Research Article

การวิเคราะห์ปริมาณเบอจีนินในสารสกัดลำต้น *Mallotus repandus* โดยโครมาโทกราฟีของเหลวสมรรถนะสูงวัฏภาคย้อนกลับ

ยลดา ศรีเศรษฐ์[1], วรัญญา จตุพรประเสริฐ[2], กนกวรรณ จารุกำจร[3]*

[1] นักศึกษาหลักสูตรปรัชญาดุษฎีบัณฑิต สาขาวิชาวิจัยและพัฒนาเภสัชภัณฑ์ คณะเภสัชศาสตร์ มหาวิทยาลัยขอนแก่น จังหวัดขอนแก่น 40002

[2] ปร.ด. ผู้ช่วยศาสตราจารย์ คณะแพทยศาสตร์ มหาวิทยาลัยมหาสารคาม จังหวัดมหาสารคาม 44000

[3] ปร.ด. รองศาสตราจารย์ กลุ่มวิจัยฤทธิ์ทางยาของผลิตภัณฑ์ธรรมชาติโดยเทคโนโลยีชีวภาพทางเภสัชศาสตร์
คณะเภสัชศาสตร์ มหาวิทยาลัยขอนแก่น จ. ขอนแก่น 40002

*** ติดต่อผู้นิพนธ์:** กนกวรรณ จารุกำจร คณะเภสัชศาสตร์ มหาวิทยาลัยขอนแก่น อำเภอเมือง จังหวัดขอนแก่น 40002 โทรศัพท์: 043-202305, โทรสาร: 043-202379, อีเมล: kanok_ja@kku.ac.th

บทคัดย่อ

การวิเคราะห์ปริมาณเบอจีนินในสารสกัดลำต้น *Mallotus repandus* โดยโครมาโทกราฟีของเหลวสมรรถนะสูงวัฏภาคย้อนกลับ

ยลดา ศรีเศรษฐ์[1], วรัญญา จตุพรประเสริฐ[2], กนกวรรณ จารุกำจร[3]*

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เบอจีนินจัดอยู่ในกลุ่มของสารโพลีฟีนอล เป็นสารสำคัญของต้น *Mallotus repandus* ซึ่งมีฤทธิ์ทางเภสัชวิทยาในการต้านอนุมูลอิสระ การปกป้องตับ การต้านเชื้อแบคทีเรีย การต้านอักเสบและการควบคุมระบบภูมิคุ้มกัน **วัตถุประสงค์:** เพื่อพัฒนาและประเมินวิธีโครมาโทกราฟีของเหลวสมรรถนะสูงวัฏภาคย้อนกลับ (RP-HPLC) สำหรับหาปริมาณเบอจีนินในสารสกัดลำต้น *Mallotus repandus* **วิธีการศึกษา:** เทคนิค RP-HPLC วัฏภาคนิ่งใช้ร่วมกับคอลัมน์ชนิด Phenomenex Luna C18 (5 μm, 250 mm x 4.6 mm) ส่วนวัฏภาคเคลื่อนที่ประกอบด้วยอะซีโทไนไตรล์และน้ำ (10 : 90 โดยปริมาตร) อัตราการไหล 1 มิลลิลิตรต่อนาที การตรวจวัดเบอจีนินที่ความยาวคลื่น 272 นาโนเมตร **ผลการศึกษา:** เวลารีเทนชั่นของเบอจีนินเป็น 10.885 นาที โดยไม่มีพีคของสารรบกวน การวิเคราะห์เบอจีนินในช่วงความเข้มข้น 20-320 ไมโครกรัมต่อมิลลิลิตรแสดงความสัมพันธ์เชิงเส้นตรงที่ดี (R^2 = 0.9999) ความถูกต้องแสดงด้วยค่าร้อยละการคืนกลับเท่ากับ 93.22 ± 1.49% ความเที่ยงตรงภายในวันและระหว่างวันแสดงด้วยร้อยละของค่าเบี่ยงเบนมาตรฐานสัมพัทธ์เท่ากับ 0.57 - 1.48% และ 0.19 - 1.70% ตามลำดับ ขีดจำกัดของการตรวจวัดและขีดจำกัดของการวิเคราะห์เชิงปริมาณเท่ากับ 4.56 และ 15.20 มิลลิกรัมต่อมิลลิลิตร ตามลำดับ สารสกัดส่วนน้ำของลำต้น *Mallotus repandus* จากการสกัดด้วยวิธีซอกห์เลตและการหมักมีปริมาณเบอจีนินสูงที่สุดเท่ากับ 12.67 ± 0.26% และ 19.38 ± 0.63% ของน้ำหนักแห้ง ตามลำดับ รองลงมาคือสารสกัดส่วนเมธานอล (9.28 ± 0.49% และ 8.73 ± 0.72%) และส่วนเอธานอล (2.16 ± 0.10% และ 1.73 ± 0.05%) ตามลำดับ **สรุปผลการศึกษา:** วิธีการวิเคราะห์เบอจีนินด้วยเทคนิค RP-HPLC นี้ มีความจำเพาะ ความสัมพันธ์เชิงเส้นตรง ความถูกต้อง ความเที่ยงตรง และความไวที่ดีและเป็นที่ยอมรับได้ วิธีนี้สามารถใช้ในการวิเคราะห์ปริมาณเบอจีนินในสารสกัดลำต้น *Mallotus repandus*

