Original Article

Antioxidant Activity and Total Phenolic Content of the Aqueous Extracts of a Thai Blood Tonic (Bhamrung-Lohit) Remedy and Its Plant Components

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Abstract

Introduction: In the Mahachotarata scripture, there was a blood tonic remedy known as Bhamrung-Lohit remedy in Thai which has been used in female adult to treat menstrual irregularities, abnormal menstruation, and for nourishing blood and body. The aims of this study were to determine the amount of total phenolics and antioxidant activities of Bhamrung-Lohit remedy and its plant components.

Methods: All tested extracts were prepared by decoction as practiced in traditional medicine. All extracts were analyzed for their phenolic content by Folin-Ciocalteu assay and their antioxidant activities by using DPPH radical scavenging assay, ABTS radical scavenging and ferric reducing antioxidant power assay (FRAP). The correlation analysis was evaluated by curve fitting regression model.

Results: The results showed that the extract of *Caesalpinia sappan,* a Bhamrung-Lohit plant ingredient, contained the highest total phenolic contents of 781.72 ± 21.19, 269.38 ± 12.88 mg GAE/g. The aqueous extract of *Terminalia citrina* and *Bixa Orellana* demonstrated stronger antioxidant activity than positive control (BHT) by DPPH radical scavenging assay (EC_{50} = 4.51 ± 0.21 µg/ml, 4.92 ± 0.08 µg/ml, and 13.78 ± 0.23 µg/ml, respectively). In $ABTS^{\bullet+}$ assay, the extract of *Terminalia citrina* and *Caesalpinia sappan* showed stronger activity than positive control (BHT) with (EC_{50} = 4.16 ± 0.11 µg/ml, 5.19 ± 0.38, and 5.55 ± 0.23 µg/ml, respectively). The extract of *Caesalpinia sappan* and Bhamrung-Lohit remedy gave the highest TEAC and FRAP value of 1,223.14 ± 25.26 mg Trolox/g, 2,885.64 ± 57.27 mg Fe(II)/g, 338.28 ± 9.13 mg Trolox/g, and 795.06 ± 20.69 mg Fe(II)/g, respectively, it also showed stronger activities than positive control (BHT). DPPH and $ABTS^{\bullet+}$ scavenging activity relate to total phenolic content by negative exponential model. Both FRAP assay showed the linear correlation with the total phenolic content.

Conclusion: This study demonstrated that Bhamrung-Lohit remedy and its plant components showed good antioxidant activities. These results support the indication of this preparation as blood tonic.

Keywords: Bhamrung-Lohit remedy, Total phenolic content, FRAP, DPPH, ABTS

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Introduction

In the Mahachotarata scripture, there was a blood tonic remedy known in Thai as Bhamrung-Lohit (BRL) remedy which has been used in female adult to treat menstrual irregularities, abnormal menstruation and for nourishing blood and body.[1] Bhamrung-Lohit remedy has been included in the National List of Essential Medicines since 2013 for the treatments of abnormal blood and for nourishing blood and body.[2] According to Thai traditional medicine theory, if the blood circulation system is good, the body will be in good health.

Endometriosis is one of the most common gynecological diseases in women of reproductive age. It is characterized by the presence of endometrial tissue outside the uterine cavity. The women affected suffer from pelvic pain and infertility. The complex etiology is still unclear, and it is based on three main theories: retrograde menstruation, coelomic metaplasia, and induction theory.[3]

Free radicals are molecules that contain unpaired electrons, making them energetically unstable and highly reactive substances. Free radicals have been implicated in the pathology of several human diseases, including cancer, atherosclerosis, malaria, rheumatoid arthritis, and neurodegenerative diseases.[4]

The enzyme glucose-6-phosphate dehydrogenase (G6PD) is essential to erythrocytes for maintaining their normal functions such as metabolism and elimination of toxic substances.[5] Nicotinamide adenine dinucleotide phosphate (NADPH) is derived from G6PD activity which prevent early degradation of erythrocytes.[6] In addition to this, G6PD works with reduced glutathione; GSH to scavenge the free radicals within the cells.[7] Therefore, antioxidants are essential for healthy erythrocytes.

The Bhamrung-Lohit remedy has been listed as blood tonic but its antioxidant activity has not been studied. The aims of this study were to find out the amount of total phenolics and antioxidant activities of Bhamrung-Lohit remedy and its plant components.

Methods

Chemicals and Reagents

Folin-Ciocalteu's phenol reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxyl-toluene (BHT) were purchased from Fluka, Germany. Gallic acid, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), Potassium sulfate, ferrous sulfate, 2,4,6-tripyridyl-*s*-triazine (TPTZ), sodium acetate trihydrate and ferric chloride were purchased from Sigma-Aldrich®, USA. Absolute ethanol and hydrochloric acid were purchased from RCI Labscan, Thailand. Sodium carbonate and acetic acid were purchased from Merck, Germany.

