Research in Pharmaceutical Sciences, August 2023; 18(4): 449-467
Received: 10-01-2023
Peer Reviewed: 25-01-2023
Revised: 21-02-2023
Accepted: 28-02-2023
Published: 07-06-2023

School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences

Original Article

Analytical determination, antioxidant and anti-inflammatory activities of Bhamrung-Lohit a traditional Thai medicine

Chitralada Panchakul[1], Pakakrong Thongdeeying[2,3], Arunporn Itharat[2,3,*], Weerachai Pipatrattanaseree[4], Chonthicha Kongkwamcharoen[5], and Neal M. Davies[6]

[1]Faculty of Medicine, Thammasat University (Rangsit Campus), Pathumthani, 12120, Thailand.
[2]Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University (Rangsit Campus), Pathumthani, 12120 Thailand.
[3]Center of Excellence in Applied Thai Traditional Medicine Research, Thammasat University (Rangsit Campus), Pathumthani, 12120, Thailand.
[4]Regional Medical Science Center 12 Songkhla, Department of Medical Sciences, Ministry of Public Health, Songkhla, 90100, Thailand.
[5]Graduate School of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Pathum Thani 12120, Thailand.
[6]Faculty of Pharmacy and Pharmaceutical Sciences, Katz Centre for Pharmacy and Health Research, University of Alberta, Edmonton, Alberta T6G2E1, Canada.

Abstract

Background and purpose: Bhamrung-Lohit (BRL) remedy is a traditional Thai medicine (TTM). There are few reports of biological activity, the activity of its constituent plants, or quantitative analytical methods for the content of phytochemicals. In this study, we investigated antioxidant, anti-inflammatory activity, and total phenolic and flavonoid content and validated a new analytical method for BRL.
Experimental approach: Antioxidant activity was evaluated by a 2,2-diphenyl-1-picrylhydrazyl (DPPH and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging. The cellular antioxidant activity was evaluated by inhibition of the superoxide anion (O_2●-) production from HL-60 cells and anti-inflammatory activity by inhibition of nitric oxide production in RAW264.7 cells. The total phenolic and flavonoid contents were analyzed using the Folin-Ciocalteu method and an aluminum chloride colorimetric assay, respectively. Validated analytical procedures were conducted according to International Conference on Harmonization (ICH) guidelines.
Findings/Results: An ethanolic extract of BRL exerted potent DPPH radical scavenging activity and moderate antioxidant and anti-inflammatory activity. *Caesalpinia sappan* exerted the greatest effect and the highest content of total phenolics and flavonoids. The HPLC method validated parameters that complied with ICH requirements. Each peak showed selectivity with a baseline resolution of 2.0 and precision was less than 2.0% CV. The linearity of all compounds was > 0.999 and the recovery % was within 98.0%-102.0%. The validated results demonstrated specificity/selectivity, linearity, precision, and accuracy with appropriate LOD and LOQ.
Conclusion and implication: BRL remedy, a TTM demonstrated antioxidant and anti-inflammatory properties. This study is the first report on the biological activity and the validation of an HPLC method for BRL remedy.

Keywords: Anti-inflammation; Antioxidant; Bhamrung-Lohit; HPLC; Method validation.

INTRODUCTION

Oxidative stress occurs in disease and pathophysiology when redox reactions in the body are imbalanced. Reactive oxygen species (ROS) are also released in the body and can act as signaling molecules. Overproduction of ROS and defects in the homeostasis of antioxidants can cause an imbalance in the redox system causing oxidative stress in cells and tissues.

*Corresponding author: A. Itharat
Tel: +66-29269749, Fax: +66-29269705
Email: iarunporn@yahoo.com

Access this article online

Website: http://rps.mui.ac.ir

DOI: 10.4103/1735-5362.378091

Red blood cells (RBCs) are integral to the cardiovascular system and help maintain oxidative balance in the blood (1). Hemoglobin with the RBCs initiates oxidative stress by its auto-oxidation which produces the superoxide radical leading to RBC damage (2). Inflammation is an important aspect of oxidative stress (3). Nitric oxide (NO) is involved in redox reactions and is pro-inflammatory in the body, when in excess NO reacts with the superoxide anion (O_2●-) producing a peroxynitrite radical (ONOO●) which reacts with enzymes, DNA, and cell membranes and causes irreversible damage leading to tissue damage and cell death (4). Various cellular antioxidants in the cytosol including superoxide dismutase, and glutathione peroxidase reduce the overproduction of ROS and oxidative balance is maintained (5).

The functional use of antioxidant supplements is to provide beneficial effects in the amelioration of oxidative stress (6). These antioxidants can be medicinally synthesized or naturally occurring xenobiotics. All antioxidants can have potential side effects including allergic reactions, and gastrointestinal tract irregularities (7-10). The use of plants and traditional herbal remedies may provide an alternative source of new phytochemicals that impart antioxidant properties.

Table 1. The ethnobotanical data of Bhamrung-Lohit remedy and its plant components.

Plant species	Family	Plant part	Voucher specimen number	Proportion (%w/w)	Taste	Traditional use
Alyxia reinwardtii	Apocynaceae	Peel	SKP 013 01 18 01	1.41	Sweet	Tonic, anti-dizziness (49)
Amomum verum	Zingiberaceae	Fruit	SKP 206 01 20 01	1.41	Spicy	Antiemetic, carminative (50)
Anaxagorea luzonensis	Annonaceae	Heartwood	SKP 011 01 12 01	2.82	Spicy and astringent	Blood tonic, tonic, paregoric (51)
Anethum graveolens	Apiaceae	Dry fruit	SKP 199 01 07 01	1.41	Bitter and spicy	Tonic, carminative (50,52)
Angelica dahurica	Apiaceae	Root	SKP 199 01 04 01	1.41	Spicy and bitter	Tonic, antipyretic, paregoric, relieve asthma (49)
Angelica sinensis	Apiaceae	Root	SKP 199 01 19 01	1.41	Sweet and spicy	Antipyretic, gynecological (49,53,54)
Aquilaria crassna	Thymelaeaceae	Heartwood	SKP 193 01 03 01	1.41	Spicy and bitter	Blood tonic, tonic, cardiotonic (49)
Arcangelisia flava	Menispermaceae	Stem	SKP 114 11 06 01	2.82	Spicy and astringent	Carminative, anti-diarrhea, blood tonic (55)
Artemisia annua	Compositae	Aerial parts	SKP 051 01 01 01	1.41	Bitter	Antipyretic, relieve cough (56)
Atractylodes lancea	Compositae	Rhizome	SKP 051 01 12 01	1.41	Sweet and bitter	Tonic, oral prophylaxis, antipyretic (50)
Avicennia marina	Acanthaceae	Heartwood	SKP 213 01 13 01	1.41	Salty and bitter	Carminative, gynecological (57)
Bixa orellana	Bixaceae	Flower	SKP 026 02 15 01	14.08	Sweet and mellow	Blood tonic, Treatment of anemia (55)
Caesalpinia sappan	Leguminosae	Heartwood	SKP 072 03 19 01	14.08	Bitter and astringent	Blood tonic, treatment of menstruation pain (50)(56)
Cinnamomum verum	Lauraceae	Peel	SKP 096 03 03 01	1.41	Spicy and sweet	Carminative, relieve dyspepsia (58)
Cuminum cyminum	Apiaceae	Dry fruit	SKP 199 03 03 01	1.41	Spicy	Carminative (50)

