Phenolic compounds from kenaf (*Hibiscus cannabinus* L.) seeds by ultrasound-assisted extraction

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ABSTRACT

Introduction: There is great interest in the replacement of synthetic antioxidants with natural sources, especially from plant materials. Kenaf seed potentially contained phenolic compounds. **Materials and Methods:** Kenaf seed extract (KSE) was extracted by ultrasonic-assisted extraction. Then, the purification was carried out using silica gel column chromatography. Finally, the phenolic compounds were determined by high-performance liquid chromatography. **Result:** There were 11 phenolic acids identified from the purified KSE, which were identified as sinapic acid (3563.67 mg/100 g), ferulic acid (2085.55 mg/100 g), catechin hydrate (1189.02 mg/100 g), tannic acid (1032.24 mg/100 g), naringin (385.75 mg/100 g), 4-hydroxybenzoic acid (382.51 mg/100 g), gallic acid (380.18 mg/100 g), 4-hydroxybenzaldehyde (372.00 mg/100 g), protocatehuic acid (207.04 mg/100 g), syringic acid (189.13 mg/100 g), and cinnamic acid (85.51 mg/100 g). **Conclusion:** The KSE was found to exhibit an antioxidant capability, and it is potentially suitable to be used as an alternative source of antioxidants.

Keywords: Antioxidant, chromatography, kenaf, phenolic, purification

INTRODUCTION

Kenaf (*Hibiscus cannabinus* L.) is an herbaceous annual crop in the malvaceae family. kenaf is well-known in Asia and Africa for its multipurpose use, and it has been widely cultivated in some Mediterranean areas.[1] According to Nyam, *et al.*, (2009), eight main phenolic acids were identified in kenaf seed oil, namely, gallic acid, p-hydroxybenzoic acid, caffeic acid, vanillic acid, syringic acid, p-coumaric, and ferulic acids.[2] There is a growing interest in phenolic acids due to their antioxidant potential to scavenge free radicals.[3] The presence of free radicals within the body can have a significant role in the development and progression of various diseases.[4] The addition of plant antioxidants to processed foods is becoming increasingly important in the food industry as an alternative to synthetic antioxidants.[5]

Ultrasonic-assisted extraction was used to extract the kenaf seed because it is offering advantages such as improved efficiency, reduced extraction time, low solvent consumption, and a high level of automation relative to conventional extraction techniques.[6]

To the best of our knowledge, very little information has been reported on the purification of phenolic compounds in kenaf seeds. Hence, the present study investigated the possible anti-oxidative compounds present in an ultrasound-assisted kenaf seed extract (KSE).

MATERIALS AND METHODS

Seed material

Dried kenaf (*H. cannabinus* L.) seed (1 kg) was obtained from the Malaysian Agricultural Research and Development Institute (MARDI, Serdang, Malaysia) and ground into powder with a grinder (SHARP, Japan).

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Methods

Pulsed ultrasound-assisted extraction (PUAE)

Ground kenaf seed (50 g) was added to 500 ml of 80% ethanol to undergo ultrasonic extraction by ultrasonic homogenizer labsonic (Sartorius, Germany). The ultrasonic extraction was performed over a 5 min pulse duration period and a 5 min pulse interval period. The extraction was repeated for three cycles. The resulting KSE was centrifuged at 3500 rpm for 10 min. The supernatant of the KSE was collected and underwent filtration, while the pellet was discarded. The filtered extract was evaporated using a rotary evaporator (Buchi, Switzerland) and kept in a centrifuge tube, which was wrapped with aluminum foil and stored in the freezer at −20°C for future use.

Liquid-liquid extraction

Dried KSE (15 g) was dissolved in 15 ml of water with the help of a sonicator bath (OSIM, Singapore). The dissolved sample was transferred into a separatory funnel and mixed with 150 ml of hexane. The mixture was shaken vigorously and left to settle into two layers. The bottom layer was collected in a 250 ml beaker, and extraction was continued with ethyl acetate. The top layer was collected according to the solvent in the 500 ml schott bottles. Each extraction was repeated 3 times.

After the hexane and ethyl acetate extractions, 50 ml of water was used to rinse the separatory funnel, which was collected as the third extracted solvent. The three different solvents were rotary evaporated. The dried extract was re-dissolved with ethanol to prepare 10,000 ppm (10 mg/ml) for a 2,2-diphenyl-1 picrylhydrazyl (DPPH) test to determine which extracted solvent contained the highest DPPH free radical scavenging activity.