Plant Materials

All 38 plant materials in Bhamrung-Lohit remedy were purchased from Charernsuka Osot pharmacy (Nakornpathom, Thailand). The plants were cleaned with water, sliced into small pieces and dried in a hot air oven at 45ºC and powdered. The ingredients were weighed according to Bhamrung-Lohit remedy and mixed thoroughly. Bhamrung-Lohit remedy consists of *Caesalpinia sappan* L., *Bixa orellana* L. each of 13.33%, *Dactylopius coccus* 10.67%, *Zingiber officinale* Roscoe, *Piper retrofractum* Vahl, *Plumbago indica* L., *Piper ribesioides* Wall., *Piper sarmentosum* Roxb., *Arcangelisia flava* (L.) Merr., *Urceola rosea* (Hook. & Arn.) D.J.Middleton, *Anaxagorea luzonensis* A. Gray, *Mammea siamensis* Kosterm., *Mimusops elengi* L., *Mesua ferrea* L., *Nelumbo nucifera* Gaertn. each of 2.67%, *Myristica fragrans* Houtt. (aril), *Myristica fragrans* Houtt. (seed), *Amomum verum* Blackw., *Syzygium aromaticum* (L.) Merr. & L.M.Perry., *Nigella sativa* L., *Lepidium sativum* L., *Cuminum cyminum* L., *Foeniculum vulgare* Mill., *Anethum graveolens* L., *Angelica dahurica* (Hoffm.) & Hook.f. ex French. & Sav., *Atractylodes lancea* (Thunb.) DC., *Ligusticum sinense* Oliv., *Angelica sinensis* (Oliv.) Diels., *Artemisia annua* L., *Terminalia chebula* Retz., *Terminalia citrina* Roxb. ex Fleming, *Terminalia bellirica* (Gaertn.) Roxb., *Alyxia reinwardtii* Blume, *Cinnamomum verum* J. Presl, *Dracaena cochinchinensis* (Lour.) S.C.Chen, *Senna garrettiana* (Craib) H.S.Irwin & Barneby., *Avicennia marina* (Forssk.) Vierh., *Aquilaria crassna* Pierre ex Lecomte each of 1.33% and mixed together. Plant component voucher specimens from Bhamrung-Lohit remedy were deposited at the herbarium of Southern Center of Thai Medicinal Plants at Faculty of Pharmaceutical Science, Prince of Songkhla University, Songkhla, Thailand (Table 1).

Table 1 The ethnobotanical data of Bhamrung-Lohit remedy

Species	Plant part	%Yield (w/w)	Voucher specimen number	Traditional use
Caesalpinia sappan L. LEGUMINOSAE	Heartwood	3.98%	SKP 072 03 19 01	Blood tonic, Treatment of menstruation pain
Bixa orellana L. BIXACEAE	Flower	14.50%	SKP 026 02 15 01	Blood tonic, Treatment of anemia
Dactylopius coccus DACTYLOPIIDAE	Lac*	6.32%	SKP 215 12 12 01	Blood tonic
Zingiber officinale Roscoe ZINGIBERACEAE	Rhizome	7.49%	SKP 206 26 15 01	Carminative, Antiemetic
Piper retrofractum Vahl PIPERACEAE	Flower	5.26%	SKP 146 16 18 01	Carminative, Promote blood circulation, Antiemetic
Plumbago indica L. PLUMBAGINACEAE	Root	25.68%	SKP 148 16 09 01	Promote blood circulation, Gynecology, Carminative
Piper ribesioides Wall. PIPERACEAE	Nest	5.85%	SKP 146 16 14 01	Carminative
Piper sarmentosum Roxb. PIPERACEAE	Root	4.49%	SKP 146 16 19 01	Carminative
Arcangelisia flava (L.) Merr. MENISPERMACEAE	Stem	5.41%	SKP 114 11 06 01	Carminative, Anti-diarrhea
Urceola rosea (Hook. & Arn.) D.J.Middleton APOCYNACEAE	Stem	6.77%	SKP 013 05 18 01	Carminative
Anaxagorea luzonensis A.Gray ANNONACEAE	Heartwood	3.72%	SKP 011 01 12 01	Blood tonic, Tonic, Paregoric
Mammea siamensis Kosterm. GUTTIFERAE	Flower	21.50%	SKP 083 13 19 01	Cardiotonic, Tonic
Mimusops elengi L. SAPOTACEAE	Flower	16.15%	SKP 171 13 05 01	Cardiotonic, Antipyretic
Mesua ferrea L. CALOPHYLLAEAE	Flower	11.37%	SKP 083 13 06 01	Blood tonic, Cardio Tonic, Antipyretic
Nelumbo nucifera Gaertn. NELUMBONACEAE	Pollen	14.34%	SKP 125 14 14 01	Cardiotonic, Tonic
Myristica fragrans Houtt. MYRISTICACEAE	Aril	4.27%	SKP 121 13 06 01	Blood tonic, Tonic, Carminative
Myristica fragrans Houtt. MYRISTICACEAE	Seed	16.01%	SKP 121 13 06 01	Tonic, Carminative, Antiemetic
Amomum verum Blackw. ZINGIBERACEAE	Fruit	5.16%	SKP 206 01 20 01	Antiemetic, Carminative
Syzygium aromaticum (L.) Merr. & L.M.Perry MYRTACEAE	Flower	17.54%	SKP 123 19 01 01	Paregoric of tooth and pyorrhea, Carminative, Promote blood circulation
Nigella sativa L. RANUNCULACEAE	Dry fruit	7.93%	SKP 160 14 19 01	Antiemetic, Carminative
Lepidium sativum L. BRASSICACEAE	Dry fruit	9.00%	SKP 057 12 19 01	Gastrointestinal agent
Cuminum cyminum L. APIACEAE	Dry fruit	16.78%	SKP 199 03 03 01	Carminative

Table 1 The ethnobotanical data of Bhamrung-Lohit remedy (Cont.)

