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Quantification of gallic acid and ellagic acid from longan (*Dimocarpus longan* Lour.) seed and mango (*Mangifera indica* L.) kernel and their effects on antioxidant activity

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Abstract

Gallic acid (GA) and ellagic acid (EA) have been identified in longan seed and mango kernel by the use of reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with photodiode array detection (DAD). The ethanolic extract of longan seed contained 23.3 and 156 mg/100 seeds of GA and EA, respectively. The ethanolic extracts of mango kernel contained approximately 87% more GA than the longan seed ethanolic extracts but about 32% less EA. After heat treatment and acid hydrolysis, mango kernel had higher concentrations of GA and EA, contributing to more potent antioxidant activity. The results demonstrated rich sources of GA and EA in longan seed and mango kernel which might provide a novel source of these natural antioxidants.

Keywords: HPLC; Ellagic acid; Gallic acid; Longan seed; Mango kernel

1. Introduction

Gallic acid (GA) and its dimeric derivative, known as ellagic acid (EA), exist either in the free form or bound as gallo- (GT) and ellagitannins (ET), respectively. These hydrolyzable tannins (HTs) are present in a rich variety of plants and are present in tea, red wine, fruits, beverages and various medicinal plants (Hatano, 1995; Namiki, Yamashita, & Osawa, 1993; Okuda, 1995; Okuda, Yoshida, & Hatano, 1992, 1993; Tanaka, 1999). GT is considered to be a generally regarded as safe (GRAS) food additive and ellagic acid has been allowed for use as a food additive, functioning as an antioxidant in some countries, including Japan.

HTs are easily hydrolyzed in vivo by the action of acid and/or enzymes, releasing GA or EA units (Brune, Rossander, & Hallberg, 1989; Clifford & Scalbert, 2000). GA is known to have anti-inflammatory, antimutagenic, anticancer and antioxidant activity (Gichner, Pospisil, Veleminsky, Volkeova, & Volke, 1987; Huang et al., 1985; Inouc et al., 1995; Kroes, Van den Berg, Ufford, Van Dijk, & Labadie, 1992; Madsen & Bertelsen, 1995; Mirvish, Cardesa, Wallcave, & Shubik, 1975; Nakatani, 1992). EA has been found to exhibit antimutagenic, antiviral, anticancer, antitumor and antioxidant properties, along with whitening of the skin (Bhargava & Westfall, 1968; Khanduja, Gandhi, Pathania, & Shal, 1999; Mandal & Stoner, 1990; Stoner & Gupta, 2001; Wood et al., 1982).

Longan seed and mango kernel have previously been shown to possess potent antioxidant activities which could be ascribed to their phenolic contents (Soong & Barlow, 2004). GA, EA and gallates have been determined in mango kernel by paper chromatography (Puravankara, Boghra, & Sharma, 2000). Gallotannins and condensed tannin-related polyphenols were also reported to be present in mango kernel

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by thin-layer chromatography (Arogba, 2000). Berardini, Carle, and Schieber (2004) has recently characterized gallotannins in mango kernels by the use of HPLC-ESI-MS.

To the best of the authors' knowledge, the phenolics composition of longan seed has not yet been characterized and the quantification of GA and EA in longan seed and mango kernel has not been reported. An acid-catalyzed hydrolysis process was employed to liberate phenolic acids from their bound forms. The objectives of this study were to optimize the methods of extraction and hydrolysis of phenolic acids from mango kernel and to investigate the relationship between the phenolic acids and the antioxidant capacity. The identification and quantification of GA and EA from longan seed and mango kernel extracts, as well as mango kernel acid hydrolysates, were undertaken by the use of RP-HPLC coupled with DAD.

2. Materials and methods

2.1. Materials

2.1.1. Fruits

Longan (*Dimocarpus longan* Lour.) and mango (*Mangifera indica* L.) were purchased on several separate occasions from local markets in Singapore.

2.1.2. Chemicals

ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid)], EA, L-ascorbic acid and potassium persulfate were purchased from Sigma (MO, USA). GA was purchased from Acros Organics (NJ, USA). Acetic acid, ethanol, HPLC grade methanol and hydrochloric acid were purchased from Merck (Darmstadt, Germany).

