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Antioxidant activities of extract from Makmao seed waste

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Abstract

Makmao is a nutritious tropical fruit that is usually processed into products such as juice, wine, and jam. During the process, Makmao seeds are generated as a major form of waste. The aim of this research was to evaluate the antioxidant properties of Makmao seeds extracts using seed from different cultivars (Yaikumtar, Lungkumlar, and Thepnimit) with two ripeness degrees (mature and fully ripe) of Makmao fruits and using different solvents (hexane and ethanol) for extraction. The results show that the highest % yield (15.58±2.42 by weight) was obtained from fully ripe Lungkumlar seed extracted with ethanol, while the ethanol extract from fully ripe Yaikumtar seed possessed the highest content of phenolic and flavonoid. Considering the anthocyanin content, Lungkumlar seed at a fully ripe stage extracted with ethanol contained the highest amount of monomeric anthocyanin. The ethanol extract obtained from mature Yaikumtar seed showed the highest antioxidant activity determined by 2, 2-Diphenyl-1-picryl hydrazyl (DPPH), 3-ethylbenzothaia zoline-6-sulfonic acid (ABTS) and Ferric reducing antioxidant power assay (FRAP) (6.66±2.52, 7.80±2.08, and 5.84±1.07 mg TEAC/g extract, respectively) compared to the other extracts. According to the findings, the ethanolic extract from fully ripe Yaikumtar seed possessed the highest content of phenolic and flavonoid, and a relatively high antioxidant activity compared to the other extracts in this study. It could be further explored for its use as a potential natural antioxidant in the food products.

Keywords: Makmao, Seed, Extract, Phenolic, Antioxidant

1. Introduction

Makmao (*Antidesma* sp.) is cultivated in the north eastern part of Thailand and is also found in other tropical regions of Asia, Africa, and Australia, etc. [1]. Its spike contains about 30-50 drupes bearing a single seed each. The ripe fruit is red in color and turns to dark purple when it is fully ripe. The harvesting period of Makmao is usually between August to October. Makmao has a sweet and sour taste and contains phytochemicals with high antioxidant activity, thus, the consumptions of Makmao products such as juice, wine, jam have been gradually increasing. It was reported that more than 350 mg of Makmao fruits have been used annually in the food processing industry in Sakhon Nakhon province, Thailand [2]. During the process of converting the fruits into food products, seeds are generated as one of the major by-products, which constitute about 30% waste (approximately 100 mg/year). Waste and by-products from the fruit industry have been reported to contain potentially valuable bioactive compounds which could be exploited for the development of functional foods and pharmaceuticals. Makmao pomace has been reported to possess beneficial health effects including antioxidative, antihypertensive and cytoprotective activity [1,3]. However, there are scarcely studies on the properties and utilizations of Makmao seed [1].

Extraction of bioactive compounds from fruits and their by-products has attracted interest in order to obtain valuable natural extracts for applications in different industries. Yield and composition of the extracts are highly influenced by cultivars and ripeness of fruits. Samappito and Butkhup [4] reported that different cultivars of Makmao fruit contained various kinds of organic acids which could affect the sensory properties of their

products. In addition, fifteen cultivars of Makmao fruits contained different compounds of procyanidin which are responsible for their potent antioxidant activities and ability to scavenge free radicals [4]. Among other Makmao cultivars, Yaikumtar, Lungkumlar, and Thepnimit are locally grown in Sakhon Nakhon province, Thailand and are generally used for juice and wine processing due to their attractive characteristics of fruit including sweet taste and good flavor. It was previously revealed that the fruit of Yaikumtar, Lungkumlar, and Thepnimit cultivars contains considerable levels of total phenolic content (3.02, 2.70, 3.08 mg gallic acid equivalent/g dry weight, respectively) and total flavonoid content (1.39, 1.39, 1.65 mg catechin equivalent/ g dry weight, respectively) [5]. In addition, changes in bioactive compounds and antioxidant activities during the ripening of fruits have been widely investigated [6-10]. Several biochemical and physiological processes occur during fruit ripening and affect the phenolic compositions and their bioactivity. Moreover, the types and amount of bioactive compounds in the extracts significantly depend on extraction procedures. Solvent extraction is a separation process using a solvent to extract a desired component from plant material. Polarity of solvent is an influencing factor that greatly impacts the efficiency of the extraction process. Ethanol is usually used as a polar solvent for antioxidant extraction because it is cheap and nontoxic. Hexane is a nonpolar organic solvent that widely used for the extraction of lipophilic compounds. The hydrophilic and lipophilic fractions could possess different antioxidant activity [11]. Thus, this research aims to investigate the antioxidant properties of Makmao seeds extracts using seed from different cultivars (Yaikumtar, Lungkumlar, and Thepnimit) and ripeness degree (mature and fully ripe) of Makmao fruits and using ethanol and hexane as solvents. The use of extract from Makmao seed waste for producing potentially bioactive compounds could contribute towards their sustainable development.