DPPH radical scavenging capacity assay

The KSE antioxidant activity was determined using the DPPH radical scavenging capacity assay, as described by Liu *et al*., (2007),[7] with slight modifications. A 200-µL aliquot of KSE at a concentration of 1 mg/mL was mixed with 2.8 mL of ethanol, followed by the addition of 0.004% DPPH. After 30-min incubation, the absorbance was measured against a blank reagent (ethanol) at 517 nm with an ultraviolet-visible spectrophotometer (Model XTD 5, Secomam, France). All DPPH radical scavenging activities of the KSE were expressed as percentage inhibition. Inhibition percentage (IP) was calculated by using the formula below:

$$IP = (blank - KSE)/blank \times 100$$

Where blank and KSE were the absorbance values of the blank and the KSE.

Sample preparation for dry loading

A dried KSE (1.5 g) was mixed with ethyl acetate in a round-bottomed flask until the sample dissolved. Silica gel (5 g) was then added and the flask was gently shaken. The mixture of sample and silica gel was rotary evaporated until dry. The powdered sample was collected in a universal bottle and stored in a dark place.

Silica gel column chromatography

This analysis was performed in a glass column (0.45 m × 16 mm) packed with silica gel conditioned with the eluent solvent. Silica gel (Merck, Silica gel 60, 0.063-0.200 mm, 70-230 mesh ASTM) (60 g) was mixed with hexane to yield a silica gel slurry. The gel slurry was continuously stirred while being poured into the column to allow the silica particles to settle at the same rate. The hexane level was kept at approximately 5 cm above the silica gel bed to prevent cracking. The column was continuously rinsed with hexane.

A thin layer of acid-washed sand was added above the silica gel before the sample was loaded into the column. The sample was loaded into the column and left to settle before the separation began. The separation solvents ranged from non-polar to polar solvents as shown in Table 1. Thin layer chromatography (TLC) was used to monitor the separation process. The solvent was expected to shift to the next solvent, according the sequence of hexane, mixture of hexane and ethyl acetate, ethyl acetate, mixture of ethyl acetate and methanol, and lastly methanol when no spot appeared in the TLC plate. The solvent eluted from the glass column was collected in universal bottles. After the compound separation in the glass column was completed, all of the universal bottles under the same solvent were combined in the schote bottles and then rotary evaporated.

TLC

The pooled fractions collected from silica gel chromatography were prepared in 10,000 ppm (10 mg/ml) before spotting onto the silica gel aluminum plate (Merck, 20 cm × 20 cm, pre-coated with silica gel 60 F_{254}). A different mobile phase was used to dilute each eluted solvent fraction to optimize

Table 1: Solvents used for silica gel column chromatography

Solvent fraction in silica gel column chromatography	Solvent ratio used for silica gel column chromatography	Solvent ratio used for TLC (hexane: Ethyl acetate)
Hexane	1:0	90:10
Hexane: Ethyl acetate	5:5	80:20
Ethyl acetate	1:0	70:30
Ethyl acetate: Methanol	5:5	20:80
Methanol	1:0	10:90

TLC: Thin layer chromatography

the separation of spots as shown in Table 1. The TLC plate was visualized by using an ultraviolet chamber at 254 nm.

High performance liquid chromatography (HPLC)

The gradient elution method was used for HPLC. A reverse phase HPLC column (Purospher star 5 μm × 250 mm × 4.6 mm) was used. The mobile phase was HPLC grade methanol, 0.1% phosphoric acid and ultra-pure water. The elution solvents were designated as A (aqueous with 0.1% phosphoric acid) and B (methanol with 0.1% phosphoric acid). The samples were eluted according to the following binary gradient: The gradient began with 5% B to reach 50% B at 5 min, 55% B at 65 min, and 5% B at 70 min. The injection volume was set at 20 μl. The flow rate of the mobile phase solvent was 1.0 ml/min. The column temperature was set at 30°C. The detector wavelength was 210 nm using a diode array detector. Prior to the HPLC analysis, the whole mobile phase was filtered through a 0.45 μm nylon membrane filter using a vacuum pump and compressor to protect against the foreign particles that clog the column.

The five fractions of dried KSE remaining following the column chromatography were prepared in 10,000 ppm (10 mg/ml) by dissolving them in the solvent of the first gradient before injecting the sample into the HPLC. The dissolved samples were filtered through a 0.45 μm nylon syringe filter before being injected into the HPLC. There were 11 types of phenolic acids determined by this study, namely, gallic acid, tannic acid, catechin hydrate, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, syringic acid, sinapic acid, ferulic acid, naringin, protocatehuic acid and cinnamic acid. The results were expressed in mg/100 g extract.

