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In Vitro Antioxidants Activities of Extract of Tri-Kasorn-Mas remedy and its plant ingredients

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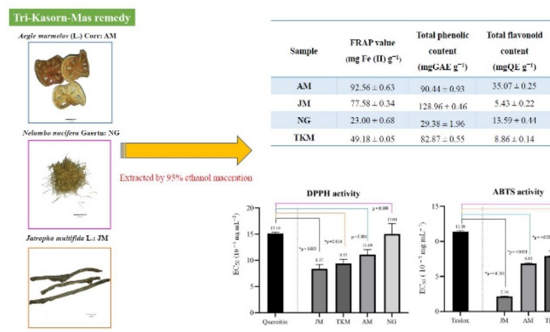
Abstract



Tri-Kasorn-Mas remedy is one of the Thai traditional medicines notified in the list of Herbal Medicinal Products A.D. 2021 of Thailand for the treatment of fatigue and maintaining body balance of patients. However, there have been no scientific reports on the antioxidant activities and antioxidant capacity. This study aimed to investigate antioxidant activities, and total phenolic and flavonoid contents of the three extracts of the Tri-Kasorn-Mas remedy and their mixture. The remedy plants and each ingredient were extracted by 95% ethanol maceration. These studies determined antioxidant activity by DPPH radical scavenging assay, ABTS radical cation decolorization assay, Ferric ion reducing antioxidant power assay (FRAB) and chemical contents by total phenolic and flavonoid content methods. The yield of Tri-Kasorn-Mas remedy was 26.68%. The results found that the Tri-Kasorn-Mas remedy showed strong antioxidant activity with an EC_{50} value of $9.37 \pm 0.82 \times 10^{-3}$ mg mL^{-1} by DPPH radical scavenging assay and $7.91 \pm 0.01 \times 10^{-3}$ mg mL^{-1} by ABTS radical cation decolorization assay. These extracts also were stronger activity than the positive control. In addition, *J. multifida* was the highest antioxidant activity than other ingredients. The results of this study can be preliminarily concluded that the Tri-Kasorn-Mas remedy has antioxidant activity and total phenolic compounds that are in high values.

Keywords: Tri-Kasorn-Mas remedy; Antioxidant; Total phenolic content; Total flavonoid content

1. Introduction

Free radicals are derived from several small molecules such as oxygen and nitrogen, etc. These molecules are the predominant part of groups of molecules call reactive oxygen species (ROS), which lead to the mechanism of oxidative stress in the human body. Additionally, free radicals can cause several diseases such as cardiovascular disease, cancer, an inflammatory disorder, aging, etc. Also, the study of antioxidants in natural sources or herbal medicine is the most popular to promote and prevent body tonic and inflammatory disorders [1].

Recently, Thai traditional practitioners have using a combination of herbs more than a single herb because according to the knowledge of Thai conventional wisdom we used a combination of herbs that can relieve several symptoms such as fever, headache, diuretic, and blood tonic, etc. [2]. Besides, Thai traditional medicine wisdom explains the three components of herbs called Tri-Pi-Kut. Tri-Pi-Kut mixed with an equal ratio of single herb became the one remedy. Interestingly, the Thai traditional medicine textbook named Sap-Phra-Kunya scripture that has one remedy called Tri-Kasorn-Mas [3].

Tri-Kasorn-Mas remedy is one of the Thai traditional medicines notified in the list of Herbal Medicinal Products A.D. 2021 of Thailand [4] and has been empirically used in Thai traditional medicine to treat loss of appetite, dyspepsia, diarrhea, vomiting, and body tonic. The components of Tri-Kasorn-Mas remedy are composed of *Jatropha multifida* L. (Fin-Ton), and *Nelumbo nucifera* Gaertn. (Bua-Luang) and *Aegle marmelos* (L.) (Ma-Toom) in the ratio (1 : 1 : 1). However, there have been no scientific reports on the antioxidant activities of several mechanisms such as single electron transfer (free radical scavenging) and measurement of the chemical contents that related to antioxidant capacity. Also, the aim of this study that we investigated the total phenolic and flavonoid contents of the Tri-Kasorn-Mas remedy and its plant ingredients. Moreover, its antioxidant activities using three different methods i.e., DPPH, ABTS, and FRAP assay were evaluated.