2. Materials and methods

2.1 Chemicals and materials

Three cultivars of Makmao fruits, namely Yaikumtar, Lungkumlar, and Thepnimit at fully ripe stage (dark purple in color) and mature stage (red in color) were harvested from Phuphan District, Sakon Nakhon, Thailand in August, 2017. The seeds were separated from the fruits by hand and dried in a hot air oven (Binder FD53, USA) at 60 ˚C for 6 h (4.78% final moisture content), then kept in polyethylene bags at -20 ˚C for further experiment. 2, 2-Diphenyl-1-picryl hydrazyl (DPPH), 6-hydroxy-2, 5, 7, 8-tetramethyl-chroman-2-carboxylic acid (trolox), catechin hydrate, and TPTZ (2, 4, 6-tripyridyl-s-triazine) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Gallic acid was obtained from Fluka (Buchs, Switzerland). Folin–Ciocalteu phenol reagent was purchased from Merck (Darmstadt, Germany). Sodium hydroxide was obtained from Fisher Chemical (Mumbai, India). Potassium chloride, ferric chloride, potassium acetate, potassium persulfate, aluminum chloride hydrated, and methanol were purchased from Ajax Finechem (Auckland, New Zealand). Hexane was obtained from Macron Fine Chemicals (PA, USA). Ethanol 99.9% was purchased from QRëC (New Zealand). All chemicals were of analytical grade. Double-distilled and deionized water was used in the preparation of all solutions.

2.2 Sample extraction

The dried ground Makmao seeds (10 g) were extracted with 250 mL of either hexane or ethanol, using Soxhlet apparatus (Gerhardt EV 16, Germany) for 8 h. The solvent was removed by rotary evaporator (Buchi R205, Switzerland) at 60 ˚C, 335 mbar. The extract was kept in an amber glass bottle under N2 atmosphere at -20 ˚C until analysis. The % yield of the extract was calculated as follows:

% Yield = Weight of the extract (g) x 100/Weight of dried seed (g)

2.3 Total phenolic content

The total phenolic content (TPC) of the extract was evaluated using Folin Ciocalteu assay, according to a previously reported procedure with some modifications [12]. Briefly, 50 μL of the extract was mixed with 250 μL of Folin-Ciocalteu reagent, 0.75 mL of 20% sodium carbonate, and 3 mL of distilled water. The mixture was incubated for 2 h at ambient temperature. Then, the absorbance of the samples was determined at 765 nm using a spectrophotometer (Biochrom, Libra S12, USA). The TPC was calculated from the calibration curve of gallic acid (0-100 μg/mL) using the following equation $y = 0.0112x + 0.027$ ($R^2 = 0.9998$). The results were expressed in milligram gallic acid equivalent (GAE) per 1 g extract.

2.4 Total flavonoid content

Aluminum chloride colorimetry assay was used to determine the total flavonoid content (TFC) [12]. In brief, the extract (0.5 mL) was mixed with 95% ethanol (1.5 mL), 10% aluminum chloride (0.1 mL), 1 M potassium acetate (0.1 mL) and distilled water (2.8 mL). After 30 min incubation at room temperature, the absorbance of the samples at 415 nm was determined using a spectrophotometer (Biochrom, Libra S12, USA). The TFC was calculated from the calibration curve of (+)-catechin (5-100 μg/mL) using the following equation $y = 0.0051x + 0.0022$ ($R^2 = 0.9992$). The results were expressed in mg (+)-catechin equivalent (CE) per 1 g extract.

