 CH	6.60 s	56.4 CH	4.59 dd (10.2, 4.4)
8a			130.4C	
9	155.2C		145.7C	
10	107.0C		152.0C	
11			110.9 CH	6.93 d (8.8)
12			118.6 CH	7.54 d (8.8)
12a			124.9C	
13			123.7C	
14			140.1C	
15			100.8 CH_2	6.04 s 5.98 s
1′	30.9 CH_2	2.84 s	50.1 CH	1.20 br d (11.0)
2′	47.1C		22.9 CH_2	2.07 m 2.01 m
3′	204.9 CH	9.49 s	28.6 CH_2	2.29 br d (16.0) 2.07 m
4′	21.5 CH_3	1.03 s	135.6C	
5′	21.5 CH_3	1.03 s	124.7 CH	4.92 s
6′			39.9 CH	1.65 m
7′			46.5 CH	1.36 m
8′			22.0 CH_2	1.50 m 1.54 m
9′			42.2 CH_2	1.79 m 1.80 m
10′			72.5C	
11′			42.2 CH_2	1.96 br d (10.2) 2.05 m
12′			25.7 CH	1.69 m
13′			21.3 CH_3	0.74 d (7.0)
14′			14.6 CH_3	0.45 d (7.0)
15′			20.7 CH_3	1.15 s
OCH_3-5	62.8 CH_3	3.79 s		
OCH_3-7	55.7 CH_3	3.82 s		
OCH_3-9			55.7 CH_3	3.92 s
OCH_3-10			60.9 CH_3	3.94 s
N-CH_3			42.8 CH_3	2.60 s

Compound **2** was found as a yellow oil, and assigned the molecular formula $C_{36}H_{43}NO_5$ by HRESIMS analysis. The ^1H NMR spectrum showed six aromatic protons. Two doublet signals (J = 8.8 Hz) at δ 6.93 and δ 7.54 were assigned as H-11 and H-12, and correlated with carbons at δ 110.9 (C-11) and δ 118.6 (C-12), respectively, in the HMQC experiment (Table 1). Another two doublet signals (J = 8.8 Hz) at δ_H/δ_C 7.46/123.3 (H-5) and δ_H/δ_C 7.72/119.7 (H-6) were also observed. Two singlet signals at δ 7.60 and δ 7.08 were assigned as H-1 and H-4, respectively. In the HMBC experiment, two methoxy groups at δ 3.94 and δ 3.92 correlated with carbons at δ 152.0 (C-10) and δ 145.7 (C-9), respectively (Fig. 2). An *N*-methyl group showed signals at δ_H/δ_C 2.60/42.8 which correlated with carbons at δ 140.1 (C-14) and δ 56.4 (C-8) in the HMBC experiment. A methylenedioxy group displayed signals at δ 6.04 and 5.98. These data indicated that this molecule contained dihydrobenzo[c]phenanthridine moiety.

The remaining signals in the ^{13}C NMR and DEPT spectra showed 15 carbon signals including, three methyl, five methylene, five methine (four aliphatic and one olifinic), and two quaternary (one aliphatic and one olifinic) carbons. The ^1H NMR revealed signals of an isopropyl group at δ 0.74 (3H, d, J = 7.0, CH_3-13′) and 0.45 (3H, d, J = 7.0, CH_3-14′) and a methine proton at δ 1.69 (m, H-12′). These two methyl protons correlated with carbons at δ 25.7 (C-12′) and δ 46.5 (C-7′) in the HMBC spectrum. The methyl group at δ 1.15 (s, CH_3-15′) correlated with carbons at δ 50.1 (C-1′), δ 42.2 (C-9′) and an oxygenated carbon at δ 72.5 (C-10′) in the HMBC experiment. In this spectrum, the olifinic proton H-5′ (δ_H/δ_C 4.92/124.7) showed the correlations with carbons at δ 50.1 (C-1′), 28.6 (C-3′), and 42.2 (C-11′). From these data indicated that this molecule contained cadinane moiety. The COSY spectrum displayed a correlation between H-8 and H-11′, which indicated the connection of cadinane sesquiterpene and phenanthridine moieties at these points. This is the first time to find dihydrochelerythrine link with cadinane sesquiterpene derivative in this plant. The specific