2. Materials and Methods

Plant Materials and Extractions

In June 2021, *J. multifida* bark and *A. marmelos* fruits were collected from Uttaradit provinces and *N. nucifera* pollens were obtained from Thai traditional medicine stores in Phitsanulok provinces, Thailand. After that, the individual samples were cleaned and dried in a hot air oven at 40 °C. Then, the dried samples were powdered mechanically, extracted by maceration with 95% ethanol for 3 days, and filtered. This was repeated two times with the residue and was concentrated under reduced pressure by a rotary evaporator and the percentage of yield was calculated. The 95% ethanol crude extracts were stored at – 20 °C until testing.

Determination of Total Phenolic Content

Total phenolic contents in the extracts were determined by the modified Folin-Ciocalteu method using microplate readers. In accordance with Sachindra *et al.* report in 2010 with some modifications [5], first, 50×10^{-3} mL of the extract was mixed with 50×10^{-3} mL of Folin-Ciocalteu's reagent in a 96-well microplate. After waiting for 5 minutes, 50×10^{-3} mL of sodium carbonate was added, and the plate was allowed to stand for 30 minutes. Absorbance values of samples were measured at 765 nm. Gallic acid (GA) was used as the standard phenolic substance to compare with the extracts. Total phenolic content was expressed as milligrams of gallic acid equivalent per gram of dry weight of each extract (mgGAE g^{-1}). All measurements were performed in triplicate.

Determination of Total Flavonoid Content

Total flavonoid content was performed by the aluminium chloride colorimetric technique modified by Chang *et al*. [6]. The 50×10^{-3} mL of each extract was mixed with the 10×10^{-3} mL of 10% (w v^{-1}) aluminum chloride complex and left for 5 minutes. The 10×10^{-3} mL of 1 M sodium hydroxide was added, and the volume was supplemented to 150×10^{-3} mL with distilled water. Then, the reaction was incubated for 30 minutes in the dark room at temperature 25 °C. The absorbance was measured at 415 nm. Total flavonoid contents were expressed as milligrams of quercetin equivalents per gram of dry weight of each extract (mgQE g^{-1}).

Determination of antioxidant activity using the DPPH radical scavenging assay.

Antioxidant activity was determined using 2,2-Diphenyl-1-picrylhydrazyl (DPPH), according to the modified method of Yamasaki *et al*. [7]. In brief, the 180×10^{-3} mL of 6×10^{-5} M DPPH solution in absolute ethanol was mixed with 20×10^{-3} mL of different concentrations of the extracts and incubated for 30 minutes in a dark room at room temperature. The absorbance was measured at 520 nm using microplate readers. Quercetin was used as a positive control. Initially, DPPH (EC_{50}) was determined as the antioxidant concentration to reduce 50% DPPH activity, a parameter widely used to measure antioxidant activity. The antioxidant scavenging activity was calculated as a percentage inhibition in the formulae below:

$$\text{The Percentage of Inhibition} = \left[\frac{\text{Abs.control} - \text{Abs.sample}}{\text{Abs.control}}\right] \times 100 \quad (1)$$

Determination of antioxidant activity using ABTS radical cation decolorization assay

ABTS radical scavenging assay was conducted according to the method of Re *et al.* [8]. The preparation of ABTS [2,2 -azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical solution followed by the discoloration assay. ABTS radical solution is prepared by mixing 7 mM ABTS solution with 2.45 mM potassium persulfate. The reaction mixture was allowed to stand in the dark room at room temperature for 12 – 16 hours to complete radical generation. Then, the ABTS radical solution was diluted with deionized water to reach an absorbance of 0.68 – 0.72 at 734 nm before being used. The discoloration assay was assessed by mixing 180×10^{-3} mL of ABTS solution with 20×10^{-3} mL of various dilutions of each extract in 96 well microplates. The reaction mixture was allowed to stand for 6 minutes, and the absorbance was measured at 734 nm by using a microplate reader. Trolox was used as a positive control. The scavenging activity of extracts against ABTS radicals was calculated by the percentage inhibition of scavenging activity using the formula below and expressed as EC_{50} ($\times 10^{-3}$ mg mL^{-1}).

$$\text{The Percentage of Inhibition} = \left[\frac{\text{Abs.control} - \text{Abs.sample}}{\text{Abs.control}}\right] \times 100 \quad (2)$$

Determination of Antioxidant Activity Using Ferric ion reducing antioxidant power assay (FRAP) [9].