2.5 Total monomeric anthocyanin content

The total monomeric anthocyanins content (TAC) was measured by pH differential method [13]. Briefly, 0.3 mL of the extract was put into 2.7 mL of different buffer solutions including KCl buffer (0.025 M, pH = 1.0) and sodium acetate buffer (0.4 M, pH 4.5). The obtained solutions were measured absorbance at 510 and 700 nm. Total anthocyanin in form of cyanidin-glucoside (Cyd-3-glu) was calculated using the following equation:

TAC (mg/g) = (A × MW × dilution factor × 1000)/(ε×L)

Where:

$A = (A_{510\,nm} - A_{700nm})\ pH1.0 - (A_{510\,nm} - A_{700})\ pH4.5$,

MW for cyd-3-glu = 449.2 g mol^{-1}, ε = 26900 molar extinction coefficient in M^{-1} cm^{-1} for cyd-3-glu, Dilution factor = 10

2.6 DPPH radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging effect of the sample extract was determined by measuring the color change of DPPH radical (purple) to reduced DPPH (pale yellow) [14]. Briefly, 0.1 mm DPPH methanolic solution (2.9 mL) was added to the extracts (0.1 mL). After thoroughly mixing, the solution was kept in the dark for 30 min. To prepare the control, 2.9 mL of DPPH solution was mixed with 0.1 mL of methanol. After 30 min incubation, the absorbance at 517 nm was recorded using a spectrophotometer (Biochrom, Libra S12, USA). The DPPH radical scavenging activity was calculated from the calibration curve of trolox (0-0.1 mm) using the following equation $y = -5.87x + 0.52$ ($R^2 = 0.984$). The results were expressed in mg trolox equivalent antioxidative capacity (TEAC) per 1 g extract.

2.7 ABTS+ radical scavenging capacity

The ABTS cation radical scavenging capacity of the extract was evaluated according to the previous method [14]. In brief, 700 μM of 2,2'azinobis (3-ethylbenzothaia zoline-6-sulfonic acid (ABTS) radical cation solution was prepared by adding 0.0166 g potassium persulphate and 0.096 g of ABTS radical. The volume was then adjusted to 250 mL with distilled water. After throughly mixing, the solution was left to stand for 12 h. The sample extract (30 μL) was mixed with ABTS radical cation solution (3 mL). After 6 min incubation, the absorbance at 734 nm was measured using a spectrophotometer (Biochrom, Libra S12, USA). The TEAC values were calculated from the calibration curve of trolox (0-0.1 mm) using the following equation $y = -3.93x + 0.6476$ ($R^2 = 0.9909$). The results were expressed in mg TEAC/g extract.

2.8 Ferric reducing antioxidant power assay (FRAP)

The FRAP was also used to evaluate the antioxidant activity of the extract [14]. To prepare the FRAP reagent, 300 mm acetate buffer (adjusted pH to 3.6 by acetic acid), 10 mmTPTZ solution (in 40 mm HCl) and 20 mm ferric chloride solution were mixed in the proportions of 1:1:10 (v/v), respectively. The extract (150 μL) was mixed with the FRAP reagent (2.85 mL) and incubated for 10 min. The absorbance at 595 nm was recorded using a spectrophotometer (Biochrom, Libra S12, USA). Samples were determined in triplicates and the results were expressed in mg TEAC/g extract. TEAC values were calculated from the Trolox standard curve ($y = 7.14x + 0.0586$, $R^2 = 0.9983$).

2.9 Color measurement

The colors of Makmao extracts were measured in CIE L^*, a^*, b^* using a colorimeter (Hunter *Lab*, Color flex, USA).

2.10 Statistical analysis

All data shown in this study represent the mean values ± standard deviation of triplicate measurements. The statistical differences between mean values were analyzed by analysis of variance (ANOVA) using IBM SPSS Statisics 21.0 and compared using Duncan's multiple-range test with a significance level of 0.05.