Statistical analysis

All experimental and/or measurements were replicated three times and expressed as a mean ± the standard error of the mean. In single factor experiments, significant differences ($P < 0.05$) among different extraction parameters were determined by a one-way analysis of variance with MINITAB statistical software (Version 15.1.1.0, Minitab Inc., USA).

RESULTS AND DISCUSSION

PUAE

Ultrasonic extraction was used because of its high extraction efficiency, low energy use and low solvent consumption. The pulsed mode was selected in this study because it has less effect on the antioxidant activity in the samples. According to Pan, *et al*.[8], (2011), PUAE is superior to Cisco unified application environment (CUAE) because of its low electrical energy consumption, high extraction time reduction, increased antioxidant yield and higher antioxidant activity. In addition, the temperature of extracted KSE can be more easily controlled under PUAE than CUAE because of the lower heat generation and accumulation.

LLE

The KSE contains large amounts of carbohydrates and/or lipoidal material, and the phenolic concentration in the crude extract may also be low. Liquid-liquid partitioning was carried out to concentrate and collect polyphenol-rich fractions before analysis. The ethanol extract was first extracted with hexane to separate non polar compounds; the yield from hexane was 24.67% (w/w). It was then extracted with ethyl acetate, which is a common solvent for extracting phenolics; the extract yield from ethyl acetate was 11.53% (w/w). The remaining aqueous portion accounts for a 60.33% yield with an operational loss of 3.47%. Table 2 summarizes the antioxidant activity and the yields of different extracts. The antioxidant activity of ethyl acetate extracts (77.46%) was higher than those of hexane (51.94%) and aqueous extracts (10.90%). In Table 2, the initial KSE (before solvent extraction) had a weaker scavenging activity (66.68%) than the ethyl acetate fraction. This finding showed that solvent extraction prior to column chromatography is necessary to remove some of the impurities.

Column chromatography

From the results of the DPPH for LLE fractions, the ethyl acetate fraction had the highest antioxidant activity. Hence, the ethyl acetate fraction of the crude KSE was selected to undergo separation and purification by column chromatography. The ethyl acetate fraction was loaded into the column by dry loading. Non-polar phenolic compounds in the KSE were eluted before the more polar phenolic compounds. Each of the collected fractions from the elution process was monitored with TLC.

Table 2: DPPH free radical scavenging activity and yield of each liquid-liquid extraction fraction of extracts from kenaf (*H. cannabinus* L.) seed

Solvent	Scavenging activity (%)	Yield (g)	% yield (w/w)
Crude	66.68±0.4^{b}		
Hexane	51.94±1.7^{c}	3.70±0.8^{d}	24.67
Ethyl acetate	77.46±2.2^{a}	1.73±0.1^{c}	11.53
Water	10.90±1.3^{d}	9.05±0.4^{b}	60.33
Total		14.48±0.3^{a}	96.53

Each value is presented in means±standard deviation (n=9). Values within a column with different superscripts are significantly different (P<0.05), DPPH: 2,2-diphenyl-1 picrylhydrazyl, *H. cannabinus: Hibiscus cannabinus*

TLC

In the present study, each fraction collected after running column chromatography was subjected to TLC analysis. All the fractions collected under the same solvent polarity were pooled together and subjected to the TLC analysis. Based on (Figure 1), the second TLC analysis showed more than one band on the TLC plate, and some of the bands were overlapping. TLC was used for preliminary qualitative identification. To further quantify the phenolic compounds, all five fractions were subjected to HPLC to identify the phenolic acids present in each pooled fraction.

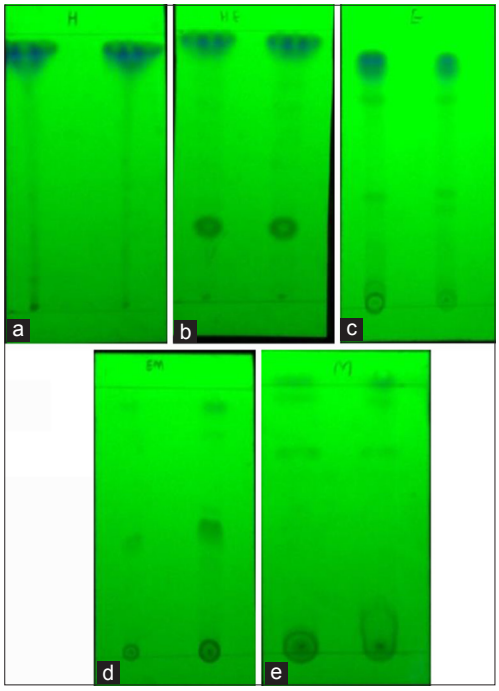

Figure 1: Thin layer chromatography (TLC) profiles of five purified kenaf (Hibiscus cannabinus L.) seed extract from hexane fraction to methanol fraction with the first and second column replicate in each TLC plate. (a) Hexane fraction; (b) hexane and ethyl acetate fraction; (c) ethyl Acetate fraction; (d) ethyl acetate and methanol fraction; (e) methanol fraction