2.2. Sample pretreatment

The longan seeds and mango kernels from ripened and ready-to-eat fruits were freeze-dried (−50 °C, 24 h). Portions of the fresh mango kernels were heated at 160 ± 2 °C in an oven. All samples were separately ground using a stainless-steel grinder and then stored in vacuum-packaged polyethylene pouches at −20 °C until required for analysis.

2.3. Sample preparation

(A) Approximately 200 mg of each freeze-dried longan seed and mango kernel were accurately weighed and refluxed with a total volume of 50 ml of ethanol: water (50:50, v/v) in a water bath at 70 °C for one hour.

(B) An amount of 200 mg of each freeze-dried and heated (160 ± 2 °C) mango kernel were accurately weighed and refluxed with a total volume of 50 ml of 50% (v/v) aqueous methanol in a water bath at 70 °C for 1 h.

The above extracts were passed through Whatman filter paper (no. 4). All filtrates were evaporated to dryness under vacuum below 40 °C. The residue was either dissolved in 50 ml of ethanol: water (50:50, v/v) and measured by the ABTS assay or dissolved in 3 ml of HPLC grade methanol and filtered through a 0.45 μm filter prior to injection (20 μl) to the HPLC system.

2.4. Hydrolysis

An amount of 200 mg of each freeze-dried and heated (160 ± 2 °C) mango kernel was weighed and rinsed with 40 ml of 50% (v/v) aqueous methanol into a round bottom flask. To this solution, 10 ml of 6 M hydrochloric acid was added by careful mixing (final HCl concentration 1.2 M). The solution was stirred using a magnetic stirrer at 35 °C for 16 h and refluxed at 85 °C for 2 h according to the method of Häkkinen, Kärenlampi, Heinonen, Mykkänen, and Törrönen (1998) and Hertog, Hollman, and Venema (1992), respectively. The extracts were passed through Whatman filter paper (no. 4). All filtrates were evaporated to dryness under vacuum below 40 °C. The residue was either dissolved in 50 ml of ethanol: water (50:50, v/v) and measured in the ABTS assay or dissolved in 3 ml of HPLC grade methanol and filtered through a 0.45 μm filter prior to injection (20 μl) into the HPLC system.

2.5. ABTS cation radical-scavenging assay

The ABTS cation radical-scavenging assay was carried out using an UV–vis spectrophotometer (Shidmazu UV-1601) with a Shidmazu CPS-240A temperature controller. The procedure was adapted from a previous report by Robert et al. (1999). Briefly, stock solution was prepared by reacting 7 mM ABTS with 2.45 mM potassium persulfate to generate the ABTS cation chromophore. The mixture was diluted with absolute ethanol to give an absorbance of 1.5 2 at 414 nm. An aliquot of the samples (10–40 μl) was added to 3 ml of ABTS reagent and the absorbance reading was taken after initial mixing and up to 90 min, until it reached a plateau. Total antioxidant capacity was calculated relative to the reactivity of ascorbic acid as a standard under the same conditions and the results expressed as μmol/g AEAC.

2.6. HPLC assay

The HPLC system consisted of a Shimadzu HPLC (Model LC-10Atvp two pumps and DGU-14A Degasser) equipped with a photo-diode array detector (Model SPD-M10A_{vp}; Shimadzu, Kyoto, Japan) interfaced with an IBM Pentium-III personal computer. The separation was performed on a Shim-Pack VP-ODS column

(250 × 4.6 mm i.d.; Shimadzu, Kyoto, Japan) with a guard column (GCP-ODS, 10 × 4.6 mm i.d.). The temperature of the column oven was set at 40 °C. Solvent gradients were formed, by the dual pumping system, by varying the proportion of solvent A [water–acetic acid (97:3, v/v)] to solvent B (methanol). Solvent B was increased to 10% in 10 min and subsequently increased to 70% in 40 min at a flow rate of 0.9 ml min^{-1}. The elution system was a modified method of Suárez, Picinelli, and Mangas (1996). The phenolic compounds were detected at both 280 and 360 nm.

