ฤทธิ์การต้านอนุมูลอิสระและปริมาณสารประกอบฟีนอลรวมจากผลไม้ป่าของไทย

Antioxidant Activities and Total Phenolic Contents from Thai Wild Fruits

Pakit Kumboonma[1*] and Sudarat Sombatsri[1]

[1]Department of Applied Chemistry, Faculty of Science and Liberal Arts,

Rajamangala University of Technology Isan, Nakhon Ratchasima, 30000

[*]Corresponding Author, E-mail: pakit.ku@rmuti.ac.th

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บทคัดย่อ

งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาปริมาณของสารประกอบฟีนอลรวมและฤทธิ์ยับยั้งอนุมูลอิสระของผลไม้ป่าของไทย 10 ชนิด ได้แก่ ตะขบป่า สมัดน้อย เม่าไข่ปลา ไข่เน่า ระเวียง ตูมกา ตะโก มะกอกป่า ย่านางและตะคร้อ นำตัวอย่างทั้งหมดมาสกัดด้วยไดคลอโรมีเทน เอธิลอะซิเตตและเมธานอล จากนั้นนำสารสกัดทั้งหมดมาหาปริมาณของสารประกอบฟีนอลรวมด้วยวิธี Folin-Ciocalteu และศึกษาฤทธิ์การต้านอนุมูลอิสระด้วยวิธี 2,2-diphenyl-1-picrylhydrazyl (DPPH) วิธี reducing power และวิธี iron (II) chelation จากการทดลองพบว่าสารสกัดเอธิลอะซิเตตของตะขบป่ามีปริมาณสารประกอบฟีนอลรวมสูงสุด คือ 915.63±4.66 mg GAE/g นอกจากนั้นสารสกัดเอธิลอะซิเตตของตะขบป่ายังมีฤทธิ์ยับยั้งอนุมูลอิสระ DPPH ดีที่สุด โดยมีค่าการยับยั้ง (IC_{50}) เท่ากับ 3.94±0.01 µg/mL และมีค่า reducing power และ ferrous chelation ที่สูงเช่นเดียวกัน โดยมีค่าเท่ากับ 375.14±8.39 mmol/g และ 124.51±1.07 mmol/g ตามลำดับ ในขณะที่สารสกัดเมธานอลของมะกอกป่ามีฤทธิ์ยับยั้งอนุมูลอิสระที่ดีเช่นเดียวกัน โดยมีค่าการยับยั้งอนุมูลอิสระ DPPH เท่ากับ 3.97±0.06 µg/mL และมีค่า reducing power และ ferrous chelation ที่สูง โดยมีค่าเท่ากับ 366.02±12.27 mmol/g และ 105.70±1.21 mmol/g ตามลำดับ ดังนั้นตะขบป่าและมะกอกป่าสามารถที่จะเป็นแหล่งของสารต้านอนุมูลอิสระและสามารถพัฒนานำไปใช้ทางเภสัชวิทยาได้

ABSTRACT

The objective of this research was to study the total phenolic contents and the antioxidant activities of selected ten Thai wild fruits including *Flacourtia indica*, *Micromelum minutum*, *Antidesma ghaesembilla*, *Vitex glabrata*, *Catunaregam tomentosa*, *Strychnos nuxblanda*, *Diosyios rhodcalyx*, *Spondias pinnata*, *Tiliacora triandra* and *Schleichera oleosa*. All samples were extracted with dichloromethane, ethyl acetate and methanol. The total phenolic contents using the Folin-Ciocalteu reagent and the antioxidant activities using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, reducing power and iron (II) chelation of all extracts were evaluated. The total phenolic contents of the ethyl acetate extract of *F. indica* possessed the highest quantity of total phenolic compounds as 915.63±4.66 mg GAE/g extract. Moreover, the ethyl acetate extract of *F. indica* showed the strongest DPPH inhibition with the IC_{50} of 3.94±0.01 µg/mL. In addition, the ethyl acetate

extract of *F. indica* exhibited strong ferric reducing power and ferrous chelation activity with the value of 375.14±8.39 mmol/g extract and 124.51±1.07 mmol/g extract, respectively. The methanolic extract of *S. pinata* indicated very strong DPPH inhibition with the IC_{50} of 3.97±0.06 µg/mL. Furthermore, the methanolic extract of *S. pinata* showed strong reducing power and ferrous chelation activity with the values of 366.02±12.27 mmol/g extract and 105.70±1.21 mmol/g extract, respectively. Therefore, these fruits can be further used as sources of antioxidants for food and pharmacological application.

