Research Article

การศึกษาทางพฤกษเคมี ปริมาณฟีนอลิกทั้งหมดและฤทธิ์ต้านอนุมูลอิสระของตำรับยาบำรุงเลือดที่ได้จากแขวงเวียงจันทน์ สปป. ลาว

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บทคัดย่อ

การศึกษาทางพฤกษเคมี ปริมาณฟีนอลิกทั้งหมดและฤทธิ์ต้านอนุมูลอิสระของตำรับยาบำรุงเลือดที่ได้จากแขวงเวียงจันทน์ สปป. ลาว

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ว. เภสัชศาสตร์อีสาน 2562; 15(1) : 181-194

รับบทความ : 10 เมษายน 2561 **แก้ไขบทความ:** 8 ตุลาคม 2561 **ตอบรับ:** 29 ตุลาคม 2561

สาธารณรัฐประชาธิปไตยประชาชนลาวหรือ สปป.ลาว ยังคงเป็นแหล่งทรัพยากรสมุนไพรหลากหลายชนิด ที่ใช้ในยาแผนโบราณเพื่อป้องกันและรักษาโรคหรือเพื่อส่งเสริมสุขภาพ **วัตถุประสงค์:** การศึกษานี้มีวัตถุประสงค์เพื่อทำการศึกษาและเปรียบเทียบองค์ประกอบทางพฤกษเคมี ปริมาณฟีนอลิกทั้งหมด และฤทธิ์ต้านอนุมูลอิสระของตำรับยาบำรุงเลือดจากแขวงเวียงจันทน์ สปป.ลาว **วิธีการศึกษา:** นำสมุนไพรในตำรับยาบำรุงเลือดที่ประกอบด้วยสมุนไพร 4 ชนิด คือ *Knema globularia* (Myristicaceae) หรือเลือดแรด, *K. furfuracea* (Myristicaceae) หรือเลือดควายใบใหญ่, *Endosamara racemosa* (Fabaceae) หรือหางไหลและ *Dendrophthoe pentandra* (Loranthaceae) หรือกาฝากมะม่วงไปทำการศึกษาทางพฤกษเคมี และทำการสกัดด้วยน้ำโดยการนำไปต้มตามแบบที่ใช้จริงและสกัดเอทานอลด้วยการหมัก สารสกัดที่ได้นำไปศึกษาเพื่อหาปริมาณฟีนอลิกและสมรรถภาพการต้านอนุมูลอิสระในวิธี DPPH ABTS FRAP และ ORAC. **ผลการศึกษา:** การศึกษาทางพฤกษเคมีของตัวอย่างพืชทั้งหมด พบผลบวกในการตรวจสารกลุ่มฟลาโวนอยด์ เทอร์พีนอยด์ สเตอรอยด์และแทนนิน ปริมาณฟีนอลิกทั้งหมดและฤทธิ์ต้านอนุมูลอิสระ พบค่าสูงสุดในสารสกัดเอทานอลของ *E. racemosa* โดยมีค่าเท่ากับ 273.02 มิลลิกรัมสมมูลย์กรดแกลลิกต่อกรัมสารสกัดและ IC_{50} เท่ากับ 26.80 มิลลิกรัมต่อมิลลิลิตร (วิธี DPPH) 49.43 มิลลิกรัมต่อมิลลิลิตร (วิธี ABTS), 297.14 มิลลิกรัมสมมูลย์โทรลอกซ์ต่อกรัมสารสกัด (วิธี FRAP) ตามลำดับ สารสกัดเอทานอลจาก *D. pentandra* ให้ค่าการต้านอนุมูลอิสระในวิธี ORAC สูงสุด (3385.77 ไมโครโมลสมมูลย์โทรลอกซ์ต่อกรัมสารสกัด) ตำรับยาบำรุงเลือดและสมุนไพรแต่ละตัวในตำรับมีฤทธิ์ต้านอนุมูลอิสระสูง แต่ยังต่ำกว่าสารสกัดเดี่ยวของ *E. racemosa* **สรุปผลการศึกษา:** ผลการศึกษานี้สนับสนุนการใช้ยาแบบดั้งเดิมของตำรับสมุนไพรที่ถูกเลือก สมุนไพรที่ทำการศึกษาโดยเฉพาะ *E. racemosa* เป็นสมุนไพรที่มีศักยภาพเป็นแหล่งของสารต้านอนุมูลอิสระที่ดี

คำสำคัญ: สปป.ลาว, ต้านอนุมูลอิสระ, ฟีนอลิกทั้งหมด, การตรวจพฤกษเคมี, ตำรับยาบำรุงเลือด

Phytochemical screening, total phenolic contents and antioxidant capacities of selected blood tonic formula from Vientiane province, Lao PDR

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Abstract

Phytochemical screening, total phenolic contents and antioxidant capacities of selected blood tonic formula from Vientiane province, Lao PDR

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IJPS, 2019; 15(1) : 181-194

Received: 10 April 2018 **Revised:** 8 October 2018 **Accepted:** 29 October 2018