คำสำคัญ: *Mallotus repandus*, เบอจีนิน, โครมาโทกราฟีของเหลวสมรรถนะสูงวัฏภาคย้อนกลับ

Quantitative Determination of Bergenin in *Mallotus repandus* (Willd.) Muell. Arg. Stem Extract by Reverse Phase-High Performance Liquid Chromatography

Yollada Sriset[1], Waranya Chatuphonprasert[2] Kanokwan Jarukamjorn[3]*

[1] Ph.D. Candidate in Research and Development in Pharmaceuticals, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002

[2] Ph.D. (Research and Development in Pharmaceuticals) Assistant Professor, Faculty of Medicine, Mahasarakam University, Mahasarakham 44000

[3] Ph.D. (Pharmaceutical Sciences) Associate Professor, Research Group for Pharmaceutical Activities of Natural Products using Pharmaceutical Biotechnology (PANPB), Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002

*** Corresponding author:** Kanokwan Jarukamjorn, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002 Thailand

Tel: 043-202305, Fax: 043-202379 E-mail: kanok_ja@kku.ac.th

Abstract

Quantitative Determination of Bergenin in *Mallotus repandus* (Willd.) Muell. Arg. Stem Extract by Reverse Phase-High Performance Liquid Chromatography

Yollada Sriset, Waranya Chatuphonprasert Kanokwan Jarukamjorn[3]*

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Bergenin, a polyphenol compound, is a major active constituent of *Mallotus repandus* (Willd.) Muell. Arg. which possesses pharmacological activities including anti-oxidant, hepatoprotective, anti-microbial, anti-inflammatory, and immunomodulatory activities. **Objectives:** To develop and validate the reverse phase-high performance liquid chromatography (RP-HPLC) for quantification of bergenin in *M. repandus* stem extract. **Methods:** The RP-HPLC was employed with Phenomenex Luna C18 column (250 mm x 4.6 mm, 5 μm) as stationary phase. The mobile phase consisted of acetonitrile : water (10 : 90 v/v), at a flow rate of 1 mL/min. The wavelength at 272 nm was used for detection of bergenin. **Results:** The retention time of bergenin was 10.885 min with no peak interference. The method at the bergenin concentration range of 20-320 μg/mL was in a good linearity (R^2 = 0.9999). The accuracy measured as %recovery was 93.22 ± 1.49%. The precision of within-day and between-day expressed as % relative standard deviation were of 0.57-1.48% and 0.19-1.70%, respectively. Limit of detection and limit of quantification were 4.56 and 15.20 μg/mL, respectively. By the soxhlet extraction and maceration, the aqueous extract of *M. repandus* stem had the highest bergenin content of 12.67 ± 0.26% and 19.38 ± 0.63% dry% weight, respectively, followed by methanolic (9.28 ± 0.49% and 8.73 ± 0.72%) and ethanolic extracts (2.16 ± 0.10% and 1.73 ± 0.05%), respectively. **Conclusion:** The RP-HPLC method for determination of bergenin was achieved with good and acceptable specificity, linearity, accuracy, precision, and sensitivity. The utilization was assured by determination of bergenin content in the *M. repandus* stem extracts.