Dactylopius coccus	Dactylopiidae	Lac	SKP 215 12 12 01	5.63	Astringent	Blood tonic (50,59)
Dracaena cochinchinensis	Asparagaceae	Heartwood	SKP 065 04 12 01	1.41	Bitter	Cardiotonic, anti-inflammatory (50)
Foeniculum vulgare	Apiaceae	Dry fruit	SKP 199 06 22 01	1.41	Sweet and spicy	Tonic, carminative (50)
Lepidium sativum	Brassicaceae	Dry fruit	SKP 057 12 19 01	1.41	Spicy and bitter	Gastrointestinal agent (60)
Ligusticum sinense	Apiaceae	Rhizome	SKP 199 12 19 01	1.41	Bitter	Carminative, blood tonic, gynecology (50)
Mammea siamensis	Guttiferae	Flower	SKP 083 13 19 01	2.82	Bitter	Cardiotonic, tonic (49,50)
Mesua ferrea	Calophyllaeae	Flower	SKP 083 13 06 01	2.82	Bitter	Blood tonic, cardiotonic, antipyretic (49,50)
Mimusops elengi	Sapotaceae	Flower	SKP 171 13 05 01	2.82	Sweet and astringent	Cardiotonic, antipyretic (50,61)
Myristica fragrans	Myristicaceae	Aril	SKP 121 13 06 01	1.41	Spicy	Blood tonic, tonic, carminative (50)
Myristica fragrans	Myristicaceae	Seed	SKP 121 13 06 01	1.41	Bitter and astringent	Tonic, carminative, antiemetic (50)
Nelumbo nucifera	Nelumbonaceae	Pollen	SKP 125 14 14 01	2.82	Astringent	Cardiotonic, tonic (50)
Nigella sativa	Ranunculaceae	Dry fruit	SKP 160 14 19 01	1.41	Spicy and bitter	Antiemetic, carminative (50)
Piper retrofractum	Piperaceae	Flower	SKP 146 16 18 01	2.82	Spicy and bitter	Promote blood circulation, antiemetic (50)
Piper ribesioides	Piperaceae	Vine	SKP 146 16 14 01	2.82	Spicy	Carminative (50)
Piper sarmentosum	Piperaceae	Root	SKP 146 16 19 01	2.82	Spicy	Carminative (50)
Plumbago indica	Plumbaginaceae	Root	SKP 148 16 09 01	2.82	Spicy	Promote blood circulation, gynecology, carminative (50)
Senna garrettiana	Leguminosae	Heartwood	SKP 072 19 07 01	1.41	Bitter	Carminative, gynecology (55)
Syzygium aromaticum	Myrtaceae	Flower	SKP 123 19 01 01	1.41	Spicy	Paregoric of tooth and pyorrhea, carminative, promote blood circulation (50)
Terminalia bellirica	Combretaceae	Fruit	SKP 049 20 02 01	1.41	Sour and astringent	Laxative, carminative, relieve cough (62)
Terminalia chebula	Combretaceae	Fruit	SKP 045 20 03 01	1.41	Astringent	Laxative, anti-diarrhea, relieve cough (55)
Terminalia citrina	Combretaceae	Fruit	SKP 049 20 03 01	1.41	Bitter and astringent	Laxative, antipyretic (63)
Urceola rosea	Apocynaceae	Stem	SKP 013 05 18 01	2.82	Astringent	Carminative (64)
Zingiber officinale	Zingiberaceae	Rhizome	SKP 206 26 15 01	2.82	Spicy	Carminative, antiemetic (56)

In traditional Thai medicine (TTM), the herbal remedy "Bhamrung-Lohit" (BRL) is listed in the Thailand National List of Essential Medicine (NLEM) since 2013 for nourishing blood and treating extensive menstrual blood loss in women (the Thai word "Bhamrung" means nourishment and "Lohit" means blood). BRL remedy consists of 38 plant ingredients which are combined in different proportions according to Table 1. *Caesalpinia sappan* and *Bixa orellana* are the two major plant ingredients in the remedy (11).

This study aimed to investigate the biological activity related to antioxidant properties of BRL remedy and its plant components as follows: chemical-based antioxidant activities by DDPH and ABTS●+ scavenging activity, cellular-based antioxidant activity through inhibition of O_2●- from the promyelocyte cell, HL-60 cell assay and anti-inflammatory activity by inhibition of NO production from the macrophage RAW264.7 cells. A quantitative high-performance liquid chromatography (HPLC) method for determination of the major analytes brazilin, ellagic acid, eugenol, piperine, and myristicin in ethanolic extract of BRL remedy was developed and validated. This study is the first report of antioxidant activity, anti-inflammatory activity, and quantitative HPLC methodology. The study provides helpful information for further study of BRL remedy and the validated method can be used as a quality control standard for further development of BRL remedy extract.

MATERIALS AND METHODS

Plant materials and preparation of BRL extract

All plant materials were purchased from Charernsuka Osot Pharmacy (Nakornpathom, Thailand). The plant specimens were compared to the reference plant standards deposited at the Herbarium of Southern Center of Thai Medicinal Plants at the Faculty of Pharmaceutical Science, Prince of Songkhla University, Songkhla Province, Thailand. The voucher IDs are shown in Table 1. Plant materials were washed, dried, and cut into small pieces, weighed according to proportions and mixed homogeneously. Each plant ingredient and the BRL remedy were macerated with 95% ethanol for three days. The extract was filtered and evaporated at 45 °C under a vacuum. The residue of BRL was then re-extracted an additional two times. All three extracts were combined and dried in a vacuum oven at 45 ℃.

Chemicals and reagents

Brazilin, ellagic acid, eugenol, myristicin, piperine, gallic acid, and propyl gallate (purity > 98%) were purchased from Sigma-Aldrich (MO, USA). Folin-Ciocalteus's phenol reagent, ٢,٢-diphenyl-١-picrylhydrazyl (DPPH), and butylated hydroxytoluene (BHT) were purchased from Fluka (Munich, Germany). Dimethyl sulfoxide (DMSO), acetonitrile, and phosphoric acid were purchased from RCI Lab Scan (Bangkok, Thailand). Purified water was prepared by Milli Q® system from Millipore (Bedford, MA, USA).

Murine macrophage leukemia cell line (RAW 264.7: ATCC® TIB-71™) and Human promyelocytic leukemia cells (HL-60) were purchased from American Type Culture Collection (VA, USA). Roswell Park Memorial Institute medium 1640 (RPMI-1640), fetal bovine serum (FBS), penicillin-streptomycin, phosphate buffer saline (PBS), and trypsin-EDTA were purchased from Biochrom (MA, Germany). Lipopolysaccharide (LPS), 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-2H-tetrazolium bromide (MTT) and nitroblue tetrazolium (NBT) were purchased from Sigma-Aldrich. (MO, USA).

DPPH radical scavenging activity

The antioxidant activity assessed by DPPH assay was conducted according to the modified method of Yamasaki *et al.* (12). Briefly, the stock solution of the extract was dissolved in absolute ethanol and also diluted to obtain various sample solutions at different concentrations. The sample solution (100 µL) was added with an equal volume of 6×10^{-5} M DPPH (in absolute ethanol) and kept in darkness at room temperature for 30 min. The absorbance of the reaction was measured at 520 nm. BHT was used as a positive control. The scavenging activity of the sample is the ability to reduce the color intensity of DPPH.

Inhibition (%) was calculated using the following equation:

$$\text{Inhibition (\%)} = \frac{\text{Abs. control} - \text{Abs. sample}}{\text{Abs. control}} \times 100 \quad (1)$$

ABTS●+ radical scavenging activity

ABTS●+ radical scavenging assay was conducted according to the method of Re *et al*. (13). Briefly, sample solutions of each extract were prepared at various concentrations in absolute ethanol. ABTS●+ radical solution was prepared by dissolving potassium persulfate with distilled water to produce the radical solution at a concentration of 2.45 mM. The sample solution (20 µL) was mixed with ABTS●+ solution (180 µL) and incubated at room temperature for 6 min. The absorbance of the solution reaction was measured by a microplate reader at a wavelength of 734 nm. The percentage of ABTS●+ scavenging activity was calculated. BHT was used as a positive control. The calculation of percent scavenging activity was calculated using the equation (1).