Lao People's Democratic Republic or Lao PDR is still a plenty source of medicinal plants used in traditional medicine for treating or preventing the illness as well as health maintenances. **Objectives:** The aims of this work were to investigate and compare the phytochemical screening, total phenolic content and antioxidant activities of blood tonic formula from Vientiane province, Lao PDR. **Methods:** The blood tonic formula and each herb that consisted of *Knema globularia* (Myristicaceae), *K. furfuracea* (Myristicaceae), *Endosamara racemosa* (Fabaceae) and *Dendrophthoe pentandra* (Loranthaceae) to determine phytochemical screening test. Then, prepared the water extracts by boiling to imitate real use and ethanol extracts by maceration. The crude extracts were determined total phenolic contents and antioxidant activities using DPPH ABTS FRAP and ORAC assays. **Results:** *In vitro* phytochemical screening for all plants showed positive results for flavonoids, terpenoids, steroids and tannins. The ethanol extract of *E. racemosa* displayed the highest total phenolic content (273.02 mg GAE/ g extract) and also exhibited the highest antioxidant activity with IC_{50} values of 26.80 µg/ml (DPPH assay), 49.43 µg/ml (ABTS assay) and 297.14 mg trolox equivalent/g extract (FRAP assay), respectively. Ethanol extract of *D. pentandra* showed the highest ORAC antioxidant activity (3385.77 µM trolox equivalent/ g extract). This blood tonic formula and its individual herbs showed the relatively high antioxidant activity but lower than *E. racemosa* extract. **Conclusion:** The results of this study support the ethnomedicinal uses of the selected formula. The selected medicinal plants especially *E. racemosa* are potential sources of antioxidants.

Keywords: Lao PDR, Antioxidant, Total Phenolic, Phytochemical screening, Blood tonic formula

Introduction

In Lao PDR, traditional medicine is commonly used at childbirth and to treat sick family members. The reasons for the use of them include its perceived efficacy, accessibility, and lower cost compared to modern medicine. Their utilization is officially supported and strongly promoted for keeping with a strong cultural heritage but also reflects the recognition that traditional medicine has a role in addressing the problem of access to, and cost of, modern medicine (World Health Organization and Ministry of Health, 2012). Ministry of Health, Lao PDR set up the Traditional Medicine Research Center (TMRC), with the purpose of studying and incorporating "the use of medicinal plants and Lao traditional medicine into current and more modern healthcare systems" (Libman *et al.*, 2006). Several researchers have investigated the effects of phytochemicals especially from poly-herbal formula in protection against degenerative diseases including, atherosclerosis, diabetes, cardiovascular diseases, as well as to promote and maintain health (Saeed *et al.*, 2012) by focusing on increases in the antioxidant defense system (Xu and Howard, 2012). In various pathophysiological processes implicated with the free radicals (Wallace, 1997). Antioxidants are compounds that can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged (Oroian and Escriche, 2015). Antioxidant activities of medicinal plants, such as those possessing anticancer activities, antiviral actions, heat-clearing property, nutritious and tonic functions have been evaluated (Gen *et al.*, 2010).

Vientiane is the one province in Lao PDR. It has plenty of natural resources included the medicinal plant preservation garden and there are traditional healers have used the medicinal plants in the area to treat the ailments and they mostly possess knowledge passed down from generation to generation. One particular blood tonic formula extensively used in the area and the formula consisted of four herbs, *Knema globularia* (Myristicaceae): Leuad Nok or Leuat Raet, *K. furfuracea*: Waa Lued or Leuat Kuay Bai Yai, *Endosamara racemose* (Fabaceae): Doo Lued or Hang Lai and *Dendrophthoe pentandra* (Loranthaceae): Phark Mone or Ka Fak Ma Muang (Figure 1). It was formulated by ancient traditional healer in Vientiane province and used as a blood tonic, cardiotonic, neurotonic, emmenagogue, increasing milk volume after childbirth and orexigenic. This formula is prepared by decoction of 4 herbs with ratio 1:1:1:1 and drink a glass of cooled filtrate instead of water until the symptoms are relieved (Institute of Traditional Medicine, 1997). According to the extensive literature reviews, *K. furfuracea* is used the stem barks for the treatment of sores and pimples (Pinto *et al.*, 1990) and the barks of *K. globularia* are used as blood tonic (Wiart, 2006). Plants in the genus *Knema* were possessed the potent free radical scavenging activities and phytochemical screening demonstrated the presence of different types of compounds which could be responsible for the biological activities (Phadungkit *et al.*, 2010); and found the neuroprotective effect and protection against H_2O_2- and Aβ-induced cell death (Ismail *et al.*, 2015). *Dendrophthoe pentandra* was found the isolated compound as flavonol glycoside (quercetrin) which is an active antioxidant (Artanti *et al.*, 2006) as well as a potential source of antidiabetes compounds (Artanti *et al.*, 2012; Fitrilia *et al.*, 2015). In Indonesia, *D. pentandra* is used to treat hypertension, diabetes, coughs, use–as a diuretic agent and possess potent anticancer activity (Endharti and Permana, 2017).

Until recently, these medicinal plants in Lao PDR have no studied in their activities. Therefore, the main objectives of this study were to perform the phytochemical screening test, to determine the total phenolic contents and the antioxidant activities of this blood tonic formula. Blood tonic formula denotes the combination of physical and energetic properties functions to nourish, vitalize the whole body while providing functional support to the kidney, spleen, heart and lung (Wang *et al.*, 2010). Overall results from antioxidant assays of this study will be useful as the affirmation for the benefits of blood tonic formula as health maintenance products.