Keywords: *Mallotus repandus*, bergenin, reverse phase-high performance liquid chromatography (RP-HPLC)

Introduction

Mallotus species are a member of Euphobiaceae family. The most regions of distribution are tropical and subtropical areas in Asia such as Thailand, Cambodia, Vietnam, China, and India (Rieivre *et al.*, 2010). *M. repandus*, *M. philippensis*, *M. japonicas*, and *M. paniculatus* are examples of plants in this genus which have been usually employed as herbal drugs. *Mallotus* species contain abundant compounds such as polyphenols, diterpenoids, triterpenoids, and steroids (Tistaert *et al.*, 2012). In particular, bergenin, a polyphenol compound, is derivative of dihydroisocoumarin. The main chemical structure of bergenin included trihydroxybenzoic acid glycoside and *C*-glycoside of 4-*O*-methyl gallic acid (Fig. 1) which has been claimed to possess anti-oxidant (Nazir *et al.*, 2011; Yun *et al.*, 2015; Khan *et al.*, 2016), hepatoprotective (Lim *et al.*, 2001), anti-microbial (Nazir *et al.*, 2011), anti-inflammatory (Gao *et al.*, 2015), and immunomodulatory activities (Nazir *et al.*, 2007).

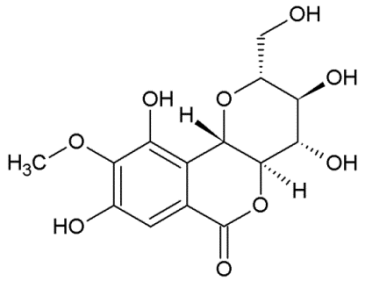

Fig.1. Chemical structure of bergenin

M. repandus (Willd.) Muell. Arg. is a potential herb which has long been basically used in Thai traditional medicine for the treatment of tendon inflammation and muscle pains including homeostatic maintenance (Wuttidhammaved, 2007). *M. repandus* had been reported to demonstrate several pharmacological activities including anti-inflammatory (Hasan *et al.*, 2014), analgesic (Hasan *et al.*, 2014), and anti-oxidant activities (Lin *et al.*, 1995). Bergenin was reported as the main compound of *M. repandus* (Li, 2006), but there is still no clear scientific evidence regarding quantitative determination of bergenin in *M. repandus*. Thus, it is of interest to quantitatively analyze the bergenin content in order to assure quality control and to support pharmacological activity of *M. repandus*.

The previous studies reported analytical methods for bergenin; for example, bergenin from *M. philippinensis* and *Bergenia* species (*B. ciliate*, *B. stracheyi*, and *B. ligulata*) were quantified by high performance thin layer chromatography-mass spectrometry (HPTLC-MS) and high performance liquid chromatography (HPLC), respectively (Singh *et al.*, 2007; Haribabu *et al.*, 2012). Though there was not reported in the prior study about quantitative determination of bergenin in *M. repandus*, HPLC might be an appropriate technique due to be simultaneously qualitative and quantitative determination with a reasonable precise and accurate outcome (Aguilar, 2004).

The current study aimed to establish the quantitative method for bergenin using reverse phase (RP)-HPLC. The validation of the method was fully performed, in which the analytical parameters, namely specificity, linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ) were established. To confirm utilization of the method, the bergenin content in the methanolic, ethanolic, and aqueous extracts of *M. repandus* stem by both soxhlet extraction and maceration were analyzed.

Materials and Methods

Chemicals and reagents

Standard bergenin (Cat. No. BP0258, purity > 98%) was a product of Biopurify Phytochemicals (Chengdu, China). Acetonitrile (HPLC grade) was purchased from Merck (Darmstadt, Germany). All other laboratory chemicals were of the highest purity available from chemical suppliers.

Plant material

M. repandus stem was obtained from Yasothon Panich herbal store (Yasothon, Thailand) in May, 2016. The specimen was identified as PANPB-MR 2016-001 and deposited in the Herbarium of Faculty of Pharmaceutical Sciences, Khon Kaen university.

Preparation of *M. repandus* extract

The stems of *M. repandus* were dried at 50°C and ground into fine powder. The dried powder (10 g) was extracted by two different methods comprising soxhlet extraction and maceration. For the soxhlet extraction, the powder was extracted with methanol, or ethanol for 3 h, or

water for 6 h using a soxhlet apparatus. For the maceration, the powder was macerated three times with methanol, ethanol, or water for 3 days. Both of the methanolic and ethanolic extracts were evaporated by using a rotary evaporator and then freeze-dried while the aqueous extract was only freeze-dried. A-50 mg aliquot of the dried powder (methanol, ethanol, and water parts) was accurately weighed about 50 mg and dissolved in 1 mL of methanol, ethanol, or water, respectively. After the mixture was vortexed for 5 min, it was centrifuged at 2,000 *g* for 20 min. The supernatant was collected, followed by filtration through a 0.45 μm-membrane filter before subjected to the RP-HPLC system. Each sample was analyzed for 5 replicates.

