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Antioxidant activity of *Rafflesia kerrii* flower extract

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Summary ***Rafflesia kerrii* has been used in Thai traditional remedies for treatment of several diseases. However, scientific data particularly on biological activities of this plant is very rare. The present study explores an antioxidant activity of *R. kerrii* flower (RKF). Extracting solvent and extraction procedure were found to play an important role on the activity of RKF extract. The extract obtained from water-ethanol system showed higher antioxidant activity than that from water-propylene glycol system. Fractionated extraction using different solvents revealed that methanol fractionated extract (RM) possessed the highest antioxidant activity with Trolox equivalent antioxidant capacity (TEAC) and inhibitory concentration of 50% inhibition (IC_{50}) values of approximately 39 mM/mg and 3 µg/mL, respectively. Phytochemical assays demonstrated that RM contained extremely high quantity of phenolic content with gallic antioxidant equivalent (GAE) and quercetin equivalent (QE) values of approximately 312 mg/g and 16 mg/g, respectively. Ultraviolet-visible spectroscopy (UV-VIS) and high-pressure liquid chromatography (HPLC) indicated that gallic acid was a major component. RM which was stored at 40°C, 75% RH for 4 months showed slightly significant change ($p < 0.05$) in phytochemical content and antioxidant activity with zero order degradation. The results of this study could be concluded that *R. kerrii* flower was a promising natural source of strong antioxidant compounds.**

Keywords: *Rafflesia kerrii*, antioxidant, hydroalcoholic extract, fractionated extract, stability

1. Introduction

Rafflesia kerrii is a unique plant in family Rafflesiaceae which is the family of holoparasitic flowering plants most famous for being the world's largest single flower. As for all holoparasitic plants, this family relies upon their host plant for both water and nutrients (*1*). The plants in genus *Rafflesia* grow in a tropical rainforest area. Most species like *R. arnoldii*, *R. kerrii, R. azlanii*, and *R. hasseltii* are found in Malasia whereas *R. kerrii* is found in Thailand with a local name of "Bua Phut". *R. kerrii* is a second largest species after *R. arnoldii* (*2*). It lives as a holoparasite plant depends entirely on a host plant known as Liana due to the lack of root, stem and leaf. *R. kerrii* flower has a diameter of about 70 cm.

Phytochemical and biological properties of *R. hasseltii* which is similar to *R. kerrii* have been reported to contain alkaloids and phenolic compounds (*3*) which exhibited broad spectrum of antimicrobial activity (*4*) and accelerating the wound healing process in rats (*5*). For *R. kerrii*, the report on phytochemical and biological aspects is still rare. Kanchanopoom *et al*. (*6*) reported that *R. kerrii* was rich of tannin and phenylpropanoid glycoside. The flower of *R. kerrii* has been used by local people to restore the uterus of post-natal's women, cure infectious disease, reduce fever, and even as sexual stimulant without scientific support. Recently, some authors demonstrated the anticancer activity of *R. kerrii* extract on skin cancer cell lines (*7*). It is realized that cancer incidence is due to the oxidative stress that is resulted from an imbalance between formation and neutralization of prooxidants. Oxidative stress is initiated by free radicals like hydroxyl, peroxyl and superoxide radicals, which become stable through electron pairing with biological macromolecules such as proteins, lipids and DNA in healthy human cells and cause protein and DNA damage along with lipid peroxidation. The damage caused by oxidative stress has been implicated as a potential contributor to a severe disease like cancer (*8*). Substances which

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possess antioxidant activity can prevent damage to cells caused by these free radicals and can be used for cancer prevention. In the present study, antioxidant activity of *R. kerrii* flower is explored for the first time. The effect of extracting solvent as well as extraction procedure on antioxidant activity and phytochemical content of the extracts was evaluated. The stability of the extract which possessed the highest antioxidant activity and kept in the stress condition of a high temperature and humidity was also investigated.

2. Materials and Methods

2.1. *Chemicals*

Trolox, potassium persulfate, 2,'-azinobis-(3-ethylbenzothiazoline-6-sulfonicacid) diammonium salt (ABTS), 2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, quercetin and sodium carbonate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Organic solvents (AR grade) were from Merck (Darmstadt, Germany). Water was deionized and purified by Milli-Q system (Millipore, Bedford, MA, USA). All other chemicals were of the highest grade available.