Species	Plant part	%Yield (w/w)	Voucher specimen number	Traditional use
Foeniculum vulgare Mill. APIACEAE	Dry fruit	12.52%	SKP 199 06 22 01	Tonic, Carminative
Anethum graveolens L. APIACEAE	Dry fruit	10.79%	SKP 199 01 07 01	Tonic, Carminative
Angelica dahurica (Hoffm.) Benth. & Hook.f. ex Franch. & Sav. APIACEAE	Root	16.22%	SKP 199 01 04 01	Tonic, Antipyretic, Paregoric, Relieve asthma
Atractylodes lancea (Thunb.) DC. COMPOSITAE	Rhizome	20.11%	SKP 051 01 12 01	Tonic, Oral prophylaxis, Antipyretic
Ligusticum sinense Oliv. APIACEAE	Rhizome	15.37%	SKP 199 12 19 01	Carminative, Blood tonic, Gynecology
Angelica sinensis (Oliv.) Diels APIACEAE	Root	24.63%	SKP 199 01 19 01	Antipyretic, Gynecology
Artemisia annua L. COMPOSITAE	Aerial parts	15.13%	SKP 051 01 01 01	Antipyretic, Relieve cough
Terminalia chebula Retz. COMBRETACEAE	Fruit	19.91%	SKP 045 20 03 01	Laxative, Anti-diarrhea, Relieve cough
Terminalia citrina Roxb. ex Fleming COMBRETACEAE	Fruit	31.04%	SKP 049 20 03 01	Laxative, Antipyretic
Terminalia bellirica (Gaertn.) Roxb. COMBRETACEAE	Fruit	17.37%	SKP 049 20 02 01	Laxative, Carminative, Relieve cough
Alyxia reinwardtii Blume APOCYNACEAE	Peel	14.37%	SKP 013 01 18 01	Tonic, Anti-dizziness
Cinnamomum verum J. Presl LAURACEAE	Peel	6.73%	SKP 096 03 03 01	Carminative, Relieve dyspepsia
Dracaena cochinchinensis (Lour.) S.C.Chen ASPARAGACEAE	Heartwood	5.00%	SKP 065 04 12 01	Cardiotonic, Anti-inflammatory
Senna garrettiana (Craib) H.S.Irwin & Barneby LEGUMINOSAE	Heartwood	8.72%	SKP 072 19 07 01	Carminative, Gynecology
Avicennia marina (Forssk.) Vierh. ACANTHACEAE	Heartwood	7.94%	SKP 213 01 13 01	Carminative, Gynecology
Aquilaria crassna Pierre ex Lecomte THYMELAEACEAE	Heartwood	4.82%	SKP 193 01 03 01	Blood tonic, Tonic, Cardio tonic
Bhamrung-Lohit remedy	-	15.19%	-	Blood tonic

*Lac = Cochineal from insect secretion

Preparation of Extracts

Each plant powder and the BRL remedy mixture were separately extracted by decoction methods. Each extract was prepared by boiling the plant powder in distilled water for 15 minutes, the process was repeated twice. The extracts were combined, filtered and dried in a freeze dryer. The yield of dry extracts was calculated based on dry weight.

Determination of Total Phenolics

Total phenolic content was determined by using Folin-Ciocalteu's assay.[8] The aqueous extract was dissolved in water. The extract solution (20 µl) was added into 96-well plate, and 100 µl Folin-Ciocalteu's reagent was added and mixed. After 5 minutes standing, 80 µl of sodium carbonate solution (7.5% w/v) was added. The samples were mixed and incubated at room temperature for 30 minutes. Then, the absorbance was measured at 765 nm in a spectrophotometer. Standard solutions using gallic acid (12.5, 25, 50, 100, 200, and 400 µg/ml) were prepared and calibration curve was generated. The content of total phenolic compound was calculated against the calibration curve. The results were expressed as mg gallic acid equivalents/ gram of sample (mg GAE/g).

DPPH Radical Scavenging Activity

Antioxidant activity was determined by using DPPH, according to the modified method of Yamasaki et al., 1994.[9] The extract was dissolved distilled water to obtain the final concentration of 100 µg/ml. Each extract was further diluted to obtain at least 4 dilutions, to give final concentration of 100, 50, 10, and 1 µg/ml. Each concentration was tested in triplicate. Then an aliquot of the extract and sample solutions (100 µl) was mixed with an equal volume of $6x10^{-5}$ M DPPH in absolute ethanol, and kept in darkness at room temperature for 30 minutes. The absorbance 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:

$$\%\text{Inhibition} = [(\text{Abs.}_{\text{control}} - \text{Abs.}_{\text{sample}})/\text{Abs.}_{\text{control}}] \times 100$$

Where $\text{Abs.}_{\text{control}}$ was the absorbance of the solvent and $\text{Abs.}_{\text{sample}}$ was the absorbance of the tested compound. EC_{50} values were calculated from regression analysis of the graph between percentage of inhibition against concentration of the extracts.

$ABTS^{\bullet+}$ Radical Scavenging Activity

$ABTS^{\bullet+}$ radical scavenging assay was conducted according to method of Re et al., 1999.[10] Briefly, sample solutions of each extract were prepared at various concentrations (same as DPPH assay) in water or absolute ethanol according to the type of extract. $ABTS^{\bullet+}$ radical solution was prepared by dissolving potassium persulfate with distilled water to produce the radical solution at concentration of 2.45 mM. The sample solution (20 µl) was mixed with $ABTS^{\bullet+}$ solution (180 µl) and incubated at room temperature for 6 minutes. The absorbance of the solution reaction was measured by a microplate reader at wavelength of 734 nm. The percentage of $ABTS^{\bullet+}$ scavenging activity was calculated. BHT was used as a positive control. The calculation of percent scavenging activity was done by the following formula:

$$\%\text{Inhibition} = [(\text{Mean of OD}_{\text{control}} - \text{Mean of OD}_{\text{sample}})/ \text{Mean of OD}_{\text{control}}] \times 100$$

Effective concentration of sample required to inhibited $ABTS^{\bullet+}$ by 50% (EC_{50}) was obtained by regression analysis of the dose-response curve of %inhibition versus concentration, and EC_{50} was calculated using prism program.