Cellular antioxidant by NBT dye reduction assay

The cellular antioxidant of ethanolic extract of BRL remedy was evaluated by inhibition of O_2●- in differentiated HL-60 cells according to the method of Surarit *et al*. (14). Briefly, human promyelocytic leukemia cells (HL-60) were cultured in RPMI 1640 supplemented with 10% heated FBS, 50 IU/mL penicillin and 50 µg/mL streptomycin. HL-60 cells were seeded at 10,000 cells and incubated to obtain morphologically and functionally mature granulocytes by culturing in DMSO 1.3% in RPMI for 7 days at 37 ºC, 5% CO_2 and 95% humidity. The stock solution of the extract was prepared in DMSO. The extract was diluted in a medium to produce working solutions at various concentrations. The concentration of DMSO in the treated cell was not more than 0.2% v/v. Propyl gallate was used as a positive control.

The differentiated HL-60 was dissolved in Hanks' balanced salt solution in a 50 mL centrifuge tube to produce a concentration of 1×10^6 cells. Various concentrations of the extract (500 µL) were added into each tube and incubated in an incubator at 37 ºC with 5% CO_2 and 95% humidity for 15 min. After that, phorbol 12-myristate 13-acetate solution was added (final concentration of 250 ng/mL) and an aliquot of 250 µL of NBT solution (1.25 mg/mL) was added. Then, the mixture was incubated for 60 min and then, 2 mL of 1 N HCl was added. After mixing and centrifugation, the supernatant was removed and the produced NBT formazan was dissolved in 300 mL DMSO and the solution was transferred into 96 well plates. The plate was measured at 572 nm using a microplate reader. The inhibition of each concentration of the extract against superoxide formation measured by NBT reduction was calculated using equation (1).

Anti-inflammatory activity by inhibition of nitric oxide production from RAW 264.7 cells and cytotoxicity by MTT assay

Anti-inflammatory activity by inhibition of nitric oxide production in RAW264.7 cells was determined according to the method of Tewtrakul and co-workers with slight modifications (15). Briefly, RAW264.7 cells were cultured in DMEM medium with 10% FBS, penicillin (100 units/mL) and streptomycin (100 µg/mL) and incubated at 37 °C under 5% CO_2. The cells were seeded into a 96-well plate and incubated for 24 h. The cells were treated with LPS at a final concentration of 5 ng/mL. A sample solution was added and incubated for 24 h. The supernatant was transferred to a new 96-well plate and Griess's reagent was added. The plate optical density (OD) was measured with a microplate reader at the wavelength of 570 nm. The viability of treated cells was determined by MTT solution. The OD was measured at 570 nm. The % inhibition and % toxicity were calculated.

Determination of total phenolic content

The total phenolic content of the extracts was determined by the modified Folin-Ciocalteu method (16). A 20 µL aliquot of the extracts was mixed with 100 µL of Folin-Ciocalteu's reagent and 80 µL of sodium carbonate solution. The plate was mixed and allowed to stand at room temperature to develop color for 30 min. The absorbance of the

reaction was measured at 765 nm. Total phenolic content was calculated from a calibration curve of gallic acid standard solutions (ranging from 2.5 to 100 µg/mL) and expressed as mg gallic acid equivalents (GAE)/g of dried plant.

Determination of total flavonoid content

Total flavonoid content was measured by the aluminum chloride colorimetric assay (17). Briefly, an aliquot (1 mL) of the extracts or standard solution of quercetin (20, 40, 60, 80, 100, 120, and 240 mg/L) was added to a volumetric flask. To the flask were added 75 µL of 5% $NaNO_2$ and 150 µL of 10% AlCl3. After 5 min, 500 µL of 1 M NaOH was added and the volume was adjusted by H_2O. The solution was mixed well and the absorbance was measured against the prepared reagent blank at 510 nm. The concentration of total flavonoid content in the test samples was calculated from the calibration plot and expressed as mg quercetin equivalent/g of dried plant.

HPLC analysis

The analytical method for determination of anti-inflammatory markers in BRL ethanolic extract was carried out by using an HPLC system (Shimadzu, Japan) consisting of a solvent degasser (G1322A), an autosampler (G1329A), a quaternary solvent pump (G1311A), a photodiode array detector (G1315D), and a column oven (G1316A). The chromatographic data were processed by the Chemstation® software revision B.04.01 SP1.

Chromatographic conditions

All markers were separated along a C18 HPLC column (5 µm, 4.6 mm × 250 mm; Zorbax®, Agilent, USA) protected by a C18 guard cartridge (5 µm, 4.6 mm × 3 mm; Zorbax®, Agilent, USA). The mobile phase consisted of acetonitrile (A) and 0.1% v/v phosphoric acid in water (B). The gradient elution of the mobile phase was programmed and presented in Table 2.

The flow rate was set at 1.0 mL/min. The operating temperature was maintained at room temperature. Ten µL of sample and standard solutions were injected into the chromatographic system and the anti-inflammatory markers were detected at a wavelength of 210 nm (brazilin, eugenol and myristicin), 254 nm (ellagic acid), and 320 nm (piperine).

Preparation of standard and sample solution

Each stock standard solution of brazilin, ellagic acid, eugenol, myristicin, and piperine was prepared at a concentration of 1.0 mg/mL in methanol. The working standard mixtures were prepared in methanol according to the concentration from the linear range of each standard. For sample solutions, the ethanolic of BRL extract was dissolved in methanol to produce a sample solution at the concentration of 10 mg/mL.

Validation of the HPLC method

The developed HPLC method was validated as per International Conference on Harmonization (ICH 2005) guidelines (18). The validation parameters included selectivity, linearity, accuracy, precision, the limit of detection (LOD), and the limit of quantitation (LOQ). All validation parameters passed the specification limits.

Table 2. Mobile phase gradient elution program for developed analytical assay. The mobile phase consisted of acetonitrile (A) and 0.1% v/v phosphoric acid in water (B).

Time (min)	Mobile phase composition (%)
0-5	5 A: 95 B
5-45	5 A: 95 B – 50 A: 50 B
45-60	50 A: 50 B – 95 A: 5 B
60-65	95 A: 5 B – 100 A: 0 B
65-70	5 A: 95 B

Statistical analysis

The results of the anti-inflammatory activities were reported as mean ± standard error of the mean (SEM) from three replicated experiments. IC_{50} values were calculated by using regression analysis using the GraphPad Prism software (CA, USA). Mean differences among groups were analyzed by Student's t-test. Statistical analysis was conducted by using GraphPad Prism software (CA, USA). The results of the HPLC analysis were expressed as mean ± SD. The hierarchical cluster analysis (HCA) and heat map were analyzed using Heatmap Illustrator (HemI) version 1.0 software (19). Statistical comparisons between groups will be calculated *via* independent sample t-test and one-way analysis of variance (ANOVA) followed by the LSD post hoc test. Differences were considered significant at $P < 0.05$.

RESULTS

Antioxidant activity and Anti-inflammatory activity by inhibition of nitric oxide production

The results of DPPH, ABTS●+ radical scavenging activities, the cellular antioxidant by NBT assay, and anti-inflammatory activity by inhibition of NO production are shown in Table 3 and Fig. 1.