2.2. *Plant materials*

All plant samples (Table 1) were collected as a fresh condition from local area of Southern Thailand. The samples were washed with clean water and dried under the controlled temperature of 50°C. The dried samples were then pulverized and kept in the vacuum desiccators until used.

2.3. *Preparation of hydroalcoholic extracts*

The dried plant samples were separately macerated in water-ethanol (5:95) or water-propylene glycol (PG) (1:1) solvent system for 3 days. After that, the mixtures were filtered through Whatman filter paper No. 1. The filtrates were kept at 4°C until used.

2.4. *Preparation of fractionated extracts*

The dried powder of *R. kerrii* flower (RKF) was sequentially macerated at room temperature in different polar solvents. First, it was macerated with n-hexane (48 h × 3). The residue after the third filtration was dried at room temperature for 24 h in order to remove n-hexane. After that, the dried residue was further macerated with ethyl acetate, n-butanol and methanol, respectively, in the same procedure as n-hexane. The filtrates of each solvent were collected and dried using a rotary evaporator under vacuum. The fractionated extracts of each solvent were kept at 4°C until used.

Table 1. Plant names and part used for extraction

Latin name	Common name	Part used	Abbreviation
Rafflesia kerrii	Bua Phut	Flower	RKF
Rosa damascene	Damask rose	Flower	RDF
Etlingera elatior	Torch ginger	Flower	EEF
Terminalia chebula	Cheburic myrobalan	Fruit	TCF
Terminalia bellirica	Beleric myrobalan	Fruit	TBF

2.5. *Determination of antioxidant activity*

2.5.1. *ABTS assay*

The ABTS assay was carried out according to a procedure described by Saeio *et al*. (*9*) with minor modification. Briefly, the free radical ABTS was firstly generated by reacting ABTS solution with potassium persulfate. The mixture was allowed to stand in the dark at room temperature for 12 h and diluted with absolute ethanol to obtain the absorbance of approximately 0.7 units at 750 nm. The extracts were diluted appropriately concentration with absolute ethanol. The ethanolic solution of each sample was added to ABTS free radical solution. The mixture was left to stand for 5 min at room temperature then the absorbance at 750 nm was recorded using a microtiter plate reader. Trolox was used as the calibration curve. The result was expressed as Trolox equivalent antioxidant capacity (TEAC) in millimolar concentration of Trolox which antioxidant capacity was equivalent to 1 mg of the test extract.

2.5.2. *DPPH assay*

The DPPH assay was determined using a procedure described by Okonogi *et al*. (*10*) with minor modification. Briefly, the solution of DPPH free radicals was firstly prepared by dissolving the free radicals in absolute ethanol to a concentration of 100 µM. The extracts were diluted with absolute ethanol of different concentrations. The ethanolic solution of each sample was added to DPPH solution. The mixture was left to stand for 20 min in the dark at room temperature. The amount of DPPH remaining in each period of stand was determined at 540 nm using a microtitre plate reader. Antioxidant activity was calculated as % inhibition of DPPH free radicals. The concentration of the extract that showed 50% inhibition (IC_{50}) value was recorded.

2.6. *Determination of total phenolic content*

Total phenolic content of RKF extracts was determined using Folin-Ciocalteu assay described by Sato *et al*. (*11*) with some modification. Briefly, an exact amount of the test extract was dissolved in absolute ethanol and mixed with Folin-Ciocalteu reagent. After 2 min of mixing, sodium carbonate was added. The mixture was allowed to stand for 2 h at room temperature before the absorbance was measured at 750 nm. Gallic acid was

used as the standard for the calibration curve. Total phenolic content was expressed as gallic acid equivalent (GAE) in mg of gallic acid to 1 g of the test extract.

