Evaluation on Phytochemical Constituents and Antioxidant Activities of Various Formula from Ko-Klan Remedies by Aqueous Infusion Preparation Method

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Abstract:

Ko-Klan remedy is Thai folklore medicine appear on list of Thai herbal medicinal products which have been widely used to relief of muscle pain. The remedy were separated to 3 recipes; first, *Mallotus repandus*: *Rhinacanthus nasutus: Elephantopus scaber: Aegle marmelos* ; 1: 1:1:1 (w:w:w:w), second, *Mallotus repandus: Rhinacanthus nasutus: Elephantopus scaber: Aegle marmelos*; 5:2.5:1.5:1.5 (w:w:w:w), third, *Mallotus repandus: Rhinacanthus nasutus: Elephantopus scabe: Ceasalpinia sappan: Cryptolepis buchanani: Piper interruptum*; 2:1:1:2:2:2 (w:w:w:w). Aims of this study were evaluated to total phenolic (TPC) and flavonoids (TFC) content, and antioxidant activities of various formulas from Ko-Klan remedy extracts by water infusion preparation. The TPC and TFC were determined to chemical composition. The antioxidations were tested using by 2,2-diphenyl-1-picrylhydrazy (DPPH) radical scavenging, and 2,2 -azinobis-(3-ethylbenzothiazoline-6-sulphonate) ($ABTS^+$) assay. The results showed that, the 1st remedy were higher contents of TPC and TFC (12.7927±0.0762 mgQE/gExt and 40.7925± 0.5372 mgGE/gExt), the 2nd remedy was highest antioxidant activity by ABTS assay (IC_{50} = 0.0022± 0.00002 mg/mL), the 3rd remedy were highest free radical scavenging by DPPH assay (IC_{50} = 0.0122± 0.00005 mg/mL). The finding studies suggest that all of the remedy were antioxidation higher than Ascorbic acid and Trolox as standard substances. Further study, some chemical compound(s) and pharmaceutical activity were clarified to medicinal plant use on health promotion.

Keywords: Ko-Klan, Phenol, Flavonoids, Antioxidantion, Infusion, DPPH, ($ABTS^+$).

Introduction

Ko-Klan remedies are Thai folklore medicine on list Thai herbal medicinal products which have been widely used for relief of muscle pain. There are according in National List of Essential Medicines, 2012 [1]. Recent studies, the authers were selected to 3 formulas from Ko-Klan remedies hich but not have some sciencetific report in each recipe. The reviews of some literature revealed to the plants in the each formulas of the recipe. *M. repandus* the main medicinal plant of remedy, is scandent and hard wood plant. It composed of phytochemicals such as polyphenols, terpenoid, benzopyran, coumarin and steroid [2]. *M. repandus* stem had the highest bergenin (polyphenol) content of 12.67±0.26% and 19.38±0.63% of dry% weight, respectively [3]. Chemical structure of phenolic compound contents of flavonoid, tannins, coumarin, lignans, quinone and curcuminoids [4]. Moreover, by DPPH assay and FRAP assay, the methanol extract of *M. repandus* had IC_{50} = 24.45 µg/mL and 99.01±4.56 GEAC/g, respectively [5]. *R. nasutus* is s shrub plant, composing of phytochemicals such as naphthoquinone, lignans, flavonoid, tritepene and steroid. By DPPH assay and FRAP assay, the methanol extract of *R. nasutus* had IC_{50} = 55.56±7.71 µg/mL and IC_{50}= 215.19±20.69 µg/mL, respectively [6]. *E. scaber* is herbaceous plant [7,8] whole plant composing of phytochemicals such as alkaloid, flavonoid, tannins [9], terpinoid, steroid [10], tritepene and flavone [11]. By DPPH assay, the 70% ethanol extract of leaf and the separate extraction by ethyl acetate (ESEAF) revealed that ESEAF 1,000 µg/mL had IC_{50} 69.70±0.01 µg/mL. By superoxide anion radical scavenging activity (SOD), there is antioxidant property of IC_{50} 3.79±0.16 µg/ [12]. The methanol extract of root had superoxide anion radical scavenging activity of IC_{50}= 48±5 µg mL-1, hydroxyl radical scavenging activity of IC_{50}=72±12 µg mL-1 and lipid peroxidation inhibition of IC_{50}=103±18 µg mL-1 [13]. By The 3 hours 80 °C refluxed ethanol extract of whole plant had SC_{50}= 12.4 µg/ml-1. By

