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Antioxidant capacity of extracts and fractions from mango (*Mangifera indica* Linn.) seed kernels

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Abstract: This study investigated the antioxidant capacity and chelating activity of the mango seed kernel (MSK) extracts prepared by shaking (SMSK) and acid hydrolysis (AMSK) including fractions. The MSK was dissolved in methanol and separated using a Sephadex LH-20 column with monitoring by UV absorption. The results showed that both MSK showed significantly ($P<0.05$) higher antioxidant capacity, assessed by the DPPH• and ABTS•+ scavenging assays and ferric thiocyanate test, than α-tocopherol. AMSK showed higher phenolic antioxidant activities than SMSK. The chelating efficiency of AMSK was greater than that of ascorbic acid. The chromatographic profile of MSK extracted by shaking (5 fractions) and acid hydrolysis (2 fractions) was different. Fraction 2 of AMSK (93.68±1.24% yield, db) showed a remarkable antioxidant and chelating activity in comparison with tannic acid and methyl gallate. The present study demonstrated that mango seed kernel obtained from acid hydrolysis is a potential material for use as a natural phenolic antioxidant.

Keywords: Thai, mango seed kernel, antioxidant, phenolic, chelating

Introduction

Mango has been cultivated for about 4,000 years and its production and consumption has gradually increased as its popularity has grown. At least 87 countries grow over 26,286,255 MT per annum (Youngmok *et al*., 2004). Among internationally traded tropical fruits, mango ranks only second to pineapple in quantity and value. Mango production is highest in India, at 41% of the world's production (10,800,000 MT), followed by China, Thailand, Mexico, Pakistan, Indonesia, the Philippines, Nigeria, and Brazil (Dube *et al*., 2004). Mangoes are consumed as fresh fruits and after processing into pickles, chutneys, canned or dried goods, juices, or nectars. During processing of mango, by-products such as peel and kernel are generated. Kernel contributes about 17-22% of the fruit (Soong and Barlow, 2004).

Mango seed kernel was also shown to be a good source of phytosterols including campesterol, β-sitosterol, and stigmasterol and it also contains tocopherols. Maisuthisakul (2008) has recently reported the phenolic compounds in eleven cultivars of Thai mango seed kernels. Mango seed kernel showed potent tyrosinase inhibitor, antioxidant activity and chelating activity (Maisuthisakul and Gordon, 2009). Moreover, the extraction conditions affected the content of phenolic compounds and the activities of mango seed kernel extracts (Maisuthisakul, 2009). Therefore, the purpose of the present study was to investigate the antioxidant properties and phenolic content of different fractions from mango seed kernel (MSK) from various extractions in an attempt to make a systematic comparison of their antioxidant activity for further studies.

Materials and Methods

Materials

Three batches of sun dried seeds separated from ripened mango (*M. indica* cultivar Chok-Anan) as by-products from March to June 2008 were donated by Woraporn Co., Ltd., a mango processing manufacturer in Thailand. The seeds were washed and sun dried in the greenhouse for three days and the kernels were removed manually from the seeds for further extraction. The moisture content of dried mango seed kernel on a dry weight basis according to AOAC (1990) was 9.81±0.34 %. The dried material was kept in a freezer at -20°C for no longer than two months.

Folin Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and sodium carbonate, were purchased from Sigma Chemical Co., Ltd (St. Louise, USA). Tannic acid was purchased from

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Acros Organics (New Jersey, USA). The other chemicals and solvents used in this experiment were analytical grade purchased from Sigma-Aldrich Co., Ltd (Steinheim, Germany).

