Original Article

การพัฒนาสารสกัดดัดแปลงและวิธีวิเคราะห์ไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโตกราฟี สำหรับการวิเคราะห์หาปริมาณรูทีนในสารสกัดดัดแปลงจากเมล็ดลำไย

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บทคัดย่อ

การพัฒนาสารสกัดดัดแปลงและวิธีวิเคราะห์ไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโตกราฟีสำหรับการวิเคราะห์หาปริมาณรูทีน ในสารสกัดดัดแปลงจากเมล็ดลำไย

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บทนำ: ลำไยเป็นสมุนไพรที่มีการศึกษาพบว่ามีฤทธิ์แก้ไขภาวะบกพร่องทางการเรียนรู้และความจำ ในงานวิจัยนี้พบว่าสิ่งสกัดเมล็ดลำไยในชั้นเอทิลอะซิเทตมีฤทธิ์แก้ไขภาวะบกพร่องทางการเรียนรู้และความจำที่ถูกเหนี่ยวนำให้เกิดในหนูเมาส์ ด้วยการอุดกั้นหลอดเลือดแดงคอมมอนแคโรติดทั้งสองข้าง **วัสดุและวิธีการทดลอง:** นำสิ่งสกัดเมล็ดลำไยในชั้นเอทิลอะซิเทตมาแยกสารบริสุทธิ์ด้วยวิธีคอลัมน์โครมาโตกราฟี และนำสารบริสุทธิ์ที่ได้ไปทดสอบฤทธิ์แก้ไขภาวะบกพร่องทางการเรียนรู้และความจำ การศึกษาครั้งนี้ได้พัฒนาวิธีวิเคราะห์โดยใช้เทคนิคไฮเปอร์ฟอร์แมนลิควิดโครมาโตกราฟี เพื่อหาปริมาณรูทีน ซึ่งใช้เป็นสารบ่งชี้ ในสารสกัดดัดแปลง โดยใช้รีเวอร์เฟสคอลัมน์เป็นวัฏภาคนิ่ง วัฏภาคเคลื่อนที่ใช้ระบบไอโซเครติกใช้สารเฮสเปอริดินเป็นสารมาตรฐานอินเทอร์นอล **ผลการศึกษา:** เมื่อนำสารบริสุทธิ์ที่แยกมาพิสูจน์เอกลักษณ์ทางกายภาพ และวิธีเสปกโตรสโกปี แยกสารได้ 4ชนิด คือ เคอร์ซิทิน, กาลิค แอซิด, โพรพิว กาเลต และ รูทีน นำสารทั้ง 4ชนิดไปทดสอบฤทธิ์ พบว่ารูทีนเป็นสารที่มีฤทธิ์ดีที่สุดตัวหนึ่ง การศึกษานี้ได้เสนอรูทีนเป็นสารบ่งชี้ในสารสกัดดัดแปลง ในการพัฒนาและประเมินวิธีวิเคราะห์มีการตรวจสอบความใช้ได้ของวิธีตามข้อกำหนดไอซีเอช ได้ความเป็นเส้นตรงมีค่าสัมประสิทธ์สหสัมพันธ์เท่ากับ 0.9940 ช่วงความเป็นเส้นตรง 0.31 ถึง 1.54 ไมโครกรัมต่อมิลลิลิตร ร้อยละของการกลับคืนอยู่ในช่วง 99.30-101.98 และร้อยละของความเบี่ยงเบนมาตรฐานสัมพัทธ์ไม่เกิน 2 ลิมิตการตรวจวัดและลิมิตการวิเคราะห์เชิงปริมาณ มีค่าเท่ากับ 0.13 และ 0.38ไมโครกรัมต่อมิลลิลิตร ตามลำดับ **สรุปผล:** ในการศึกษาครั้งนี้ได้พัฒนาวิธีการเตรียมสารสกัด เพื่อกำจัดสารที่ไม่คงตัวออกจากสารสกัด ทำให้ได้สารสกัดดัดแปลงของเมล็ดลำไยที่คงตัว เพื่อนำไปใช้ในการศึกษาทางเคมีและพรีคลินิกต่อไป ในการพัฒนาและประเมินวิธีการวิเคราะห์ พบว่าวิธีวิเคราะห์ดังกล่าวได้ผ่านการตรวจสอบความใช้ได้ของวิธี ตามข้อกำหนดไอซีเอช