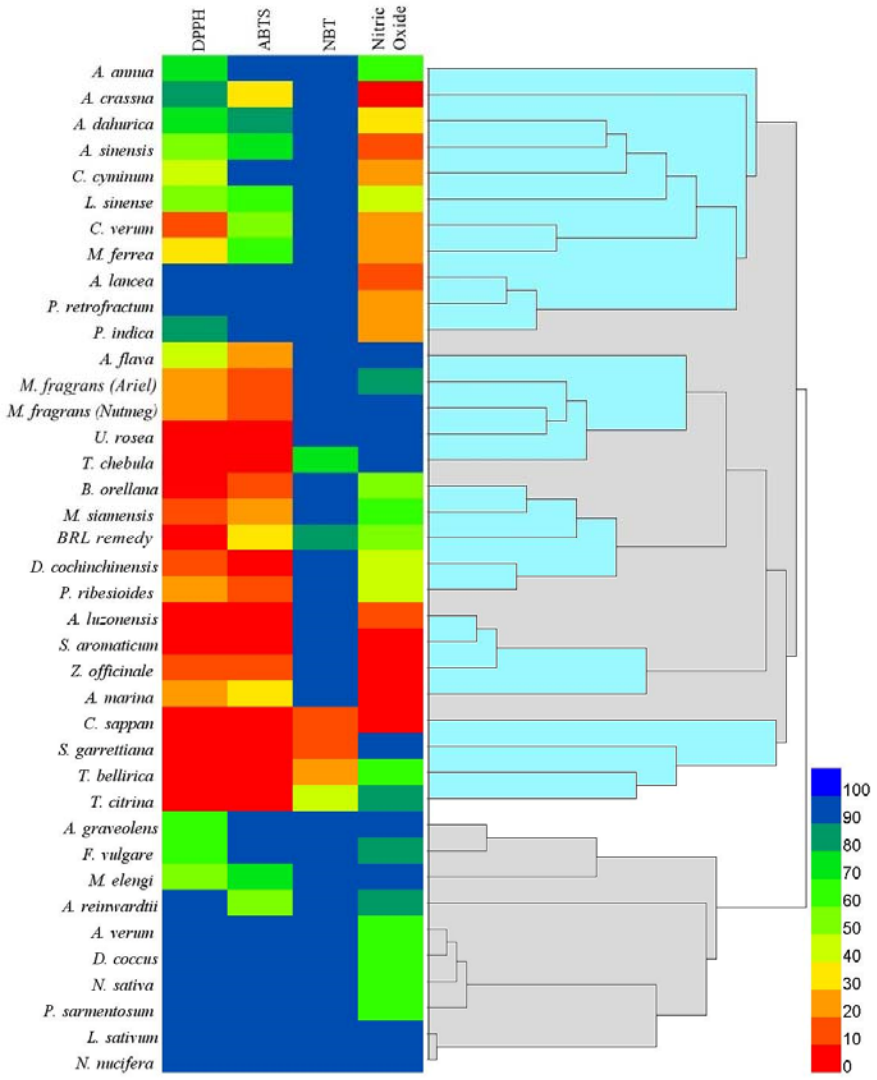


Fig. 1. Heat map and hierarchical cluster analysis of plant and Bhamrung-Lohit remedy extracts. The color scale bar showed a range of IC_{50}, the red color bar represents more potent activity (0-10 µg/mL) incrementing to a blue color bar which represents weak activity (90-100 µg/mL).

Table 3. Antioxidant and anti-inflammatory activities of ethanolic extract of Bhamrung-Lohit remedy and its plant ingredients. Data are expressed as mean ± SEM; n = 3.

Plant genus and species	Antioxidant (EC_{50}; µg/mL)			Inhibitory activity of nitric oxide (IC_{50}; µg/mL)
	DPPH	ABTS	NBT	
Artemisia annua	72.64 ± 3.48	> 100	> 100	64.11 ± 5.97
Aquilaria crassna	84.85 ± 3.85	32.81 ± 3.73	> 100	2.33 ± 2.33
Angelica dahurica	71.06 ± 3.18	84.15 ± 6.28	> 100	36.47 ± 1.92
Arcangelisia flava	43.95 ± 0.58	28.93 ± 2.61	> 100	91.13 ± 5.50
Anethum graveolens	64.26 ± 1.73	> 100	> 100	95.27 ± 2.69
Atractylodes lancea	90.17 ± 0.64	> 100	> 100	15.66 ± 1.45
Anaxagorea luzonensis	5.23 ± 0.34	5.15 ± 0.66	> 100	11.26 ± 0.8
Avicennia marina	25.19 ± 0.87	30.37 ± 1.8	> 100	7.59 ± 2.12
Alyxia reinwardtii	> 100	58.85 ± 4.71	> 100	89.93 ± 3.29
Angelica sinensis	56.37 ± 1.44	75.12 ± 5.87	> 100	14.24 ± 1.41
Artemisia verum	> 100	> 100	> 100	64.75 ± 4.47
Bixa orellana	8.32 ± 0.48	18.36 ± 1.56	> 100	57.62 ± 2.8
Cuminum cyminum	48.5 ± 4.52	> 100	> 100	27.19 ± 1.08
Caesalpinia sappan	4.51 ± 0.14	4.98 ± 0.18	12.07 ± 0.44	7.57 ± 1.46
Cinnamomum verum	16.55 ± 2.22	55.85 ± 1.41	> 100	21.19 ± 6.58
Dactylopius coccus	> 100	> 100	> 100	64.99 ± 3.4
Dracaena cochinchinensis	17.36 ± 0.64	7.55 ± 0.45	> 100	42.21 ± 0.57
Foeniculum vulgare	64.08 ± 3.56	> 100	> 100	86.30 ± 3.40
Lepidium sativum	> 100	> 100	> 100	> 100
Ligusticum sinense	52.23 ± 2.53	63.18 ± 4.79	> 100	46.67 ± 3.29
Mimusops elengi	55.84 ± 1.01	75.6 ± 5.83	> 100	> 100
Mesua ferrea	31.88 ± 1.93	67.91 ± 1.93	> 100	27.46 ± 0.78
Myristica fragrans (Arail)	21.27 ± 0.42	18.4 ± 1.57	> 100	81.81 ± 1.98
Myristica fragrans (Nutmeg)	21.91 ± 0.78	14.98 ± 1.29	> 100	> 100
Mammea siamensis	13.22 ± 1.13	28 ± 2.93	> 100	64.49 ± 4.19
Nelumbo nucifera	> 100	> 100	> 100	> 100
Nigella sativa	> 100	> 100	> 100	67.17 ± 3.17
Plumbago indica	80.92 ± 5.99	> 100	> 100	22.58 ± 2.6
Piper retrofractum	> 100	> 100	> 100	21.40 ± 4.40
Piper ribesioides	22.82 ± 1.9	17.61 ± 1.89	> 100	42.38 ± 5.59
Piper sarmentosum	95.66 ± 1.86	> 100	> 100	66.48 ± 3.95
Syzygium aromaticum	7.2 ± 0.15	2.94 ± 0.22	> 100	3.06 ± 0.67
Senna garrettiana	4.87 ± 0.33	4.46 ± 0.95	10.84 ± 1.14	98.64 ± 0.04
Terminalia bellirica	3.84 ± 0.35	5.94 ± 0.59	23.81 ± 0.56	68.83 ± 0.14
Terminalia chebula	5.37 ± 0.58	8.51 ± 0.69	78.16 ± 1.49	93.49 ± 2.24
Terminalia citrina	2.42 ± 0.27	3.93 ± 0.3	49.15 ± 2.18	83.90 ± 6.10
Urceola rosea	5.02 ± 0.16	7.64 ± 0.52	> 100	> 100
Zingiber officinale	11.83 ± 0.71	10.86 ± 1.43	> 100	9.29 ± 0.13
Bhamrung-Lohit remedy	6.01 ± 0.21	37.77 ± 2.48	84.94 ± 7.47	57.63 ± 4.01
Butylated hydroxytoluene	13.78 ± 0.23	N/A	N/A	N/A
Trolox	N/A	5.55 ± 0.70	N/A	N/A
Propyl gallate	N/A	N/A	19.52 ± 0.31	N/A
Prednisolone	N/A	N/A	N/A	0.44 ± 0.04

N/A, Not applicable.