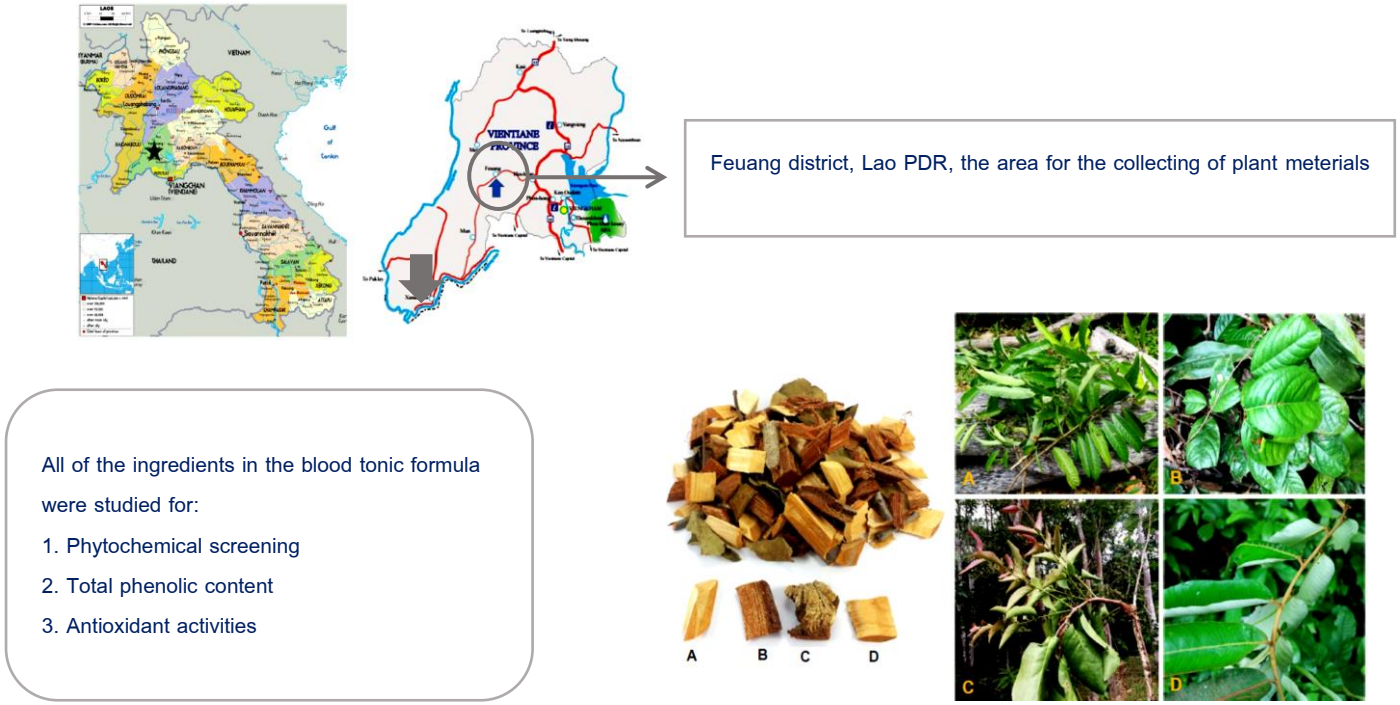

Figure 1: The map of the area where the blood tonic formula was collected in this study (The country map and region map). Blood tonic formula consisted of 4 medicinal plants including *Knema globularia* (A), *Endosamara racemosa* (B), *Dendrophthoe pentandra* (C) and *K. furfuracea* (D).

Materials and methods

Chemicals

Ethanol, methanol, gallic acid, sodium carbonate, 2,2'-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-Tris(2-pyridyl)-s- triazine (TPTZ), ferric (III) chloride, 2,2'- azino- bis(3-ethylbenzothiazoline- 6- sulfonate) (ABTS), potassium persulphate, 2,2'- azobis(2- methylpropaneimidamide) dihydrochloride (AAPH) and fluorescein were purchased from Sigma Aldrich (USA) except Folin- Ciocalteau reagent was purchased from Loba (India).

Plant materials

Plant materials were collected in Feuang district, Vientiane province, Lao PDR. All of them were identified and confirmed the scientific name by botanist (Prof. Dr. Bounhong Southavong, from Institute of Traditional Medicine, Ministry of Health) and taxonomist (Soulivanh Lanorsavanh, from Faculty of Natural Sciences, National University of Laos). The part used and voucher codes were summarized on Table 1.

Table 1: Plant materials in this study

Herbs	Voucher codes	Part used
Dendrophthoe pentandra (L.) Miq. (Loranthaceae) or DP	FD130	Whole plant
Endosamara racemosa (Roxb.) R. Geesink (Fabaceae) or ER	FD131	Vine
Knema furfuracea (Hook. f. & Thomson) Warb. (Myristicaceae) or KF	FD095	Stem-twig
K. globularia (Lam.) Warb. (Myristicaceae) or KG	FD128	Stem-twig

Crude extract preparation

All plant materials were washed, dried with sunlight, cut to small pieces and dried again in hot air oven at 40 ºC until plants were completely dried. The dried plants were ground to be coarse powder by grinder; the powders were kept in the zip-locked bags and stored in dry area (Elkington *et al.*, 2009). 60 g of each plant powders were macerated with 1000 mL of ethanol 95%, at 25 ºC for 7 days. The maceration with solvent was conducted three times. The extract solvents were filtered and evaporated by rotary evaporator (BUCHI Labortechnik AG, Switzerland) until the solvents were completely removed. For the water extraction, 60 g of each plant powders were boiled in 2 liters of water to imitate the actual preparation (conduct only 1 time with the temperature around 85-90 ºC) for 30 minutes. The extract solvents were then filtered and the water was removed by using freeze dryer (SCANVAC coolsafe 110-4 Pro, Denmark), store all crude extracts at 4 ºC until the antioxidant assay experiments (Sheikh *et al.*, 2013).