2.7. Construction of reference standards calibration curve

Authentic standards of GA and EA were dissolved in HPLC-grade methanol to prepare the calibration curves. The standard response curve for each phenolic was a linear regression fitted to triplicate values obtained at each of five concentrations (12.5–200 ppm).

3. Results and discussion

3.1. Antioxidant activity of mango kernel extracts and acid hydrolysates

Phenolic compounds occur in plants mainly as aglycones, glycosides or esters, or are bound to the cell wall. Acid hydrolysis can release the combined flavonoids and phenolic acids (Krygier, Sosulski, & Hogge, 1982; Sosulski, Krygier, & Hogge, 1982) but requires relatively high concentrations of mineral acids under refluxing conditions (Hertog et al., 1992; Merken & Beecher, 2000). Different plant materials contain different phenolic compounds in different forms, resulting in variable susceptibility to degradation. Therefore, the method of choice for hydrolysis must be a compromise between efficient production of aglycones from the plant material and degradation of aglycones.

As may be seen from Table 1, mango kernel methanolic extracts showed an increase in antioxidant activity with increasing severity of hydrolysis. Methanolic extracts of freeze-dried and 160 °C-heated mango kernels, showed a 24% and 32% rise in AEAC, respectively, after hydrolysis at 85 °C. The results suggested that some conjugated phenolics might be released by acid hydrolysis and the free form might provide more potent antioxidant activity. Further investigation is required as the finding is in conflict with some other reports. Hagerman et al. (1998) demonstrated a higher antioxidant ability of condensed and hydrolyzable tannins at quenching peroxyl radicals over simple phenols. Yamaguchi, Yoshimura, Nakazawa, and Ariga (1999) has reported that the higher the polymerization degree of flavanols, the stronger is the superoxide-scavenging activity.

3.2. Identification and quantification of GA and EA by HPLC assay

RP-HPLC coupled with UV–vis DAD was employed to separate, identify and quantify phenolic compounds in longan seed and mango kernel. The concentrations were determined by calculating the HPLC peak areas which are proportional to the amount of analytes in a peak and presented as the mean of two determinations which were highly repeatable. Fig. 1 shows the chromatogram of authentic standards of GA and EA. These phenolic acids have been identified in longan seed and mango kernel extracts according to their retention times and spectral characteristics of their peaks against those of standards (Figs. 2–7), as well as by spiking the samples with standards.

As shown in Table 2, ethanolic extracts of mango kernel contained approximately 87% more GA than the longan seed ethanolic extracts but about 32% less

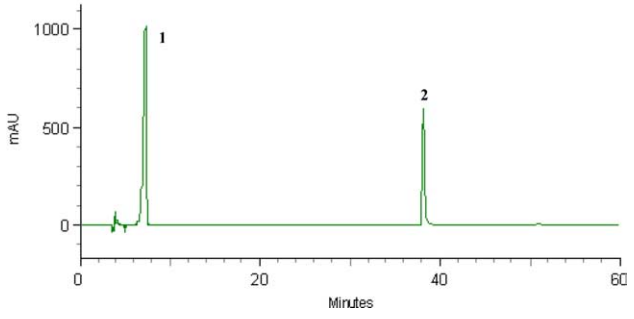

Fig. 1. HPLC chromatogram of authentic standards (1) GA and (2) EA monitored at 280 nm.

Table 1
AEAC of hydrolyzed and unhydrolyzed mango kernel extract

Mango kernel	AEAC[a] (μmol g^{-1})
Freeze-dried/Methanol extraction at 70 °C, 1 h	575 ± 23.5
Freeze-dried/Methanol extraction & hydrolysis at 35 °C, 16 h	600 ± 32.5
Freeze-dried/Methanol extraction & hydrolysis at 85 °C, 2 h	713 ± 74.9
160 °C-heated/Methanol extraction at 70 °C, 1 h	1130 ± 216.0
160 °C-heated/Methanol extraction & hydrolysis at 35 °C, 16 h	1250 ± 61.9
160 °C-heated/Methanol extraction & hydrolysis at 85 °C, 2 h	1496 ± 190

[a] Mean of three determinations ± SD (standard deviation).