FRAP was evaluated by the measurements of Fe (III)/TPTZ – complex by a colorimetric method using a spectrophotometer. FRAP reagent was prepared using 10 ml of acetate buffer 300 mM, adjusted to pH 3.6 by the addition of acetic acid, containing 1 ml of 10 mM 2, 4, 6-tripyridyls-triazine (TPTZ) in 40 mM HCl, and 1 ml of 20 mM ferric chloride hexahydrate dissolved in distilled water: chloride solution in the proportion of 10 : 1 : 1 respectively. The FRAP reagent was prepared fresh daily and was warmed to 37 °C for 4 minutes in a water bath. About 10 mg of crude extracts were weighed in centrifuge tubes and diluted to a concentration of 1 mg mL^{-1}. Aliquots of sample solutions (20×10^{-3} mL) were added to 180×10^{-3} mL of the FRAP reagent, mixed well, and allowed to stand at room temperature for 8 minutes. The absorbance was measured at 593 nm. Increased absorbance of the reaction mixture indicated increasing of the reducing the power of the sample. The results were expressed as mg Fe (II) per gram of extract sample.

Statistical analysis

All experiments were presented as mean ± standard error of the mean (SEM) in triplicate. One-way ANOVA following Dunnett's test was used to compare sample groups and the positive control (P-value < 0.05). Statistical analysis was calculated by the Prism Software program.

3. Results and Discussion

These studies determined antioxidant activities using DPPH radical scavenging assay, ABTS radical cation decolorization assay, and Ferric ion reducing antioxidant power assay (FRAB). The chemical contents were studied in total phenolic and total flavonoid contents.

Table 1 The percentage yield of Tri-Kasorn-Mas remedy and component plants.

Scientific name	Common name	Used Part	Code	% Yield
Aegle marmelos L.	Bael Fruit Tree	Fruit	AM	19.98
Jatropha multifida L.	Coral bush	Bark	JM	35.94
Nelumbo nucifera Gaertn.	Indian Lotus	Pollen	NG	40.81
–	Tri-Kasorn-Mas remedy	–	TKM	25.68

Table 2 Antioxidant activities, total phenolic and flavonoid contents of Tri-Kasorn-Mas remedy and its plant ingredient extracts (n = 3).

Sample	Antioxidant activity (EC_{50} : × 10^{-3} mg mL^{-1})		FRAP value (mg Fe (II) g^{-1})	Total phenolic content (mgGAE g^{-1})	Total flavonoid content (mgQE g^{-1})
	DPPH radical scavenging assay	ABTS radical cation decolorization assay			
AM	11.09 ± 0.98	6.82 ± 0.14	92.56 ± 0.63	90.44 ± 0.93	35.07 ± 0.25
JM	8.37 ± 0.87	2.16 ± 0.01	77.58 ± 0.34	128.96 ± 0.46	5.43 ± 0.22
NG	15.04 ± 1.96	9.47 ± 0.03	23.00 ± 0.68	29.38 ± 1.96	13.59 ± 0.44
TKM	9.37 ± 0.82	7.91 ± 0.01	49.18 ± 0.05	82.87 ± 0.55	8.86 ± 0.14
Quercetin	15.16 ± 0.22	NT	–	–	–
Trolox	NT	11.38 ± 0.13	–	–	–

The percentage yield of Tri-Kasorn-Mas remedy was 25.68% w w^{-1} of 95% ethanolic extract. This result is high value than the previous study (7.99% w w^{-1}) [10]. The percentage yield of all ethanolic extracts of plant ingredients showed in ranging from 19.98 – 40.81%. The highest percentage yield of the ethanolic extracts was *N. nucifera.* This result is high value than the previous study (7.58% w w^{-1}) [11]. The lowest percentage yield of the ethanolic extracts was *A. marmelos* but the result was still higher than the physicochemical specification of *A. marmelos* [12]. The results showed in Table 1.