Phytochemical screening test

Each of the selected herbs was subjected to preliminary phytochemical screening to identify its phytochemical constituents. Ethanol or methanol extract prepared by 10 g of dried powder taken in 50 mL of solvent (methanol or ethanol) in a conical flask, plugged with cotton wool and then kept on a rotary shaker for 24 hours. After 24 hours, the extract solvents were filtered, the filtrates were collected, evaporated and the extracts were stored at 4°C in airtight bottles (Department of Pharmacognosy and Toxicology, 2015). Powders or extract of the each plant materials was then analyzed by specific reactions; the color of the extracts and precipitates were observed for identification. The details of each phytochemical screening test were as follows:

Alkaloids test (Fitrilia *et al.*, 2015)

The methanol extract of each plant was evaporated to dryness in a boiling water bath. The residue was dissolved in 10 mL of 2 N HCl. The mixture was filtered and the filtrate was divided into 3 equal portions. One portion was treated with a few drops of Mayer's reagent; one portion was treated with equal amount of Dragendorff's reagent and the other portion was treated with equal amount of Hager reagent. The creamish precipitate, reddish orange precipitate and yellowish precipitate indicated the presence of respective alkaloids.

Tannins test (Iqbal *et al.*, 2015)

5 g of sample powder was boiled with 10 mL of deionized water, and filtered. A few drops of 5% ferric chloride were then added. Black or blue-green coloration or precipitate was taken as positive result for the presence of tannins.

Saponins test (Iqbal *et al.*, 2015)

10 g of sample powder was boiled with 30 mL of deionized water and then filtered. 3 mL of the aqueous extract solution were mixed with 10 mL of distilled water in a test-tube. The test-tube was stoppered and shaken vigorously for about 5 minutes; it was allowed to stand for 30 minutes and observed for honeycomb froth, which was indicative of the presence of saponins.

Anthraquinones test (Iqbal *et al.*, 2015)

10 mL of 1 N potassium hydroxide and 5 mL of hydrogen peroxide were added to 1 g of sample powder, mixed and gently boiled for 5 minutes. Filtered while hot, then cooled the filtrate and neutralized with 10 drops of glacial acetic acid. Equal volume of benzene was added, then the extract was partitioned and the layer of benzene was collected. Benzene layer was shaken with 5 mL of ammonium hydroxide. A rose pink to red color was produced in the ammoniac layer indicate the presence of anthraquinones.

Cardiac glycosides test (Gul *et al.*, 2017)

5 g of sample powder was boiled with 20 mL of 80 % ethanol for 5 minutes and filtered. 3 drops of 10 % lead acetate were added to the filtrate, boiled and stirred thoroughly, then filtered. Filtrate was shaken with 5 mL of chloroform in a separating funnel. The chloroform layer was evaporated to dryness in a small evaporating dish. The

residue was dissolved in a glacial acetic acid containing a trace of ferric chloride; this was transferred to the surface of 2 mL concentrated sulfuric acid in a test tube. The upper layer and interface of the two layers were observed for bluish-green and reddish-brown respectively as indicative of the presence of cardiac glycosides.

Flavonoids test (Gul *et al*., 2017)

Ethanol extract was mixed with few fragments of magnesium ribbon and concentrated HCl was added drop wise. Pink or magenta red color appeared after few minutes which indicated the presence of flavonoids.

Steroids test (Saeed *et al*., 2012)

10 g of sample powder was boiled with 30 mL of hydrochloric acid in ethanol on water bath for 30 minutes, then, filtered. Filtrate was extracted for three times with 30 mL of petroleum ether. Petroleum ether layer was collected, transferred to evaporating dish and evaporated to dryness. A solution of glacial acetic acid (4 mL) with 1 drop of 2% $FeCl_3$ mixture was mixed with the 10 mL of plant extract and 1 mL of H_2SO_4 concentrated. A brown ring formed between the layers which showed the entity of cardiac steroidal glycosides.

Terpenoids test (Saeed *et al*., 2012)

5 mL of chloroform was added to 0.05 g of ethanol extract, followed by 3 mL of sulfuric acid. Reddish brown color between the interfaces of solvent indicated the presence of terpenoids.

Total Phenolic contents

The total phenolic contents of the selected herbs and blood tonic formula were determined by using Folin-Ciocalteu reagent following a slightly modified method of Sen *et al*. (2013). Gallic acid was used as a reference standard. By serial dilutions, various concentrations of gallic acid ranged from 12.5-100 µg/mL were prepared from its stock dilution. The plant extracts were prepared in the concentration of 500 µg/mL. For sample measurement, 100 µL of Folin-Ciocalteau's reagent and 80 µL of 7% sodium carbonate were added to the 20 µL of sample in 96 well plates. The solutions were mixed and incubated at room temperature for 30 minutes. Absorbance at 765 nm was measured. Data presented are average values of three measurements for each sample. The total phenolic contents were expressed as mg of gallic acid equivalent (GAE)/g extract.