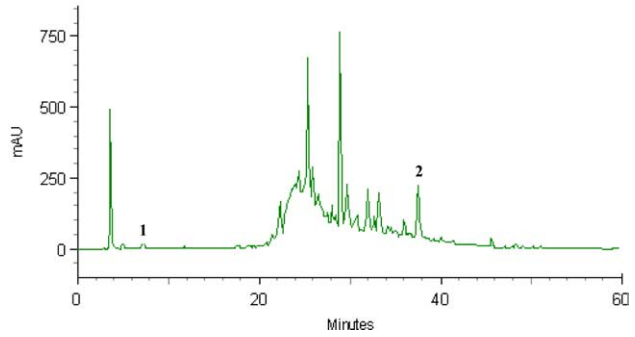

Fig. 2. HPLC chromatogram (monitored at 280 nm) of (1) GA and (2) EA extracted from freeze-dried longan seed with ethanol at 70 °C for 1 h.

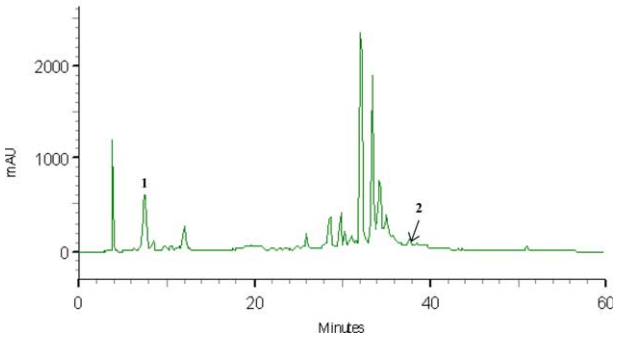

Fig. 5. HPLC chromatogram (monitored at 280 nm) of (1) GA and (2) EA extracted from 160 °C-heated mango kernel with methanol at 70 °C for 1 h.

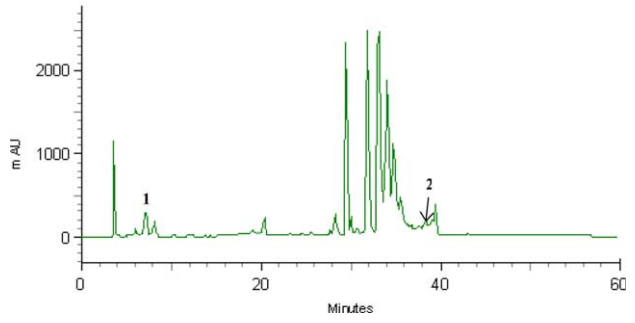

Fig. 3. HPLC chromatogram (monitored at 280 nm) of (1) GA and (2) EA extracted from freeze-dried mango kernel with ethanol at 70 °C for 1 h.

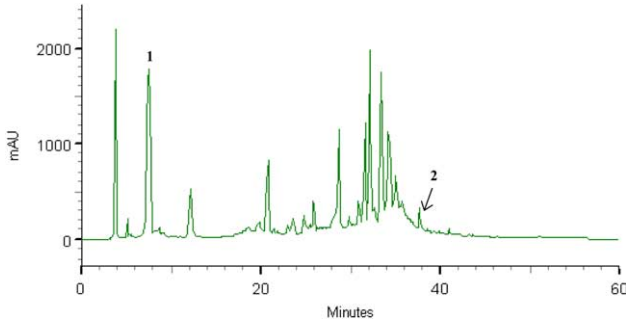

Fig. 6. HPLC chromatogram (monitored at 280 nm) of (1) GA and (2) EA from 160 °C-heated mango kernel methanolic extract hydrolyzed at 85 °C for 2 h.

EA. The concentration of GA and EA is about 90% higher in ethanolic extract of mango kernel than its methanolic extract. This result indicates that ethanol solution is a better extraction solvent for the extraction of both GA and EA. The higher yield of these compounds might contribute to the higher antioxidant activity of mango kernel ethanolic extract (previously reported by Soong & Barlow, 2004) when compared with its methanolic extract (Table 2).