In table 2, total phenolic contents of all extracts were also determined as mg equivalent to gallic acid per gram of each extract (mg GAE g^{-1}). *J. multifida* showed the highest total phenolic content with a value of 128.96 mg GAE g^{-1} followed by *A. marmelos* (90.44 mg GAE g^{-1}). Tri-Kasorn-Mas remedy showed the 82.87 mg GAE g^{-1}.

Total flavonoid content was expressed as milligrams of quercetin equivalents per gram of dry weight of each extract (mgQE g^{-1}). *A. marmelos* showed the highest total flavonoid content with a value of 35.07 mgQE g^{-1}. Tri-Kasorn-Mas remedy showed the 8.86 mgQE g^{-1}.

These studies determined antioxidant activities by DPPH radical scavenging assay. The positive control, quercetin showed EC_{50} value of 15.16 × 10^{-3} mg mL^{-1}. The result found that the Tri-Kasorn-Mas remedy and all plant ingredient extracts showed excellent activity. Tri-Kasorn-Mas remedy, *J. multifida* and *A. marmelos* were significantly stronger activity than the positive control. In addition, *N. nucifera* display good activity and does not differ significantly from quercetin. The result showed in Table 2 and fig 1.

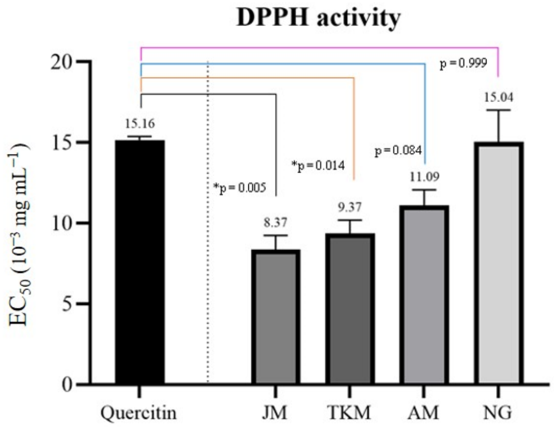


Fig. 1 The comparison of its plant ingredients extracts and positive control on DPPH radical scavenging assay *Significant differences ($p < 0.05$) compared to positive control, quercetin.

The ABTS radical scavenging assay of the extracts is showed in Fig 2. The positive control trolox exerted potent activity with EC_{50} value of 11.38×10^{-3} mg mL^{-1}. The result demonstrated that, EC_{50} of Tri-Kasorn-Mas remedy and all plant ingredient showed extracts significantly were lower than the positive control. Preferably, the ethanolic extract of *J. multifida* showed the highest activity with EC_{50} value of 2.16×10^{-3} mg mL^{-1}. While Tri-Kasorn-Mas remedy extract showed the EC_{50} value of 7.91×10^{-3} mg mL^{-1}.

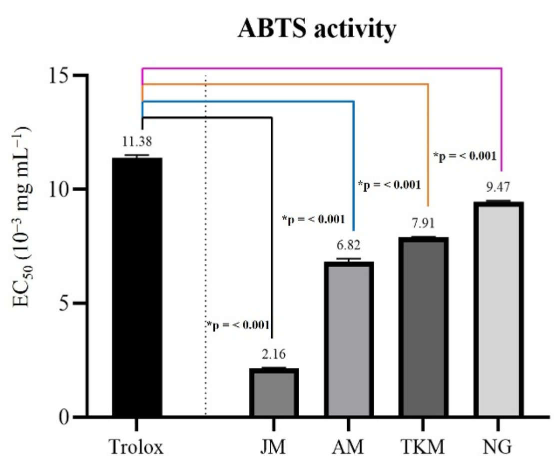


Fig. 2 The comparison of its plant ingredients extracts and positive control on ABTS radical cation decolorization assay. *Significant differences ($p < 0.05$) compared to positive control, trolox.