Antioxidant activities

DPPH assay (Dhanani *et al*. 2017)

Free radical scavenging of DPPH radical was measured *in vitro* adapted from the method of Baba and Malik (2015). The DPPH• solution 0.2 mM was prepared in 100 mL of methanol, wrapped and stored in refrigerator at 4 ºC until used. The reaction was carried out by mixing DPPH• solution with the crude extracts in various concentrations (31.25-1.000 mg/mL), incubated for 30 minutes in dark area at 25 ºC. Absorbance was read at 517 nm. Trolox was a standard and methanol was used as a control. For estimation of scavenging activity was based on the percentage of DPPH radical scavenged as equation (1) and then, the extract concentration corresponding to 50 percent inhibition (IC_{50}) was calculated from the curve of radical scavenging activity percentage against extract concentration. Each sample was assayed in triplicate for each concentration.

$$\text{Scavenging effect}(\%) = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100 \qquad (1)$$

$A_{Control}$ = was the absorbance of the control reaction

A_{sample} = was the absorbance in the presence of the sample

ABTS assay

The method of free radical scavenging test of ABTS was adapted from Thaipong *et al*. (2006). The $ABTS^{\bullet+}$ solution was prepared by reacting 7 mM of ABTS with 2.48 mM of potassium persulfate in 10 mL of deionized water, incubated in the dark at room temperature (25-27 ºC) for 12 hours prior to the study. Working solution was prepared by diluting $ABTS^{\bullet+}$ solution with methanol to obtain the absorbance of 0.7±0.02 at 734 nm. The samples and standard (Trolox) were prepared in various concentrations (31.25-1.000 mg/mL).

50 µL of samples/standard/control (methanol) were added into 96 well-plate and were reacted with working solution 100 µL, incubated in dark area for 15 minutes and measured at 734 nm. Results were reported in mg Trolox equivalent/ g extract (mg TE/ g extract) . Estimation of scavenging activity was based on the percentage of ABTS radical scavenged as (1) and then, the IC_{50} of each sample was calculated as described in DPPH assay.

FRAP assay

The method of Ferric Reducing Antioxidant Potential assay (FRAP assay) was adapted from Benzie and Straint (1996) and Songsermsakul *et al.* (2013). The FRAP reagent was prepared from dissolving 20 mM of Ferric chloride in deionized water (solution A), preparing 10 mM of TPTZ in 40 mM of HCl (solution B) and then, 300 mM of acetate buffer pH 3.6 in 16 mL of deionized water (solution C); mixed solution A, solution B and solution C in a volume ratio of 1:1:10. Exceptionally, the FRAP reagent was daily prepared and stored at 37°C before use. 150 µL of FRAP reagent was added to 50 µL of the sample/ standard/ blank. Determination was absorbed in 593 nm and recorded the result. The standard curve was constructed using Trolox and the results were expressed as mg TE/g extract.

ORAC assay

The ORAC assay was carried out according to the method of Zulueta *et al.* (2009). Sodium fluorescein stock solution (4.19×10^{-3} mM) was made using 75 mM potassium phosphate buffer (pH 7.4) and stored at 4°C. The reaction was performed by thermal decomposition of (2, 2'-azobis (2-amidino-propane) dihydrochloride) (AAPH) at 37 ºC. Fluorescein's stock solution was prepared by dissolving 44 mg of fluorescein in 100 mL of phosphate buffer, covered with tinfoil and kept in refrigerator. 0.167 mL of fluorescein stock solution was diluted in 25 mL of phosphate buffer and used as working solution. 600 mg of AAPH was dissolved in 10 mL phosphate buffer. 20 mM Trolox was prepared daily to use as a standard and kept at -20 ºC. The reaction was performed in 96 black-flat bottom wells. The standard curve was constructed and the results were expressed as mg TE/g extract.

50 µL of fluorescein solution was added to 50 µL of sample/standard then mixed and incubated for 15 minutes and 25 µL of AAPH was added last into the well. The reaction was measured immediately after the adding of AAPH. The ORAC values were calculated by the equation (2)

$$\text{Net AUC} = \text{AUC sample} - \text{AUC blank} \quad (2)$$

Statistical analysis

All data from the experiment were presented as mean ± standard deviation (SD). For *in vitro* antioxidant assays included total phenolic contents, the results were analyzed by one way ANOVA and P-value ≤ 0.05 was considered as significantly difference. Correlations between total phenolic contents and antioxidant activities were calculated using Pearson's correlation coefficient.

Results

Phytochemical screening test

The results of phytochemical screening test were showed on Table 2. The powders of blood tonic formula and all its ingredients were consisted of the terpenoids, steroids and flavonoids; almost all samples contained tannins, followed by saponins; only *Endosamara racemosa* (ER) contained alkaloids but no samples had anthraquinones and cardiac glycosides.

Table 2: The results of phytochemical screening test. Key: (+) Presence and (-) Absence. Blood tonic formula (BT), *Knema globularia* (KG), *Endosamara racemosa* (ER), *K. furfuracea* (KF) and *Dendrophthoe pentandra* (DP)

Methods	BT	KG	ER	KF	DP
Alkaloids					
Mayer	+	+	+	+	+
Dragendorff	-	-	+	-	-
Hager	+	+	+	+	+
Tannin	+	-	+	+	+
Saponin	+	-	+	-	+
Anthraquinone	-	-	-	-	-
Cardiac glycosides	-	-	-	-	-
Flavonoids	+	+	+	+	+
Steroids	+	+	+	+	+
Terpenoids	+	+	+	+	+

Percentage yield of crude extracts

The selected formula and their ingredients were extracted with water and 95% ethanol. The percentage yields were reported by the ratio of crude extract and weight of sample (w/w). The yield of ethanol extract of *E. racemosa* (ER) is highest among the ten crude extracts whereas the yield of ethanol extract of *K. globularia* (KG) is the lowest compared to the other crude extracts (Table 3).