คำสำคัญ: การตรวจสอบความใช้ได้ของวิธี,ไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโตกราฟี, ลำไย, สารสกัดดัดแปลง, รูทีน

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Abstract

Development of Modified Extract and HPLC Analytical Method for Determination of Rutin in Modified Extract of Longan seed

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Introduction: Several studies of chemicals in longan (*Dimocarpuslongan* Lour.) seed have been suggested to have positive effect on deficit in learning and memory. In our study, the ethyl acetate extracts of longan seeds found the positive effect on learning and memory impairment after bilateral carotid arteries (2-VO) occlusion in mice. **Materials and Methods:** The Fractionation of the ethyl acetate part by conventional chromatographic technique together with the screening activities was performed. A HPLC-analytical method was developed for determination of rutin, a chemical marker in modified extract using reverse phase column with isocratic elution. Hesperidin was used as an internal standard. **Results:** We obtained pure four major compounds with physical and spectroscopic data acquired indicated as quercetin, gallic acid, propyl gallate and rutin. Rutin showed to be a one of the most active principle compounds at dose 100mg/kg; we proposed rutin as chemical marker for the modified herbal extract. The method was validated according to ICH guideline and showed linearity range of 0.31-1.54μg/ml of rutin (r^2=0.9940). The percentage recovery (%R) was in the range of 99.30-101.98 and the relative standard deviation (%RSD) was not more than 2. The limit of detection and limit of quantification of rutin were 0.13 and 0.38 µg/ml, respectively. **Conclusion:** We prepared relatively stable herbal extract from longan seeds by removing the unstable phenolic portion for further chemical and preclinical studied. Also, High-performance liquid chromatography technique was used for quality control of modified extract of longan seed. Proper validation of HPLC method according to ICH guidelines.

Keywords : longan seed, validation, HPLC, modified extract, rutin

Introduction

DimocarpuslonganLour. (longan) is a subtropical fruit, which belongs to the Sapindaceae family. Longan, known as “Lumyai” in Thailand, is widely grown in China, Taiwan, and South East Asia including Thailand and Vietnam (Rangkadilok *et al.,* 2007). Dried pulp of longan has been used traditionally to treat symptoms such as deficiency of spleen and heart, palpitations, insomnia, and poor memory (Losuwanarak *et al.,* 2009). Studies have shown that longan seed contains many antioxidant compounds such as gallic acid, ellagic acid and corilagin (Zheng *et al.,* 2009), these antioxidant compounds could be beneficial for memory deficit (Losuwanarak *et al.,* 2009). However, Rangkadilok suggested that not only previously reported polyphenols contribute to the high antioxidant activity of longan seed but also other phenolic constituents might also play important roles (Rangkadilok *et al.,* 2007). A previous study demonstrated the beneficial effect of the crude ethanolic extract of longan seed on scopolamine induced deficit in learning and memory in mice. The results shown oral administrations of longan seed ethanolic extract at the dose of 1,000 mg/kg significantly reduced the latency to find the platform in Morris water maze test

with reference to saline-treated group (Losuwanarak *et al.,* 2009). Additionally, there is a study that reported sub-chronic administration of aqueous extract of longan fruit enhances learning and memory (Park *et al.,* 2010).

All these studies indicate that longan seed extract may benefit for learning and memory impairment. However, there is limited information of the pharmacologically chemical marker compound and the processes to employ the modified herbal extracts. The objectives of this study were to develop the modified extracts to identifying and quantifying the chemical marker compound for longan seed.

Here, we describes four polyphenols, quercetin, gallic acid, propyl gallate and rutin were obtained from longan seeds. We prepared the modified extraction of longan seed and also developed HPLC method with validation according to International Conference on Harmonization guideline (ICH Steering Committee, 2006) to analyze the chemical marker in the modified herbal extract. Quercetin 3-O- rutinoside (rutin) was used as a marker compound in this study.

