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Determination of Daidzein and Genistein Contents in *Mangifera* Fruit

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ABSTRACT

The aim of this study was to determine the daidzein and genistein contents in *Mangifera* fruits. Three *Mangifera* species namely 'bacang' (*Mangifera foetida*), 'kuini' (*M. odorata*) and 'bambangan' (*M. pajang*) each from two different locations were selected. The extraction of isoflavones was carried out at 80°C for 30, 60 and 90 min. HPLC method was performed with a flow rate of 1.00 ml/min using three different separation columns to determine isoflavone contents. The Zorbax Eclipse RP C18 reverse-phase column was found to give the best resolution for isoflavone separation in *Mangifera* fruits. Moreover, extraction time of 90 min was found to increase the isoflavone aglycone contents. At optimised condition, 'kuini' had relatively high daidzein (9.4-10.5 mg/100 g) and genistein (1.6-1.7 mg/100 g) contents. Daidzein content of 'bambangan' (8.3-8.7 mg/100 g) was higher than 'bacang', but the genistein content of 'bambangan' (0.4-0.6 mg/100 g) was similar to that of 'bacang' (0.4-0.8 mg/100 g). However, there was a variation in daidzein and genistein contents in *Mangifera* fruits between two geographical locations.

INTRODUCTION

Phytoestrogens are naturally occurring chemical constituents of plants that have estrogenic, antiestrogenic or antiandrogenic activities in animals and human. They are found widely distributed in fruits (Monache *et al.*, 1994; Liggins *et al.*, 2000), vegetables (Liggins *et al.*, 2000a), legumes (Mazur *et al.*, 1998; Sobolev & Cole, 1999), seeds (Liggins *et al.*, 1998) and other parts of plants (Kato, Yoshida & Gottlieb, 1992; Wu, Wang & Simon, 2003).

Isoflavones are one of the major groups of phytoestrogens found in the human diet. Isoflavones includes daidzein, glycitein, genistein, O-desmethylangolensin, equol, formononetin, and biochanin A. The

major food sources of isoflavones are soy bean and soy-based products (Klejdus *et al.*, 2004). Formononetin and biochanin A are methylated isoflavones found in clovers and are metabolised to daidzein and genistein. Daidzein is also metabolised to O-desmethylangolensin and to equol in the gut. Isoflavone is also found naturally in the form of glucoside and aglycone. Isoflavone aglycones (daidzein and genistein) can be derived from six other isoflavone glucosides, while its glucosides consist mainly of daidzin, genistin, and their conjugate forms (Figure 1).

The *Mangifera* species belongs to the Anacardiaceae family in the order of Sapindales. There are about 600 species in 70 genera in the family which are mostly

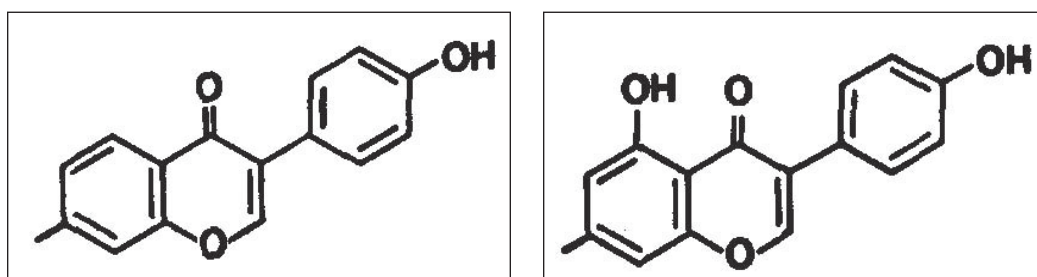


Figure 1. Classification and structures of isoflavones (Setchell *et al.*, 1987).

Name	Abbreviation	R
Daidzin	Dn	$C_5H_8O_5-H$
Genistin	Gn	$C_5H_8O_5-H$
Acetyldaidzin	ADn	$C_5H_8O_5-COCH_3$
Acetylgenistin	AGn	$C_5H_8O_5-COCH_3$
Malonylgenistin	MGn	$C_5H_8O_5-COCH_2COOH$
Malonyldaidzin	MDn	$C_5H_8O_5-COCH_2COOH$

subtropical and tropical woody trees and shrubs. There are *Mangifera* fruits of over 2,500 varieties. Mango (*Mangifera indica*) is a commercially important member of this family. Eight *Mangifera* species which originated from the South-east Asian region are *Mangifera caesia* (jack, binjai), *M. foetida* (horse mango, bacang), *M. horsefieldia* (hambawang), *M. indica* (chokanan, maha), *M. lagenifera* (machang), *M. odorata* (kuini), *M. pajang* (bambangan), and *M. torquenda* (buah loam, kemantan) (Porcher, 1995). 'Bacang', 'kuini' and 'bambangan' have yellow-orange colour pulp, probably due to carotenoid compounds. Similarly, as in *Mangifera indica* that was mentioned by Morton (1987), these fruits have sour and pungent tastes, and are reported to have many medicinal uses. Most studies on *Mangifera* fruits have focused on the antioxidant compounds such as carotenoids and vitamin C; however, other health promoting compounds for example, isoflavones have not been explored.