Table 2
Cytotoxic and antimalarial activities of all compounds.

compound	Cytotoxicity (IC_{50} μg/mL)				Antimalarial (IC_{50} μg/mL)
	KB	NCI-H187	MCF-7	Vero cells	
2	32.2	5.8	inactive[a]	17.6	3.8
3	31.0	7.4	25.4	inactive[a]	inactive[a]
5	17.1	inactive[a]	23.4	inactive[a]	inactive[a]
6	inactive[a]	inactive[a]	inactive[a]	47.5	inactive[a]
9	inactive[a]	inactive[a]	23.2	inactive[a]	inactive[a]
10	12.3	10.5	inactive[a]	11.3	inactive[a]
11	2.4	0.8	11.6	4.9	inactive[a]
12	inactive[a]	inactive[a]	inactive[a]	inactive[a]	5.4
13	inactive[a]	inactive[a]	8.7	inactive[a]	inactive[a]
17	15.5	23.1	17.8	inactive[a]	inactive[a]
other	inactive[a]	inactive[a]	inactive[a]	inactive[a]	inactive[a]
Ellipticine	1.3	0.7	0.4	6.5	
Dihydroartemisinin					3.7 nM

[a] Inactive at > 50 μg/mL

rotation of this compound was $[\alpha]_D^{21}$ +63.2 (c 0.1, $CHCl_3$) which was the same sign as *epi*-zanthomuurolanine; $[\alpha]_D^{20}$ + 86.9 (c 0.22, $CHCl_3$) (Yang et al., 2008). In addition, the CD of this compound was similar to *epi*-zanthomuurolanine which showed a negative value at 219 nm and positive values at 251 and 281 nm (Yang et al., 2008). The specific rotation and CD results indicated that the configuration of **2** was *rel*-8*S*,1′*S*,6′*S*,7′*S*,10′*S*. Thus, the structure of **2**, named toddayanis was determined as shown.

Toddayanis (**2**) showed cytotoxicity against the KB, NCI-H187 and Vero cell lines with IC_{50} values of 32.2, 5.8 and 17.6 μg/mL, respectively (Table 2). Coumarin **3** displayed cytotoxicity against KB, NCI-H187 and MCF-7 with IC_{50} ranging from 7.4 to 31.0 μg/mL while **4** showed inactive activity. The results suggest that a prenylalkoxy group is required for the cytotoxicity. Compound **11** exhibited strong cytotoxicity against NCI-H187 (IC_{50} = 0.8 μg/mL) which was nearly equal to the ellipticine standard (IC_{50} = 0.7 μg/mL). However, this compound showed cytotoxic activity against Vero cells with IC_{50} value of 4.9 μg/mL while the ellipticine showed an IC_{50} value of 6.5 μg/mL. Compounds **5** and **13** exhibited cytotoxicity against MCF-7 cell line with IC_{50} values of 23.4 and 8.7 μg/mL but showed inactive against Vero cells. These findings reveal that **5** and **13** may be lead compounds for anticancer agents.

In addition, all compounds were evaluated for antimalarial activity against *Plasmodium falciparum* (K1, multidrug resistant strain). Compound **2** showed strong antimalarial activity with an IC_{50} value of 3.8 μg/mL. Unfortunately, this compound exhibited cytotoxicity against normal, Vero cells (IC_{50} = 17.6 μg/mL). It is interesting to note that alkaloid **12** showed strong antimalarial activity with an IC_{50} value of 5.4 μg/mL and displayed inactivity against Vero cells. These results suggest that **12** is likely to be the lead compound for the development of antimalarial agents.