Extraction of mango seed kernel phenolics

The kernels (80 g) were blended for 1 min with ethanol at -20°C and the containers were then flushed with nitrogen and shaken (shaking method) for 4.5 hours in the dark at 30°C (Maisuthisakul *et al*., 2007). Another set of samples was refluxed with 1.2 M hydrochloric acid in ethanol for 3 h (acid hydrolysis method). The supernatant, after filtration through cheesecloth and Whatman No 4 filter paper, was evaporated under vacuum. The sample was dried in a freeze dryer and stored in aluminum foil after flushing with nitrogen at -20°C until analysis. The dried extracts and reference samples (α-tocopherol, ascorbic acid, methyl gallate and tannic acid) were used to estimate the antioxidant properties by the DPPH, ABTS and the thiocyanate methods. The MSK extracts were also used to evaluate total phenolic content and chelating activity

Fractionation of mango seed kernel phenolics

Sephadex LH-20 gel (Sigma Chemical Co., St. Louis, MO) was used for fractionation by column chromatography. The ethanol-soluble fraction of MSK extract from shaking and acid hydrolysis methods (19.8 g) was redissolved in methanol (3.000 ml) and loaded onto the column (9×85 cm2), which was equilibrated with methanol. The column was eluted with methanol at a flow rate of 60 ml/hour. Fifty-milliliter fractions were collected and their absorbance values were determined at 280 nm. Eluted fractions were then pooled based on the elution profiles. After evaporation of methanol, the samples were lyophilized and the residue was weighed. The total phenolic content, DPPH radical scavenging activity, antioxidant activity in a linoleic acid emulsion system and chelating activity of each major fraction were estimated.

Determination of antioxidant properties

The total free radical-scavenging capacity of MSK or reference samples was determined by using the DPPH and ABTS methods. The antioxidant activity in a linoleic acid emulsion system was also determined.

The free radical scavenging activity of MSK or reference samples was evaluated using the stable radical DPPH according to the method of Masuda *et al*. (1999). The radical scavenging activity (%) was plotted against the plant extract concentration (μg/ml) to determine the concentration of extract that reduced activity by 50% (EC_{50}). These values were changed to antiradical activity (AAR) defined as $1/EC_{50}$, since this parameter increases with antioxidant activity. All determinations were performed in triplicate.

The ABTS radical scavenging activity was determined according to Re *et al*. (1999). The activity of each antioxidant was determined at three concentrations, within the range of the dose-response curve of Trolox, and the radical-scavenging activity was expressed as the Trolox equivalent antioxidant capacity (TEAC), defined as mMol of Trolox per gram of sample. All determinations were performed in triplicate.

The antioxidant activity in a linoleic acid emulsion system of MSK or reference samples was determined using the thiocyanate method (Hu *et al*., 2004), with some modifications. Each sample in absolute ethanol (0.5 ml) was mixed with 0.5 ml of 5.21% linoleic acid, 1 ml of 0.05 M phosphate buffer (pH 7), and 0.5 ml of distilled water and placed in a screw capped tube. The reaction mixture was incubated in the dark at 40°C in an oven. Aliquots of 0.1 ml were removed every 24 h during incubation and the degree of oxidation was measured by sequentially adding ethanol (9.7 ml, 75%), ammonium thiocyanate (0.1 ml, 30%) and ferrous chloride (0.1 ml, 0.02 M in 3.5% HCl). After the mixture was rested for 3 min, the peroxide value was determined by monitoring absorbance at 500 nm until the absorbance of the control reached the maximum. The degree of linoleic acid peroxidation was calculated using the following formula: antioxidant activity = $[(A_{control} - A_{sample})/A_{control}] \times 100$. The antioxidant activity was plotted against sample concentration in order to determine the concentration required to achieve a 50% inhibition of linoleic acid oxidation [AA_{50}]. All tests and analyses were carried out in triplicate and averaged.

Determination of total phenolic content

The total phenolic content of extracts was determined using the Folin-Ciocalteu's phenol reagent (modified from Kähkonen *et al*., 1999). The concentration of total phenolic compounds in all plant extracts was expressed as mg of tannic acid equivalent per g dry weight of MSK using a linear equation. All determinations were performed in triplicate.