Liquid and gas chromatography techniques have been used to analyse and

detect phytochemicals in plants, foods and food products (Setchell, Welsh & Lim, 1987; Hutabara, Mulholland & Greenfield, 1998; Apers *et al.*, 2004; Kuo & Ding, 2004; Peñalvo, Nurmi & Adlercruetz, 2004). Optimisation of the available methods in phytoestrogen analysis can further improve detection limits. There are various chromatographic methods available for the analysis of phytoestrogen compounds in foods, such as high-performance liquid chromatography (HPLC) (Setchell *et al.*, 1987; Nurmi & Adlercruetz, 1999; Griffith & Collison, 2001; Apers *et al.*, 2004) and gas chromatography (GC) (Mazur *et al.*, 1996; Kuo & Ding, 2004). Each method used in analysis of phytoestrogen has its limitations.

The present study aims to determine the daidzein and genistein contents in *Mangifera* fruits using the HPLC method. The effects of extraction time and separation column on these compounds were also investigated. Optimisation of HPLC conditions was carried out prior to analysis.

MATERIALS AND METHODS

Chemicals and standards

Acetonitrile (HPLC grade) was purchased from Merck (Missouri, USA); HPLC grade methanol was obtained from Scharlau (Barcelona, Spain); acetic acid and hydrochloric acid were obtained from J. T. Baker (New Jersey, USA); sodium acetate was obtained from May & Baker (Dagenham, UK). Distilled water was prepared from Favorit W4L water distillation system (Nottingham, UK). Daidzein and genistein (100%, HPLC purity) were purchased from Sigma Chemical (Missouri, USA).

Samples

A total of six types of fruits from the three *Mangifera* species (*Mangifera foetida*, *M. odorata* and *M. pajang*) were obtained from the Malaysian Agricultural Research and Development Institute (MARDI), Serdang, Selangor, Malaysia. The fruits were harvested from two different locations in Malaysia. The locations of each *Mangifera* species obtained differed in soil mineral composition. Both places have similar weather. The sources of the *Mangifera* species are shown in Table 1. In this study, three *Mangifera* fruits of each species from each location were collected conveniently during August and September 2005 due to the difficulty in obtaining them casually due to the seasonal nature of fruiting. The maturity of *Mangifera* fruit was determined based on the firmness and visual peel colour (yellow-orange colour). All fruits were stored in a freezer (-20°C) for less than a month prior to analysis.

For analysis, the whole fruit (with peel) of the three samples from each of the *Mangifera* species were cleaned and washed under running tap water. Then the peel and kernel were removed manually. The flesh was cut into small pieces, and stored at -80°C for 24 h before freeze-drying using a

Virtis freeze dryer (New York, USA). The lyophilised flesh was mashed and ground into fine powder using a dry blender, and the resulting sample was stored at -20°C for less than a week before further analysis.

Isoflavones extraction

The isoflavone compounds were extracted using a method described by Peñalvo *et al.* (2004). One gram of each sample powder was weighed and transferred into a test tube, and 10 ml of 1.0 M acidified aqueous 80% ethanol was added. The mixture was shaken vigorously in a shaking water bath (Memmert, Schwabach, Germany). The test tube was covered with aluminum foil and incubated for 30, 60 and 90 min at 80°C. Following incubation, the mixture was cooled to room temperature. Then it was centrifuged for 2 min at 2140×g using a tabletop Hettich Universal 32 R centrifuge (Tuttlingen, Germany). The sample residue was re-extracted three times according to the above steps. The supernatants were pooled, filtered through a Whatman No.1 filter paper (Kent, United Kingdom) and evaporated to dryness using a Büchi R-200 rotary evaporator (Flawil, Switzerland). The resulting extract was dissolved in 5 ml of 80% ethanol and filtered through a 0.45 µm nylon membrane before injecting it in to the HPLC system. Due to limited resources, all extractions were done in duplicate.

All standard concentrations were prepared from stock solutions of daidzein or genistein. For preparing stock solution, 1 mg of daidzein or genistein was weighed in a test tube. One ml of 80% methanol was added, dissolved and filtered through a nylon filter (0.45 µm). Stock solutions of the standard were stored at -20°C prior to HPLC analysis.