3. Experimental

3.1. General experimental procedures

Melting points were determined on a SANYO Gallenkamp (UK) melting point apparatus and are uncorrected. UV spectra were measured on an Agilent 8453 UV–vis spectrophotometer (Germany). IR spectra were recorded as KBr disks or thin films, using Perkin Elmer Spectrum One FT-IR spectrophotometer (UK). The NMR spectra were recorded on a Varian Mercury plus spectrometer (UK) operating at 400 MHz (^{1}H) and at 100 MHz (^{13}C). Mass spectra were determined on a Micromass Q-TOF 2 hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer with a Z-spray ES source (Micromass, Manchester, UK). Thin layer chromatography (TLC) was carried out on MERCK silica gel 60 F_{254} TLC aluminium sheet. Column chromatography was done with silica gel 0.063-0.200 mm or less than 0.063 mm. Preparative thin layer chromatography (PLC) was carried out on glass supported silica gel plates using silica gel 60 PF_{254} for preparative layer chromatography. All solvents were routinely distilled prior to use.

3.2. Plant material

The roots of *T. asiatica* were collected in June 2014 from Khon Kaen Province. The plant was identified by Dr. Pranom Chantaranothai, Faculty of Science, Khon Kaen University. A botanically identified voucher specimen (KKU0042011) was deposited at the herbarium of the Department of Chemistry, Faculty of Science, Khon Kaen University, Thailand.

3.3. Extraction and isolation

Air-dried and finely powdered root (7 kg) of *T. asiatica* was sequentially extracted at room temperature for three days with hexane (2 × 10 L), EtOAc (2 × 10 L), and MeOH (2 × 10 L). The extracts were evaporated *in vacuo* to obtain three dry extracts, crude hexane (147 g), EtOAc (89 g), and crude MeOH (121 g). The crude hexane extracts (60 g) was subjected to column chromatography on silica gel 60 and subsequently eluted with a gradient of four solvents (hexanes, EtOAc, CH_2Cl_2, and MeOH) by gradually increasing the polarity of the elution solvents system. On the basis of their thin layer chromatography (TLC) characteristic, the fractions which contained the same major compounds were combined to give thirteen fractions, F_1–F_{13}. Fraction F_3 was purified by silica gel flash column chromatography (FCC) eluted with an isocratic system of hexane:EtOAc (97:3) to give fifteen fractions, $F_{3.1}$–$F_{3.15}$. Subfraction $F_{3.15}$ was purified by crystallization from MeOH to give **11** (8.5 mg, 0.0001%). Fraction F_4 was purified by crystallization (EtOAc) to afford yellow solid of compound **5** (340 mg, 0.0048%). Fraction F_6 was further purified by FCC using hexane:EtOAc (80:20) as eluent to obtain seven fractions, $F_{6.1}$-$F_{6.7}$. The purification of $F_{6.5}$ by FCC (hexane:EtOAc; 90:10) yielded **4** (88.4 mg, 0.0013%). Purification of $F_{6.7}$ was carried out on silica gel FCC and eluting with CH_2Cl_2:hexane to give five subfractions $F_{6.7.1}$–$F_{6.7.5}$. Fraction $F_{6.7.2}$ was purified by FCC over silica gel, eluted with an isocratic system of hexane:EtOAc (90:10) to give **10** (4.9 mg, 0.0001%). Fraction $F_{6.7.5}$ was purified by preparative TLC using hexane:CH_2Cl_2 (70:30) as developing solvent to obtain **14** (1.1 mg, 0.00001%). Further purification of F7 by FCC (hexane:EtOAc) yielded seven subfractions, $F_{7.1}$–$F_{7.7}$. Subfractions $F_{7.1}$ and $F_{7.2}$ were