Ferric ion Reducing Antioxidant Power (FRAP) Assay

FRAP assay is an antioxidant power estimation method depending upon the reduction of the ferric tripyridyltriazine (Fe^{3+}-TPTZ)

complex to the ferrous triyridyltriazine (Fe^{2+}-TPTZ) at acidic pH.[11] The color of solution changes from colorless solution (Fe^{3+}-TPTZ) to blue colored solution (Fe^{2+}-TPTZ) which can be measured at wavelength of 593 nm. Briefly, the freshly prepared FRAP solution consist of 300 mM acetate buffer pH 3.6, 10 mM TPTZ solution in 40 mM HCl and 20 mM $FeCl_3$. The sample solution of each extract was prepared at concentration of 1 mg/ml in water. An aliquot 20 µl of sample solution was added to 180 µl of FRAP solution and mixed. The reaction mixture was allowed in room temperature for 8 minutes. The absorbance of the reaction was measured at wavelength of 593 nm. For calculation of FRAP value. Six concentrations of ferrous sulphate ($FeSO_4.7H_2O$) solutions were prepared for creation of calibration curve. The FRAP value of extract was calculated by the linear regression equation of the calibration curve and the value was expressed as mg Fe^{2+} equivalent/g of crude extract. For trolox equivalent antioxidant capacity (TEAC) value, calibration curve of trolox was created from six concentration solutions. The TEAC value of sample was calculated by comparing the absorbance of sample reaction with the linear regression equation. TEAC value was expressed as mg trolox equivalent/g of crude extract.

Statistical Analysis

The experiments were carried out in triplicate and the results were expressed as mean ± standard error of mean (SEM). Calculation of EC_{50} values were done using the GraphPad Prism (GraphPad®, USA). The correlation analysis was evaluated by regression analysis using Microsoft® Excel.

Results

Plant Material Extractions

The percent yields of the plant extracts are shown in Table 1. The yields of aqueous extracts ranged from 3.72% to 31.04%, *T. citrina* gave the highest yield of 31.04%, *A. luzonensis* gave the lowest yield of 3.72% and Bhamrung-Lohit remedy gave 15.19% yield.

DPPH Radical Scavenging Activity

The DPPH radical scavenging activity of the extracts are shown in Table 2. The positive control, BHT, showed EC_{50} value of 13.78 µg/ml. There were 16 plants extracts exerted DPPH scavenging activity more potent than positive control. Bhamrung-Lohit remedy extract also showed the DPPH scavenging activity more potent than BHT with EC_{50} of 7.47 ± 0.28 mg/ml. *T. bellirica* extract showed the highest DPPH scavenging activity with EC_{50} of 4.51 ± 0.21 mg/ml. The relative ratio between EC_{50} of BHT and the active extracts are shown in Table 3. The highest antioxidant extract, *T. citrina,* exerted 3.06 times more active than BHT. There were seven plant extracts which were more than twice times active than BHT; *T. bellirica* (2.83), *B. orellana* (2.80), *S. aromaticum* (2.57), *C. sappan* (2.43), *S. garettiana* (2.40), *T. chebula* (2.33), and *U. rosea* (2.18). Bhamrung-Lohit remedy showed 1.84 times more potent than BHT.

$ABTS^{\bullet+}$ Radical Scavenging Activity

The $ABTS^{\bullet+}$ radical scavenging assay of the extracts are shown in Table 2. The positive control, BHT, exerted potent activity with EC_{50} value of 5.55 µg/ml. *T. citrina* exerted the highest $ABTS^{\bullet+}$ scavenging activity with EC_{50} of 4.16 ± 0.11µg/ml. Four plant extracts exerted $ABTS^{\bullet+}$ scavenging activity more than BHT. The ratio between EC_{50} of BHT were calculated and shown in Table 3. *T. citrina* about exerted $ABTS^{\bullet+}$ scavenging activity more than BHT 1.33 times following with *C. sappan* (1.07), *T. bellirica* (1.04), and *S. aromaticum* (1.01). Bhamrung-Lohit remedy also exerted $ABTS^{\bullet+}$ scavenging activity with EC_{50} of 12.23 ± 1.03 mg/ml.

Determination of Total Phenolics

Total phenolic contents of all extracts were also determined as mg equivalent to gallic acid per gram of each extract (mg GAE/g). *C. sappan* showed the highest total phenolic content with value of 781.72 mg GAE/g, following with *T. citrina* (620.30 mg GAE/g) and *S. garrettiana* (553.22 mg GAE/g). Bhamrung-Lohit remedy showed the total phenolic content was 269.38 mg GAE/g. Six plant extracts contained total phenolic contents between 100 - 199 mg GAE/g; *D. coccus* (199.14), *M. ferrea* (172.34), *M. siamensis* (144.28), *M. elengi* (113.00), *A. mariana* (101.85), and *N. nucifera* (101.82) (Table 2).