2.7. *Determination of total flavonoid content*

Total flavonoid content of RKF extracts was determined by aluminium chloride colorimetric assay (*12*). The 100 µL sample solution was mixed with 20 µL 10% $AlCl_3$, 20 µL 1 M sodium acetate and 860 µL DI water. The mixture was allowed to stand for 30 min at room temperature before the absorbance at 415 nm was measured. Quercetin was used as the standard calibration curve. Total flavonoid content was expressed as quercetin equivalent (QE) in mg of quercetin to 1 g of the test extract.

2.8. *Ultraviolet-visible spectroscopy (UV-VIS) and high-pressure liquid chromatography (HPLC) analysis*

The most effective fractionated extract of RKF was examined under visible and UV light for proximate analysis. A solution of the test extract was prepared and filled in a cuvette with a solvent kept as blank in UV-VIS spectrophotometer (UV-2450, Shimadzu, Japan) and the maximum absorption (λ_{max}) was recorded.
The HPLC analysis was performed using an HP1100 system with a thermostatically controlled column oven and a UV detector (Hewlett-Packard, Palo Alto, CA, USA). A reversed phase column Zorbax SB-C18 (250 × 4.6 mm *i.d*., 5µm, Algilent, CA, USA) was connected with a Zorbax SB-C18 guard column (125 × 4.6 mm *i.d*., 5 µm, Algilent, CA, USA). Isocratic condition of a 4:1 volume ratio mixture of acetronotrile and 0.3% v/v o-phosphoric acid in DI water was used as a mobile phase at ambient temperature with a flow rate of 1 mL/min. An exact quantity of 20 µL of sample was injected. Samples and mobile phases were filtrated through a 0.45 µm Millipore filter, type GV (Millipore, Bedford, MA, USA) prior to HPLC injection. Gallic acid was used as a standard for identification of phenolic compounds in the extract.

2.9. *Stability test*

The stability of RKF extract which possessed the highest antioxidant activity was investigated by keeping the extract in a stress condition of 40°C and 75% relative humidity for 4 months. The physical characteristic of the extract was observed. The antioxidant activity and the total phenolic content were determined during the storage time.

2.10. *Statistical analysis*

All experiments were carried out in triplicate. The results were expressed as mean values ± S.D. To determine statistical difference between means ($p < 0.05$), ANOVA and Duncan's test were calculated using SPSS statistical software package v.10.

3. Results and Discussion

3.1. *General*

The antioxidant activity of the plant extracts is according to the constituents existing in them. The amount of such compounds existing in each plant is usually different. In this study, five different medicinal plants including *R. kerrii* were firstly investigated for their antioxidant activity. The hydroalcoholic (water-PG) extracts of these samples were used for comparison of their antioxidant power. Several methods could be used for determination of antioxidant activity. Each method is usually responded for the different mechanism of antioxidant action (*13,14*). Free radicals are a major cause of the propagation stage of oxidation process. The higher potential of free radical scavenging causes the stronger inhibition of the spreading of oxidation. Therefore, ABTS and DPPH assays were used for antioxidant activity testing in the present study because these two methods can determine the free radical scavenging activity of the test samples directly.

3.2. *Antioxidant activity of water-PG extract*

Water-PG extract of RKF in comparison with that of other four medicinal plants demonstrated that RKF extract possessed the highest antioxidant activity with the maximum TEAC value of 3 mM/mg and the minimum IC_{50} values of 0.06 mg/mL as shown in Table 2. Phytochemical study demonstrated that RKF possessed the highest total phenolic content with GAE value of 23 mg/g while the highest total flavonoid content belonged to TCF with QE value of 6 mg/g. The antioxidant power of the four other plants was previously reported (*15-21*). However, antioxidant activity of *R. kerrii* has not yet been reported elsewhere. The present work is the first study that demonstrates the antioxidant activity of *R. kerrii*. The highest antioxidant activity of RKF among the other high-antioxidant plants found in this study suggested that RKF is the potential natural source of antioxidant compounds and suitable for further investigation.

