In vitro Simulated Digestion on the Biostability of *Hibiscus cannabinus* L. Seed Extract

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Abstract

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We investigate the biostability of phenolic acids from a kenaf (*Hibiscus cannabinus* L.) seed extract using an *in vitro* model simulating the physicochemical (pH, temperature and bile salts) and biological (gastric and pancreatic enzymes) gastrointestinal conditions. Some of the antioxidants in the kenaf seed extract were not relatively stable in the intestinal phase of the gastrointestinal tracts. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity and total phenolic content (TPC) assays displayed similar trends as the biostability of phenolic acids, which decreased during the digestion process. The overall percentage loss of selected phenolic acids was 8.4–49.4% in the intestinal phase. For the overall *in vitro* digestion system, significant correlations between phenolic acids, total phenolic content (TPC) and antioxidant activity (DPPH) were obtained in all digestion phases with the exception of the gastric phase.

Keywords: pulsed ultrasound-assisted extraction (PUAE); phenolic acids; kenal; antioxidant activities

Kenaf (*Hibiscus cannabinus* L.) is a valuable fibre and medicinal plant of the family Malvaceae, which is native to India and Africa (Ji *et al.* 2011). Kenaf has been developed for use in the pulp and paper industry, oil adsorption and potting media, board making, filtration media, and animal feed (Keshk *et al.* 2006). It contains various active components that have long been prescribed in traditional folk medicine in Africa and India. Recently, kenaf seed oil was proved to contain anti-proliferative activity towards cancer cell lines (Yazan *et al.* 2010). According to Nyam *et al.* (2009), kenaf seed contains 9.6% moisture, 6.4% ash, 20.4% oil, 21.4% nitrogenous matter, and 12.9% crude fibre whilst the dry press cake contains 33% protein, 6.0% oil, 17.4% crude fibre, and 6.0% ash.

In this study, ultrasound-assisted extraction was used to extract the kenaf seed because it utilises acoustic cavitation to cause the molecular movement of solvent and sample (Herceg *et al.* 2012), offering advantages like improved efficiency, reduced extraction time, low solvent consumption, and high level of automation as compared to conventional extraction techniques (Chen *et al.* 2007). It has been reported that the application of ultrasound-assisted extraction in grape peel can enhance the recovery of functional compounds up to 30% as compared to conventional solvent extraction (Cho *et al.*, 2006). There is no study regarding the bioactive compounds in kenaf seed extract. However, Nyam *et al.* (2009) showed that seven main phenolic acids have been identified in the kenaf seed oil, including gallic acid, *p*-hydroxybenzoic acid, caffeic acid, vanillic acid, syringic acid, *p*-coumaric acid, and ferulic acid. Several studies have reported that phenolic acids in plants significantly contribute to their antioxidant properties (Lachman *et al.* 2005; Canadanovic-Brunet *et al.* 2011). The antioxidant behaviour of a compound is the result of its capacity to inhibit the initiation of free radical or chain breaking in the process propagating oxidation (Ahmed & Shakeel 2012).

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One of the principal topics concerning the beneficial effects of polyphenols is their bioaccessibility and metabolic fate. Bioavailability of polyphenols to peripheral tissues can be diminished by high intestinal and biliary secretion of their conjugates (Silberberg *et al.* 2006). Therefore, the bulk of dietary polyphenols and their conjugates must remain in the gut lumen and in the gastrointestinal tract where these compounds may exert important beneficial effects, such as inhibition of abnormal cell proliferation and protection against cancer development (Bermudez-Soto *et al.* 2007). *In vitro* methods are very useful for evaluating the effects of factors such as digestion conditions or interaction with other compounds on the stability and properties of polyphenols which may affect their bioaccessibility and final metabolic fate (Giménez-Bastida *et al.* 2009). In the present study, the stability and antioxidant activity of phenolic acids from ultrasound-assisted extraction of kenaf seed extract were investigated using an *in vitro* model that simulated various chemical (pH, temperature and bile salts) and biological (gastric and pancreatic enzymes) gastrointestinal conditions.

MATERIAL AND METHODS

Sample. Dried kenaf (*Hibiscus cannabinus* L.) seed was obtained from the Malaysian Agricultural Research and Development Institute (MARDI, Serdang, Malaysia) and ground into powder form with a grinder (SHARP, Tokyo, Japan). The particle size of the kenaf seed powder was less than 1 mm.