Determination of chelating activity

The ferrous ion chelating activity of studied samples was estimated based on the decrease in the maximal absorbance of the iron (Fe^{2+})-ferrozine complex according to previously reported methods

(Dinis *et al*., 1994), with some modifications. Briefly, 1 ml of a solution of a test compound at various concentrations dissolved in ethanol was incubated with 0.5 ml of $FeCl_2 \cdot 4H_2O$ (1.0 mM). The reaction was initiated by the addition of 1 ml of ferrozine (5.0 mM), and then the total reaction volume was adjusted to 4 ml with ethanol. After the mixture had reached equilibrium (10 min), the absorbance at 562 nm was read. The control was prepared without the test compound. Fe^{2+} chelating activity of the test compound was calculated from the following formula: chelating activity (%) = $[(A_{control} - As_{ample})/A_{sample}] \times 100$. The chelating activity (%) was plotted against the plant extract concentration (µg/ml) to determine the concentration of extract necessary to reduce chelation by 50% (CA_{50}). Samples with a lower CA_{50} had a stronger chelating activity. All tests and analyses were carried out in triplicate and averaged.

Statistical analysis

Each experiment, from sample preparation to analysis, was repeated in triplicate, and the data were analyzed by SPSS software program (SPSS Inc., Chicago, IL, USA). The general linear model procedure was applied and Duncan's multiple range test was used to compare the mean values at $P<0.05$. Mean values and pooled standard error of the mean (SEM) were then estimated.

Results and Discussion

Antioxidant capacity of mango seed kernel from different extraction methods

The effect of extraction on antioxidant property of mango seed kernel was investigated. Several methods have been used to determine antioxidant capacity of plants. Our present study involved three methods to evaluate the antioxidant activity of the MSK extract, namely, DPPH radical scavenging activity, ABTS cation radical scavenging activity and ferric thiocyanate assay. Total phenolic content and chelating activity were also determined.

The extract prepared by shaking showed lower antioxidant properties than that of acid hydrolysis (Table 1) which was comparable to the antioxidant activity of methyl gallate and higher than that of α-tocopherol. Some phenolic compounds in mango seed kernel occur as insoluble bound phenolic and esterified phenolic compounds (Maisuthisakul, 2008). Acid hydrolysis enhances the extraction of total bound phenolics, thus breaking more easily the linkages between phenolics and other constituents such as sugar and protein (Nuutila *et al*., 2002). This caused the higher phenolic content of the extract prepared by acid hydrolysis (285.70 mg TAE/g) than that of the extract prepared by shaking (110.03 mg TAE/g) (Table 2). The close correlation between antioxidant activity and phenolic content of extracts obtained from various natural sources has been demonstrated by many workers (Velioglu *et al*., 1998; Parejo *et al*., 2002; Meyers *et al*., 2003).

The antioxidant activity assessed by the ferric thiocyanate method involving oxidation of linoleic acid showed the same order as metal chelating activity (Table 1 and 2). The ferric thiocyanate method was originally designed for measuring lipid peroxide content in an emulsion system, whereby the end-point measure is the amount of Fe^{2+} that is oxidized to Fe^{3+} by lipid peroxides.The Fe^{3+}-thiocyanate complex produces a deep red colour, which is detectable at 500 nm. The advantage of using ammonium thiocyanate over other coloring reagents is that binding of iron by thiocyanate ion is specific to Fe^{3+} only, and that the Fe^{3+}–thiocyanate complex produces a single absorbance peak at 500 nm. For chelating activity measurement, the known coloring reagent, ferrozine, has a binding affinity towards Fe^{2+} only (Moon and Shibamoto, 2009). Metal chelating activity may affect from the rate at which hydroperoxides form.

Table 1. The antioxidant capacities of mango seed kernel extracts obtained by different extraction methods ¥

Conditions	Antiradical activity (A_{AR}, $1/EC_{50}$)	ABTS activity (mMol of Trolox/g)	Antioxidant efficiency ($1/AA_{50}$)
Shaking	1.75 ^{c}±0.15	1.03 ^{a}±0.02	0.014 ^{a}±0.000
Acid hydrolysis	4.16 ^{e}±0.54	1.41 ^{d}±0.01	0.019 ^{c}±0.000
α-tocopherol	2.68 ^{d}±0.04	1.30 ^{c}±0.03	0.017 ^{b}±0.000
Ascorbic acid	0.70 ^{a}±0.00	1.12 ^{b}±0.00	0.048 ^{d}±0.000
Methyl gallate	4.01 ^{e}±0.01	1.40 ^{d}±0.01	0.019 ^{c}±0.000
Tannic acid	0.95 ^{b}±0.01	3.10 ^{e}±0.01	0.054 ^{e}±0.000

¥dry weight basis of mango seed kernel; Means of three replications ± SD (standard deviation); Different superscript letters mean significant differences (P<0.05) between conditions in each column.