Standard preparation

Bergenin standard was accurately weighted about 1 mg and diluted with 1 mL of methanol to obtain standard stock solution of 1 mg/mL. A standard stock solution was serially diluted with methanol to have concentrations of 20, 40, 80, 160, and 320 μg/mL, respectively.

RP-HPLC system

The separation was performed on a RP-HPLC system 1100 series Agilent (Waldbronn, Germany), consisting of an isocratic pump, a variable wavelength programmable UV detector system, a manual injector, and ChemStation as the operating software. Phenomenex® Luna C18 column (particle size 5 μm, 250 mm x 4.6 mm i.d., Phenomenex®, Torrance, CA, USA) was used as the stationary phase. The mixture of acetonitrile and water (10 : 90 v/v) was employed as the mobile phase and filtered through a 0.45 μm-membrane filter prior to use. The flow rate of mobile phase was constant at 1 mL/min. The wavelength was set at 272 nm. The 20 μL-fixed loop Rheodyne® was equipped.

Method validation

The chromatographic method was developed and validated in accordance with the International Conference on Harmonization (ICH) guideline (2005), including analytical parameters of specificity, linearity, accuracy, precision, and sensitivity (LOD and LOQ).

Specificity was determined by the relative retention time of bergenin in the extract to the standard.

Linearity was estimated by correlation coefficient (R^2) of calibration graph from a series of bergenin standard (20, 40, 80, 160, and 320 μg/mL). The calibration line was constructed by plotting peak area versus concentration to obtain a linear equation. The slope and y-intercept of calibration line were presented.

Accuracy was evaluated by percentage of recovery (%recovery) from triplicates of different concentrations of bergenin standard (30, 60, and 120 μg/mL).

Precision was assessed as within-day and between-day variations by subjecting a series of five bergenin standard (20, 40, 80, 160, and 320 μg/mL) for 5 replicates and 5 different days, respectively (n=5). The percentage of relative standard deviation (%RSD) of peak area was calculated to demonstrate the within-day and between-day precision.

LOD and LOQ were calculated from the standard deviation of the linear regression line (S_B) and y-intercept (Y_B) using the equations: $Y = 3S_B + Y_B$ for LOD and $Y = 10S_B + Y_B$ for LOQ. Then the value Y was substituted in the linear regression equation to achieve LOD or LOQ, respectively

Results

The validation method was performed to confirm an optimization of RP-HPLC system. This method was demonstrated to be good specificity, linearity, accuracy, precision, and sensitivity for quantitative determination of bergenin.

The RP-HPLC method was assured a good specificity for bergenin. The retention time of bergenin standard was 10.885 min. No peak interference around the retention time of bergenin was noted (Fig. 2B), compared with the blank methanol (Fig 2A). The good linearity between the concentration range of bergenin from 20 to 320 μg/mL and the peak area at UV 272 nm was achieved. The linear regression equation of calibration curve, $Y = 14.688X - 55.893$ with R^2 0.9999 (Table 1), was established in the accepted criteria of the ICH guideline that R^2 not less than 0.990 (ICH, 2005). The method indicated the good accuracy with the %recovery of 93.22 ± 1.49% (Table 1) that fit to the ICH guideline (2005) of 90-110%. The %RSD of both within-

day and between-day precision in the concentration range of bergenin from 20 to 320 μg/mL were 0.57 - 1.48% and 0.19 - 1.70%, respectively (Table 1), confirmed the high precision of the method and met the ICH guideline that %RSD not more than 2% (ICH, 2005). The method showed a good sensitivity with the LOD and LOQ of bergenin at 4.56 μg/mL and 15.20 μg/mL, respectively (Table 1).