purified by preparative TLC using hexane:CH_2Cl_2 (60:40) as developing solvent to give compound **8** (6.3 mg, 0.0001%) and **9** (68.4 mg, 0.0010%), respectively. Subfraction $F_{7.7}$ was purified by silica gel FCC and eluted with an isocratic system of hexane:CH_2Cl_2:EtOAc (70:25:5) to afford compounds **3** (50.6 mg, 0.0007%) and **1** (3.7 mg, 0.0001%). Fraction F_9 was subjected to silica gel FCC, eluted with an isocratic system of hexane:CH_2Cl_2 (50:50) to obtain ten subfractions, $F_{9.1}$–$F_{9.10}$. Subfraction $F_{9.8}$ was purified by silica gel FCC, eluted with an isocratic system of hexane:EtOAc (80:20) to give fourteen subfractions, $F_{9.8.1}$-$F_{9.8.14}$. Subfraction $F_{9.8.6}$ was purified by silica gel FCC, eluted with an isocratic system of hexane:CH_2Cl_2:EtOAc (30:65:5) to afford compound **2** (5.2 mg, 0.0001%). Subfraction $F_{9.8.8}$ was subjected to Sephadex LH-20 CC and an isocratic system with MeOH to give compound **16** (7.2 mg, 0.0001%). Subfraction $F_{9.8.11}$ was purified by preparative TLC using hexane:CH_2Cl_2:MeOH (40:58:2) to afford compound **17** (6.1 mg, 0.0001%). Subfraction $F_{9.10}$ was subjected to Sephadex LH-20 CC, eluted with isocratic MeOH to give eight fractions, $F_{9.10.1}$-$F_{9.10.8}$. Subfraction $F_{9.10.6}$ was purified by PLC using hexane:CH_2Cl_2:EtOAc (45:40:15) to afford compound **7** (26 mg, 0.0004%). Subfraction $F_{9.10.8}$ was subjected to PLC using hexane:EtOAc (80:20) to give five subfractions, $F_{9.10.8.1}$–$F_{9.10.8.5}$. Subfraction $F_{9.10.8.4}$ was purified by silica gel FCC and eluted with an isocratic system of hexane:EtOAc (70:30) to afford compound **18** (11.6 mg, 0.0002%). Fraction F_{10} was purified by FCC over silica gel, eluted with an isocratic system of hexane:EtOAc (70:30) to give sixteen subfractions, $F_{10.1}$–$F_{10.16}$. Subfraction $F_{10.10}$ was purified by silica gel FCC, eluted with an isocratic system of hexane:CH_2Cl_2:EtOAc (50:40:10) to afford compound **13** (16.9 mg, 0.0002%). Subfraction $F_{10.13}$ was purified by PLC using CH_2Cl_2:MeOH (1:99) as an eluent to afford **12** (8.5 mg, 0.0001%). Fraction F_{12} was purified by FCC and eluting with a gradient system of CH_2Cl_2:EtOAc afforded **15** (25.5 mg, 0.0004%) and **6** (26 mg, 0.0004%).

3.4. Spectroscopic data of compounds

Toddayanin (**1**): Pale yellow oil; UV ($CHCl_3$) λ_{max} (log ε) 242 (3.92), 256 (3.83), 328 (4.09) nm; IR (neat) ν_{max} 2964, 1729, 1609, 1461, 1382, 1131, 1084, 825 cm^{-1}; HRESIMS *m/z* 291.1233 $[M+H]^+$ (calcd. 291.1232); ^1H and ^{13}C NMR spectroscopic data, see Table 1.

Toddayanis (**2**): Yellow oil; $[\alpha]_D^{21}$ +63.2 (*c* 0.1); UV ($CHCl_3$) λ_{max} (log ε) 242 (4.50), 286 (4.59) nm; IR (neat) ν_{max} 3387, 2933, 1672, 1463, 1277, 1038, 942, 756 cm^{-1}; HRESIMS *m/z* 570.3217 $[M+H]^+$ (calcd. 570.3219); ^1H and ^{13}C NMR spectroscopic data, see Table 1.