Pulsed ultrasound-assisted extraction (PUAE). A 500 ml Scott bottle was prepared and filled with 80% ethanol to a total volume of 500 ml. A total of 50 g of ground kenaf seed was added to the solvent, followed by ultrasound extraction (Ultrasonic Homogeniser Labsonic P 400W; Sartorius AG, Goettingen, Germany) for 30 min with a 5 min pulse duration period and a 5 min pulse interval period. The extraction was repeated for 3 cycles. This condition was an optimum extraction parameter which was determined in a preliminary study. The kenaf seed extract collected was centrifuged at 3500 rpm for 10 minutes. The supernatant of the kenaf seed extract was collected and filtered; the pellet was discarded. The filtered supernatant was subjected to rotary evaporation (Rotavapor R-200; Buchi AG, Flawil, Switzerland).

In vitro digestion. Kenaf seed extract was digested *in vitro* according to Bermudez-Soto *et al.* (2007) with slight modifications. Approximately 10 ml of pH 2 simulated gastric juice (0.4 g pepsin in 10 ml 0.1M HCl) was added to 1 g of kenaf seed extract in a 50 ml tube. The sample was mixed gently, flushed with nitrogen gas and sealed. The tubes were incubated for 1 h at 37°C in a shaking water bath (95 rpm). Approximately 2.5 ml from each tube was transferred into 15-ml polypropylene tubes, flushed with nitrogen and kept on ice, while the remainder of the sample in the 50 ml tube was digested as described below. The gastric phase was terminated by adding 0.5M $NaHCO_3$ to increase the pH to 6. Approximately 0.5 ml porcine pancreatin stock and 0.5 ml bile extract stock were added. The pH was adjusted to 6.9 with 1M NaOH, and the volume was increased to 15 ml with distilled water. The sample was flushed with nitrogen, sealed and incubated for 2 h in a water bath shaker (Memmert, Schwabach, Germany) at 37°C. During 2 h of incubation, a 2.5 ml aliquot from the gastric phase was centrifuged (3500 *g* at 4°C) in an Eppendorf centrifuge for 35 minutes. Approximately 1 ml of the clear supernatant was transferred to a clearly labelled Eppendorf tube, flushed with nitrogen and frozen (–20°C) immediately at the end of the 2 h incubation; this procedure was repeated with the digestion in the tubes. The simulated gastric and small intestine conditioned samples were thawed when required and centrifuged (3500 *g* at 4°C) for 35 minutes. The supernatant was assayed for antioxidant activity.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay. The antioxidant activity of the kenaf seed extract was determined by using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay as described by Liu *et al.* (2007) with slight modifications. A 200-µl aliquot of kenaf seed extract with 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 the blank reagent (ethanol) at 517 nm with a UV-VIS spectrophotometer (Model XTD 5; Secomam, Ales, France).

Total phenolic content (TPC). The total phenolic content of kenaf seed extract was determined with the Folin-Ciocalteu assay, based on the method described by Rapisarda *et al.* (1999) with slight modifications. A 300 ml aliquot of a 10 mg/ml sample was added to 1.5 ml of 10-fold diluted Folin-Ciocalteu reagent (FCR) and mixed well. The mixture was allowed to stand at room temperature for 5 minutes. Next, 1.2 ml of sodium carbonate (7.5%, w/v) solution was added, and the sample was mixed thoroughly with a vortex mixer and allowed to stand for 30 minutes. After 30 min, the absorbance was measured against a reagent blank (ultra-pure water mixed with FCR and sodium carbonate) at 765 nm with a UV-VIS spectrophotometer (Model XTD 5; Secomam, Ales,

France). The results were expressed as mg of gallic acid equivalents (GAE) per 100 g of sample.