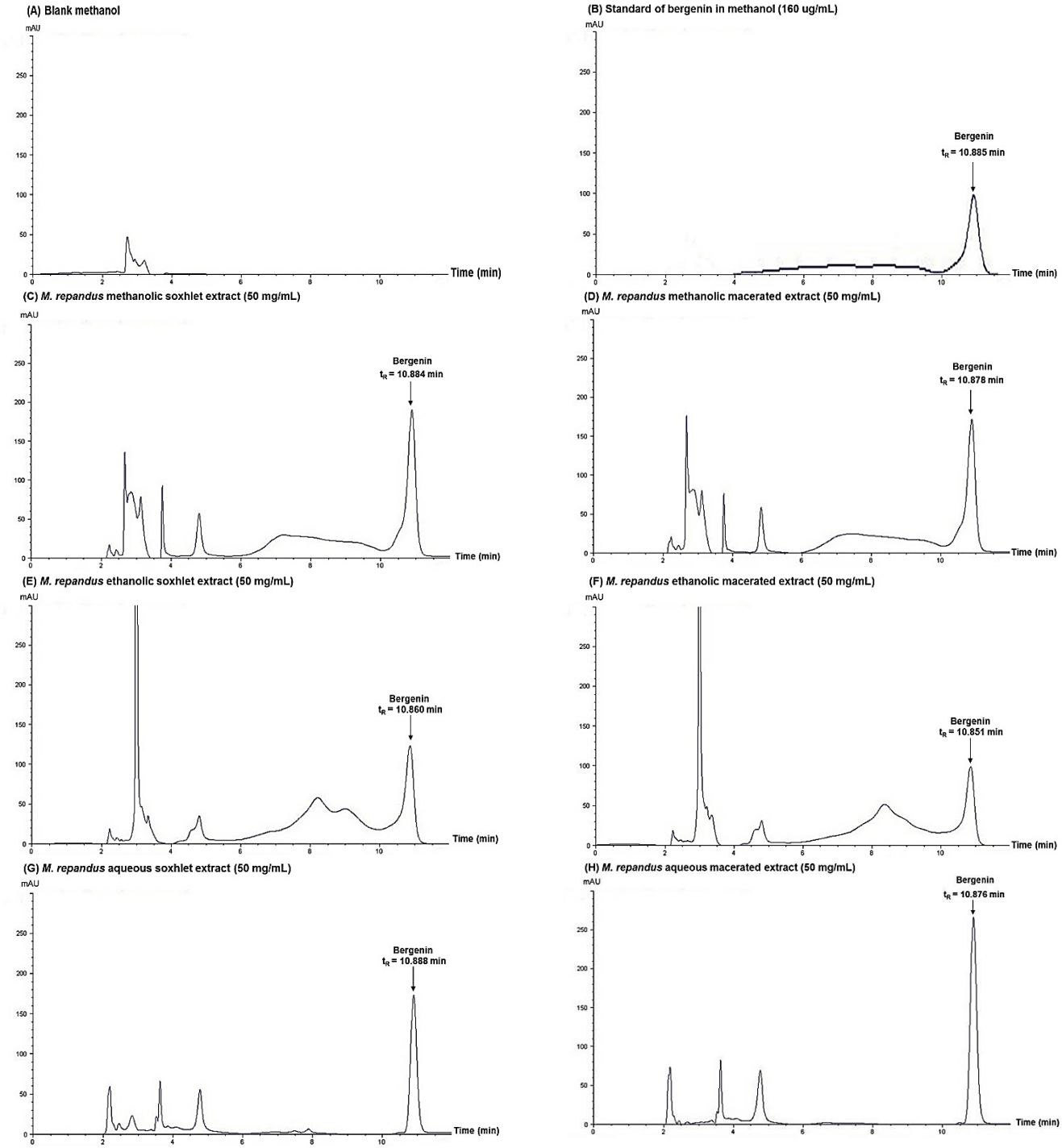

Fig. 2. Chromatogram of (A) blank methanol, (B) bergenin standard (160 μg/mL), and *M. repandus* (50 mg/mL) (C) methanolic soxhlet extract, (E) ethanolic soxhlet extract, (G) aqueous soxhlet extract, (D) methanolic macerated extract, (F) ethanolic macerated extract, and (H) aqueous macerated extract at the wavelength of 272 nm

Table 1 Validation parameters of the analytical method for quantification of bergenin in *M. repandus* extract