Methods

General experimental procedures

Thin layer chromatography (TLC) was performed on precoated silica gel plate (GF_{254} 10 x 10 cm) with detector under UV light (254 and 365 nm), spray of $FeCl_3$ solution in methanol and anthrone solution in ethanol. Plates were developed in three system i) butanol : acetic acid (9:3) ii) chloroform : methanol : acetic acid (30:15:5) and iii) methanol: ethyl acetate: acetic acid (1:6:3).IR spectra were obtained on a Shimadsu IR 440 infrared spectrometer in KBr disc.^{1}H NMR (300MHz) and ^{13}C (125MHz) spectra were recorded on a JEOL JMN-A Spectrometer in DMSO-d_6. The activity (learning and memory impairment after bilateral common carotid arteries occlusion (2-VO) in mice test (Wanakhachornkrai, 2006) was carried out at Department of Physiology, Faculty of Pharmaceutical sciences, Chulalongkorn University.

Plant materials

Longan seeds (cultivars Edor) were freshly harvested from the garden, Chiangmai province, Thailand in June, 2008. The seeds were washed and dried in the air at room temperature for one week and ground into powder.

Fractionation of extract

Diagram of extraction and separation is shown in diagram 1. The whole dried ground seed of longan (1 kg) was macerated four times in six liters with methanol, each time for three days. The collected filtrate was concentrated under reduced pressure at temperature not over 60 °C. The result of methanol extract was re-dissolved in methanol and partitioned with hexane. The collected hexane and methanol were concentrated and evaporated under reduced pressure at temperature not over 60°C. The methanol residue was dissolved with distilled water and partitioned with ethyl-acetate and the collected ethyl-acetate was concentrated. The collected distilled water residue was partitioned with butanol and the collected butanol residue was concentrated and evaporated under reduced pressure at temperature not over 60°C. Each fraction (ethyl-acetate and butanol) was tested for the effect on learning and memory deficit in 2-VO mice (Wanakhachornkrai, 2006).

Separation of extract

The selected fraction (ethyl acetate part, 0.701g), the most active part was separated on sephadex column and eluted with methanol. Five sub-fractions (B1-B5) were obtained.

Fraction B1 was separated on silica gel column using a gradient of hexane/ethyl acetate giving five sub-fractions (B1.1-B1.5) and fraction B1.3 was repeatedly separated on silica gel column and eluted with a gradient of dichloromethane/methanol giving three sub-fractions (B1.3.1-B1.3.3). The fraction B1.3.3, the yellow powder, was identified as quercetin (11mg, 0.0027%w/w).

Fraction B2 was separated on silica gel column using a gradient of hexane/ethyl acetate giving three fraction (B2.1-B2.3) and fraction B2.2, the white crystal was identified as gallic acid (0.063g, 0.0154%w/w). Fraction B2.1 was repeatedly separated on silica gel column with an isocratic of dichloromethane/ methanol (4:1) and giving two fractions (B2.1.1-B2.1.2). The fraction B2.1.1, the white crystal which was identified as propyl gallate (0.007g, 0.0017%w/w).

Fraction B3 was separated on sephadex column eluted with dichloromethane/methanol (1:1), giving three fractions (B3.1-B3.3). Fraction B3.2 was separated on silica gel column with an isocratic of dichloromethane/methanol/water (4:1:0.1) giving three fractions (B3.2.1-B3.2.3).The fraction B3.2.2, the yellow crystal which was identified as rutin (0.009g, 0.0022%w/w).

Each pure compound was test for the effect on learning and memory deficit in 2-VO mice (Wanakhachornkrai, 2006). The result was shown that rutin is a one of the active principle (at dose 100mg/ kg B.W.)and will be used as chemical marker for both qualitative and quantitative assessment.

Preparation for modified herbal extract

Diagram 2 shows the diagram of modified extraction, base on acid-base extraction theories. The ethyl acetate fraction (fraction B from scheme 1) was dissolved in ethyl acetate and then partitioned with 5% sodium bicarbonate for three times. The ethyl acetate layer was concentrated, thus giving the yellow-brown precipitate (fraction MB-1). The fraction MB-1 was dissolved in ethyl acetate and rapidly partitioned with 0.5N sodium hydroxide for three times, giving fraction MB-2. Fraction MB-2 as a modified herbal extracts contains rutin used for quantifying as a chemical marker compound.