Table 2. Antioxidant activity and phytochemical content of water-PG extracts of five medicinal plants

Plant extracts	Antioxidant activity		Phytochemical content	
	TEAC (mM/mg)	IC_{50} (ug/mL)	GAE (mg/g)	QE (mg/g)
RKF	3.3 ± 0.2	57.6 ± 2.2	23.2 ± 0.6	6.1 ± 0.3
RDF	0.9 ± 0.0	330.4 ± 30.7	5.3 ± 0.1	2.3 ± 0.3
EEF	0.2 ± 0.0	1,563.8 ± 83.3	1.6 ± 0.5	1.3 ± 0.2
TCF	2.4 ± 0.2	87.3 ± 2.5	18.5 ± 1.1	5.6 ± 1.7
TBF	2.4 ± 0.2	80.6 ± 1.2	17.3 ± 0.9	6.1 ± 0.5

3.3. *Effect of extracting solvent*

Extraction of active compounds from plants is generally carried out using various types of solvents such as hexane, ethyl acetate, butanol, acetone, ethanol, methanol and water. As an alternative to the extraction processes, water-cosolvent systems have been investigated for use in the extraction of various plant compounds (*22*). Water-ethanol and water-methanol systems have been investigated for extraction of several phytochemicals from medicinal plants (*23,24*) but it is still less data for water-PG which might be due to the high viscosity of PG that could affect the diffusion of a substance. However, PG has a better benefit than ethanol when the solution form of extract has been used according to its non volatile property. In the present study, two extracting water-cosolvent systems, water-ethanol (5:95) and water-PG (1:1) systems were compared. The results found that the physical status of the RKF extracts was different. The water-ethanol extract was semi-solid while the water-PG extract was liquid state due to not feasibly complete evaporation of PG from the extract. The water-ethanol extract showed extremely higher TEAC and lower IC_{50} values than the water-PG extract as shown in Table 3, indicating its higher antioxidant activity. Phytochemical study demonstrated that the total phenolic content and the total flavonoid content of the water-ethanol extract were obviously higher than the water-PG extract. The results suggested that the phenolic and flavonoid compounds existing in the extracts played an important role on antioxidant activity of RKF. The lower antioxidant property of RKF water-PG extract was considered to be due to the poorer ability of this solvent system to dissolve the antioxidant compounds from RKF sample in addition with the dilution effect of the solvent system.

3.4. *Effect of extraction procedure and extracting solvent*

Numerous extraction methods have been investigating in order to extract the active components optimally from various plant samples. The techniques range from a simple solvent extraction to the modern methods such as supercritical fluid extraction (*25*), pressurized liquid extraction (*26*), microwave-assisted extraction (*27*), solid phase micro-extraction (*28*) and ultrasound-assisted extraction (*29*). Among these, two common and mostly used procedures are a simple extraction using only one solvent (usually 95% ethanol) and a fractionated extraction using many solvents sequenced by their polarity. The extract of the former procedure is usually called "crude extract" where that of a fractionation is called "fractionated crude extract" or "fractionated extract". As the suitable polarity to many compounds, the crude extract always contains more kinds of compounds than the fractionated extract. In fractionated extraction, only compounds of similar polarity and high dissolution to the respective extracting solvent are extracted. Therefore, the fractionated extracts are considered to be less kind of compounds than the crude extracts. Many reports found that the fractionated extracts exhibited stronger activity than the crude extracts (*30,31*). However, several authors demonstrated the synergistic effect among the major active compounds and some minor components in the extract (*32*). As extraction procedure gives different kinds of compounds in the extracts, therefore, the effect of extraction procedure on antioxidant activity of RKF was undertaken in this study. It was found that the yield and antioxidant activity of the crude extract of RKF, namely RE and the fractionated extracts, namely RH, RA, RB and RM for hexane, ethyl acetate, butanol and methanol, respectively, were different. From this result RE, RA and RB were classified as a moderated activity group whereas RH was the lowest activity extract. It was found that RM fractionated extract possessed the strongest antioxidant with the highest TEAC value of 39 mM/mg and IC_{50} value of 3 µg/mg as shown in Table 4. This result suggested that the fractionated extraction was the suitable extraction procedure for RKF. This procedure could effectively separate the potential compounds with high antioxidant activity from RKF. The results also revealed that RKF contained phenolic and flavonoid compounds. The highest quantity of total phenolic content and total flavonoid content were obtained from RM with GAE value of 312 mg/g and QE value of 16 mg/g, as shown in Table 5. The results suggested that the phenolic compounds existing in the extract played an important role on antioxidant activity of RKF.