คำสำคัญ: ผลไม้ป่า ปริมาณฟีนอล สารต้านอนุมูลอิสระ
Keywords: Thai wild fruits, Phenolic contents, Antioxidant

INTRODUCTION

Reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radicals, peroxyl radical, nitric oxide and peroxynitrite are natural products from metabolic processes of an aerobic environment (Finkel et al., 2000). Normally, living organisms can maintain a proper level of highly reactive ROS using both enzymes and non-enzymatic mechanisms (Liu et al., 2010). However, the over production of ROS can increase from imbalances in their detoxifications and productions which lead to oxidative stress (Ellnain-Wojtaszek et al., 2003). Oxidative stress involves in many acute and chronic diseases including cancer, cardiovascular pathologies, rheumatoid arthritis and neurode-generative disorders such as Alzheimer's and Parkinson's diseases (Bouayed et al., 2007). Any compounds that can scavenge ROS are considered as important therapeutic agents. Antioxidants are known to play an important role in protection against oxidative damage. Antioxidants are compounds that can delay or inhibit the oxidant of lipids or other molecules by inhibiting the initiation or propagation of oxidizing chain reactions (Velioglu et al., 1998). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Osawa et al., 1994). Epidemiological studies have shown that food rich in oxidant played a significant role in the prevention of certain diseases such as cancer and cardiovascular diseases (Ismail et al., 2004). However, synthetic antioxidants such as BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene) have been restricted for use in food industry because of some side effects (Namiki et al., 1990). Therefore, there is great interest in finding new and safe antioxidants from nature.

Thailand is a tropical country with a large diversity of fruits, including lychee, mangosteen, durian, papaya and mango. Fruits contain many different kinds of antioxidant compounds, including flavonoids, phenolics, carotenoids and vitamins, which are all considered beneficial to human health. These fruits have been shown to be good sources of antioxidants such as vitamin E, beta-carotene and lycopene (Charoensiri et al., 2009). Besides the commonly consumed local fruits, some under-utilized species are important in the diets of rural communities. Some wild fruits of Thailand are rarely eaten, and are unknown or at least unfamiliar, especially in urban communities. These wild fruit species that are unique to the distinct climate in Thailand may offer potential benefits to human health. Despite this potential, Thai wild fruits have not received much attention as antioxidant sources

compared to commercial fruits. There is also a lack of information about their antioxidant properties, such as their phenolic compounds. Therefore, the purpose of this study was to evaluate various selections of Thai wild fruits with respect to their total phenolic content and antioxidant activities in order to find new potential sources of natural antioxidants. Evaluation of the relationship between phenolic content and antioxidant activity was also carried out including the relationship of solvent for extraction to their antioxidant activity and phenolic content.

RESEARCH METHODOLOGY

1. Materials

Ten types of Thai wild fruits were studied, namely, ta-khob-par (*Flacourtia indica* (Burm. f.) Merr), sa-mud-noi (*Micromelum minutum* (G. Forst) Wight & Arn), mao-khai-pla (*Antidesma ghaesembilla* Gaertn), khai-nao (*Vitex glabrata*), ra-wiang (*Catunaregam tomentosa* (Blume ex DC.) Triveng*)*, toom-kar (*Strychnos nux-blanda* A.W. Hill), ta-ko (*Diosyios rhodcalyx*), ma-kok-par (*Spondias pinnata* (L.f.) Kurz), ya-nang (*Tiliacora triandra* (Colebr.) Diels) and ta-khro (*Schleichera oleosa* (Lour.) Oken). The fresh fruits were collected on June 2013 from Nong-ra-wiang forest in Nakhon Ratchasima province. All samples were identified by Mr. Luis E. Garcia.