Development of High Performance Liquid Chromatography analytical method

The development of HPLC-analytical method for determination of rutin in modified extract using the column Alltech (Alltima C18, 4.6x150mm, 5µm), with isocratic 0.5% formic acid: acetonitrile (30:70%v/v) as a mobile phase (isocratic system, flow rate 0.9ml/min). Hesperidin was used as an internal standard. Detector was photodiode array detected at 365 nm and injection volume was 20 µl. The rutin standard was quantitatively diluted with methanol to the concentration of 0.3, 0.6, 0.9, 1.2 and 1.5µg/ml and added hesperidin as the internal standard to concentration to 0.8µg/ml. For the sample preparation, fraction MB-2 was dissolved in methanol to concentrated 5mg/ml and then rutin standard and hesperidin were spiked in each sample solution. The developed HPLC analytical method was validated according to the International Conference on Harmonization guideline (ICH guideline) (ICH Steering Committee, 2006).

Results

Extraction, Isolation and identification the active principle

Ground dried longan seed (1Kg) macerated with methanol and then subjected to liquid-liquid extraction (diagram 1). The ethyl acetate part (at dose 200mg/kg) demonstrated the benefit effect on learning and memory impairment in 2-VO mice.Using the fractionation of the ethyl acetate part by chromatographic technique together with the screening activities, we found quercetin, gallic acid, propyl gallate and rutin to yield 11 mg (0.003% w/w), 63 mg (0.015 %w/w), 7 mg (0.002% w/w) and 9.0 mg (0.002% w/w), respectively. Rutin showed to be the most active principle compound at dose 100 mg/kg and used to be a chemical marker for the modified herbal extract.