Table 2. The chelating efficiency and total phenolic content of mango seed kernel extracts obtained by different extraction methods ¥

Conditions	chelating efficiency ($1/CA_{50}$)	Total phenolic content (mg of TAE/ g)
Shaking	0.019 ^{a}±0.000	110.03 ^{a}±0.06
Acid hydrolysis	0.031 ^{c}±0.001	285.70 ^{b}±0.28
Ascorbic acid	0.024 ^{b}±0.001	ND
Methyl gallate	0.031 ^{c}±0.001	ND
Tannic acid	0.038 ^{d}±0.003	ND

¥dry weight basis of manko seed kernel; Means of three replications±SD (standard deviation); Different superscript letters mean significant differences (P<0.05) between conditions in each column; ND means not determined.

Table 3. Characterization of crude MSK extract obtained from shaking (SMSK) condition and fractions F1-F5 ¥

	Recovery yield (%, db)	Total phenolic content (mg of TAE/ g)	Antiradical activity (%inhibition) at 50 µg/ml	Antioxidant efficiency (%inhibition) at 100 µg/ml	chelating efficiency (%inhibition) at 50 µg/ml
SMSK	-	110.03 ^{d}±0.06	77.49 ^{c}±0.43	65.21 ^{c}±1.22	49.63 ^{e}±0.83
F1	6.64 ^{a}±0.16	17.23 ^{b}±0.12	65.32 ^{a}±0.89	35.28 ^{a}±0.75	12.32 ^{a}±0.45
F2	3.61 ^{a}±0.22	185.51 ^{e}±1.23	84.38 ^{d}±0.54	81.69 ^{d}±0.63	42.98 ^{d}±0.38
F3	31.19 ^{c}±0.11	286.25 ^{f}±1.52	97.43 ^{e}±1.39	93.21 ^{e}±2.98	91.51 ^{f}±0.73
F4	21.47 ^{d}±0.09	55.43 ^{c}±0.44	75.63 ^{b}±0.28	64.55 ^{c}±0.76	41.15 ^{c}±0.29
F5	37.27 ^{b}±0.18	15.19 ^{a}±0.15	65.26 ^{a}±0.73	48.63 ^{b}±0.94	22.34 ^{b}±0.66
Methyl gallate	-	-	97.64 ^{e}±0.42	93.55 ^{e}±0.15	91.41 ^{f}±0.13
Tannic acid	-	-	98.21 ^{e}±0.06	95.24 ^{e}±0.32	91.55 ^{f}±0.24

¥dry weight basis of manko seed kernel; Means of three replications±SD (standard deviation); Different superscript letters mean significant differences (P<0.05) between conditions in each column.

Table 4. Characterization of crude MSK extract obtained from acid hydrolysis (AMSK) and fraction F1-F2 ¥

	Recovery yield (%, db)	Total phenolic content (mg of TAE/g)	Antiradical activity (%inhibition) at 10 µg/ml	Antioxidant efficiency (%inhibition) at 50 µg/ml	chelating efficiency (%inhibition) at 50 µg/ml
AMSK	-	285.70 ^{b}±1.28	87.35 ^{b}±0.98	50.65 ^{b}±1.25	87.66 ^{b}±1.64
F1	14.31 ^{a}±0.37	187.24 ^{a}±0.77	64.94 ^{a}±1.15	32.78 ^{a}±1.22	42.23 ^{a}±1.22
F2	85.69 ^{b}±1.24	287.53 ^{b}±2.27	91.15 ^{c}±0.66	58.35 ^{c}±0.34	91.11 ^{c}±1.56
Methyl gallate	-	-	91.11 ^{c}±0.12	58.44 ^{c}±0.11	91.24 ^{c}±0.14
Tannic acid	-	-	91.21 ^{c}±1.38	59.23 ^{c}±1.11	91.55 ^{c}±0.24

¥dry weight basis of manko seed kernel; Means of three replications±SD (standard deviation); Different superscript letters mean significant differences (P<0.05) between conditions in each column.