3. Results and discussion

3.1 % Yield and bioactive compound contents

Makmao seeds are generated as wastes from Makmao juice and wine industry, but to our knowledge, there have been very few reports of Makmao seed extract properties [1,15]. In this study, the effect of cultivar (Yaikumtar, Lungkumlar, Thepnimit), ripeness (mature, fully ripe) and extraction solvent (ethanol, hexane) on the bioactive compounds content and antioxidant properties of Makmao seed extracts were analyzed. %Yield and bioactive compound contents of the extracts from seeds of different cultivars and ripeness of Makmao are shown in Table 1. Yields of the extract from Makmao seed were highly influenced by the cultivar and ripeness of the fruits in which the highest % yield was found in seed from fully ripe Lungkumlar. Mahmood et al. [16] reported that the extraction yield of strawberry and mulberry increased as the degree of maturity developed from unripe to fully-ripen. Moreover, plant could contain both hydrophilic and lipophilic compounds, polar and less polar compounds, thus, the polarity of extraction solvent also plays an important role in the extraction yield. The results show that the higher % yield was obtained by using ethanol as a solvent compared to hexane. Ethanol has been widely used for bioactive compounds extraction due to its relatively high polarity and safety for food applications of the extracts [17].

Table 1 % Yield, total phenolic, total flavonoid and anthocyanin content in Makmao seed extracts.

Cultivar	Ripeness	Extraction solvent	% Yield	Total phenolic (mg GAE/g extract)	Total flavonoid (mg CE/g extract)	Total anthocyanin (mg cyd-3-glu /g extract)
Yaikumtar	Mature	Ethanol	8.79±0.67^{d}	4.80±0.57^{b}	5.82±1.18^{b}	nd
		Hexane	5.63±1.49^{e}	0.17±0.07^{e}	0.25±0.03^{d}	nd
	Fully ripe	Ethanol	13.23±0.95^{bc}	6.33±0.37^{a}	7.28±0.64^{a}	0.34±0.10^{a}
		Hexane	5.79±0.22^{e}	0.25±0.06^{e}	0.43±0.16^{d}	nd
Lungkumlar	Mature	Ethanol	13.15±1.07^{bc}	3.74±0.10^{c}	3.41±0.54^{c}	0.09±0.03^{b}
		Hexane	6.36±0.42^{e}	0.40±0.04^{e}	0.35±0.11^{d}	nd
	Fully ripe	Ethanol	15.58±2.42^{a}	3.29±0.40^{c}	5.92±0.37^{b}	0.41±0.01^{a}
		Hexane	6.68±0.01^{e}	0.31±0.10^{e}	0.43±0.10^{d}	nd
Thepnimit	Mature	Ethanol	11.13±0.35^{c}	4.66±0.39^{b}	4.21±0.25^{c}	nd
		Hexane	6.39±2.66^{e}	0.17±0.04^{e}	0.19± 0.03^{d}	nd
	Fully ripe	Ethanol	13.93±0.05^{ab}	2.82±0.29^{d}	5.58±1.00^{b}	nd
		Hexane	5.59±0.67^{e}	0.22±0.03^{e}	0.73±0.26^{d}	nd

nd, not detected.

Different superscript letters within the column are significantly different at 0.05.

As seen in Table1, the TPC and TFC of the extract ranged from 0.17±0.07 to 6.33±0.37 mg GAE/g extract and 0.19±0.03 to 7.28±0.64 mg CE/g extract, respectively. The TAC of the extract was varied from an amount of not detected to 0.41±0.01 mg cyd-3-glu /g extract. Comparing among the extracts, the highest TPC and TFC were found in the ethanolic extract from fully ripe Yaikumtar seed of 6.33±0.37 meq gallic acid/g extract and 7.28±0.64 meq catechin/g extract, respectively. Considering the TAC, the ethanol extract from fully ripe Lungkumlar and Yaikumtar seed contained the highest amount of 0.41±0.01 and 0.34±0.10 meq cyanidin triglucoside/g extract, respectively. In contrast, the extract from ripe Thepnimit seed extracted with hexane contained the lowest TPC (0.17±0.07 mg GAE/ g extract), TFC (0.19±0.03 mg CE/g extract), and TAC (not detected). Jorjong and coworkers [5] reported that Yaikumtar makmao fruit contained TPC and TFC of 3.02 mg GAE/ g extract and 1.39 mg CE/g extract which were relatively low compared to our study possibly due to different extraction methods. It was previously reported that Makmao fruits mainly contained three different kinds of flavonoids including catechin, procyanidin B1 and procyanidin B2, of which the grand total ranged from 40.61 to 65.15 mg/ g fresh weight, depending on their cultivars [18]. These bioactive components in Makmao are responsible for their potent antioxidant activities and ability to scavenge free radicals [18]. Considering the effect of type of extraction solvent, ethanol which is a polar solvent, effectively extracted phenolic compounds, flavonoids and anthocyanin from Makmao seed. In contrast, hexane, a nonpolar organic