Table 2 The total phenolic content and *in vitro* antioxidant activities of Bhamrung-Lohit remedy and its plant components (n = 3)

Species	Total phenolic content (mg GAE/g)	EC_{50} of DPPH assay (µg/ml)	EC_{50} of ABTS assay (µg/ml)	FRAP assay	
				TEAC (mg Trolox eq./g)	FRAP (mg Fe^{2+} eq./g)
Bhamrung-Lohit remedy	269.38 ± 12.88	7.47 ± 0.28	12.23 ± 1.03	338.28 ± 9.13	795.06 ± 20.69
Caesalpinia sappan L.	781.72 ± 21.19	5.68 ± 0.17	5.19 ± 0.38	1,223.14 ± 25.26	2,885.64 ± 57.27
Bixa Orellana L.	343.69 ± 8.12	4.92 ± 0.08	5.67 ± 0.11	373.21 ± 15.77	874.24 ± 35.74
Dactylopius coccus	199.14 ± 6.37	10.30 ± 0.74	17.68 ± 0.51	137.93 ± 5.28	340.95 ± 11.97
Zingiber officinale Roscoe	15.04 ± 0.63	≥100	≥100	23.37 ± 3.24	81.27 ± 7.33
Piper retrofractum Vahl	14.71 ± 0.59	≥100	≥100	20.42 ± 0.55	70.45 ± 1.24
Plumbago indica L.	15.16 ± 1.24	53.74 ± 3.11	≥100	25.37 ± 1.91	85.81 ± 4.33
Piper ribesioides Wall.	63.78 ± 1.66	44.87 ± 2.21	65.58 ± 1.68	88.32 ± 4.30	228.49 ± 9.75
Piper sarmentosum Roxb.	22.21 ± 0.09	59.37 ± 1.84	≥100	26.26 ± 1.03	87.83 ± 2.33
Arcangelisia flava (L.) Merr.	50.64 ± 2.79	49.15 ± 2.91	70.33 ± 0.65	91.30 ± 4.91	235.24 ± 11.12
Urceola rosea (Hook. & Arn.)	390.11 ± 8.40	6.56 ± 0.39	10.43 ± 0.03	417.87 ± 6.38	975.48 ± 14.45
Anaxagorea luzonensis A. Gray	480.84 ± 5.08	7.31 ± 0.49	9.86 ± 0.20	402.09 ± 6.07	939.71 ± 13.77
Mammea siamensis Kosterm.	144.28 ± 4.07	13.40 ± 0.88	22.32 ± 1.11	205.94 ± 5.32	495.09 ± 12.07
Mimusops elengi L.	113.00 ± 7.55	15.87 ± 1.07	27.60 ± 2.58	144.23 ± 9.00	316.91 ± 17.92
Mesua ferrea L.	172.34 ± 6.51	12.92 ± 0.94	23.12 ± 0.43	220.90 ± 8.65	529.00 ± 19.60
Nelumbo nucifera Gaertn.	101.82 ± 4.29	13.59 ± 0.12	33.77 ± 1.01	150.83 ± 1.37	370.18 ± 3.10
Myristica fragrans Houtt. (aril)	8.32 ± 0.60	74.22 ± 3.93	≥100	91.01 ± 3.41	198.47 ± 0.40
Myristica fragrans Houtt. (seed)	11.67 ± 0.45	≥100	≥100	21.57 ± 4.23	77.19 ± 9.58
Amomum verum Blackw.	31.37 ± 3.57	87.62 ± 0.18	69.38 ± 5.77	51.74 ± 1.51	145.57 ± 3.42
Syzygium aromaticum (L.) Merr. & L.M.Perry	429.33 ± 5.44	5.36 ± 0.21	5.48 ± 0.23	850.88 ± 33.97	2,023.00 ± 77.00
Nigella sativa L.	19.51 ± 0.76	79.38 ± 4.46	≥100	27.08 ± 1.29	89.68 ± 2.93
Lepidium sativum L.	45.88 ± 1.56	61.96 ± 0.30	63.59 ± 2.79	63.55 ± 2.65	172.36 ± 6.00
Cuminum cyminum L.	37.05 ± 0.55	43.47 ± 3.69	84.37 ± 3.63	58.11 ± 3.12	160.01 ± 7.07
Foeniculum vulgare Miller subsp. var. vulgare	33.95 ± 0.96	44.29 ± 2.57	71.52 ± 2.59	44.90 ± 2.41	130.06 ± 5.46
Anethum graveolens Linn.	32.07 ± 1.69	61.36 ± 1.69	64.92 ± 5.55	29.09 ± 2.74	108.22 ± 7.78
Angelica dahurica Benth.	10.74 ± 0.63	≥100	≥100	12.89 ± 1.64	57.51 ± 3.71
Atractylodes lancea (Thunb.) DC.	13.40 ± 0.79	≥100	≥100	24.69 ± 2.17	84.27 ± 4.92
Ligusticum sinense Oliv. cv. Chuanxiong Hort	24.03 ± 0.18	52.71 ± 2.36	92.74 ± 5.72	36.24 ± 1.49	104.87 ± 5.91
Angelica sinensis (Oliv.) Diels	8.13 ± 1.19	≥100	≥100	4.45 ± 0.03	38.4 ± 0.07
Artemisia annua L.	32.86 ± 1.00	43.29 ± 1.75	55.72 ± 3.53	50.26 ± 1.46	148.92 ± 6.97
Terminalia chebula Retz.	278.46 ± 11.80	5.92 ± 0.25	5.96 ± 0.45	248.73 ± 2.63	592.09 ± 5.96
Terminalia citrina Roxb. ex Fleming	620.30 ± 8.45	4.51 ± 0.21	4.16 ± 0.11	307.00 ± 0.51	724.18 ± 1.15
Terminalia bellirica (Gaertn.) Roxb.	322.33 ± 14.45	4.87 ± 0.27	5.31 ± 0.15	478.89 ± 8.57	1,113.77 ± 19.42
Alyxia reinwardtii Blume.	64.32 ± 0.38	≥100	74.02 ± 3.50	55.39 ± 1.79	153.86 ± 4.05
Cinnamomum verum J. Presl	307.61 ± 4.47	7.00 ± 0.71	9.99 ± 1.10	324.49 ± 15.48	763.82 ± 35.09

Table 2 The total phenolic content and *in vitro* antioxidant activities of Bhamrung-Lohit remedy and its plant components (n = 3) (Cont.)