3.5. Bioassay

3.5.1. Cytotoxicity assay

Cytotoxicity assays against human epidermoid carcinoma (KB, ATCC CCL−17), breast adenocarcinoma (MCF-7, ATCC HTB − 22) and human small cell lung cancer (NCI-H187, ATCC CRL−5804) cell lines were performed employing Resazurin Microplate Assay (REMA) (Sigma-Aldrich, Dye content 75%) (Brien et al., 2000) while cytotoxicity assay against Vero cells (African green monkey kidney, ATCC CCL−81) was performed by Green Fluorescent Protein (GFP) based assay (Hunt et al., 1999). Ellipticine (Fluka, purity ≥99%) and doxorubicin (Fluka, purity ≥98%) were included as the reference substances.

3.5.2. Antimalarial assay

Antimalarial activity was performed against *Plasmodium falciparum* (K1, multidrug resistant strain) which was cultured continuously according to the method of Trager and Jensen (Trager and Jensen, 1976). Quantitative assessment of antimalarial activity *in vitro* was determined by means of the microdilution radioisotope technique, based on the method described by Desjardins (Desjardins et al., 1979). The inhibitory concentration was that which caused 50% reduction in parasite growth as indicated by the *in vitro* uptake of [^{3}H] hypoxanthine by *Plasmodium falciparum*. The standard compound was dihydroartemisinin (Sigma, purity ≥97%).

Acknowledgements

We thank Thailand Research Fund (RSA5980022), The Center of Excellence for Innovation in Chemistry (PERCH-CIC), Office of the Higher Education Commission, Ministry of Education and Khon Kaen University for financial support. The Bioassay Laboratory of the National Center for Genetic Engineering and Biotechnology, Pathumthani, Thailand is also gratefully acknowledged for biological activity assays.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2016.08.008.