DPPH assay, ethanol extract of whole plant had xanthine oxidase inhibition of IC_{50}= 93.1 µg/mL-1 [14]. *A. marmelos* is perennial plant that using fruit for medicinal purpose. Composing of phytochemicals such as coumarin, eugenol, tannins and mucilage [15]. By FRAP assay, the 95% ethanol extract and aqueous extract of *A. marmelos* fruit had 111.61 ± 0.59 Fe2+ and TEAC value = 39.04 ± 0.23 mg trolox equivalent and 95% ethanol extract had 27.35 ± 2.54 mg trolox equivalent. By ABTS assay, aqueous extract and ethanol extract of *A.marmelos* fruit had EC_{50} = 36.40 ± 1.23 µg/mL and 68.07 ± 5.23 µg/mL respectively [16]. Moreover, there is highly phenolic of 83.8± 37.6 mg/GAE per 100 ml [17]. *C. sappan* is perennial plant that using core for medicinal purpose. Composing of phytochemicals such as flavonoid and sterol. By infusion method, aqueous extract of *C. sappan* had total phenolic of 147.02 ± 0.63 mgGAE/g. By DPPH assay, aqueous extract of *C. sappan* had total phenolic of 33.91 ± 1.50 % [18]. *P. interruptum* is climber plant [19]. The 80% ethanol extract of *P. interruptum* had total phenolic 7.3 ± 0.8 mg GAE/g. By DPPH assay, the 80% ethanol extract of *P. interruptum* had IC_{50}=138.7 ± 2.2 µg/mL and lipid peroxidation inhibition of IC_{50}=38.7 ± 0.1 µg/mL [20]. *C. buchanani* was used boiled vine to for muscle pain relief. The vine of *C. buchanani* composes of phytochemicals such as buchananine, cardenolides and cardenolide glycoside. As far as we know, no previous report of antioxidant in *C. buchanani* but there are various pharmacology effect such as chondro protective activities, anti-inflammatory activities, analgesic activity and hepatoprotective activity [21].

Free radical is single electron in atom or molecule, unstable and rapidly react with biomolecules. Free radical in form of ROS and RNS such as super oxide anion, hydroxyl radicals nitric oxide, nitrogen dioxide which born from oxygen using in organism. Quantity of free radical that imbalance with antioxidant bring about to oxidative stress and causing diseases such as cancer, heart disease, Alzheimer, diabetes and neurotoxin. Naturally, organism can produce antioxidant that control quantity of free radical in the body for reducing destructive effects of free radical. For protecting body from free radical, eating antioxidant food such as vegetables, fruits and medicinal plants for increase antioxidant is necessary [22].

Although there are some previous reports about antioxidant in medicinal plants of Ko-Klan remedies, but there is no previous research in each formulas from Ko-Klan remedies. The aims of this research were investigated to phytochemical constituents and antioxidant activities from various formulas by water infusion extraction.

Materials and methods

Collection of plant materials

The 1st remedy composed with *Mallotus repandus* (stem): *Rhinacanthus nasutus* (whole): *Elephantopus scaber* (whole)*: Aegle marmelos* (fruit); 1: 1:1:1 (w:w:w:w). The 2nd remedy composes with *Mallotus repandus* (stem)*: Rhinacanthus nasutus* (whole)*: Elephantopus scaber* (whole)*: Aegle marmelos* (fruit); 5:2.5:1.5:1.5 (w:w:w:w). The 3rd remedy composed with *Mallotus repandus* (stem)*: Rhinacanthus nasutus* (whole)*: Elephantopus scabe* (whole)*: Ceasalpinia sappan (core): Cryptolepis buchanani* (stem)*: Piper interruptum* (stem); 2:1:1:2:2:2 (w:w:w:w). *R. nasutus* and *E. scaber* were collected from Maha Sarakham Province, northeastern Thailand. The specimens were identified and deposited at the Faculty of Medicine, Mahasarakham University, Thailand (code; *R. nasutus*:MSU. MED; RN0001/KM and *E. scaber*: MSU.MED; ES0001/KM). Remaining plants were purchased from Thong In pharmacy Co.,Ltd. All of the fresh materials were cleaned and dried at 50°C for 48 h in a hot air oven and then powdered.

Preparation of extracts

The aqueous infusion extraction was prepared using by boiled water at 80 °C for 20 min (1:200 w/v). The residue powder was excluded by using filter papers. The filtrate was evaporated using by a rotary evaporator (Heidolph Laborota 4000, Germany) and freeze-dried to obtain dark brown extract. The extracts were kept in the fridge at -4 °C until be used.