Species	Total phenolic content (mg GAE/g)	EC_{50} of DPPH assay (µg/ml)	EC_{50} of ABTS assay (µg/ml)	FRAP assay	
				TEAC (mg Trolox eq./g)	FRAP (mg Fe^{2+} eq./g)
Dracaena cochinchinensis (Lour.) S.C.Chen	306.65 ± 13.04	11.12 ± 0.39	8.87 ± 0.13	237.69 ± 2.29	567.06 ± 5.18
Senna garrettiana (Craib) H.S.Irwin & Barneby	553.22 ± 10.63	5.73 ± 0.11	6.37 ± 0.11	402.24 ± 23.60	939.61 ± 30.88
Avicennia marina (Forssk.) Vierh.	101.85 ± 15.81	27.39 ± 0.57	31.57 ± 1.17	229.88 ± 2.87	309.46 ± 2.79
Aquilaria crassna Pierre ex Lecomte	69.46 ± 3.27	84.65 ± 4.09	44.78 ± 2.26	150.26 ± 8.44	204.72 ± 13.22
BHT	-	13.78 ± 0.23	5.55 ± 0.23	279.34 ± 8.96	661.48 ± 20.32

Note: BHT was used as a positive control for antioxidant activity

Ferric ion Reducing Antioxidant Power (FRAP) Assay

Antioxidant activity by FRAP assay is the determination of reduction power of the extract. With regard to TEAC, the antioxidant activity (reduction power) of the extracts were calculated from standard curve of Trolox and presented in the unit of Trolox equivalent per gram of extract (mg Trolox eq./g). For FRAP value, the antioxidant activity of extracts were calculated from the standard curve of $FeSO_4$ and presented in unit of mg Fe^{2+} per gram of extract (mg Fe^{2+} eq./g). Standard curves of both Trolox and $FeSO_4$ had the coefficient of determination (r^2) more than 0.999. BHT was used as the positive control. The results of TEAC and FRAP value are shown in Table 2 and the relative ratio between the extracts and BHT are shown in Table 3. The positive control, BHT showed TEAC of 279.34 ± 8.96 mg Trolox eq./g and FRAP value of 661.48 ± 20.32 mg Fe^{2+} eq./g. *C. sappan* extract showed the highest TEAC with value of 1,223.14 ± 25.26 mg Trolox eq./g. It was shown to have 4.38 times the antioxidant activity of BHT. *T. bellirica* showed the highest FRAP value of 2,885.64 ± 57.27 mg Fe^{2+} eq./g, which was 4.68 times of BHT. Bhamrung-Lohit remedy also had higher antioxidant activity than BHT with TEAC value of 338.28 ± 9.13 mg Trolox eq./g (1.21 times) and FRAP value of 795.06 ± 20.69 mg Fe^{2+} eq./g (1.20 times). There were 9 extracts that had higher antioxidant activity than BHT.

Table 3 Total phenolic content and antioxidant activity of Bhamrung-Lohit remedy and its plant components in comparision with BHT

Species	Total phenolic content	EC_{50} of DPPH / BHT	EC_{50} of ABTS / BHT	FRAP assay	
				TEAC / BHT	FRAP / BHT
Bhamrung-Lohit remedy	++	1.84	0.45	1.21	1.20
Caesalpinia sappan L.	+++++	2.43	1.07	4.38	4.36
Bixa Orellana L.	+++	2.80	0.98	1.34	1.32
Dactylopius coccus	+	1.34	N.D.	0.49	0.51
Urceola rosea (Hook. & Arn.)	+++	2.10	0.53	1.50	1.47
Anaxagorea luzonensis A.Gray	++++	1.89	0.56	1.44	1.42
Mammea siamensis Kosterm.	+	1.03	N.D.	0.74	0.75
Mimusops elengi L.	+	0.87	N.D.	N.D.	0.48
Mesua ferrea L.	+	1.07	N.D.	0.79	0.80
Nelumbo nucifera Gaertn.	+	1.01	N.D.	N.D.	0.56
Syzygium aromaticum (L.) Merr. & L.M.Perry	++++	2.57	1.01	3.05	3.06
Terminalia chebula Retz.	++	2.33	0.93	0.89	0.89
Terminalia citrina Roxb. ex Fleming	+++++	3.06	1.33	1.10	1.09
Terminalia bellirica (Gaertn.) Roxb.	+++	2.83	1.04	1.71	4.68
Cinnamomum verum J. Presl	+++	1.97	0.55	1.16	1.15
Dracaena cochinchinensis (Lour.) S.C.Chen	+++	1.24	0.62	0.85	0.86
Senna garrettiana (Craib) H.S.Irwin & Barneby	++++	2.40	0.87	1.44	1.42
Avicennia marina (Forssk.) Vierh.	+	N.D.	N.D.	N.D.	N.D.

Note: N.D. = Not determine
+ = amounts phenolic compound in the range of 100-199 mg GAE/g.
++ = amounts phenolic compound in the range of 200-299 mg GAE/g.
+++ = amounts phenolic compound in the range of 300-399 mg GAE/g.
++++ = amounts phenolic compound in the range of 400-599 mg GAE/g.
+++++ = amounts phenolic compound in the range of 600-799 mg GAE/g.