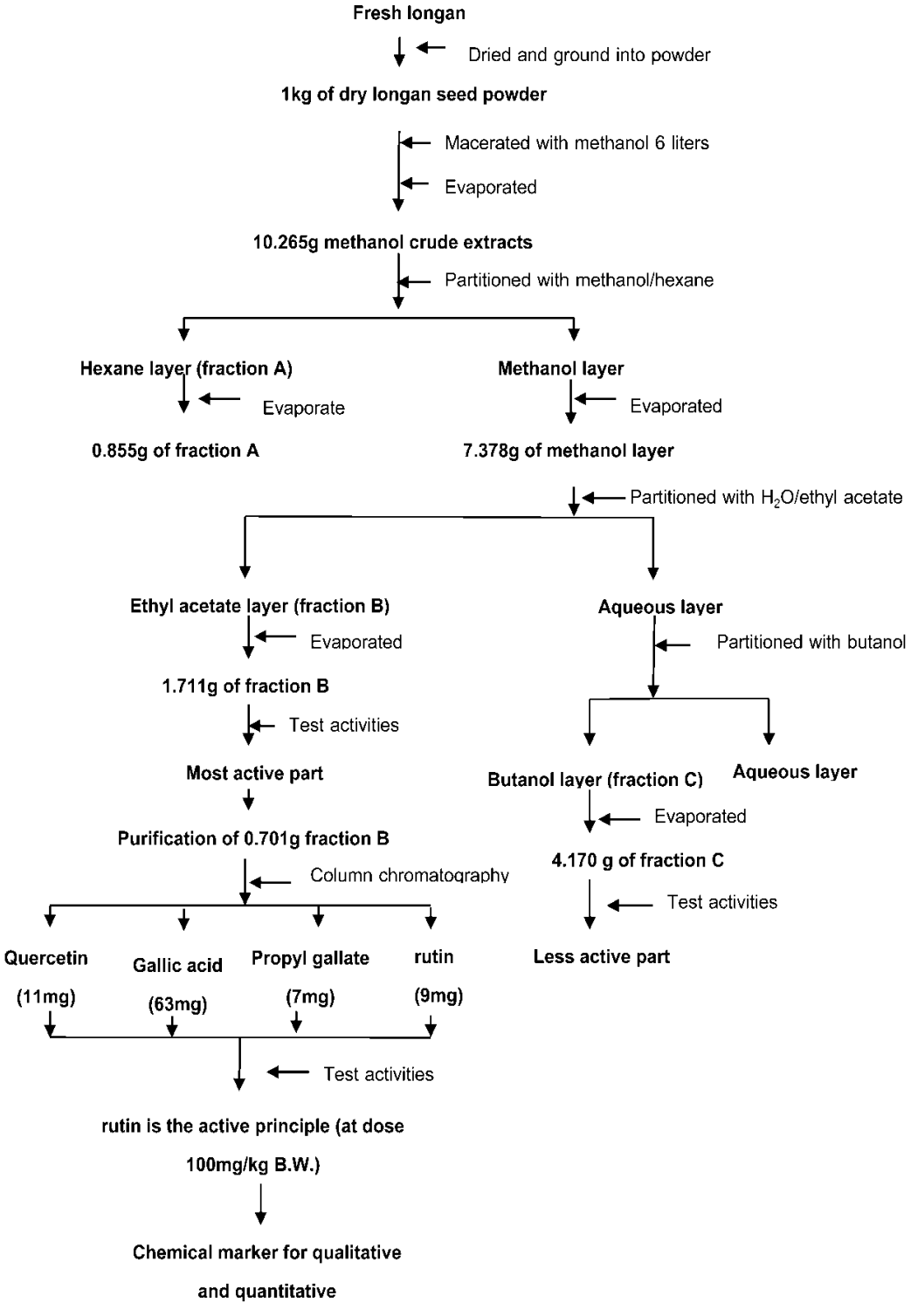

Diagram 1 Extractions and separations diagram

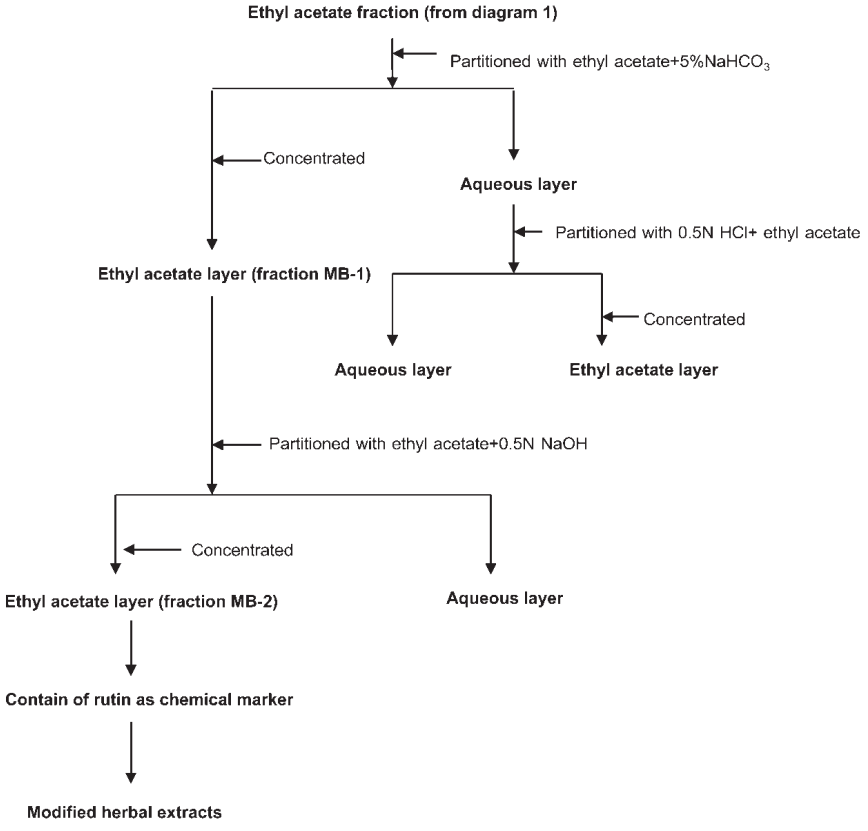

Diagram 2 Preparation of modified herbal extract

Preparation of modified herbal extract

In order to generate more stable extract and easy to maintain the chemical consistency of herbal, we removed unstable compounds such as gallic acid and propyl gallate from herbal extract (fraction B) by acid-base extraction as shown in diagram 2. Finally, from TLC (figure1), we obtained relatively more stable fractions MB-2.

Identification of purified compounds

Quercetin: IR (KBr); 3406.95(OH-stretching), 1663.56(C=O-stretching), 1521.75(C=C-stretching) cm-1.1H NMR: (300MHz,DMSO-d6): 12.47 (s,5-OH),7.66(d,2'H,2.2), 7.52(dd,6'H), 6.87 (d,5'H,8.55), 6.39(d,8H,1.9), 6.17(d,6H,2.1).13C: (125MHz,DMSO-d6): 146.78(C2), 135.71(C3), 175.82(C4), 160.70(C5), 98.16(C6), 163.9(C7), 93.33(C8), 156.12(C9), 102.99(C10), 121.93(C1'), 115.04(C2'), 145.04(C3'), 147.68(C4'), 115.58(C5'), 119.96(C6').