High performance liquid chromatography (HPLC) analysis. Phenolic acids in kenaf seed extract were quantified after each digestion phase by an HP 1100 Agilent HPLC (Waldbronn, Germany) as previously described by Baydar (2006), with slight modifications. The HPLC system included a diode array detector (DAD). Separations were performed on a reversed-phase HPLC column (Purospher STAR 5 µm × 250 mm × 4.6 mm). The column temperature and detection wavelength were set at 30°C and 210 nm, respectively. A gradient elution system of solvent A (water with 0.1% phosphoric acid) and solvent B (methanol with 0.1% phosphoric acid) was used [5% B (0 min); 50% B (5 min); 55% B (65 min); 5% B (70 min)]. The flow rate was 1 ml/minute. The volume injected was 20 µl. The chromatographic peaks of the phenolic acids were confirmed by comparing their retention times with those of the reference standards.

Statistical analysis. All experiments were performed in duplicate and measurements were repeated twice. Analysis of variance (ANOVA) was performed, and the average values were compared by Fisher's Multiple Comparison Test. Differences were considered statistically significant at $P < 0.05$. Correlations between the TPC, DPPH and HPLC values were obtained by simple linear regression analysis and Pearson's correlation. All statistical analyses were performed using Minitab 16 for Windows.

RESULTS AND DISCUSSION

In this study, the simulated stomach and duodenum digestion of a kenaf seed extract rich in phenolic acids was performed to determine the stability of these acids during gastrointestinal digestion. The samples that were removed from the digestion container were centrifuged to obtain translucent samples that could be analysed spectrophotometrically. We determined total soluble phenolics recovered in the filtered digested samples as an estimate of compounds that may be available for uptake.

DPPH and TPC were determined after each digestion phase, as presented in Table 1. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity followed a trend similar to that of TPC, which decreased during the digestion process. However, as simple carbohydrates or amino acids may be present in the extracts and may interfere with the antioxidant test or with the determination of total phenolics, the total percentage loss of DPPH and TPC varied.

Table 1. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay and total phenolic content (TPC) of kenaf seed extract after each digestion phase

Phases	DPPH (%)	TPC (mg/100 g extract)
Initial	93.8 ± 0.5^a	194.6 ± 0.8^a
Gastric	89.6 ± 0.5^b	188.5 ± 0.4^b
Intestinal	28.7 ± 0.6^c	151.3 ± 4.0^c

Each value is presented as mean ± standard deviation ($n = 4$); values within the same column with different superscripts are significantly different ($P < 0.05$)

In addition, the stability of the major phenolic acids in kenaf seed extract was investigated, and the percentage of changes during the *in vitro* digestion is summarised in Table 2. A small increase in some phenolic acids, such as ferulic acid, 4-hydroxybenzoic acid, and cinnamic acid, was observed after *in vitro* stomach digestion. This increase has been attributed to a decrease in the pH of the kenaf seed extract after the gastric incubation (pH 2.0) relative to the pH of the initial kenaf seed extract. This pH change may make these compounds become more bioaccessible. Therefore, further investigation is needed. Most dietary phenolic acids appeared to be quite stable during gastric digestion as no significant changes were observed in the phenolic acids of kenaf seed extract incubated under conditions mimicking those that must occur in the stomach. Similar results were obtained by Vallejo *et al.* (2004). They showed that after *in vitro* gastric digestion of broccoli, there were no losses of phenolic acid levels in comparison with the initial fraction, indicating that pepsin digestion had no effect on phenolic stability.

Following the stomach incubation, the kenaf seed extract was then subjected to a 2-h digestion under conditions mimicking those of the duodenum. A large proportion of the dietary polyphenols examined were lost or their structures were changed because of the digestion and pH modification. Our results demonstrated that dietary polyphenols are highly sensitive to the conditions in the small intestine and suggest that a proportion of these acids was transformed into their corresponding degradation products during digestion in the duodenum. This finding agreed with Vallejo *et al.* (2004), who found out significant decreases in phenolic concentration after the pancreatin-bile salt digestion (simulation of the small intestine digestion). It is possible that the pancreatin digestion liberates compounds (macromolecules as proteins and fibre) able to associate with phenolic compounds. Therefore, most of the antioxidants would be accessible in the intestinal phase of the gastrointestinal tract.