Total phenolic contents

The total phenolic contents (TPC) in the crude extracts of herbs and blood tonic formula are presented in Table 3. TPC in the different herbs as determined by Folin-Ciocalteu assay, was expressed as gallic acid equivalents by reference to a standard curve ($y = 0.0036x + 0.0039$, $R^2 = 0.9915$). The TPC varied from 8.76 ± 0.85 to 273.02 ± 4.84 mg GAE/g extract. The results showed that ethanol extract of *E. racemosa* (ERE) has the highest phenolic concentration (273.02 ± 4.84 mg GAE/g extract), followed by ethanol extract of *D. pentandra* (DPE); 227.28 ± 5.48 mg GAE/g extract and water extract of *E. racemosa* (ERW) ;171.54 ± 2.85 mg GAE/g extract. Water extract of *K. globularia* (KGW) presented the lowest level of TPC (8.76 ± 0.85 mg GAE/g extract). Overall comparison, the content of the total phenolics decreased in the following order: from ethanol extract ERE>DPE>BTE>KFE>KGE and from water extract ERW>DPW>KFW>BTW>KGW. Among the different medicinal plant materials, ethanol extract and water extract of *E. racemosa*; ERE and ERW, significantly contained (p<0.05) higher total phenolic contents.

Table 3: Percentage yield of extracts of the formula and individual ingredients extracted by water and 95% ethanol. Blood tonic formula (BT), *Knema globularia* (KG), *Endosamara racemosa* (ER), *K. furfuracea* (KF) and *Dendrophthoe pentandra* (DP). TPC = Total phenolic content.

Samples		Percentage yield of extracts (% w/w)	TPC (mg GAE/g extract)
BT	Water	4.20	34.69±1.67[a]
	Ethanol	6.37	158.57±3.89[1]
KG	Water	3.62	8.76±0.85[b]
	Ethanol	5.60	88.94±3.70[2]
ER	Water	11.27	171.54±2.85[c]
	Ethanol	31.72	273.02±4.84[3]
KF	Water	4.58	39.69±4.81[d,a,e]
	Ethanol	3.03	101.35±0.56[4]
DP	Water	6.32	47.28±5.84[e,d]
	Ethanol	4.00	227.28±5.48[5]

[a b c d e] The mean difference of samples in water extract is significant at the 0.05 level.

[1 2 3 4 5] The mean difference of samples in ethanol extract is significant at the 0.05 level.

Antioxidant activities

Various models of *in vitro* antioxidant activity of the extracts were assessed to identify potential sources of substances possibly useful against the effects of free radicals. For DPPH assay (Table 4), the results showed that the various extracts of *E. racemosa* had a good scavenging effect. Ethanol extract, water extract of *E. racemosa* and ethanol extract of blood tonic formula showed the highest DPPH radical scavenging activity with IC_{50} values of 26.80 ± 1.10 µg/mL, followed by 30.32 ± 0.72 and 33.15 ± 0.93 µg/mL, respectively. Overall, ethanol extract of herbs and formula showed the higher DPPH scavenging activity than water extract. Ethanol and water extract of *E. racemosa* showed higher DPPH scavenging activity than positive control (Trolox), and other extracts (p<0.05).

According to the results from ABTS assay (Table 4), ethanol extract of *E. racemosa* showed the most potent activity with an IC_{50} of 49.43 ± 0.44 µg/ml, followed by water extract of *E. racemosa*; 56.30 ± 0.62 µg/ml and ethanol extract of *D. pentandra*; 62.49 ± 0.53 µg/ml. The water extract of *K. globularia* presented the weakest activity with an IC_{50} of 688.70 ± 2.30 µg/ml. ABTS assay results were similar with those of the DPPH assay. Ethanol and water extract of *E. racemosa* showed higher ABTS scavenging activity than positive control (Trolox), and other extracts.

The results (Table 4) for FRAP and ORAC assay were calculated from calibration curves which were linear over the calibration range with R^2 value of 0.9994 and 0.995, respectively. Trolox was used as a standard for both FRAP assay and ORAC assay. Overall, the FRAP values of the extracts varied from 57.99 ± 0.90 to 325.93 ± 3.51 mg TE/g extract. Among all the extracts, ethanol extract of blood tonic formula showed the highest FRAP value (325.93 ± 3.51mg TE/g extract), followed by ethanol extract of *K. globularia* (KGE); 302.78 ± 4.24 mg TE/g extract and water extract of

E. racemosa (ERW); 301.72 ± 2.31 mg TE/g extract. Water extract of *K. globularia* (KGW) presented the lowest FRAP value (57.99 ± 0.90 mg TE/g extract). For ORAC assay, results indicated that all extracts showed antioxidative protection against peroxyl radicals. ORAC values varied from 638.88 ± 0.44 to 5297.80 ± 0.77 μM TE/g extract (Table 4). In fact, ethanol extract of blood tonic (BTE) showed the highest antioxidant capacity (5297.80 ± 0.77 μM TE/g extract), followed by ethanol extract of *D. pentandra* (3385.77 ± 0.93 μM TE/g extract), and ethanol extract of *E. racemosa* (3118.94 ± 0.73 μM TE/g extract) extracts. In comparison to all of the extracts, the ethanol extract of blood tonic displayed the most interesting antioxidant activity. It was observed that ethanol extract of blood tonic formula (BT) showed the highest value for both FRAP assay and ORAC assay (p<0.05).