2. Chemical and reagents

The compounds 2, 2-diphenyl-1-picryl-hydrazyl (DPPH), Folin-Ciocalteu's reagent, potassium hexacyanoferrate and butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich (USA). Ascorbic acid, sodium dihydrogen phosphate, ferric chloride, methanol (AR grade) and disodium ethylenediamine tetraacetate were purchased from Qrec (New Zealand). Sodium hydroxide, sodium carbonate and ferrous sulphate were purchased from Ajax (Australia). Trichloro acetic was purchased from Carlo Erba (Italy). Ferrozine reagent and gallic acid were purchased from Acros (USA). Deionize water was prepared using a TKA water purification system (Germany). All other solvent were obtained locally and distilled prior to use.

3. Instruments

The spectrophotometric measurement was performed using a Shimadzu (UV-2450) UV-visible spectrophotometer. The Z206A centrifugal (Hermle, Germany), model R-210 rotary evaporator (Buchi, Switzerland), C-MAG HS7 hotplate stirrer (IKA, Germany), WNB22 water bath (Hemert, Germany), ED224S balance (Sartorius, Germany) were used in the sample preparation step. A micropipet (Jencons, England) and membrane filter (Whatman International Ltd, UK) were also used.

4. Preparation

Upon arrival at the laboratory, the fruits were cleaned with running water and cut into small sizes, and then baked in an oven at 40 $^{\circ}$C for 48 hours. All samples were ground with grinder.

5. Extraction

The fruit powder (20 g) was extracted for 24 hours with 200 mL of each solvent (CH_2Cl_2, ethyl acetate and methanol) at room temperature on a stirrer. The mixture was filtrated through the Whatman-1 filters, and then evaporated to dryness under vacuum (35-40 $^{\circ}$C) to give crude extracts. The crude extracts were used to determine total antioxidant activities and total phenolic contents.

6. Determination of total phenolic content (TPC)

Total phenolic content was determined by Folin-Ciocalteu reagent using gallic acid as a standard (Arnnok et al., 2012). The Folin-Ciocalteu method based on the reduction of phosphor-wolframate phosphor-molybdate complex by phenolics to blue reaction products. A 0.2 mL of the crude extracts (1

mg/mL in methanol) was mixed with 0.2 mL of Folin-Ciocalteu reagent and allowed to stand at room temperature for 5 min; 2.80 mL of 7% (w/v) sodium carbonate solution was added to the mixture. After incubation at room temperature for 90 min, the absorbance was measured at 745 nm against the blank sample contained the same mixture solution without the sample extract. Using a five-point calibration curve (1-5 mgL^{-1}), the total phenolics were determined by a comparison of the values obtained with the calibration curve of gallic acid. Results were expressed as mg gallic acid equivalents in 1 g of dried sample (mg GAE/g).

7. DPPH free radical-scavenging activity

The hydrogen atom or electron-donating ability of the corresponding extracts was measured from the bleaching of purple-colored methanol solution of DPPH (Gulluce et al., 2007). The antioxidant activity of the extracts on the basis of the scavenging activity of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical, was determined by the method described by Parejo et al. (2002). Briefly, a 1 mL of various concentrations of the crude extracts (in methanol) was added to 3 mL of a solution of 0.1 mM DPPH solution (in methanol). Absorbance at 517 nm was determined after 30 min, and the percent inhibition was calculated using Eq (1) (A_c = absorbance without the crude extract, A_s = absorbance with the crude extract).

$$\text{Inhibition (\%)} = [(A_c-A_s)/A_c] \times 100 \quad (1)$$

IC_{50}, the amount of the crude extract decreasing by 50% the initial DPPH concentration, was derived from the % inhibition vs concentration plot. BHT was used as a standard.

8. Reducing power activity

The ability of the fruit extracts to reduce iron (III) was assessed by the method of Oyaizu (1986). A 1 mL of each extract (1 mg/mL in methanol) was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% aqueous potassium hexacyanoferate [$K_3Fe(CN)_6$] solution. After 30 min incubation at 50 $^{\circ}C$, 2.5 mL of 10% trichloroacetic acid were added, and the mixture was centrifuged at 3000 rpm for 10 min. A 2.5 mL of the upper layer was mixed with 2.5 mL of DI water and 0.5 mL of 0.1% aqueous $FeCl_3$, and the absorbance was recorded at 700 nm. Increased absorbance of the reaction mixture indicates an increase of reduction capability. Iron (III) reducing activity was determined as ascorbic acid equivalents (mmol ascorbic acid/g extract). The values were presented as the means of triplicate analyses.