The HCA (Fig. 1) clustered the plant constituents into two major groups, the low-activity group (group I) and the moderate to high-activity group (group II). Each sub-group in group II expressed its characteristic on the tested activities. BRL remedy showed potent activity on DPPH radical scavenging and exerted moderate activity on ABTS●+ scavenging activity, cellular antioxidant by inhibition of superoxide anion production (NBT assay), and inhibition of NO production. For the plant ingredients of the BRL remedy, *C. sappan* exerted potent activity on every tested activity with EC_{50} and IC_{50} less than 20 µg/mL. The sub-group containing *C. sappan*, *Senna garrettiana*, *Terminalia bellirica*, and *Tholymis citrina* performed inhibitory effects on all tested activities. The sub-group containing *Anaxogorea luzonensis*, *Syzygium aromaticum*, *Zingiber officinale*, and *Acaryochloris marina* showed potent activity on DPPH, ABTS●+ and

inhibition of NO production. With regard to antioxidant activity, *C. sappan* and *S. garrettiana* exerted potent activity more than the positive controls in all tested antioxidant activity methodology. *A. luzonensis*, *S. Aromaticum,* and *T. citrina* exerted potent activity greater than the positive controls on DPPH and ABTS●+ scavenging activity. For inhibitory activity on NO production, several extracts showed potent activity with IC_{50} less than 20 µg/mL including *Aguilaria crassna*, *Atracytolodes. lancea*, *A. luzonensis*, *Acaryochloris marina*, *Angelica sinensis*, *C. sappan*, *S. Aromaticum,* and *Z. officinale*. The BRL remedy showed inhibitory activity with an IC_{50} value of 57.63 ± 4.01 µg/mL. All plant constituents exerted significantly less anti-inflammatory activity than the positive control (prednisolone).

Total phenolic content and total flavonoid content

As shown in Table 4, *C. sappan* showed the highest content of total phenolic followed by *S. garrettiana* and *T. citrina*, respectively. For total flavonoid content, *C. sappan* also showed the highest content followed by *S. garrettiana* and *A. luzonensis*. BRL remedy showed total phenolic content and total flavonoid content of 207.93 ± 7.40 mgGAE/g) and 457.63 ± 11.25 mg quercetin quivalent/g, respectively.

Table 4. Total flavonoid content of ethanolic extract of Bhamrung-Lohit remedy and its plant components. Data are expressed as mean ± SEM; n = 3.

Plant genus and species	Total phenolic content (mg GAE/g)	Total flavonoid content (mg QuE/g)
Artemisia annua	50.26 ± 1.41	89.97 ± 1.09
Aquilaria crassna	104.06 ± 5.39	148.25 ± 7.19
Angelica dahurica	68.88 ± 2.38	142.47 ± 3.28
Arcangelisia flava	103.30 ± 1.54	176.85 ± 2.04
Anethum graveolens	24.36 ± 2.30	106.85 ± 5.16
Atractylodes lancea	33.88 ± 1.77	238.72 ± 7.66
Anaxagorea luzonensis	679.24 ± 18.72	1,094.82 ± 54.38
Avicennia marina	97.22 ± 8.57	211.85 ± 0.78
Alyxia reinwardtii	51.67 ± 0.57	156.85 ± 4.54
Angelica sinensis	59.88 ± 2.29	129.66 ± 0.78
Artemisia verum	36.42 ± 3.00	143.57 ± 1.88
Bixa orellana	168.13 ± 5.50	236.85 ± 6.10
Cuminum cyminum	39.19 ± 2.74	192.00 ± 0.94
Caesalpinia sappan	759.88 ± 46.44	2,535.91 ± 1.72
Cinnamomum verum	62.32 ± 5.01	431.38 ± 4.38
Dactylopius coccus	7.61 ± 0.2	95.60 ± 4.54
Dracaena cochinchinensis	373.06 ± 18.86	327.47 ± 13.91
Foeniculum vulgare	25.35 ± 2.02	92.16 ± 2.97
Lepidium sativum	13.83 ± 1.08	104.35 ± 5.79
Ligusticum sinense	53.17 ± 1.47	157.32 ± 4.07
Mimusops elengi	24.65 ± 2.10	75.13 ± 2.82
Mesua ferrea	66.33 ± 5.66	177.79 ± 2.35
Myristica fragrans (Arail)	86.24 ± 3.50	336.85 ± 0.16
Myristica fragrans (Nutmeg)	101.12 ± 3.45	291.07 ± 7.82
Mammea siamensis	98.73 ± 10.81	310.28 ± 7.03
Nelumbo nucifera	25.64 ± 0.64	181.07 ± 3.44
Nigella sativa	9.50 ± 0.44	77.79 ± 1.10
Plumbago indica	11.99 ± 0.16	150.13 ± 5.32
Piper retrofractum	22.69 ± 2.56	271.85 ± 1.10
Piper ribesioides	151.44 ± 5.08	490.13 ± 12.50
Piper sarmentosum	36.65 ± 0.84	259.50 ± 6.56
Syzygium aromaticum	548.98 ± 14.39	846.06 ± 3.75
Senna garrettiana	754.85 ± 10.62	1,364.04 ± 30.16
Terminalia bellirica	419.14 ± 2.16	135.60 ± 4.85
Terminalia chebula	297.18 ± 9.36	98.41 ± 4.53
Terminalia citrina	694.81 ± 3.27	187.31 ± 8.75
Urceola rosea	488.06 ± 22.50	1,241.07 ± 19.68
Zingiber officinale	197.64 ± 4.90	780.75 ± 19.69
Bhamrung-Lohit remedy	207.93 ± 7.40	457.63 ± 11.25

GAE, Gallic acid equivalents; QuE, quercetin equivalent.

Validation of the HPLC method

Specificity/selectivity

A new analytical method for the simultaneous determination of brazilin, ellagic acid, eugenol, myristicin, and piperine in ethanol extract of BRL remedy was developed and validated. As shown in Fig. 2, the chromatogram of the sample was compared to the chromatogram of the standard solution. The retention time of brazilin, ellagic acid, eugenol, piperine, and myristicin in the sample solution were 19.17, 23.8, 42.1, 49.0, and 52.1 min, respectively. The UV spectrum of the analyzed peaks in the chromatogram of the sample solution corresponded to the respective standards in the chromatogram of the standard solution (Fig. 2). These results indicated the specificity and selectivity of this method.

Linearity and ranges

The linearity of the developed method was determined by the coefficient of determination (R^2) calculated from the constructed calibration curves of each marker. As shown in Table 5, the R^2 values of brazilin, ellagic acid, eugenol, piperine, and myristicin were 0.9996, 0.9991, 0.9996, 0.9991, and 0.9999, respectively. The linear range of each marker varied relating to their contents in the BRL extract and their absorptivity at the detected wavelength.