Correlation Analysis

The correlation analysis was evaluated from the correlation coefficient (R) of the best fit regression model between two variables. As shown in Figure 1, DPPH and ABTS$^{\bullet+}$ radical scavenging activity related to the total phenolic content with exponential regression model in negative direction. The correlation coefficient presenting the high relationship with the value of 0.82 for DPPH and 0.95 for ABTS$^{\bullet+}$. The correlation between the reduction power by FRAP assay and the total phenolic content is shown in Figure 2. The FRAP assay, both TEAC and FRAP value, highly related to total phenolic content was calculated using linear regression model with the R value of 0.88 and 0.86, respectively.

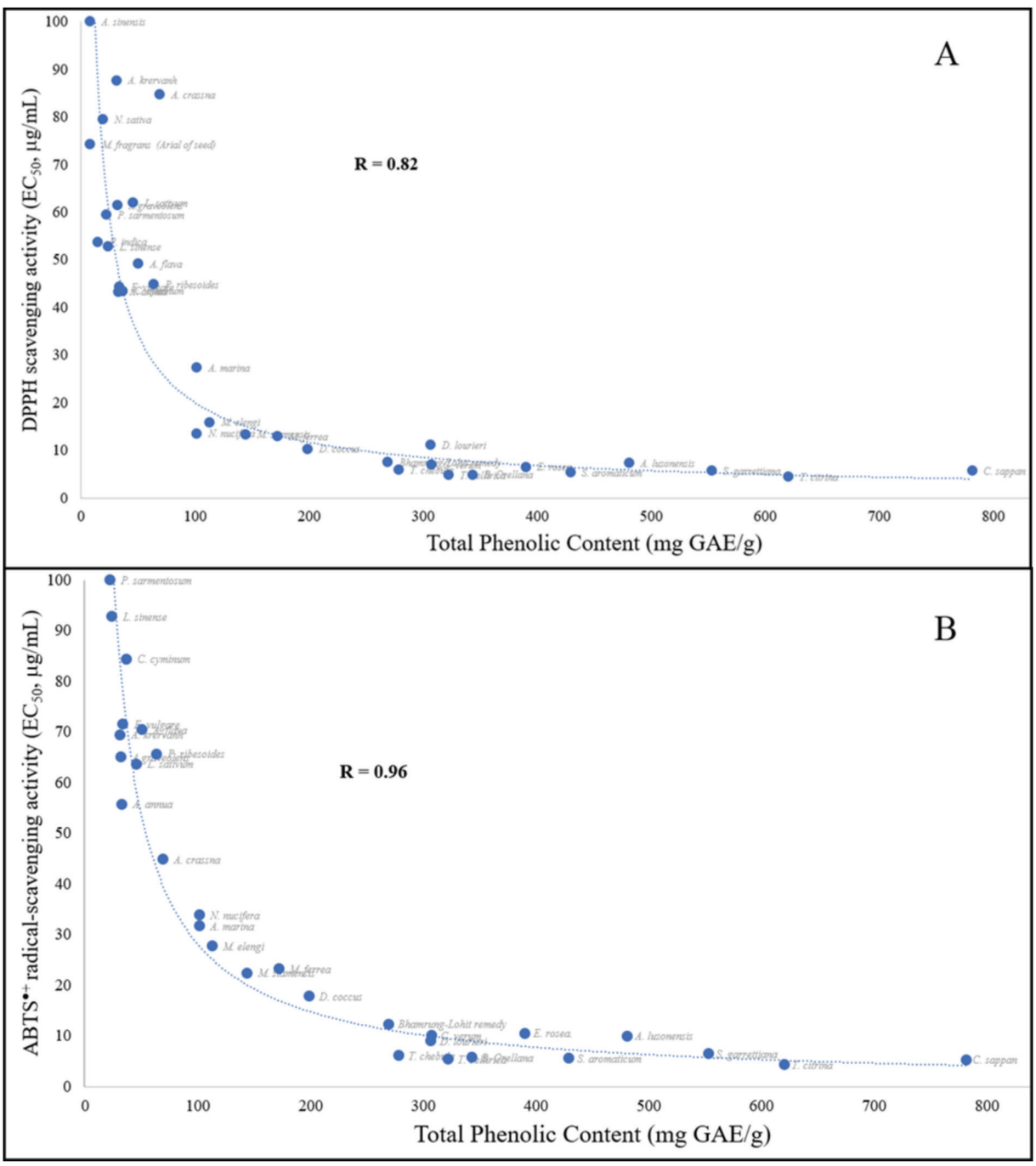

Figure 1 Correlation between DPPH scavenging activity and total phenolic content (A), ABTS$^{\bullet+}$ and total phenolic compound (B).