9. Ferrous ions chelating activity

The chelation of iron (II) by the different fruit extracts was carried out as described by Carter et al. (1971). A 0.2 mL of each extract (1 mg/mL in methanol) was added into 0.1 mL of 2.0 mM aqueous $FeSO_4$ and 0.9 mL of methanol. The controls contained all the reaction reagents except the extract or positive control substance. After 5 min incubation, the reaction was initiated by 0.4 mL of 5.0 mM ferrozine. After 10 min equilibrium period, the absorbance at 562 nm was recorded. The iron chelation activities were expressed as Na_2EDTA equivalents (mg Na_2EDTA/g extract). The values were presented as means of triplicate analyses.

RESULTS

1. Total phenolic contents

The Folin-Ciocalteu procedure has been proposed as a mean to rapidly estimate the level of TPC in foods and supplements (Konczak et al., 2010). The TPC of the evaluated Thai-wild fruits varied significantly from 227.57±2.31 to 915.63±4.66 mg gallic acid equivalent (mg GAE/g) (Table 1). Significantly

highest value of TPC was found in the ethyl acetate extract of *F. indica*, followed by the methanol extract of *S. pinnata* and the dichloromethane extract of *M. minutum*. The high TPC (>400 mg GAE)/g) was in dichloro-methane extracts of *S. pinnata* and *M. minutum*, ethyl acetate extracts of *S. pinnata* and *F.* indica, methanol extracts of *C. tomentosa* and *V. glabrata.* The high TPC was observed in the fruits that had an acidulous taste such as *F. indica* and *S. pinnata*.

2. Antioxidant activity

DPPH scavenging

The DPPH radical is a free radical compound which has been widely used to test free radical scavenging ability. Antioxidants, on interaction with the DPPH radical, transfer either an electron or hydrogen atom to DPPH, thus neutralizing its free-radical character. The reagent color changes from purple to yellow and its absorbance at wavelength 517 nm decreases. The DPPH radical-scavenging activity (IC_{50}) of thirty crude extracts of Thai wild fruits are demonstrated in Table 2.

The ethyl acetate extract of *F. indica* exhibited the highest antioxidant activity with the IC_{50} of 3.94±0.01 µg/mL. While the methanol extracts of *S. pinnata* showed strong antioxidant activity with the IC_{50} of 3.97±0.07 µg/mL. When compared to BHT (5.99±0.8 µg/mL), these extracts were found to be more effective as a free radical scavenger than the synthetic antioxidant. The methanol extracts and ethyl acetate extracts showed significantly higher antioxidant activity results than dichloromethane extracts.

Reducing power activity

The reducing power values of extracts were determined as ascorbic acid equivalents ranking from 4.56±0.52 mmol ascorbic acid/g extract to 375.14±8.39 mmol ascorbic acid/g extract in ferric reducing power assay. In the reducing power activity, a higher value indicates a stronger reducing power. The results in Table 3 show that the high activities were found in the ethyl acetate extract of *F. indica* and the methanol extract of *S. pinnata*.

Table 1 Total phenolic contents of Thai wild-fruit crude extracts.

Sample	Total phenolic contents*		
	Dichloromethane	Ethyl acetate	Methanol
F. indica	290.90±2.39	915.63±4.66	260.13±6.12
M. minutum	457.67±2.52	304.77±5.51	309.80±3.02
A. ghaesembilla	244.87±3.83	273.83±4.75	272.27±7.67
V. glabrata	335.33±5.51	303.13±3.56	408.93±3.20
C. tomentosa	236.53±2.61	309.93±1.68	423.70±3.73
S. nuxblanda	309.30±2.93	393.40±4.08	278.53±10.64
D. rhodcalyx	227.57±2.31	279.13±5.54	397.87±4.01
S. pinnata	256.03±2.10	416.40±2.51	752.77±6.71
T. triandra	347.33±2.52	260.07±8.89	291.30±2.98
S. oleosa	295.53±5.66	271.27±4.69	327.70±1.66

*Values are expressed as mg gallic acid equivalent/g extract (mg GAE/g extract)

Table 2 DPPH radical-scavenging activity of Thai wild-fruit crude extracts.