The 160 °C-heated mango kernel contained about 21 and 3 times more of GA and EA, respectively, than the freeze-dried sample. Therefore, the higher antioxidant

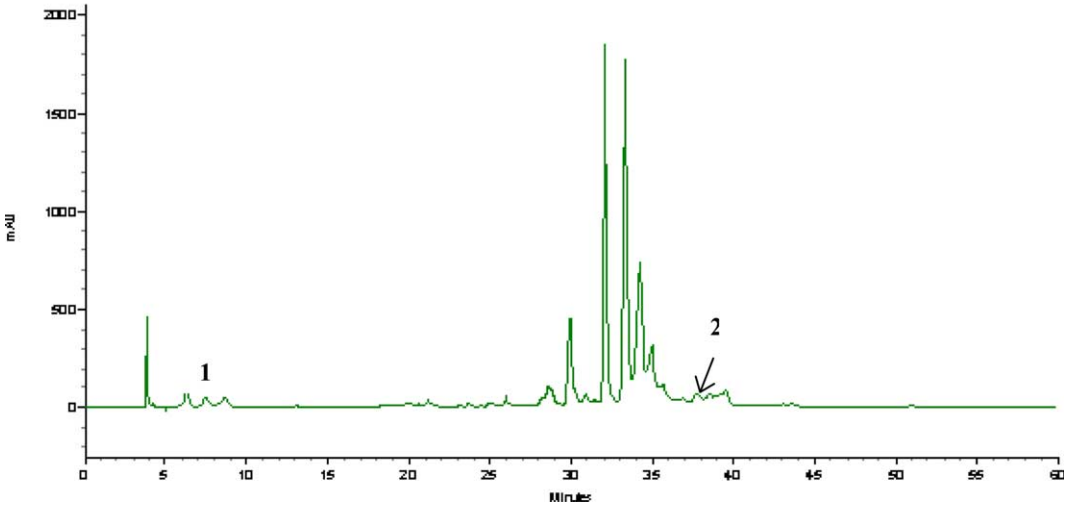

Fig. 4. HPLC chromatogram (monitored at 280 nm) of (1) GA and (2) EA extracted from freeze-dried mango kernel with methanol at 70 °C for 1 h.