Gallicacid: IR (KBr): 3363.74(OH-stretching), 3064.53(CH-stretching), 1702.00(C=O-stretching),1540.88(C=C-stretching) cm-1.1H NMR: (300MHz, DMSO-d6): 6.90(s, 2,6-H), 9.17 (s,3,5H), 8.81(br s,4H), 12.20(br s,7H). 13C: (125MHz, DMSO-d6): 120.44(C1), 108.72(C-2,6), 145.15(C-3,5), 137.99(C4), 167.48(C7).

Propyl gallate: IR (KBr): 3466.60(OH-stretching), 1694.36(C=O-stretching), 1615.92(C= C-stretching), 1H:(300MHz,DMSO-d6): 6.94(s,2,6-H), 9.24(s,3,5-H), 8.90(s,4H), 4.10(triplet,8H,6.5), 1.66(sextet,9H,6.9), 0.9(triplet,10H,7.3).13C:(125MHz,DMSO-d6): 119.60(C1), 108.49(C-2,6), 145.58 (C-3,5), 138.37(C4), 165.90(C7), 65.46(C8), 21.73(C9), 1.41(C10).

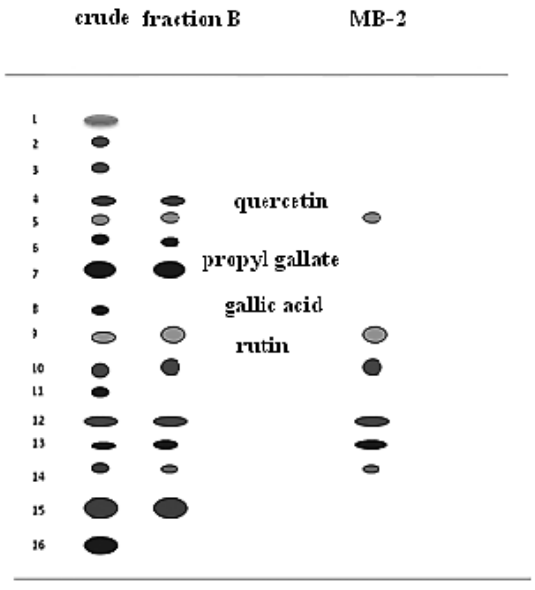

Figure 1 TLC pattern of longan seed extract shows acid-base extraction is applied to separate unwanted entities (e.g., gallic acid and propyl gallate) from the modified herbal extract

Rutin: IR (KBr); 3426.92(OH-stretching), 2924.40(CH-stretching),1655.79(C=O-stretching), 1504.47(C=C-stretching). ^{1}H: (300MHz, DMSO-*d6*): Aglycone; 6.18(6H,1.95), 6.37(8H, 2.10), 7.53(2'H,2.4), 6.81(5'H,8.0), 7.52(6'H,2.39). Glucose; 5.3(d, 1'' H, 7.51).Rhamnose ; 4.30(d, 1'''H, 1.2), 0.9(d, 6'''H, 6.0). ^{13}C: (125MHz,DMSO-d6): ^{13}C: (125MHz,DMSO-d6): Aglycone: 156.46(C2), 133.30(C3), 177.37(C4), 161.22(C5), 98.74(C6), 164.27(C7), 93.64(C8), 156.61(C9), 103.92(C10), 121.60(C1'), 115.25(C2'), 144.79(C3'), 148.47(C4'), 116.27(C5'), 121.61(C6'). Glucose; 101.22 (C1''), 74.09(C2''), 76.46(C3''), 70.58(C4''), 75.91(C5''), 67.01(C6''): Rhamnose: 100.77(C1'''), 70.39(C2'''), 70.01(C3'''), 71.86(C4'''), 68.26(C5'''), 17.79 (C6''').

Method validation of HPLC analytical method

HPLC technique for determination of rutin in longan seed extract was developed and validated. This method was validated according to ICH guideline (ICH Steering Committee, 2006). The chromatographic show retention time of rutin was at 2.97 min while that of hesperidin used as internal standard was at 7.09 min as figure2. The method shows linearity range of 0.31-1.54 μg/ml of rutin (r=0.9940) and representative linear equation was y=1.000x+0.095. The percentage recovery (%R) was in the range of 99.30-101.98 and the results shown in table 1.The relative standard deviation (%RSD) was not more than 2 and the results shown in table 2. The limit of detection and limit of quantification of rutin were 0.13 and 0.38 μg/ml, respectively.