Sample	DPPH radical scavenging activity*		
	Dichloromethane	Ethyl acetate	Methanol
F. indica	83.88±3.46	3.94±0.01	152.88±1.67
M. minutum	327.88±2.04	202.29±1.86	145.60±1.62
A. ghaesembilla	776.32±5.45	307.36±2.56	117.65±1.93
V. glabrata	605.62±1.12	149.26±4.01	17.86±0.29
C. tomentosa	836.33±2.61	142.40±3.46	5.12±0.34
S. nuxblanda	361.97±2.36	35.09±2.45	105.86±1.23
D. rhodcalyx	727.12±7.38	56.51±1.17	12.87±0.27
S. pinnata	635.71±4.18	26.88±1.01	3.97±0.07
T. triandra	248.99±4.59	228.26±2.54	46.93±1.44
S. oleosa	895.98±5.66	278.36±3.07	35.73±0.58

*Values are expressed as IC_{50} (ppm), BHT std as 5.99±0.8 ppm

Table 3 Reducing power and iron (II) chelation activity of Thai wild-fruit crude extracts.

Sample	Reducing power activity[1]			Iron (II) chelation activity[2]		
	Dichloro-methane	Ethyl acetate	Methanol	Dichloro-methane	Ethyl acetate	Methanol
F. indica	12.32±0.95	375.14±8.39	23.77±4.09	10.49±0.47	124.51±1.07	4.82±0.03
M. minutum	28.17±0.51	11.91±1.65	23.19±4.40	11.80±0.27	4.83±0.03	4.85±0.05
A. ghaesembilla	24.98±3.65	8.43±2.21	22.03±1.67	1.55±0.12	18.05±0.29	17.36±0.28
V. glabrata	15.34±0.68	33.68±1.41	32.55±4.18	19.30±0.68	11.68±0.17	4.85±0.05
C. tomentosa	9.00±1.14	15.15±3.24	47.33±2.40	9.40±0.10	12.54±0.52	11.36±0.27
S. nuxblanda	9.45±1.18	25.84±1.78	21.09±3.26	4.80±0.04	21.15±0.39	4.84±0.04
D. rhodcalyx	8.55±0.69	56.57±2.78	61.42±1.22	8.23±0.09	109.16±1.39	9.82±0.11
S. pinnata	21.87±1.41	31.55±5.23	366.02±12.27	16.04±0.57	11.31±0.26	105.70±1.21
T. triandra	28.14±0.12	4.56±0.52	34.77±6.07	17.63±0.46	1.25± 0.05	21.68±1.29
S. oleosa	2478±4.74	1381±1.53	31.52±1.17	27.75±0.54	11.49±1.36	4.88±0.14

[1]Values are expressed as mmol ascorbic acid/g extract; [2]Values are expressed as mmol Na_2EDTA/g extract.

Ferrous ions chelating activity

The ability of the fruit extracts to chelate iron (II) ions was evaluated and expressed as Na_2EDTA equivalents (mmol Na_2EDTA/g extract). A higher chelating activity is associated with a higher value. The results are presented in Table 3. The ethyl acetate extract of *F. indica* showed the best iron chelation (124.51±1.07 mmol Na_2EDTA/g extract), followed by the ethyl acetate extract of *D. rhodcalyx* (109.16±1.39 mmol Na_2EDTA/g extract), and the methanol extract of *S. pinnata* (105.70±1.21 mmol Na_2EDTA/g extract). The content of all antioxidant assays showed a good correlation with total phenolic content.