3.5. *UV-visible spectroscopy and HPLC analysis*

RM was selected to analyze in this experiment as this extract showed the highest antioxidant activity of RKF.

Table 3. Antioxidant activity and phytochemical content of RKF extracted from different extracting solvent system

Extracting solvent system	Antioxidant activity		phytochemical content	
	TEAC (mM/mg)	IC_{50} (ug/mL)	GAE (mg/g)	QE (mg/g)
Water -ethanol	22.1 ± 0.3	5.4 ± 0.3	176.5 ± 5.2	5.3 ± 1.0
Water-PG	3.3 ± 0.2	57.6 ± 2.2	23.2 ± 0.6	6.1 ± 0.3

Table 4. Comparative antioxidant activity of RKF extracted from different extraction procedure

Extraction procedure	Yield (%)	TEAC (mM/mg)	IC_{50} (µg/mL)
Single extraction			
95% Ethanol	42.0	22.1 ± 0.3	5.4 ± 0.3
Fractionated extraction			
Hexane	0.2	2.3 ± 0.0	51.8 ± 0.2
Ethyl acetate	0.5	22.5 ± 0.9	6.3 ± 0.2
Butanol	3.9	16.4 ± 0.4	10.0 ± 0.3
Methanol	32.3	38.8 ± 0.4	3.2 ± 0.6

Table 5. Comparative phytochemical content of RKF extracted from different extraction procedure

Extraction procedure	Extracts obtained	GAE (mg/g)	QE (mg/g)
Single extraction			
95% Ethanol	RE	176.5 ± 5.2	5.3 ± 1.0
Fractionated extraction			
Hexane	RH	4.9 ± 0.7	15.8 ± 0.3
Ethyl acetate	RA	182.8 ± 2.6	8.5 ± 0.2
Butanol	RB	146.5 ± 4.9	5.7 ± 0.8
Methanol	RM	311.9 ± 12.5	16.0 ± 0.0

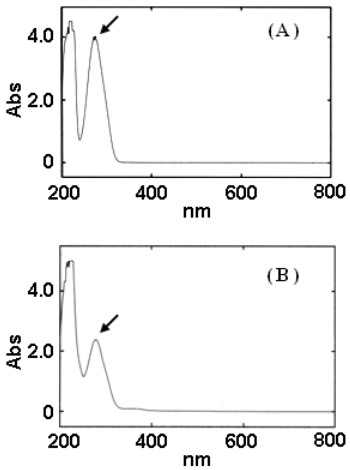

Figure 1. UV-VIS spectrum of RM (A) and gallic acid (B).

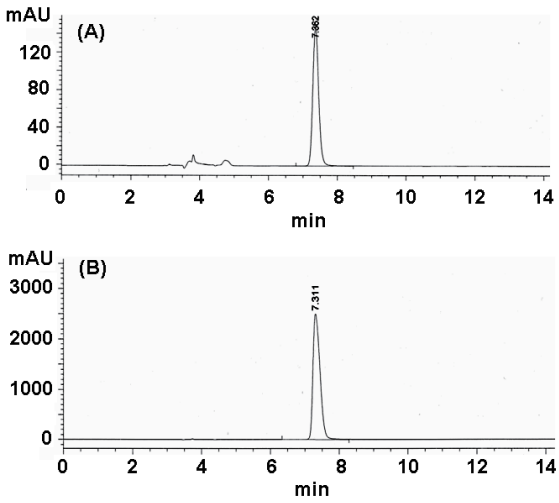

Figure 2. HPLC chromatogram of RM (A) and gallic acid (B).

The qualitative UV-VIS spectrum profile of RM was done at wavelength from 200 to 800 nm due to sharpness of the peaks and proper baseline was obtained. The UV-VIS profile of the extract showed the maximum absorption peaks at 273.2 ± 0.5, same as a standard gallic acid (274.0 ± 0.3 nm) as shown in Figure 1. This wavelength was use for UV detection in HPLC analysis. The HPLC chromatogram of RM displayed many peaks at different retention time. In comparison with a standard solution of gallic acid, the extract showed a major peak at the same retention time of gallic acid as shown in Figure 2. This result confirmed that gallic acid was one of the major active polyphenolic compounds existed in RM. Gallic acid was reported to be one of the major flavonoids possessed an antioxidant activity in grape seeds and skins (*33*). In the present study, this compound therefore was considered to play an important role on the antioxidant activity of RKF.