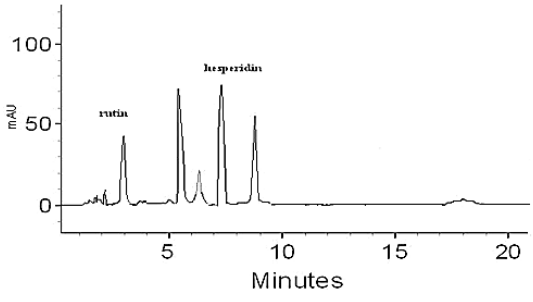

Figure 2 HPLC chromatogram of modified extract of longan seed (MB-2) (spike hesperidin internal standard).

Table 1 Result of accuracy / %recovery of HPLC analytical method

Concentration (µg/ml)	%Recovery ± SD (n=6)
0.31	99.30 ± 0.09
0.64	101.59 ± 0.11
0.93	100.44 ± 0.11
1.20	99.77 ± 0.10
1.54	101.98 ± 0.09

Table 2 Intra-day and Inter-day precision test of HLPC analytical method

C oncentration (μg/ml)	Precision (%RSD±SD) (n=6)	
	Intra-day	Inter-day
0.3	1.89±0.09	0.40±0.09
0.6	1.03±0.09	0.12±0.09
0.9	1.54±0.09	0.09±0.12
1.2	0.57±0.12	0.02±0.10
1.5	0.10±0.10	0.02±0.13

Discussion and Conclusion

Several studies shown longan seed extract would be a new source of dietary anti-amnesic for use in supplements. However, there is limited information the pharmacologically chemical marker compound and the processes employed to prepare the modified herbal extracts. The purpose of the modification of herb extract is to reproduce the chemical consistency from batch to batch which is essential for further pre-clinical and clinical studies as well as product development.

Here, four compounds quercetin, gallic acid, propyl gallate and rutin were obtained from longan seed. Base on the anti-amnesic activities using passive avoidance task and Morris water maze test in 2-VO mice model (Wanakhachornkrai, 2006), rutin was the one of the active principles at dose 100mg/kg Additionally, the previous study confirm that rutin may potential prove to have a neuroprotective effect and revealed protective the memory impairment (Kishore and Singh, 2005; Richetti *et al.,* 2011; Koda *et al.,* 2009; Pu *et al.,* 2007). Kishore and Singh reported that rutin, a bioflavonoid and a potent antioxidant or free radical scavenging is found to improve learning and memory of normal mice with no cognitive deficits in the doses of 30mg and 40mg (Kishore and Singh, 2005). Rutin (50mg/kg, single injection, intra-peritoneal) prevented the scopolamine-induced memory impairment in zebrafish and no affected general locomotor activity (Richetti *et al.,* 2011). Rutin has a protective effect on trimethyltin-induced memory dysfunction in rats and rutin could be attributable to inhibitory effect against microglial activation and its role in synapse formation via neurotropic factors in hippocampus (Koda *et al.,* 2009). Both rutin and quercetin improved spatial memory impairment in the 8-arm radial maze task and neuronal death in the hippocampal CA1 area (Pu *et al.,* 2007).

The previous studies have shown that longan seed contains many tannin compounds such as gallic acid, ellagic acid and corilagin (Zheng *et al.,* 2009).The photo-degradation of the most representative phenolic compound such as gallic acid (Lucas *et al.,* 2008). Generally, tannin develops the oxidative browning reactions during processing and storage (Hui and Barta, 2006). Rutin is a more stable against heat and oxidation than tannin compounds (Dharmananda, 2003). Moreover, screening HPLC analysis of the longan seed extracts showed that rutin present in good quantity. All these studies suggest that rutin possess the anti-amnesic compounds and suitable used as a marker compound in this study.

Therefore, this work shows a possible treatment to remove the problematic phenolic fraction of sample extraction. Acid-base extraction is applied to separate unwanted entities (e.g., gallic acid and propyl gallate) from the modified herbal extract.

High-performance liquid chromatography technique was used for quality control of the modified longan seed extract. Proper validation of HPLC method according to ICH guidelines (ICH Steering Committee, 2006) for quality control of longan seed extracts was performed. In our study showed all parameters were found in the acceptable limit ranges.

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