DISCUSSION

In recent years, the interest in plant-derived food additives has grown. Plant extracts might be used instead of synthetic antioxidants, which may influence human health when consumed chronically. Plant-derived food additives, especially polyphenols, have also been ascribed as health-promoting properties, for

example, in terms of prevention of chronic cardiovascular diseases (Harborne et al., 2000). Polyphenols are secondary plant metabolites that are extensively presence in plants and natural products. These compounds are reported as highly effective free-radical scavengers and antioxidants (Tung et al., 2009). Therefore, it is significant to determine the amount of Thai wild-fruit samples. From the experiment, the results of total phenolic analyses showed that the ethyl acetate extract of *F. indica* and the methanol extract of *S. pinnata* were more effective than the synthetic antioxidant BHT. Phenolic substances of *F. indica* fruits have been reported such as caffeic acid, ferulic acid, *p*-hydroxybenzaldehyde, vanilic acid and *p*-coumaric acid (Ndhlala et al., 2007). However, chemical constituents of *S. pinnata* have never been reported. DPPH is a compound that consists of a nitrogen free radical which is easily quenched by free radical scavenger such as phenolic compounds. The free radical scavenging activity of the extracts increased depending on extract concentration. Among all extracts, the ethyl acetate extract of *F. indica* appeared to have the highest antioxidant activity with the lowest IC_{50} value, which could be attributed to known phenolic glycoside (Bhaumik et al., 1987; Amarasinghe et al., 2007; Chai et al., 2009; Madan et al., 2009), flavonoids and condensed tannins (Ndhlala et al., 2007; Madan et al., 2009).

Reducing power is an important mechanism of phenolic antioxidant action. Several reports have demonstrated that the reducing power of plant extracts is related to their antioxidant capacity (Tanaka et al. 1998). The ferric ion reducing power of Thai wild-fruit extracts revealed that the ethyl acetate extract of *F. indica* was higher than ferric reducing power activities obtained from the other crude extracts. Transition-metal ions in biological systems could catalyze some reactions and result in the generation of some free radicals such as hydroxyl radicals. Therefore, the major strategy to avoid ROS that is associated with redox active metal catalysis involves chelating of the metal ions. The results showed that the ethyl acetate extract of *F. indica*, the ethyl acetate extract of *D. rhodcalyx* and the methanol extract of *S. pinnata* possessed high metal-chelating property.

The good correlation between the results from total phenols analysis and the anti-oxidative assays has been previously reported (Wong et al., 2006). Generally, the extracts with a high amount of phenolic compounds also exhibit high antioxidant activity. The ethyl acetate extract of *F. indica* and methanol extracts of *S. pinnata*, *C. tomentosa*, *D. rhodcalyx* and *G. Sootepensis* which showed high antioxidant activity also had high TPC. In contrast to these results, the dichlorometane extract *of M. minutum,* which exhibited high TPC did not show high antioxidant activity as observed in other extracts. It could be explained by the different response of various phenolic compounds in the antioxidant assays. According to Frankel et al. (2000), the molar response of each assay is roughly proportional to the number of the phenolic hydroxyl groups in a given substrate, but the reducing capacity is enhanced when two phenolic hydroxyl groups are oriented ortho or para.

In complex systems such as food and food preparations, various different mechanisms may contribute to oxidative processes, such as Fenton reactions, where transition metal irons play a major role, different reactive oxygen species might be generated and various target structures such as proteins, carbohydrates and lipids, can be affected. Therefore, it is important to characterize the extracts by a variety of antioxidant assays. In the reducing power assay, the general ability of the extracts to

donate electrons is tested, whereas in the DPPH assay, hydrogen atoms are involved as well. One important mechanism of antioxidant action may be the chelation of iron (II) irons which serve as catalysts in Fenton reactions. The results from the antioxidant assays showed that some of extracts can act as radical scavengers to a certain extent. The ethyl acetate extract of *F. indica* showed the highest activity in the reducing power assay and in the iron chelation activity, followed by the methanol extract of *S. pinnata*. Although the ethyl acetate extract of *D. rhodcalyx* showed high performance in the iron chelation assay, it was less effective in the iron reduction. The antioxidant results revealed that ta-khob-par (*F. indica*) was more effective antioxidant-capacity than the popular antioxidant-fruits such as grape, mango, lemon and orange (Pisoschi et al., 2009).

CONCLUSION

The antioxidant activities and total phenolic content of thirty Thai wild-fruit extracts were examined. The ethyl acetate extract of *F. indica* and the methanol extract of *S. pinnata* were found to have high total phenolics and antioxidant capacities which may be useful for treating oxidative damage in the food and pharmaceutical industries. However, further studies to isolate and identify active compounds, especially phenolics, and *in vivo* study are needed to better understand their action mechanisms as pharmacological agents.

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