Table 4: The results of Total Phenolic content (TPC) and Antioxidant properties. Blood tonic formula (BT), *Knema globularia* (KG), *Endosamara racemosa* (ER), *K. furfuracea* (KF) and *Dendrophthoe pentandra* (DP)

Samples		DPPH (μg/ml)	ABTS (μg/ml)	FRAP (mg TE/g extract)	ORAC (μM TE/g extract)
BT	Water	305.88±4.02^{a}	481.52±0.34^{a}	106.09±2.21^{a}	1442.65±0.80^{a}
	Ethanol	33.15±0.93^{1}	89.41±1.38^{1}	325.93±3.51^{1}	5297.80±0.77^{1}
KG	Water	1139.80±0.97^{b}	688.70±2.30^{b}	57.99±0.90^{b}	638.88±0.44^{b}
	Ethanol	65.60±0.60^{2}	121.23±0.74^{2}	302.78±4.24^{2,3}	2606.45±0.74^{2}
ER	Water	30.32±0.72^{c}	56.30±0.62^{c}	301.72±2.31^{c}	2985.53±0.89^{c}
	Ethanol	26.80±1.10^{3}	49.43±0.44^{3}	297.14±4.24^{3,2}	3118.94±0.73^{3}
KF	Water	541.10±0.17^{d}	495.57±0.89^{d}	79.63±0.50^{d}	1362.60±0.42^{d}
	Ethanol	69.38±1.73^{4}	84.45±0.49^{4}	257.59±0.80^{4}	2836.25±0.92^{4}
DP	Water	268.62±0.51^{e}	317.38±1.59^{e}	128.99±1.05^{e}	1969.39±0.63^{e}
	Ethanol	38.06±1.65^{5}	62.49±0.53^{5}	275.66±4.78^{5}	3385.77±0.93^{5}
Trolox		84.18±0.09*	39.38±1.84*	-	-

$^{a\,b\,c\,d\,e}$ The mean difference of samples in water extract is significant at the 0.05 level.

$^{1\,2\,3\,4\,5}$ The mean difference of samples in ethanol extract is significant at the 0.05 level.

* The mean difference is significant at the 0.05 level.

The correlation between total phenolic contents and antioxidant activities

It was observed that the total phenolic contents of water extracts correlates well with all antioxidant assays. The best correlation was found in FRAP, ORAC and ABTS assays. The correlation coefficients of TPC (water extract) with FRAP assay, ORAC assay and ABTS assay were 0.9769, 0.8751 and 0.8638 (Figure 2B, 2D and 2C), respectively. Phenolic compounds are likely to contribute to radical scavenging activity of these plant extracts. Ethanol extracts of the selected herbs were also show the good correlation between TPC and ABTS and DPPH assay. The correlation coefficients of TPC (ethanol extract) with ABTS and DPPH assay were 0.8126 and 0.7775, respectively.

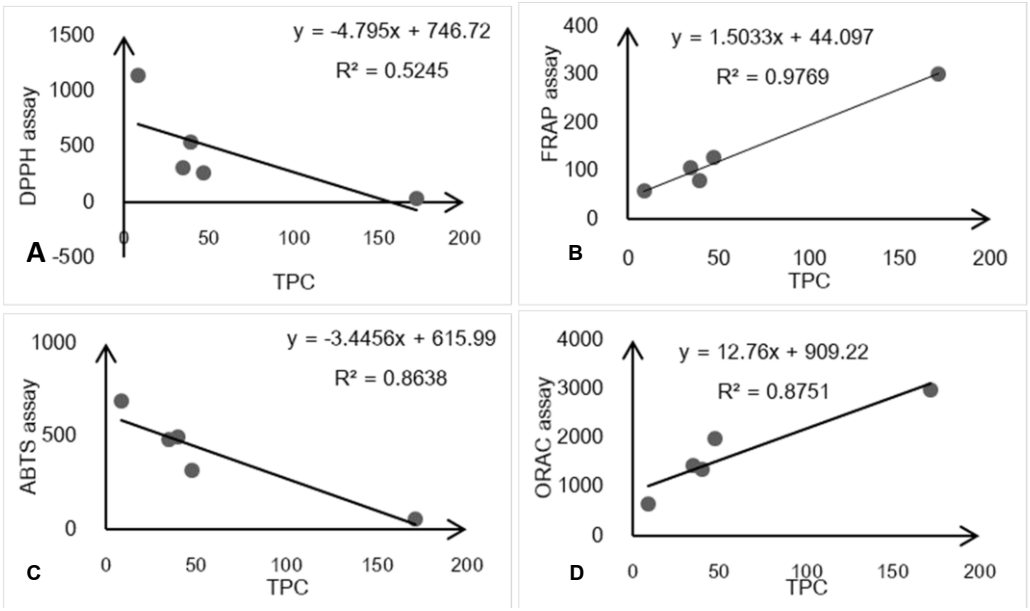

Figure 2: The correlation graphs of water extract between TPC and each of antioxidant activities. A is DPPH assay, B is FRAP assay, C is ABTS assay and D is ORAC assay.