Total phenolic content assay

Total phenolic content was determined according to a modified procedure.[9] Sample (100 µL) will be oxidized with 500 µL of 0.2 N Folin-Ciocalteu's reagent and neutralized by adding 400 µL of 7.5% Na_2CO_3. The absorbance measured at 765 nm after mixed and incubated in room temperature for 30 min. The results were expressed as gallic acid equivalents (mgGE/gExt).

Total flavonoid content assay

Flavonoid content was estimated using the aluminum chloride colorimetric method .[10] The extracts from recipe will be mixed with 100 µL of 5% aluminum chloride (w/v), 400 µL of 2.5% $NaNO_2$ After 5 min, 500 µL of 5% $AlCl_3$. The mixture will be allowed to stand at room temperature for 10 min.

The solution was mixed 2,000 µL distilled water. The results was measured at 415 nm. The TFC was calculated from a standard quercetin equivalent (mgQE/gExt).

DPPH free radical scavenging activity

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capacities of wheat extracts were estimated by the reduction of the reaction color between DPPH solution and sample extracts as previously described by prior method. DPPH was dissolved in ethanol to a 0.039 mg/mL. The recipe of extract at various concentrations was diluted with distilled water to get sample solution. 100 µL of the sample solution following which 900 µL DPPH (0.1 mM) working solution. After a 30 min reaction kept in the dark at ambient temperature then absorbance of the solution was measured at 515 nm. In this study, will be used Trolox® and ascorbic acid as standard substances. Blanks were run in each assay. DPPH radical ability was expressed as IC_{50} (mg/mL) and the inhibition percentage calculated using the following formula: DPPH scavenging activity (%) = $(A_0-A_1)/ A_0 \times 100$ where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

$ABTS^+$ radical scavenging activity

In ABTS assay, the recipe of extract will be allowed to react with $ABTS^+$, a model stable free radical derived from 2,2- azinobis (3- ethylvenzothiazolin- 6- sulphonic acid) ($ABTS^+$) assay was performed.[12] The $ABTS^+$ (900 µL) was added to the extracts (100 µL) and thoroughly mixed. The mixture was held at room temperature for 6 min, and absorbance was immediately measured at 734 nm. Trolox® and ascorbic acid solution in 80% ethanol was prepared and assayed under the same conditions. ABTS scavenging ability was expressed as IC_{50} (mg/mL) and the inhibition percentage calculated using the following formula: ABTS scavenging activity (%) = $(A_0-A_1)/ A_0 \times 100$ where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

Statistical analysis

All assays were expressed as mean± standard deviation (SD) from five separate experiments (n = 5). Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. Differences at $p < 0.05$ were considered to be significant.

Results and discussion

Total phenolic compounds and total flavonoid contents

The results showed that the extracts from all of Ko-Klan remedy had both TPC and TFC. The TPC was found that the 1st remedy (12.7927±0.0762 mgGE/gExt) was higher than the 3rd remedy and the 2nd remedy (8.3655±0.0800 and 7.6846±0.0789 mgGE/gExt), respectively. Moreover, TFC from 1st remedy (40.7925±0.5372 mgQE/gExt) had still more content than the 3rd remedy and the 2nd remedy (20.5432± 0.4095 and 17.9524±0.2972 mgQE/gExt), respectively. Phenolics were the main antioxidant components, and their total contents were directly proporty to their antioxidant activity [23]. According to Phuaklee P. *et al*.(2015), studied on 95% methanol extract and aqueous extract of *Aegle marmelos*, state that extracts had high total phenolic content 83.8± 37.6 mg/GAE per 100 ml. Furthermore, Taokaenchan N. *et al.* (2017) demonstrated that aqueous extract of *Caesalpinia sappan* by infusion methode had total phenolic content 147.02 ± 0.63 mgGAE/g. Moreover, this study is consistent with Klinthong *et al.* (2015) on P*iper interruptum* states that 80% ethanol extract of plant had 7.3 ± 0.8 mg GAE/g of total phenolic content. Nevertheless, this research had study on high electron solvent and necessary to study on other solvent including type and quantity of total phenolic content in all of Ko-Klan remedies. (As shown in Table 1)