LODs and LOQs

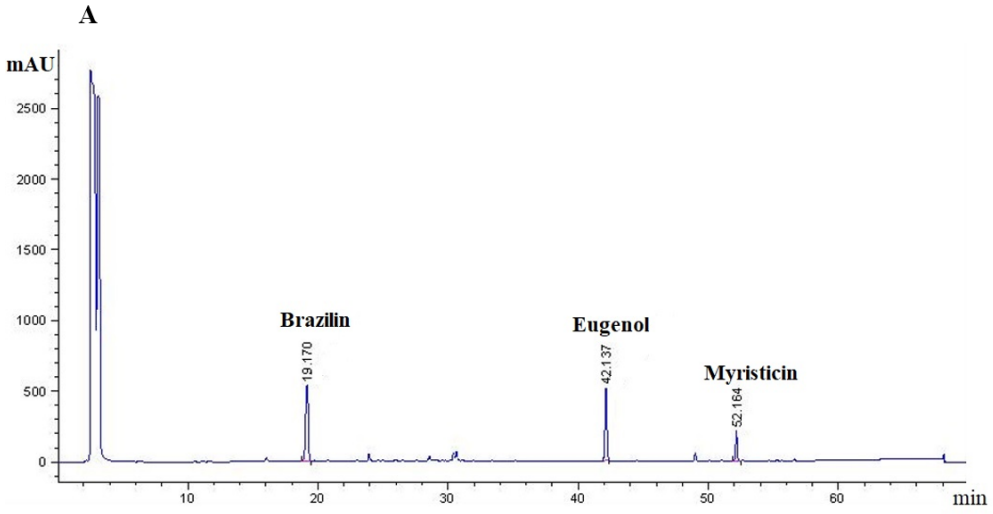
LODs and LOQs of the markers were analyzed according to the visualization shown in Table 5. The LODs of brazilin, ellagic acid, eugenol, piperine, and myristicin were 0.1, 0.5, 0.5, 1.0, and 0.2 µg/mL, respectively. The LOQs were 6.25, 1.25, 2.0, 5.0, and 1.0 µg/mL respectively.

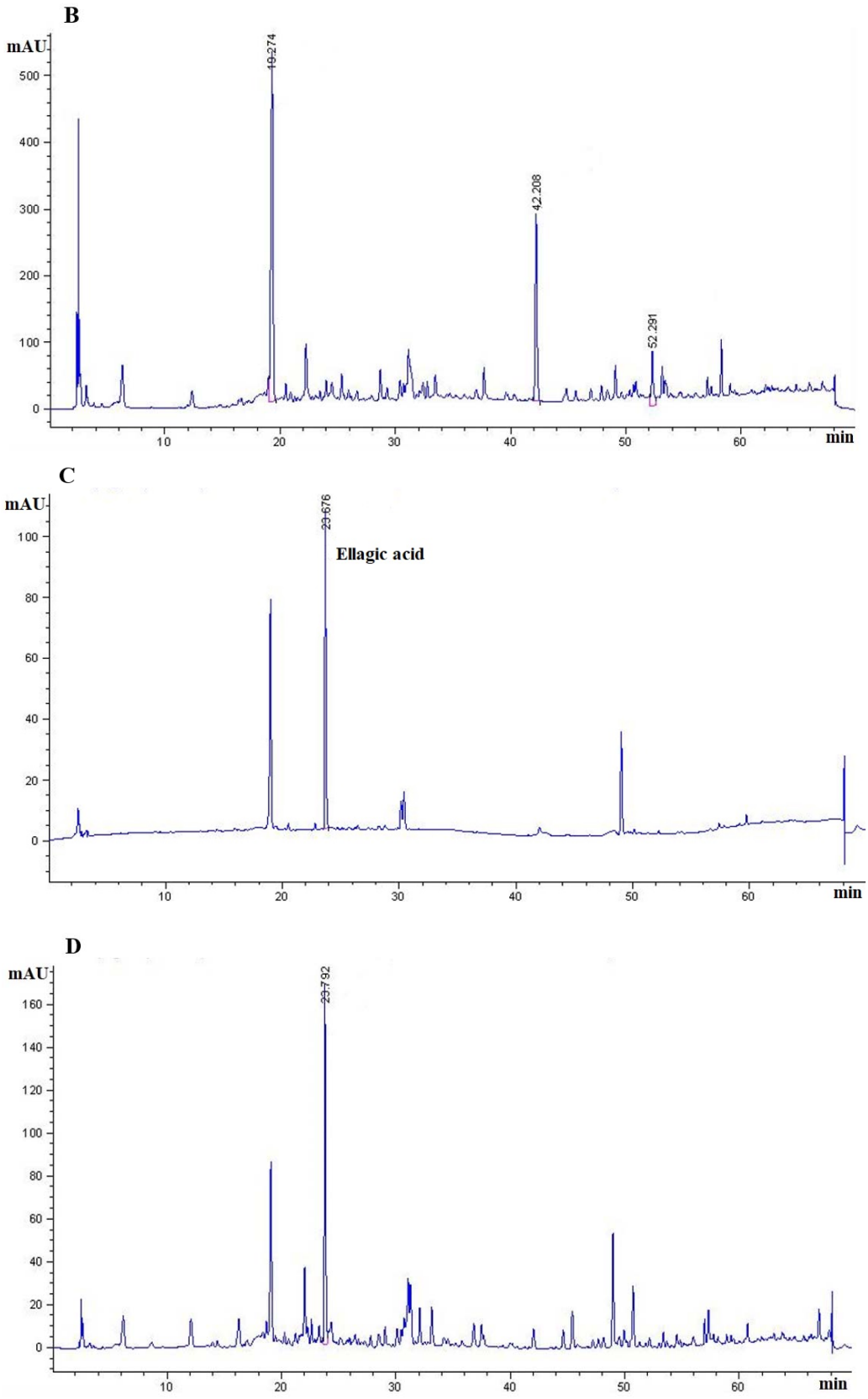
Accuracy and precision

The accuracy of the method was presented as % recovery of each compound shown in Table 6. The % recovery of all standards was within 97.62% - 101.57%. The precision of the analytical method, both intra-run and inter-run, was performed as % CV which was less than 1.99.

Content of the markers in ethanolic extract of BRL remedy

The ethanolic extract of the BRL remedy was analyzed for the content of brazilin, ellagic acid, eugenol, myristicin and piperine by using the developed and validated HPLC method. The results found the amount of brazilin, ellagic acid, eugenol, piperine and myristicin were 120.23 ± 4.90, 2.03 ± 0.07, 12.27 ± 0.19, 4.66 ± 0.12 and 0.77 ± 0.17 mg/g of extract, respectively.