For FRAP value, the antioxidant activity of extracts was calculated from the standard curve of $FeSO_4$ and presented in the unit of mg Fe^{2+} per gram of extract (mg Fe^{2+} eq g^{-1}). The results found that the Tri-Kasorn-Mas remedy showed antioxidant activity with FRAP value of 49.18 mg Fe (II) g^{-1}. Furthermore, the highest FRAP value of the plant ingredients extracts was *A. marmelos* and the lowest was *N. nucifera*.

Tri-Kasorn-Mas remedy showed strong antioxidant activity with EC_{50} value of $9.37 \pm 0.82 \times 10^{-3}$ mg mL^{-1} by DPPH radical scavenging assay and $7.91 \pm 0.01 \times 10^{-3}$ mg mL^{-1} by ABTS radical cation decolorization assay. These extracts also were stronger activity than quercetin (EC_{50} value of $15.16 \pm 0.22 \times 10^{-3}$ mg mL^{-1}), as positive control on DPPH radical scavenging assay. Trolox (EC_{50} value of $11.38 \pm 0.13 \times 10^{-3}$ mg mL^{-1}) was used as positive control on ABTS radical cation decolorization assay. These results are consistent with Phuaklee *et al*. [10]. The previous study showed that 95% ethanol extracts displayed high antioxidant activity on DPPH assay with EC_{50} value of $3.86 \pm 0.11 \times 10^{-3}$ mg mL^{-1}. The EC_{50} value of this study was less than the previous study. The climatic, seasonal and geographical conditions, age of plant, humidity of the harvested plant material, and extraction technique may be factors in the compositions of extracts of plants [13], which necessitates further studies with samples of specimens from different areas.

Surprisingly, *J. multifida* was the highest antioxidant activity by DPPH radical scavenging assay and ABTS radical cation decolorization assay with EC_{50} values of $8.37 \pm 0.87 \times 10^{-3}$ mg mL^{-1} and $2.16 \pm 0.01 \times 10^{-3}$ mg mL^{-1}, respectively. Moreover, *J. multifida* showed a total phenolic content higher than other herbs.

J. multifida is known to possess different medicinal properties including antimicrobial activity, [14, 15], anti-inflammatory and antioxidant activities [16], phytochemical and larvicidal properties [17], antioxidant, hemolytic, and toxic [18]. The stems of *J. multifida* could be regarded as an anti-influenza herbal medicine as well as a potential crude drug source for the development of anti-influenza compounds [19]. There is variation in the chemical compositions of phenols and flavonoids between leaf extract and latex of this plant and the presence of phenolic compounds acts as a factor for the antioxidant activity [20]. So, *J. multifida* is probably the main herb for its antioxidant effect of this remedy. However, the determination of chemical markers and pure compounds from Tri-Kasorn-Mas remedy is further studied.

4. Conclusion

Antioxidant activity is characterized by the inhibition or retardation of the oxidation of substances susceptible to this type of reaction, which consists of eliminating or capturing free radicals. Because synthetic antioxidants are toxic and expensive, the search for natural antioxidants has been increasing in studies and research. This activity in natural compounds has been related to the presence of phenolic compounds found in medicinal plants [20].

The results of this study can be preliminarily concluded that the Tri-Kasorn-Mas remedy has antioxidant activity and total phenolic compounds that are high values. This corresponds to Thai traditional medicine's wisdom that uses the Tri-Kasorn-Mas remedy to relieve fatigue, adjust the balance of the elements in the body, and recover from illness. Therefore, further studies of other biological activities should be undertaken to support the animal model experiments and clinical studies.

5. Suggestions

Tri-Kasorn-Mas remedy and its plant ingredients showed good activity on antioxidant effect. Therefore, further studies of other biological activities and the determination of chemical markers and pure compounds should be undertaken to support the animal model experiments and clinical studies.

6. Acknowledgement

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