DPPH free radical scavenging activity

The result showed that the 3rd remedy (IC_{50} = 0.0122±0.00005) was exerted on free radical scavenging activity higher than 2nd remedy and 1st remedy (IC_{50} =0.0153±0.00009, 0.0204±0.00018 mg/mL), respectively. The 3rd and 2nd remedy were more potent on DPPH scavenging activity than the standard controls, ascorbic acid (IC_{50} = 0.0162±0.00029 mg/mL), and Trolox (IC_{50} = 0.0444±0.00075 mg/mL). The result was consistented with a research of Wonglom *et al* (2016), revealed that methanol extract of *Mallotus repandus* had IC_{50} = 24.45 µg/mL by DPPH assay. Furthermore, according to the study of Teansuwan *et al.* (2015), by DPPH assay, ethanol extract of *Rhinacanthus nasutus* had IC_{50}= 55.56±7.71 µg/mL. This result related well with study of Chan *et al.* (2017) reported that by DPPH assay, 70% ethanol extract of *Elephantopus scaber* with separated extraction by ESEAF 1,000 µg/mL found that IC_{50} = 69.70±0.01 µg/mL. There is Superoxide Anion Radical Scavenging Activity (SOD) IC_{50} 3.79±0.16 µg/mL consistent with Pongpiriyadacha et al. (2009) which study ethanol extract of elephantopus scaber by 3 hours

of 80 °C reflux, SC_{50}= 12.4 µg/ml-1. Moreover, the research had shown xanthine oxidase enzyme inhibitory activity IC_{50}= 93.1 µg/ml-1. A similar conclusion was reached by Taokaenchan et al. (2017), aqueous extract of *Caesalpinia sappan* by infusion had 33.91 ± 1.50 % when examined by DDPH assay. Therefore, This result related well with studies of Klinthong et al. (2015)'s research on *Piper interruptum* Opiz states that by DPPH assay, 80% ethanol extract of *Piper interruptum* Opiz had IC_{50}=138.7 ± 2.2 µg/mL and lipid peroxidation inhibitory activity IC_{50}=38.7 ± 0.1 µg/mL. (As shown in Table 1)

2,2 -azinobis-(3-ethylbenzothiazoline-6-sulphonate) ($ABTS^+$) activity

In the study, using $ABTS^+$ assay, the 2nd, the 3rd and 1st remedy (IC_{50} =0.0022±0.00002, 0.0037± 0.00003, and 0.0040±0.00003 mg/mL), had more potent on free radical scavenging activity than ascorbic acid (IC_{50} = 0.0099±0.00022 mg/mL) and trolox (IC_{50} = 0.0230±0.00035 mg/mL), as the standard controls. Overall these findings are in accordance with findings reported by Abdullakasim et al. (2007), studied 95% ethanol and aqueous extracts of *Aegle marmelos* fruit by ABTS assay, EC_{50} = 36.40 ± 1.23 µg/mL. (As shown in Table 1)

Table 1 Total phenolic (TPC), flavonoid contents (TFC), DPPH and ABTS radical scavenging activities of various formula from Ko-Klan remedies by aqueous infusion preparation method

Samples	TPC (mgGE/gEtx)	TFC (mgQE/gEtx)	DPPH (IC_{50} mg/ml)	ABTS (IC_{50} mg/ml)
1st Remedy	13.7473±0.0949[a]	40.7925±0.5372[a]	0.015±0.00002[c]	0.004±0.00002[c]
2nd Remedy	11.1438±0.0875[c]	17.9524±0.2972[c]	0.014±0.00004[b]	0.002±0.00001[a]
3rd Remedy	12.1120±0.1441[b]	20.5432±0.4095[b]	0.011±0.00004[a]	0.003±0.00003[b]
Ascorbic acid	-	-	0.016±0.00029[d]	0.010±0.00022[d]
Trolox	-	-	0.044±0.00075[e]	0.023±0.00035[e]

TPC was measured with gallic acid equivalents (mgGE/gExt). TFC was measured with quercetin equivalent (mgQE/gExt). Antioxidant activities showed IC_{50} of different extracts from recipe. DPPH and $ABTS^+$ radical scavenging activity were used ascorbic acid and trolox as standard substances. Different letters indicated significantly different at $p < 0.05$.

Conclusions

The all of formulas from Ko-Klan recipe ingredient with phenolic compound and flavonoid contents. Moreover, the recipe had more effect on free radical scavenging activity. Furthermore, isolation and active compound(s) were evaluated. The pharmaceutical preliminary was confirmed usage indication in Thai traditional medicine. However, any sign or symptoms were clarified in next study. Koklan remedies can be developed as healthy drink. So, the study of proportion of herbal ingredients and flavor are necessary.

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