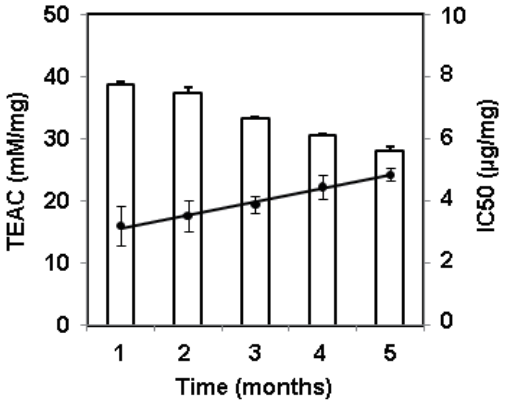

Figure 3. TEAC values (column) and IC50 values (●) of antioxidant activity of RM kept at 40°C and 75% relative humidity at various time.

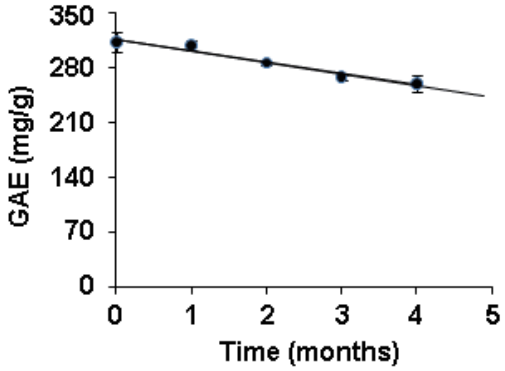

Figure 4. Total phenolic content of RM kept at 40°C and 75% relative humidity at various time.

3.6. *Stability of RM under stress condition*

Previous works observed the significant decrease in biological activities when plant extracts were subjected to a high temperature condition (*34*). In the present study, RM, the most effective antioxidant extract of RKF was subjected to a stress condition of 40°C and 75% RH for 4 months. Color change from reddish brown to slightly intense color was observed after the extract was stored for 3 months where the state of matter of the extract was still unchanged, still being as a solid mass. Antioxidant activity of RM during storage exhibited a decrease of TEAC values and an increase of IC_{50} values as shown in Figure 3, confirming the decrease of its activity. Total phenolic content of the extract depicted a decrease along the time of storage as shown in Figure 4. The decrease of these compounds was found to be a linear relationship with the antioxidant activity as shown

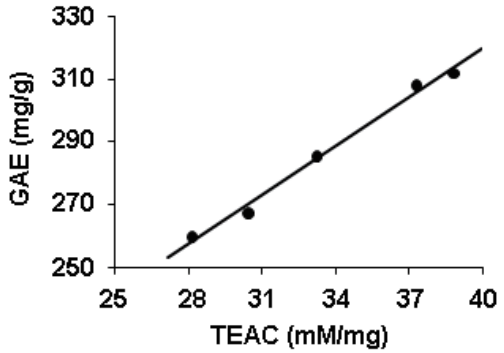

Figure 5. Relationship of total phenolic content (GAE value) and antioxidant activity (TEAC value) of RM.

in Figure 5. Therefore, the decrease of antioxidant activity of RM was considered to be due to the depletion of these active phytochemical content existing in the extract. Zero order kinetic equation (*d[A] = kdt*) was found to fit to the degradation of the existing total phenolic content in RM with the correlation coefficient (R^2) of 0.9582. According to this kinetic model, the rate constant of these phenolic compounds calculated from GAE values was 14.5 mg/month or approximately 50 µg/h. In comparison with the single antioxidant compound such as quercetin (*35*), catechins (*36*) and anthocyanin (*37*), RM seemed to exhibit higher stability than those compounds. This was considered to be due to some minor components existing in RM that might act as the desirable stabilizer for the antioxidant compounds.

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