solvent, exhibited rather poor capability of extracting bioactive compounds from Makmao seed compared to ethanol. It could, therefore, be presumed that Makmao seed mostly contained hydrophilic compounds rather than lipophilic compounds.

3.2 Antioxidant activity of the extracts

Antioxidant activity of Makmao seed extract was analyzed using three different assays namely, DPPH, ABTS and FRAP assay. As shown in Table2, the ethanol extract from mature Yaikumtar seed showed the highest antioxidant activity determined by DPPH, ABTS and FRAP assay (6.66±2.52, 7.80±2.08, and 5.84±1.07 mg TEAC/ g extract, respectively). In contrast, all of the extracts using hexane as a solvent contained lower antioxidant activities compared to those extracted using ethanol. The trends of antioxidant activity were related closely to the TPC, TFC and TAC. The use of ethanol as a solvent provided an efficient isolation of compounds possessing antioxidant properties rather than hexane. Considering the effect of ripeness, the ethanolic extract from seed of mature Makmao in all cultivars shows significantly higher antioxidant activity compared to the extract from seed of fully ripe Makmao. This is in agreement with several researches which demonstrated that antioxidant activity reached the maximum level at mature stage and then declined as ripening advanced. [7,8] Comparing among Makmao cultivars in this study, the extract from Yaikumtar seed possessed the highest antioxidant activity. The variation of antioxidant activity of Makmao extract from different ripening stages and cultivars could be owing to the changes in phenolic contents. These have been reported for their strong correlation with antioxidant activity in a variety of fruits. Wojdylo et al. [19] observed that the phenolic contents had a good relationship with the antioxidant capacity obtained from DPPH• assay ($r = 0.8352$) and FRAP assay ($r = 0.9100$) for the species of the Lamiaceae family. It has been previously revealed that the Makmao seed extract exhibited a strong antioxidant activity against the DPPH radical and the ABTS radical with IC50 ranged from 0.85 to 0.97 μg/mL and 0.94 to 1.21 μg/mL, respectively compared with IC50 of trolox that was 5.05 μg/mL[1]. Makmao seed extract also demonstrated the anti-apoptotic and anti-inflammatory properties in human breast epithelial (MCF10A) cells as described by Puangpronpitag et al. [15]. However, the findings obtained in the present study suggest that the bioactive compounds extracted from Makmao seed had relatively low antioxidant activity compared to their fruits. Jorjong et al. [5] revealed that the antioxidant activity of Makmao fruit extracts evaluated by ABTS assay were in the range of 20.68 to 46.37 mmol TE/g DW (5,176 to 11,606 mg TEAC/g DW).

Table 2 Antioxidant activities of Makmao seed extracts.

Cultivar	Ripeness	Extraction solvent	Antioxidant activity (mg TEAC/g extract)		
			DPPH	ABTS	FRAP
Yaikumtar	Mature	Ethanol	6.66±2.52^{a}	7.80±2.08^{a}	5.84±1.07^{ab}
		Hexane	0.10±0.02^{d}	0.11±0.02^{d}	0.05±0.02^{d}
	Fully ripe	Ethanol	5.04±0.26^{b}	8.35±0.65^{a}	6.38±4.41^{a}
		Hexane	0.07±0.02^{d}	0.08±0.01^{d}	0.04±0.01^{d}
Lungkumlar	Mature	Ethanol	4.52±0.10^{b}	4.96±0.51^{c}	6.87±1.82^{a}
		Hexane	0.10±0.01^{d}	0.13±0.01^{d}	0.07±0.03^{d}
	Fully ripe	Ethanol	2.44±0.46^{c}	5.09±1.18^{bc}	2.27±0.68^{cd}
		Hexane	0.05±0.01^{d}	0.08±0.00^{d}	0.04±0.01^{d}
Thepnimit	Mature	Ethanol	4.69±0.48^{b}	6.36±0.30^{b}	5.04±0.98^{ab}
		Hexane	0.09±0.01^{d}	0.17±0.09^{d}	0.05±0.01^{d}
	Fully ripe	Ethanol	2.48±0.14^{c}	5.21±0.43^{bc}	3.42±0.35^{bc}
		Hexane	0.06±0.01^{d}	0.09±0.01^{d}	0.03±0.00^{d}