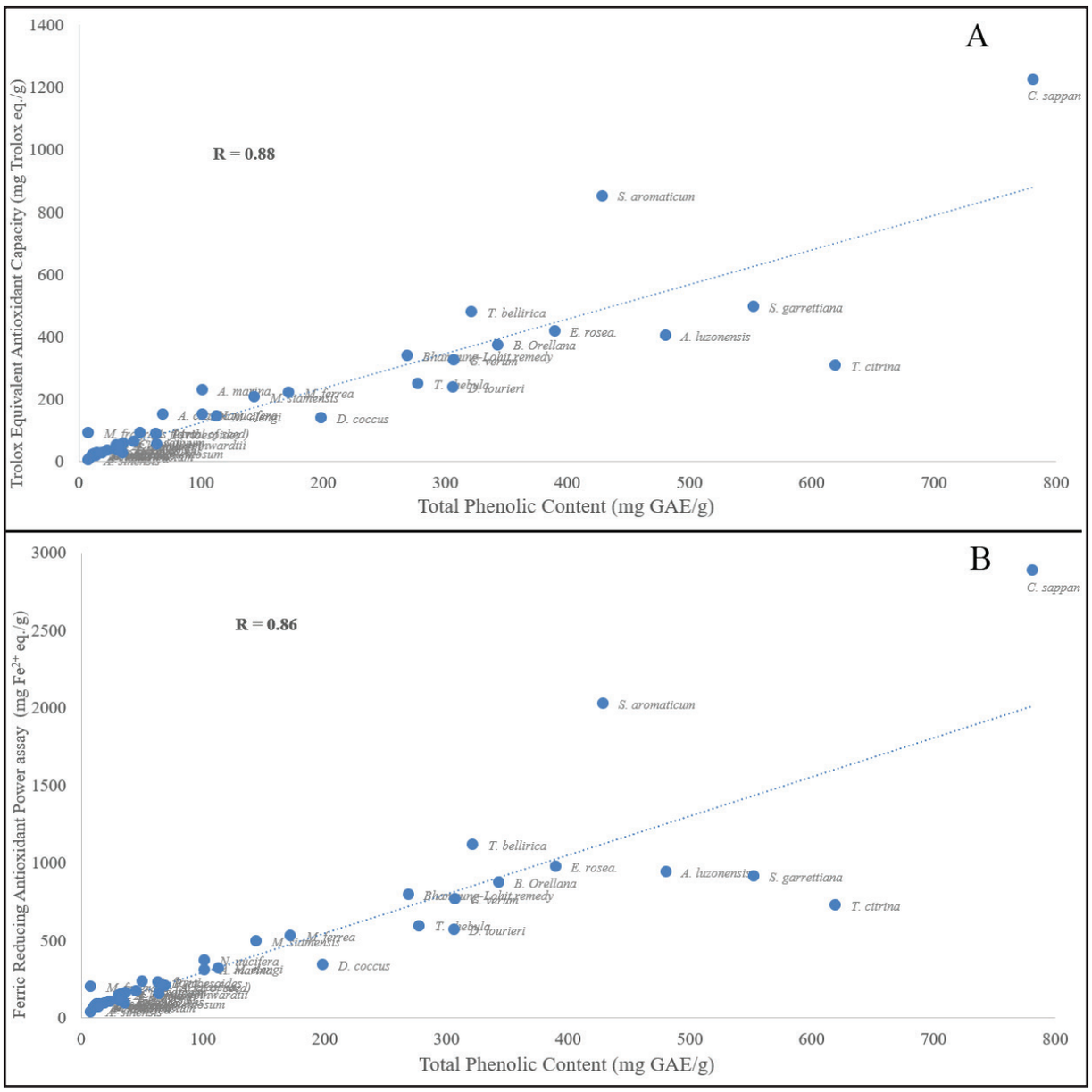

Figure 2 Correlation between FRAP assay and total phenolic content; TEAC (A) and FRAP value (B).

Discussion

Bhamrung-Lohit remedy, a traditional Thai medicine in Mahachotarata scripture has long been utilized for blood tonic in women. Our study showed the antioxidant activity of the Bhamrung-Lohit remedy and its plant ingredients. The remedy and several plant ingredients showed high antioxidant activities in all assays. Bhamrung-Lohit remedy stronger antioxidant activity than BHT in all assay except $ABTS^{\bullet+}$ scavenging activity. However, it exerted high $ABTS^{\bullet+}$ scavenging activity with EC_{50} of 12.23 µg/ml. Exhaustive review showed that the antioxidant activity of the Bhamrung-Lohit remedy has never been reported before. This study is the first study reporting the antioxidant activity of the Bhamrung-Lohit remedy. The correlation between the antioxidant activity of plant ingredients and the total phenolic content was also evaluated. DPPH and $ABTS^{\bullet+}$ scavenging activities were correlated to the total phenolic content with exponential regression model in negative direction. The FRAP assay correlated to total phenolic content with linear regression model. All correlation coefficients were more than 0.7 (in all positive and

negative direction) which showed high correlation between two variables.[12] The correlation results demonstrated that the extracts with higher phenolic contents showed stronger antioxidant activities.

The major constituents in Bhamrung-Lohit remedy were *C. sappan, B. Orellana,* and *D. coccus* which were used as blood tonics according to Thai traditional medicine (TTM). Most of them showed better activity than BHT (Table 3). The three extracts from plants which gave better activities than BHT in all tested activities were *T. bellirica, T. citrina,* and *S. aromaticum*. The Thai traditional use of *S. aromaticum* is for promoting blood circulation and carminative, i.e. correcting the imbalanced Vata. *T. bellirica* and *T. citrina* are used as laxative for the correction of Vata imbalance. Other four extracts which showed higher antioxidant activities than BHT in three of four assays were *S. garettiana*, *A. luzonensis, C. verum,* and *U. rosea. S. garettiana* has been used in gynecological problems, *A. luzonensis* for blood tonic, *C. verum,* and *U. rosea* traditionally used as carminative for the correction of Vata imbalance. Other plants including *M. sinensis, M. elengi, M. ferrea, N. nucifera,* and *D. cochinchinensis* are cardiotonic herbs in TTM. Other plant components which showed low antioxidant properties but were included in the formula for restoring normalcy to the four elements to normal condition, according to the theory of Thai Traditional Medicine theory.[13]

Our findings supported the use of Bhamrung-Lohit remedy as blood tonic due to their antioxidant properties which were essential to healthy red blood cells or erythrocytes. This study demonstrated that Bhamrung-Lohit remedy and its plants component exerted good antioxidant activities by being free radical scavengers and also good reducing properties which could promote healthy red blood cells. Therefore, these results support the use of this preparation as blood tonic.

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