HPLC

In this study, 11 components were analyzed by HPLC. Among the five fractions, the hexane fraction (F_H), the hexane: ethyl acetate fraction (F_{HE}), the ethyl acetate fraction (F_E), the ethyl acetate: methanol fraction (F_{EM}), and the methanol fraction (F_M), F_{EM} had the highest phenolic content, at approximately three-fold higher than the second highest fraction (F_E), followed by F_M, F_{HE}, and F_H. As shown in Table 3, there were 11 peaks identified in the F_{EM}, 10 peaks identified in the F_E, eight peaks identified in the F_M, three peaks identified in the F_{HE} fraction and two peaks identified in the F_H based on the retention time of each peak relative to the standard. This finding showed that most of the phenolic acids contained in KSE were more polar compounds.

Four major phenolic components were detected in the purified KSE, namely, sinapic acid, ferulic acid, catechin and tannic acid (Table 3). Sinapic acid reportedly has antioxidant efficacy as a metal chelator because of the orientation of its functional groups.[9] Ferulic acid has hypolipidemic properties and could be effective for lowering the risk of high fat diet-induced obesity.[10] In addition, this compound reduces serum cholesterol levels and protects against liver injury and is a potent inhibitor of tumor promotion, at least in vitro.[11] Catechin can significantly reduce intracellular lipid accumulation and it represses the activity of glycerol-3-phosphate dehydrogenase, an enzyme involved in lipid synthesis, and also suppressed glucose and fatty acid transport.[12] Tannic acids inhibit inflammatory gene expression and modulate the intracellular antioxidant level that protects from ischemia and cardiovascular disease.[13]

Table 3: Concentration of all phenolic acids (mg/100 g extract) in the purified kenaf (*H. cannabinus* L.) seed extract fraction

Phenolic acid	Hexane	Hex+EA	EA	EA+MeOH	MeOH	Total
Gallic acid	-	-	132.32±17^{a}	128.08±7.2^{ab}	119.79±2.0^{b}	380.18
Tannic acid	-	-	588.10±55.1^{a}	444.14±35.2^{b}	-	1032.24
Catechin hydrate	-	-	383.27±0.1^{b}	405.57±13.0^{a}	400.18±9.9^{a}	1189.02
4-hydroxybenzaldehyde	-	-	114.88±19.5^{a}	136.50±17.1^{a}	120.62±20.0^{a}	372.00
4-hydroxybenzoic acid	-	-	128.62±12.2^{b}	160.72±15.3^{a}	93.18±9.8^{c}	382.52
Syringic acid	-	-	13.61±0.3^{c}	129.14±5.9^{a}	46.38±6.1^{b}	189.13
Sinapic acid	-	389.99±7.1^{c}	352.24±19.1^{d}	2092.60±13.1^{a}	728.85±64.9^{b}	3563.67
Ferulic acid	-	-	222.53±7.7^{b}	1863.02±21.1^{a}	-	2085.55
Naringin	39.25±3.5^{d}	34.38±3.7^{d}	54.55±8.3^{c}	167.04±19.9^{a}	90.53±0.1^{b}	385.75
Protocatehuic acid	-	-	-	207.04±13.8	-	207.04
Cinnamic acid	10.66±1.5^{d}	14.61±2.6^{c}	20.07±2.1^{b}	31.44±3.2^{a}	8.72±0.6^{d}	85.51
Total	49.92	438.99	2010.18	5765.28	1608.24	9872.6

Each value is presented in means±standard deviation (n=9). Values within a row with different superscripts are significantly different (P<0.05). Hex+EA: Hexane+ethyl acetate, EA: Ethyl acetate, EA+MeOH: Ethyl acetate+methanol, MeOH: Methanol, - :Not determined, *H. cannabinus: Hibiscus cannabinus*

CONCLUSION

The major phenolic acid content in purified KSE were sinapic acid, ferulic acid, catechin and tannic acid. Other minor phenolic acids identified in this study were naringin, 4-hydroxybenzoic acid, gallic acid, 4-hydroxybenzaldehyde, protocatehuic acid, syringic acid and cinnamic acid. The high antioxidant activity of the KSE appears to be attributable to its high phenolic content. Therefore, the KSE was found to exhibit an antioxidant capability, and it is potentially suitable for use as an alternative source of antioxidants in the pharmacological field as well as food industries.

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