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Ellagic acid
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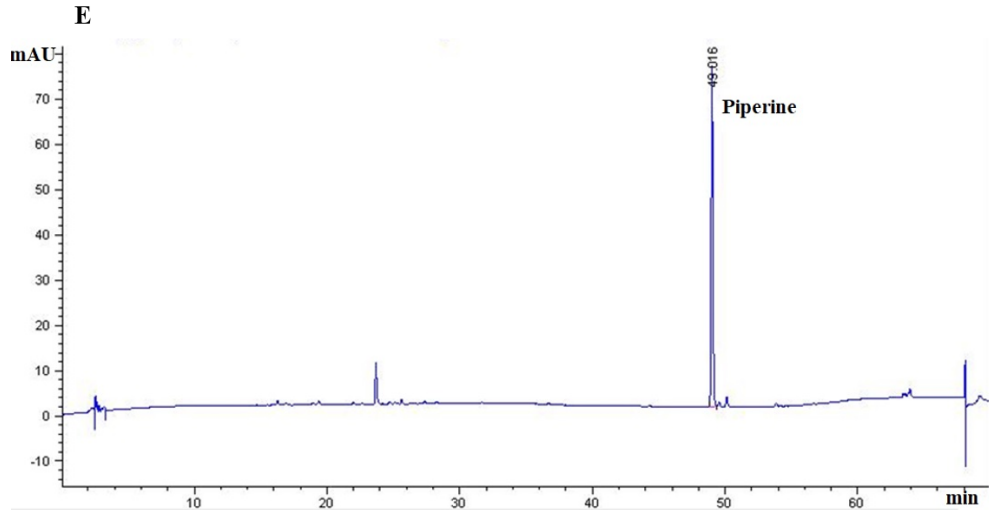
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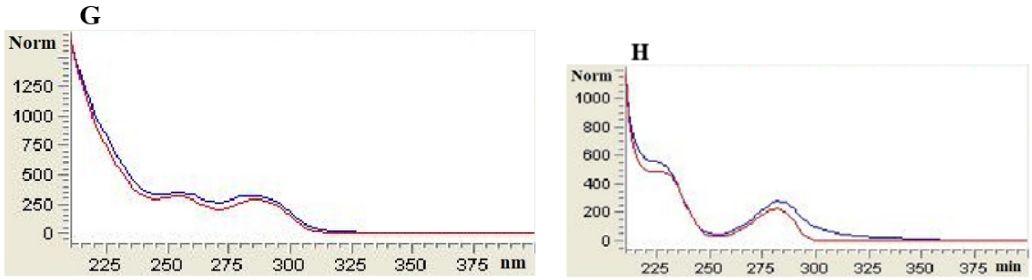
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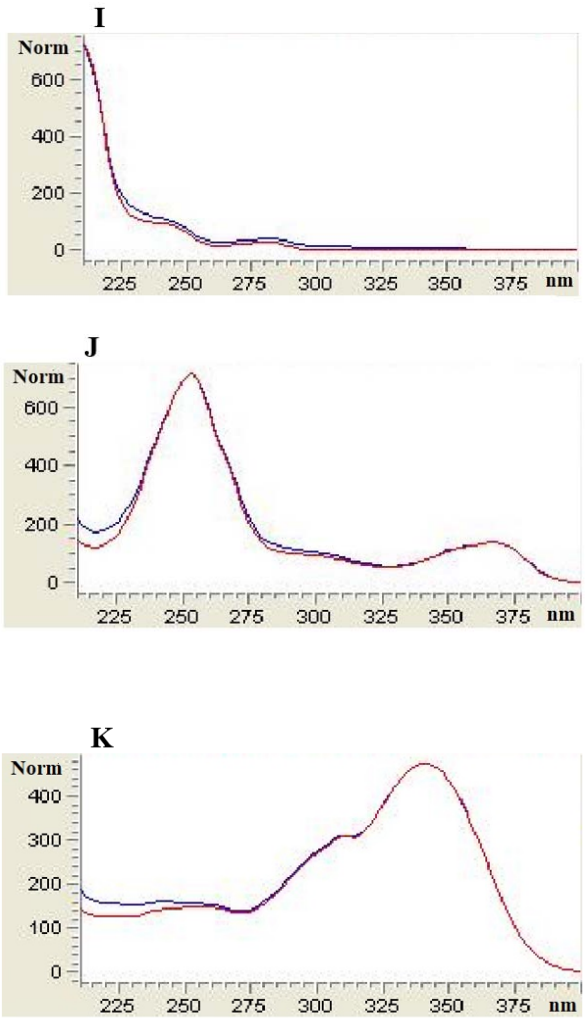

Fig. 2. The HPLC chromatogram and UV spectrum of the mixed standard solution and BRL sample solution. (A, C, E) Chromatograms of standard solution detecting at the wavelength of 210, 254, and 320, respectively; (B, D, F) chromatograms of BRL sample solution detecting at the wavelength of 210, 254, and 320, respectively. Comparative UV-spectrum of peaks in standard and sample; (G) brazilin, (H) eugenol, (I) myristicin, (J) ellagic acid, and (K) piperine. BRL, Bhamrung-Lohit.

Table 5. Linearity, LODs, and LOQs of brazilin, ellagic acid, eugenol, piperine and myristicin in Bhamrung-Lohit extract.

Compounds	Validation parameters			
	Range (μg/mL)	Linearity	LOD (μg/mL)	LOQ (μg/mL)
Brazilin	500-1800	Y = 11.621X - 988.790; r^2 = 0.9996	0.1	0.325
Ellagic	5-40	Y = 158.990X - 9.785; r^2 = 0.9991	0.5	1.25
Eugenol	25-270	Y = 59.468X - 571.650; r^2 = 0.9996	0.5	2.5
Piperine	15-60	Y = 47.834X - 6.381; r^2 = 0.9991	0.2	1.0
Myristicin	1-25	Y = 191.950X - 78.762; r^2 = 0.9999	0.2	1.0

LOD, Limit of detection; LOQ, limit of quantification.

Table 6. Accuracy of the analytical method for determination of brazilin, ellagic acid, eugenol, piperine, and myristicin in Bhamrung-Lohit extract

Compounds	Spiked concentration (µg/mL)	Intra-run (n = 3)			Inter-run (n = 9)		
		Concentration found (µg/mL; mean ± SD)	Recovery (%)	CV (%)	Concentration found (µg/mL; mean ± SD)	Recovery (%)	CV (%)
Brazilin	600	606.23 ± 11.85	100.37 - 101.04	0.96	589.49 ± 10.39	99.75 - 100.49	1.74
	900	903.88 ± 10.73		0.69	904.39 ± 7.31		0.81
	1100	1104.17 ± 4.75		0.31	1101.66 ± 18.51		1.68
Ellagic acid	10	9.76 ± 0.09	97.62 - 101.57	0.60	9.84 ± 0.17	98.37 - 100.21	1.70
	20	20.31 ± 0.38		0.68	20.01 ± 0.40		1.99
	25	24.61 ± 0.31		1.11	24.96 ± 0.36		1.43
Eugenol	50	50.23 ± 0.29	99.89 - 100.47	0.75	50.54 ± 0.66	99.19 - 100.09	1.31
	150	150.31 ± 1.02		1.26	149.92 ± 1.19		0.80
	200	199.79 ± 3.83		1.35	198.37 ± 2.83		1.43
Piperine	20	20.19 ± 0.16	100.51 - 100.97	0.34	20.00 ± 0.37	99.38 - 99.98	1.83
	30	30.25 ± 0.37		0.60	29.87 ± 0.40		1.34
	35	35.18 ± 0.62		0.25	34.78 ± 0.69		1.98
Myristicin	5	4.91 ± 0.05	98.35- 101.37	0.66	4.97 ± 0.07	99.20 - 99.86	1.49
	15	15.09 ± 0.29		0.74	14.88 ± 0.25		1.66
	20	20.27 ± 0.31		1.12	19.97 ± 0.35		1.73

DISCUSSION

Oxidative stress plays a significant role as a risk factor for diseases including cancer and cardiovascular disease (6). In the cardiovascular system, hemoglobin within red blood cells plays a key role in the initiating of oxidative stress by its auto-oxidation which produces superoxide radicals including O_2●- and H_2O_2 (2). In inflammatory processes, the upregulation and stimulation of NO which reacts with superoxide radicals leads to damage from the ONOO● radical (2). Therefore, in this study, evaluating the effects of BRL remedy and its components on both antioxidant and anti-inflammatory activity were assessed.

The ethanolic extract of BRL exerted significant antioxidant activity as measured with the DPPH scavenging activity and also showed significant activity on ABTS●+ radical scavenging, as well as a cellular antioxidant activity through inhibition of O_2●- and concurrent anti-inflammatory activity by inhibition of NO production. This study is the first report of BRL extract demonstrating both *in vitro* antioxidant and anti-inflammatory activity.