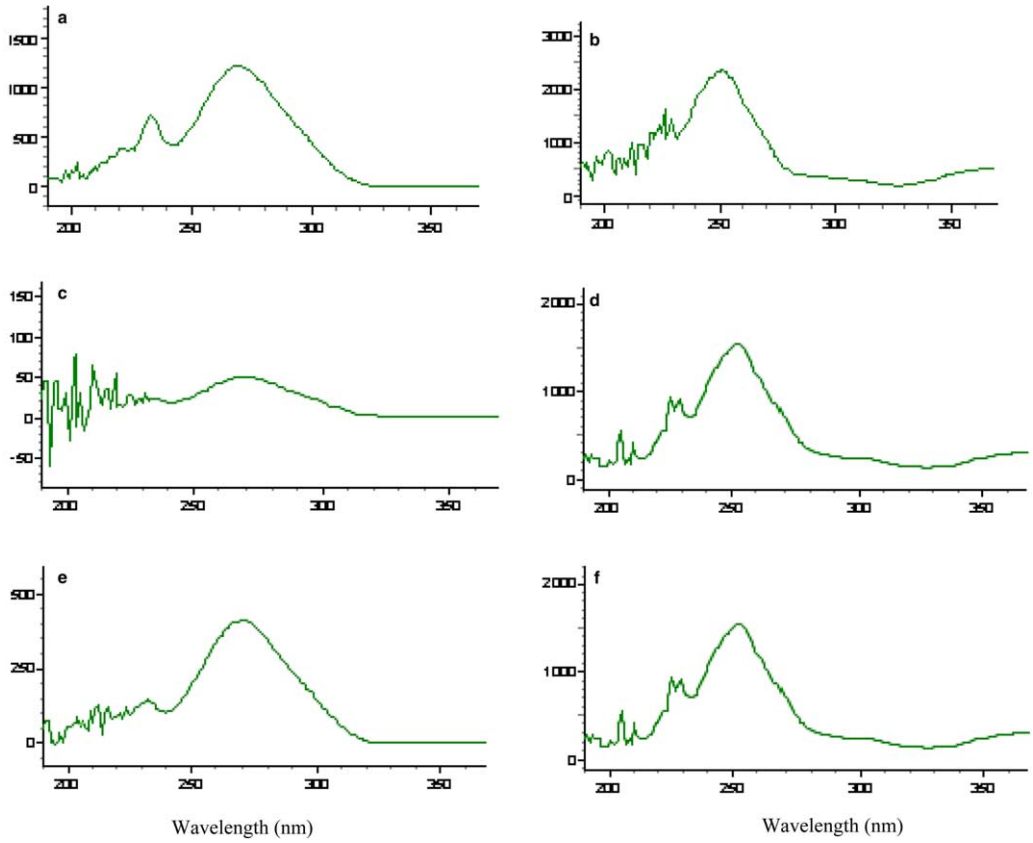

Fig. 7. Spectrum of GA and EA. (a) Standard GA (b) Standard EA (c) GA from longan seed (d) EA from longan seed (e) GA from mango kernel (f) EA from mango kernel.

Table 2
Concentrations of GA and EA recovered from longan seed and mango kernel extracts

Item	GA[a] (mg/100 g seeds)	EA[a] (mg/100 g seeds)
Longan seed		
Freeze-dried/Ethanol extraction at 70 °C, 1 h	23.3	156
Mango kernel		
Freeze-dried/Ethanol extraction at 70 °C, 1 h	185	118
Freeze-dried/Methanol extraction at 70 °C, 1 h	20.0	11.7
Freeze-dried/Methanol extraction & hydrolysis at 35 °C, 16 h	84.1	3.1
Freeze-dried/Methanol extraction & hydrolysis at 85 °C, 2 h	163	6.2
160 °C-heated/Methanol extraction at 70 °C, 1 h	414	33.6
160 °C-heated/Methanol extraction & hydrolysis at 35 °C, 16 h	535	68.7
160 °C-heated/Methanol extraction & hydrolysis at 85 °C, 2 h	838	74.5

[a] Mean of two determinations.

activity and phenolics content of heated mango kernel as previously reported (Soong & Barlow, 2004) is possibly, in part at least, attributable to the degradation of high molecular weight compounds at elevated heating temperatures, releasing the free GA and EA. In addition there may be formation of phenolic compounds during the heating process or non-enzymatic interconversion between phenolic molecules. The findings support those of the study by Berardini et al. (2004) which reported the presence of gallotannins in mango kernels.

In addition, the concentration of GA and EA in mango kernel methanolic extract increased with increasing severity of hydrolysis. The freeze-dried and 160 °C-heated mango kernel contained 8 times and 2 times more GA, respectively, after hydrolysis at 85 °C. Freeze-dried mango kernel contained minute amounts of EA so the change in concentration after hydrolysis was negligible. However, double the amount of EA was recovered from the 160 °C-heated mango kernel after hydrolysis at 85 °C. The results demonstrate that

the hydrolysis conditions may not be equally destructive to both GA and EA and again illustrate the good correlation between increment in the concentration of GA and EA and the antioxidant activity (Tables 1 and 2).

4. Conclusions

HPLC analysis has identified and quantified the GA and EA in longan seed and mango kernel extracts. Both heat treatment and acid hydrolysis were efficient in releasing free GA and EA from mango kernel extract. The results demonstrate stronger antioxidant activity of GA and EA in the free form rather than the conjugated form. In addition, they suggest a possible novel source of GA and EA which might find use as food additives or functional food products. However, further investigation of individual phenolic acids, their in vivo antioxidant activity and in the different antioxidant mechanisms is necessary before applications of longan seed and mango kernel in pharmaceutical and food products can be advocated.

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