References

Brien, J.O., Wilson, I., Orton, T., Pognan, F., 2000. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur. J. Biochem. 267, 5421–5426.
Desjardins, R.E., Canfield, C.J., Haynes, J.D., Chulay, J.D., 1979. Quantitative assessment of antimalarial activity *in vitro* by a semiautomated microdilution technique. Antimicrob. Agents Chemother. 16, 710–718.
Franke, K., Porzel, A., Masaoud, M., Adam, G., Schmidt, J., 2001. Furanocoumarins from *Dorstenia gigas*. Phytochemistry 56, 611–621.
Gao, Y., Liu, Y., Wang, Z.G., Zhang, H.L., 2014. Chemical constituents of *Heracleum dissectum* and their cytotoxic activity. Phytochem. Lett. 10, 276–280.
Genovese, S., Epifano, F., Curini, M., Dudra-Jastrzebska, M., Luszczki, J.J., 2009. Prenyloxyphenylpropanoids as a novel class of anticonvulsive agents. Bioorg. Med. Chem. Lett. 19, 5419–5422.
Heinke, R., Franke, K., Porzel, A., Wessjohann, L.A., Ali, N.A.A., Schmidt, J., 2011. Furanocoumarins from *Dorstenia foetida*. Phytochemistry 72, 929–934.
Hu, J., Shi, X., Chen, J., Mao, X., Zhu, L., Yu, L., Shi, J., 2014. Alkaloids from *Toddalia asiatica* and their cytotoxic, antimicrobial and antifungal activities. Food Chem. 148, 437–444.
Hunt, L., Jordan, M., De Jesus, M., Wurm, F.M., 1999. GFP-expressing mammalian cells for fast, sensitive, noninvasive cell growth assessment in a kinetic mode. Biotechnol. Bioeng. 65, 201–205.
Irudayaraj, S.S., Sunil, C., Duraipandiyan, V., Ignacimuthu, S., 2012. Antidiabetic and antioxidant activities of *Toddalia asiatica* (L.) Lam. leaves in streptozotocin induced diabetic rats. J. Ethnopharm. 143, 515–523.
Ishii, H., Kobayashi, J., Ishikawa, T., 1983. Toddalenone: a new coumarin from *Toddalia asiatica* (*T. aculeata*) structure establishment based on the chemical conversion of limettin into toddalenone. Chem. Pharm. Bull. 31, 3330–3333.
Jain, S.C., Pandey, M.K., Upadhyay, R.K., Kumar, R., Hundal, G., Hundal, M.S., 2006. Alkaloids from *Toddalia aculeata*. Phytochemistry 67, 1005–1010.
Karunai, R.M., Balachandran, C., Duraipandiyan, V., Agastian, P., Ignacimuthu, S., 2012. Antimicrobial activity of ulopterol isolated from *Toddalia asiatica* (L.) Lam.: a traditional medicinal plant. J. Ethnopharm. 140, 161–165.
Lakshmi, V., Kapoor, S., Pandey, K., Patnaik, G.K., 2002. Spasmolytic activity of *Toddalia asiatica* Var floribunda. Phytother. Res. 16, 281–282.
Li, W., Zhou, W., Shim, S.H., Kim, Y.H., 2014. Chemical constituents of *Zanthoxylum schinifolium* (Rutaceae). Biochem. Syst. Ecol. 55, 60–65.
Lukas, B., Schmiderer, C., Novak, J., 2015. Essential oil diversity of European *Origanum vulgare* L. (Lamiaceae). Phytochemistry 119, 32–40.
Lv, H.N., Wang, S., Zeng, K.W., Li, J., Guo, X.Y., Ferreira, D., Zjawiony, J.K., Tu, P.F., Jiang, Y., 2015. Anti-inflammatory coumarin and benzocoumarin derivatives from *Murraya alata*. J. Nat. Prod. 78, 279–285.
Phatchana, R., Yenjai, C., 2014. Cytotoxic coumarins from *Toddalia asiatica*. Planta Med. 80, 719–722.
Saxena, V.K., Sharma, R.N., 1999. Antimicrobial activity of the essential oil of *Toddalia asiatica*. Fitoterapia 70, 64–66.
Suthiwong, J., Pitchuanchom, S., Wattanawongdon, W., Hahnvajanawong, C., Yenjai, C., 2014. Terpenoids from the root bark of *Pterolobium macropterum*. J. Nat. Prod. 77, 2432–2437.
Thongthoom, T., Promsuwan, P., Yenjai, C., 2011. Synthesis and cytotoxic activity of the heptaphylline and 7-methoxyheptaphylline series. Eur. J. Med. Chem. 46, 3755–3761.
Trager, W., Jensen, J.B., 1976. Human malaria parasites in continuous culture. Science 193, 673–675.

Vazquez, R., Riveiro, M.E., Vermeulen, M., Mondillo, C., Coombes, P.H., Crouch, N.R., Ismail, F., Mulholland, D.A., Baldi, A., Shayo, C., Davio, C., 2012. Toddaculin, a natural coumarin from *Toddalia asiatica*, induces differentiation and apoptosis in U-937 leukemic cells. Phytomedicine 19, 737–746.

Wang, F., Xu, Y., Liu, J.K., 2009. New geranyloxycoumarins from *Toddalia asiatica*. J. Asian Nat. Prod. Res. 11, 752–756.

Yang, C.H., Cheng, M.J., Chiang, M.Y., Kuo, Y.H., Wang, C.J., Chen, I.S., 2008. Dihydrobenzo[c]phenanthridine alkaloids from stem bark of *Zanthoxylum nitidum*. J. Nat. Prod. 71, 669–673.

Zou, H.L., Li, H.Y., Liu, B.L., Zhou, G.X., 2015. A new cytotoxic benzophenanthridine isoquinoline alkaloid from the fruits of *Macleaya cordata*. J. Asian Nat. Prod. Res. 17, 856–860.