Concerning the plant components of BRL, *C. sappan* exerted potent activity. *S. garrettiana* also exerted potent activity in all tested antioxidant systems. *T. bellirica* and *T. citrina* exerted potent activity on DPPH and ABTS●+ radical scavenging but exerted less pronounced effects on cellular antioxidant and inhibitory activity on NO production. For the sub-group containing *A. luzonensis*, *S. aromatica*, *Z. Offinale,* and *A. marina*. All four plants were inactive in their inhibitory activity on O_2●- production determined by the NBT assay. *A. luzonensis*, *S. aromatica*, and *Z. offinale* exerted potent DPPH and ABTS●+ radical scavenging and anti-inflammatory activity while A. Marina exerted potent anti-inflammatory activity and demonstrated correspondingly less activity on DPPH and ABTS●+ radical scavenging. Eight plant constituents in particular demonstrated noteworthy bioactivity findings. Firstly, *C. sappan* and its major antioxidant compound

(brazilin) have been previously reported to possess potent antioxidant and anti-inflammatory activity similar to our findings (20-23). For *S. garrettiana*, this study is the first report of DPPH and ABTS●+ radical scavenging, inhibitory activity on O_2●- production in HL-60 cells and inhibitory activity on NO production in RAW264.7 cells. The major compound in *S. garrettiana* is trans-piceatannol, a stilbene, which has been reported to have antioxidant activity as it increased cellular antioxidant defense (24). For *T. bellirica* and *T. citrina*, Hazra and co-workers reported that *T. bellirica* exerted potent DPPH radical scavenging similar to our study. Moreover, potent activity on the scavenging of O_2●-, NO, and ONOO radicals have been reported (25). *T. bellirica* possesses potent anti-inflammatory activity through the inhibition of NO, cyclooxygenase-2, tumor necrosis factor alpha and interleukin-6 (26,27). The leaves of *T. citrina* exerted weak DPPH scavenging activity differing from our study in which the fruit was utilized (28). The bioactive constituents in *T. bellirica* and *T. citrina* are tannin, glucoside, pyranosides, triterpenoids, and acidic compounds (29). Ellagic acid in *Terminalia* spp. exerted anti-inflammatory effects by inhibition of NO production in RAW 264.7 (30). For *A. luzonensis*, few studies have been reported on activity related to antioxidation and anti-inflammatory effects and its phytochemicals. Chaiyasut and co-workers reported potent DPPH scavenging activity and high content of total phenolic compounds which is similar to our study (31). Gonda and co-workers reported the xanthone derivatives are constituents in *A. luzonensis* (32). *S. aromatica* and *Z. offinale* are common plants well known for their activity. *S. aromaticum* (clove) and its major constituent, eugenol, exerted potent antioxidant and anti-inflammatory activity (33-36). Perez-Roses and co-workers reported that eugenol exerted antioxidant activity by scavenging DPPH, NO, and H_2O_2 (37). Yeh and co-workers reported that eugenol inhibited the expression of the inducible nitric oxide synthase from the LPS-induced macrophages. It also reduced the tumor necrosis factor alpha and IL-1β as well as the nuclear factor kappa B, extracellular signal-regulated kinase 1/2, and p38 mitogen-activated protein kinases signaling pathways (38). For *Z. offinale*, several studies reported its antioxidant and anti-inflammatory activity both *in vitro* and *in vivo* (,39-41). Kim and co-workers reported that 6-gingerol, an active compound in *Z. offinale*, exerted potent activity both in vivo and in vitro, and exerted strong anti-inflammatory activity (42). For *A. marina*, several studies reported its potential antioxidant activity both *in vitro* (43) and *in vivo* (44). Lincy and co-workers reported that *A. marina* has a high content of total phenolics and flavonoids imparting antioxidant properties (43). For the phytochemicals, *A. marina* contains various constituents, including naphthalene derivatives, flavones, iridoid glucosides, phenylpropanoid glycosides, diterpenoid glucosides, flavonoids, terpenoids and steroids (45). Avicequinone C has been isolated from the methanolic extract of *A. Marina* (46).

With regard to the analyte markers analyzed by HPLC, brazilin is the major compound in *C. Sappan* (22), ellagic acid from *Terminalia* spp. (30), eugenol from *S. aromatica* (36), piperine from *Piper* spp (47). and myristicin from *M. Fragrans* (48). The present study aimed at developing and validating an HPLC method for the determination of the main major constituents of BRL extract. The method can be used as a quality control method for further development of BRL extract into health products. There are no previous analytical methods reported for quality control of the BRL remedy. The current study is the first study presenting validation results of an HPLC method for the determination of the major marker constituent brazilin, ellagic acid, eugenol, piperine, and myristicin in BRL remedy.

An HPLC method for simultaneous quantitative analysis of brazilin, ellagic acid, eugenol, piperine, and myristicin in the ethanolic extract of BRL remedy was developed and validated. In the present study, 0.1% v/v phosphoric acid was used as a component in the mobile phase to suppress the ionization of phenolic compounds, such as brazilin and ellagic acid, causing a reduction in peak tailing. All marker analytes were

separated with the C18 column with a resolution value of more than 2.0. The linear range of the calibration curve of each marker varied depending on its content in BRL extract and absorptivity. Each marker was detected at its appropriated maximum detection wavelength as follows: 210 nm for brazilin, eugenol, and myristicin, 254 nm for ellagic acid, and 320 nm for piperine. The developed HPLC method resulted in excellent specificity for the determination of the markers in BRL ethanol extract. The calibration curves showed excellent linearity with $R^2 > 0.999$ at the analyzed concentration ranges. The LODs for all markers ranged between 0.1 – 1.0 µg/mL and the LOQs ranged between 1.0 – 6.25 µg/mL. The accuracy of each marker ranged between 97.62% – 101.57%. The intra-run and inter-run precisions presented as %CV were less than 1.99%. The method validation demonstrated excellent results meeting ICH criteria for quality control of the BRL extract.

CONCLUSION

BRL remedy, a TTM remedy is being used in patients, particularly in Thailand and neighboring nations. In this study, an ethanol extract of BRL remedy exerted antioxidant and anti-inflammatory activity as measured *in vitro* by DPPH and ABTS●+ radical scavenging activity, inhibitory activity on O_2●- production and inhibitory activity on NO production. This study is the first report of a BRL extract on its anti-inflammatory activity. For quality control of the ethanolic extract of BRL, a developed HPLC method for simultaneous quantification of the major markers was validated. The validated results showed good selectivity, linearity, precision, and accuracy with appropriate LOD and LOQ. This study is the first report of validation of an HPLC method of BRL for use in quality control of the BRL extract.

Acknowledgments

The authors would like to thank the Thailand Science Research and Innovation Fundamental Fund, Center of Excellence in Applied Thai Traditional Medicine Research (CEATMR) and Bualuang ASEAN Chair Professorship, Faculty of Medicine, Thammasat University, for financial support and the use of laboratory facilities.

Conflict of interest statement

The authors declared no conflicts of interest in this study.

Authors' contribution

A. Itharat conceived and supervised the project; C. Panchakul, W. Pipatrattanaseree, and P. Thongdeeying carried out the experiments and wrote the manuscript with support from A. Itharat; C. Kongkwamcharoen revised the manuscript; N.M. Davies, the Bualuang ASEAN Chair Professor, revised, reviewed, and analyzed the data and provided scientific insights and revisions to the manuscript. The final version of the manuscript was approved by all authors.

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