nd, not detected.
Different superscript letters within the column are significantly different at 0.05.

3.3 Color of the extracts

Color is one of the main physical properties of food ingredients that impacts the consumers' acceptance. CIE L^*, a^*, b^* color coordinates were used to determine the degree of lightness (L^*), redness-greenness (+ or - a^*) and yellowness-blueness (+ or - b^*). The color of Makmao fruit changes from light-white into red or dark-purple, depending on the level of ripeness. Table3 shows the color values of Makmao seed extract. It was found that the L^* of Makmao seed extract ranged from 1.42±0.40 to 7.51±1.93. The a^* and b^* values varied from 0.34±0.11 to 1.44±0.03 and from 0.25±0.07 to 2.16±0.46, respectively. A comparison of the color of the extracts from Makmao seed show that the lightness and redness (L^* and a^* values) of the extracts were not significantly different among the different cultivars and ripeness (Table 3). However, the lightness and redness of the extracts

using hexane as a solvent were significantly lower compared to those extracted using ethanol. This is because ethanol can dissolve more anthocyanin, a water soluble pigment in Makmao, than hexane.

Table 3 Color coordinates of Makmao seed extracts.

Cultivar	Ripeness	Extraction solvent	L^*	a^*	b^*
Yaikumtar	Mature	Ethanol	7.51±1.93^{a}	1.31±0.04^{ab}	0.74±0.12^{def}
		Hexane	1.42±0.40^{c}	0.41±0.26^{de}	0.57±0.05^{efg}
	Fully ripe	Ethanol	7.69±1.49^{a}	1.08±0.26^{b}	0.25±0.07^{g}
		Hexane	3.74±1.98^{b}	0.65±0.02^{cd}	1.11±0.16^{bc}
Lungkumlar	Mature	Ethanol	7.17±0.13^{a}	1.26±0.08^{ab}	1.67±0.46^{b}
		Hexane	2.25±0.91^{c}	0.37±0.12^{de}	0.27±0.02^{fg}
	Fully ripe	Ethanol	6.57±0.50^{a}	1.34±0.09^{ab}	1.01±0.17^{bcd}
		Hexane	1.75±0.38^{c}	0.52±0.11^{cde}	1.11±0.40^{bc}
Thepnimit	Mature	Ethanol	7.18±0.13^{a}	1.44±0.03^{a}	2.16±0.46^{a}
		Hexane	1.50±0.70^{c}	0.34±0.11^{e}	0.26± -.03^{fg}
	Fully ripe	Ethanol	5.79±0.29^{a}	1.23±0.24^{ab}	1.30±0.03^{bc}
		Hexane	2.93±0.90^{bc}	0.72±0.18^{c}	1.41±0.40^{bc}

nd, not detected.
Different superscript letters within the column are significantly different at 0.05.

4. Conclusion

In summary, this current study showed that the extracts from Makmao seed could be a promising source of natural antioxidants and be utilized as a health promoting ingredient in the food, pharmaceutical, as well as cosmetic industries. The ethanolic extract from seed of Yaikumtar, particularly at fully ripe stage exhibited the highest TPC (6.33±0.37 meq gallic acid/g extract) and TFC (7.28±0.64 meq catechin/g extract) and possessed relatively high antioxidant activities compared to other extracts in this study. For the future research, further investigation is recommended on the extraction procedure in order to achieve higher yields of valuable extracts from Makmao seed and make it more economically viable. In addition, its applications in different products as a potential natural antioxidant could be further explored.

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