Antioxidant capacity of mango seed kernel from different fractions

Sephadex LH-20 column chromatography has been used by many researchers to fractionate various plant extracts (Wettasinghe *et al*., 2002; Cruz *et al*., 2005). Sephadex LH-20 is probably one of the best stationary phases available for the separation of phenolics because of their fast, yet satisfactory, separation on it. Figure 1 shows the elution profile at 280 nm of an extract from mango seed kernel.

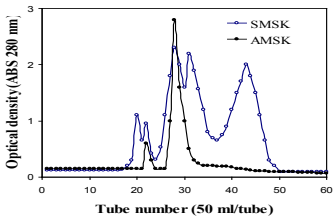

Figure 1. Chromatographic profiles of mango seed kernel extracted by shaking (SMSK) and acid hydrolysis (AMSK)

The eluate comprised 78% crude polyphenols or 8.42% of the dry weight of the MSK. Apart from the enrichment in phenolics of most samples during the extraction process, clear differences were found in its fractionation. The fractions of ethanolic extract obtained by shaking were 4 fractions denoted F1-F4. The crude ethanolic extract was a yellow color. The extract obtained by acid hydrolysis was a dark brown color, and two fractions (F1, F2) were separated from this.

Table 3 shows the recovery yield including total phenolic content, antiradical activity, and antioxidant efficiency and chelating efficiency of the fractions of mango seed kernel obtained by the shaking method. All of the subfractions showed antioxidant capacities. The inhibition percentage at 50 µg/ml for the MSK subfractions was in the range of 65.26-97.43% and decreased as follows: MSK-III > MSK-II > MSK-IV > MSK-I ≈ MSK-V. Fraction 3 had the highest phenolic content and studied activity but had a lower recovery yield than fraction 5. A higher antioxidant capacity of the fraction was expected, as this fraction was rich in phenolics. The iron-chelating activity was high in accordance with the phenolic content. Iron is one of the most important biocatalysts, and iron found in myoglobin, hemoglobin, and transferrin may cause lipid oxidation in muscle tissues. Ferrous ion is found to stimulate lipid peroxidation by generating hydroxyl radicals and by breaking down lipid peroxides to form alkoxyl radicals. All transition metal ions (iron, copper, and nickel) having two or more valency states are potent pro-oxidants (Anesini et al., 2008). In this result, the chelating activity and antioxidant efficiency (FTC method) of fraction 3 was comparable to tannic acid (TA).

TA chelates iron due to its ten galloyl groups. Iron chelators prevent various processes of oxidative stress in vivo; including damage from heart reperfusion and liver injury in chronic iron overload (George *et al*., 1999).

The antioxidant activity of fraction 2 of mango seed kernel extracted by acid hydrolysis was higher than that of the crude extract. Moreover, fraction 2, which

was also a major constituent in the AMSK (Figure 1), exhibited strong activity. The total phenolic content of the hydrolysate fractions of mango seed kernel varied (Table 4). All fractions contained phenolics, which showed antiradical, antioxidant and chelating activity. The antioxidant and metal chelating activities of AMSK extract could be attributed to the presence of phenolic compounds. Fraction 2 showed similar activities to those of methyl gallate and tannic acid.

In a previous study (Maisuthisakul, 2009), the phenolic composition (saponin, flavonoids, anthraquinones and tannins) of MSK from shaking and acid hydrolysis conditions were measured. The phenolic compounds of mango seed kernel obtained from shaking extraction contained flavonoids and tannins, whereas, the hydrolysate MSK contained only tannins. These results confirmed that flavonoids reacted during hydrolysis treatment with hydrochloric acid as shown by the chromatographic profiles (Figure 1).

Conclusions

In summary, the phenolic antioxidant properties and chelating capacity of mango seed kernel obtained by different extraction methods revealed that acid hydrolysis was an appropriate extraction process. Fraction III (shaking condition) and fraction II (acid hydrolysis condition) exhibited higher antioxidant capacities than the crude extract and were comparable to methyl gallate and tannic acid. Therefore, the extraction process affected the phenolic antioxidants of mango seed kernel.

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