Linearity (concentration ranged 20-320 μg/mL)	
Linear regression equation	Y = 14.688X – 55.893
R^2	0.9999
Accuracy	
%Recovery	93.22 ± 1.49
Precision (%RSD, concentration ranged 20-320 μg/mL)	
Within-day	0.57 - 1.48
Between-day	0.19 - 1.70
Specificity	
Wavelength at 272 nm	No peak interference
Retention time (min)	10.885
Sensitivity	
LOD (μg/mL)	4.56
LOQ (μg/mL)	15.20

Determination of bergenin in the *M. repandus* stem extracts

The developed method was utilized to analyze the bergenin content in the *M. repandus* extracts. For the soxhlet extraction, the contents of bergenin in the *M. repandus* methanolic, ethanolic, and aqueous extracts were 9.28 ± 0.49, 2.16 ± 0.10, and 12.67 ± 0.26% dry weight, respectively, (Table 2), with relative retention time to the bergenin standard of 0.9999, 0.9977, and 1.0003, respectively (Fig. 2C, 2E, and 2G). Likewise, for the maceration, the contents of bergenin in the *M. repandus* methanolic, ethanolic, and aqueous extracts were 8.73 ± 0.72, 1.73 ± 0.05, and 19.38 ± 0.63% dry weight, respectively, (Table 2), with relative retention time to the bergenin standard of 0.9994, 0.9969, and 0.9992, respectively (Fig. 2D, 2F, and 2H).

Table 2 Bergenin content in the extracts of *M. repandus*

***M. repandus* extracts**	**Bergenin content (%dry weight)**	**Retention time (min)**
Soxhlet extraction		
Methanol	9.28 ± 0.49	10.884
Ethanol	2.16 ± 0.10	10.860
Water	12.67 ± 0.26	10.888
Maceration		
Methanol	8.73 ± 0.72	10.878
Ethanol	1.73 ± 0.05	10.851
Water	19.38 ± 0.63	10.876

Discussion and conclusion

The validation of RP-HPLC method is an essential process to assure qualitative and quantitative determination of a compound in order to provide a reliable, precise, and accurate result (Shabir et al., 2003). The developed RP-HPLC method for determination of bergenin in the *M. repandus* extract was well validated for specificity, linearity, accuracy, precision, and sensitivity, in accordance with the acceptable criteria of ICH guideline (2005).

Bergenin is the major active compound in the *M. repandus* stem. Hence, the quantification of bergenin in *M. repandus* is indispensable study for the quality control of medicinal plant. Although the determination of bergenin in *M. repandus* stem has not been reported, there were several methods for the determination of bergenin in other plant such as *M. philippinensis*, *B. ciliate*, and *B. stracheyi* using HPTLC-MS and HPLC (Singh *et al.*, 2007; Haribabu *et al.*, 2012). HPLC is an appropriate and useful tool for simultaneously qualitative and quantitative determination because it is a simple and highly precise technique (Loescher *et al.*, 2014). In the present study, HPLC is considerably employed to determine the bergenin content of the *M. repandus* stem.

Soxhlet extraction and maceration are the common techniques for obtaining phytochemicals. These two methods were used in the present study. An extraction time of soxhlet technique is shorter than maceration and an amount of solvent for soxhlet extraction is less than maceration. Whereas soxhlet extraction is complicated about equipment setup, maceration is simple (Majekodunmi, 2015). The extraction of bioactive compounds from plants depends on the solvent. Regarding Thai traditional remedies, *M. repandus* stem is commonly brewed in water. Additionally, both methanol and ethanol were used as the solvent of bergenin for pharmacological studies (Lim *et al.*, 2001; Nazir *et al*., 2007; Gao *et al*., 2015; Khan *et al*., 2016). To identify and confirm the appropriate solvent, water, methanol, and ethanol were then employed as the extraction solvents for quantitative analysis of bergenin in the *M. repandus* stem. The content of bergenin was mostly found in the aqueous extract, followed by the methanolic and ethanolic extracts, corresponding to the structure of bergenin with hydrophilic glycone part, namely trihydroxybenzoic acid glycoside (Zhou *et al.*, 2008; Pushpalatha *et al.*, 2015). Therefore, bergenin was extracted the best by water, followed by methanol and ethanol, respectively. Correspondingly, in Thai traditional remedies, *M. repandus* stem is simply boiled in water.

In conclusion, the RP-HPLC method for determination of bergenin in the *M. repandus* stem was established. This method was well validated in terms of specificity, linearity, accuracy, precision, and sensitivity (LOD and LOQ), and successfully utilized for quantitative analysis of bergenin in the *M. repandus* stem extracts.

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