Table 2. Phenolic acid content (mg/100 g extract) in kenaf seed extract after each digestion phase

Phases	Initial phase	Gastric phase	Intestinal phase	% loss
Gallic acid	94.5 ± 4.2^{a}	98.2 ± 2.5^{a}	67.5 ± 1.8^{b}	28.6
Tannic acid	1033.5 ± 47.3^{ab}	1070.6 ± 66.5^{a}	844.1 ± 57.2^{b}	18.3
Catechin hydrate	64.8 ± 3.5^{a}	65.4 ± 2.6^{a}	54.9 ± 2.4^{b}	15.3
4-Hydroxybenzaldehyde	22.3 ± 1.9^{a}	22.0 ± 0.8^{a}	13.3 ± 1.1^{b}	40.4
4-Hydroxybenzoic acid	58.3 ± 3.1^{b}	74.4 ± 11.8^{a}	52.9 ± 1.6^{c}	9.2
Syringic acid	117.2 ± 6.3^{a}	126.5 ± 5.0^{a}	60.3 ± 0.9^{b}	48.5
Sinapic acid	252.3 ± 15.7^{a}	288.0 ± 23.4^{a}	167.8 ± 17.0^{b}	33.5
Ferulic acid	195.3 ± 6.5^{b}	265.7 ± 34.2^{a}	179.0 ± 13.9^{b}	8.3
Naringin	43.5 ± 4.0^{a}	43.3 ± 7.4^{a}	23.7 ± 1.3^{b}	45.5
Protocatechuic acid	11.1 ± 1.9^{a}	7.6 ± 0.4^{b}	5.6 ± 0.7^{b}	49.5
Cinnamic acid	238.4 ± 15.1^{b}	276.1 ± 7.4^{a}	162.7 ± 10.8^{c}	31.8
Total	2131.2	2337.8	1631.8	23.4

Each value is presented as mean ± standard deviation (n = 4); values within the row with different superscripts are significantly different ($P < 0.05$)

The correlations between the level of phenolic acids and their antioxidant capacities and the different extraction conditions are an interesting aspect of this study. The positive significant ($P < 0.05$) correlation between DPPH and TPC ($R^2 = 0.9806$) shown in Table 3 indicates that TPC is responsible for the DPPH radical scavenging capacity. This supports the idea that phenolic acids contribute to the antioxidant power of kenaf seed extracts. Phenolic acids (PA), as determined by HPLC, displayed a strong positive correlation ($R^2 > 0.9000$) with DPPH and TPC in all phases with the exception of the gastric phase. This may due to the transformation of phenolic acids to their derivatives at the lower pH of the gastric phase environment (Perez-Vicente *et al.* 2002). This is an important issue that requires further and thorough investigation as these derivatives may have a major impact on the bioaccessibility, bioavailability and biological activity of polyphenols.

From this study, despite the limitations of *in vitro* models and the caution that is needed when interpreting results derived from these models, methods mimicking *in vivo* human conditions, such as those simulating gastrointestinal digestion, remain a useful tool for investigating the physiological processes associated with specific food components such as polyphenolic acids. However, there are important discrepancies between *in vitro* and *in vivo* results. For example, dimeric flavan-3-ols are degraded to a great extent after a 1-h incubation in simulated gastric juice at pH 1.8 (Zhu *et al.* 2002) but remain stable in the human stomach (Rios *et al.* 2002). These differences indicate that *in vitro* methods need to be further improved and validated by additional *in vivo* animal and human studies.

CONCLUSION

Our results showed that dietary polyphenols are highly sensitive to the mild alkaline conditions in the small intestine and that a good proportion of these compounds can be transformed into other unknown and/or undetected structural forms with different chemical properties. Correlations between DPPH, TPC and PA demonstrated that there was a direct relationship between phenolic acids from kenaf seed extract, with the exception of the gastric phase. The overall percentage loss of selected phenolics in this study was 8.4–49.4%. The *in vivo* effects of the consumption of kenaf seed extract must be assessed to verify the relevance of the increase in the biostability of phenolic acids in the gut and colon with the bioaccessibility.

Table 3. Correlation between assays for all three digestion phases (n = 4)^{b}

Replication	Initial			Gastric			Intestinal		
	DPPH	TPC	PA	DPPH	TPC	PA	DPPH	TPC	PA
DPPH	1			1			1		
TPC	0.8777	1		0.9806*	1		0.9320	1	
PA	0.9088	0.9967*	1	–0.9704*	–0.9295	1	0.9106	0.9892*	1

DPPH – 2,2-diphenyl-1-picrylhydrazyl; TPC – total phenolic content; PA – phenolic acids; *significance level at $P < 0.05$

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