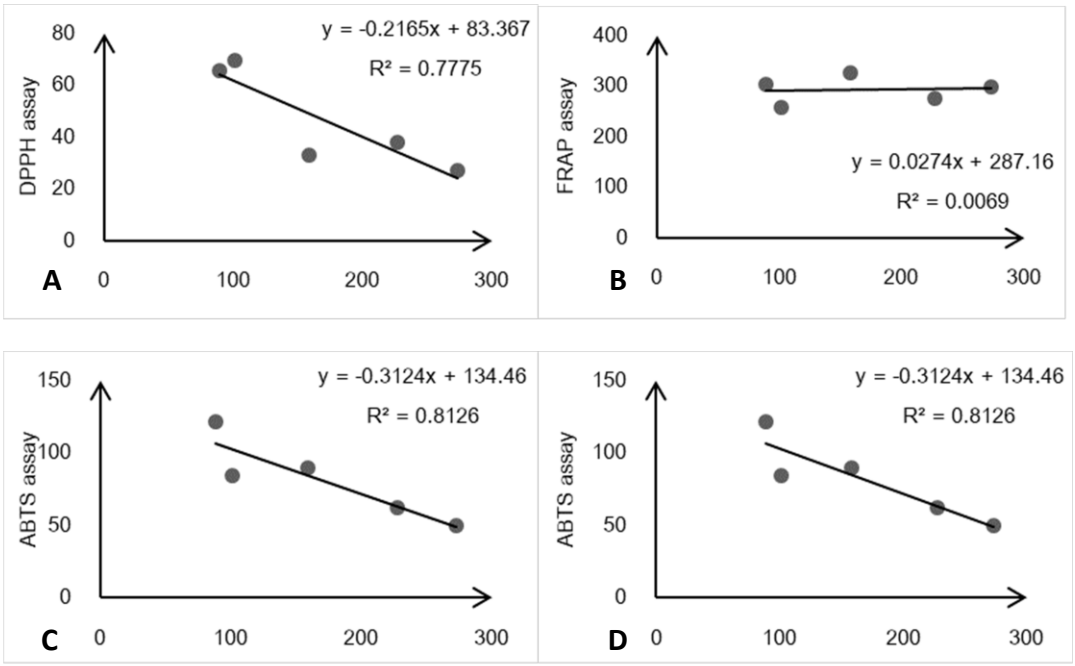

Figure 3: The correlation graphs of ethanol extract between TPC and each of antioxidant activities. A is DPPH assay, B is FRAP assay, C is ABTS assay and D is ORAC assay.

Discussion and Conclusion

In this study, the phytochemical screening of tonic formula's herb ingredients revealed that among the substances investigated, presence of phenolic compounds were detected (tannins ,flavonoids, terpenoids, steroids and alkaloids) , while anthraquinones and cardiac glycosides were not detected. The presence of some of these secondary metabolites suggests that the plants and the blood tonic formula might be of medicinal importance. The

presence of phenolic compounds such as tannins and flavonoids as well as the high total phenolic contents in most of the herbal ingredients confirmed the potential of this formula as health maintenance and the prevention of degenerative diseases. In order to realize the health benefits from potential plant sources, it is important to measure the antioxidant activities using various assays. *Endosamara racemosa* showed the highest total phenolic content and the best activity in all antioxidant assays, especially DPPH assay and ABTS assay. Blood tonic formula consisted of four herbs showed the highest activity in FRAP assay and ORAC assay. According to the additional review, *E. Racemosa* is a woody climber with 13-15 leaflets and white inflorescences flower (Picheansoonthon *et al.*, 2015). The debarked stem contains isoflavans, isomillinol, beta-amyrin and beta-sitosterol. The isoflavans showed bactericidal and insecticidal activities (Khare, 2007). Rao *et al.* (1993) extracted flavonoid compounds from methanol extract of ER bark and identified as neomillinol and millinolol. Therefore, the study results of ER is consistent with the flavonoid test in phytochemical study, the highest total phenolic content and the highest activity in two antioxidant assays. The overall results from correlation study, water extracts from the plants in this study showed strong correlation between total phenolic content and FRAP assay. Both water and ethanol extracts of *E. racemosa* were determined to have a highest level of antioxidant activity in DPPH assay and ABTS assay, proportional to their level of total phenolic content. However, water and ethanol extracts of blood tonic formula were determined to have a stronger antioxidant effect in FRAP and ORAC assay than *E. racemosa* and other extracts. Blood tonic formula is used and believed to maintain kidney and liver function as well as the action as blood purification and detoxification (Wang and Li, 2008). The water and ethanol extracts of all samples in this study were related to the preparation of blood tonic formula using a decoction or fermentation techniques (Dudonné *et al.*, 2009). DPPH assay of ethanol extract of *D. pentandra* (whole plants) in the current study showed a comparative antioxidant activity with the ethanol of extract of *D. penntanra* leaves of previous study by Fritilia *et al.* (2015) with the IC_{50} of 38.06 and 6.8 μg/ml, respectively. The overall results from four antioxidant assays (DPPH, ABTS, FRAP and ORAC assay), confirmed the high antioxidant activity of water and ethanol extracts of *E. racemosa* and blood tonic formula. Especially in DPPH assay, the ethanol extract of all selected medicinal plants showed the higher antioxidant activity than Trolox (positive control). With the exception of DP, the antioxidant and total phenolic content of ER, KG and KF have never been reported before. As a result, blood tonic formula can be used as a source of natural antioxidants.

The present study provides the useful information of phytochemical compounds, total phenolic content and antioxidant properties of four medicinal plants from Lao PDR which is the ingredient of blood tonic formula. The results exhibited a strong correlation between total phenolic content and various antioxidant assays. In addition, both of ER extracts (water and ethanol) showed higher DPPH scavenging activity than Trolox. The finding of this study supports the fact that some medicinal plants commonly consumed in Lao PDR are promising sources of potential antioxidants.

Acknowledgements

The authors wish to express their sincere appreciation for the scholarship support provided by Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand and Faculty of Pharmacy, University of Health Sciences, Lao PDR. Authors are very grateful to Prof. Dr. Bounhong Southavong and Mr. Soulivanh Lanorsavanh for identification of the plant materials in this research.

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