HPLC analysis

Isoflavone separation was performed using a HPLC system (HP1100) equipped with an Agilent 1100 series standard autosampler

Table 1. Source of three *Mangifera* species

Common Name	Scientific Name	Location
Bacang	<i>Mangifera foetida</i>	(1) Kampung Bukit, Gurun, Kedah (2) Kampung Masjid, Jalan Arau, Perlis
Bambangan	<i>Mangifera pajang</i>	(1) Kenut, Sarawak (2) Sarawak (unknown place)
Kuini	<i>Mangifera odorata</i>	(1) Jalan Pauh, Arau, Perlis (2) Jelempek, Arau, Perlis

(California, USA) and a UV-Vis diode array detection. The Zorbax Eclipse RP C18 (150 × 4.6 mm; 3 µm) column (Agilent, California, USA) was used for the separation of isoflavones. In our preliminary study, three columns were used in the pretest and the resolution of isoflavone chromatograms was compared. The columns were Purospher Star RP-18 encapped, 250 × 4.6 mm, 5µm (Merck, Darmstadt, Germany), Wakosil II C18 RS, 150 × 4.6 mm, 3µm (SGE, Texas, USA), and Zorbax Eclipse RP C18, 150 × 4.6 mm, (Agilent, California, USA). The analytical column was protected with a guard column Purospher Star RP-18e (Merck, Darmstadt, Germany) 15 × 2 mm packed with 5 µm particles. The column temperature was set at 40°C, injection volume of 5 µl, and UV-Vis diode array detection at 260 nm. The detection at 260 nm was performed based on the standard procedure by Griffith & Collison (2001).

The separation of isoflavones was performed using an isocratic programme with a mobile phase of acetonitrile, methanol, 0.2 M ammonium acetate buffer, pH 4.6 (MeCN: MeOH: buffer) (10: 50: 40 v/v/v) with a flow rate of 1.00 ml/min. A total run time of 110 min was applied, including 20 min equilibration time (Griffith & Collison, 2001). A total injection of 36 samples was separately done. All samples and standards were run in duplicate. The calibration of standards (daidzein and genistein) was carried out at concentrations of 50, 100, 500, 1000 µg/ml before sample run.

Spiking and recovery tests

The samples were spiked with two standards (daidzein and genistein), with a known concentration. The peaks of daidzein and genistein were identified by comparing the retention times. Recovery test was performed for each fruit sample to determine the analytical quality. Recoveries were measured by the addition of 100-200 µg/ml of each standard to the sample (Hutabarat *et al.*, 1998). The spiked samples were then repeated through the whole extraction procedure.

Statistical analysis

Results were expressed as mean ± standard deviation. Independent T-test and one way analysis of variance (ANOVA) coupled with LSD's post-hoc comparison were applied to determine the differences of means of the isoflavones (mg/100g) among the three *Mangifera* species from two different locations. Paired-samples T-test was applied to determine the isoflavones content of three *Mangifera* species between two different locations. The significant level was set at $p < 0.05$. The Statistical Package for Social Sciences (SPSS version 15.0) was used for statistical analysis.

RESULTS AND DISCUSSION

Chromatographic conditions

Optimised chromatographic conditions applied with HPLC-UV-DAD, Zorbax Eclipse RP C18 column and mobile phase

(MeCN: MeOH: buffer; 10: 50: 40) were used to determine the daidzein and genistein contents in *Mangifera* fruits. The addition of methanol in the mobile phase increased the separation of individual isoflavones. Methanol is the best organic solvent for isoflavone separation, as it is a better modifier compared to acetonitrile (Setchell *et al.*, 1987). However, acetonitrile was needed as an eluent for isoflavone compounds in chromatographic separation (Torres-Lapasió *et al.*, 2007). The maximum UV absorption of isoflavone was set at 260 nm as reported by Griffith & Collison (2001).

The purpose of using sodium acetate buffer (pH 4.6) for separation of isoflavones was due to the non-volatile character of the compound when compared to the ammonium buffer. In the chromatographic method, non-volatile buffer salts are frequently used to ensure proper pH of the mobile phase and to prevent tailing and poor peak shape of the compounds (Majors, 2004). The pH of the mobile phase can also affect

the retention time of isoflavone compounds. The changes in pH of the mobile phase will determine the dissociation degree of ionisation and the retention of compounds (Heyrman & Henry, 1999). In the present study, a Zorbax Eclipse RP C18 column was the best separation column for the pH range of 2-9. It gave better peak shape and high resolution for acidic, basic and neutral compounds (Agilent, 2006). In addition, a flow rate of 1.00 ml/min was appropriate for the use of the 4.6 nm internal diameter column in HPLC analysis (IonSource, 2001).

Identification of isoflavones and recovery

The peaks of isoflavones (daidzein and genistein) of *Mangifera* species (Figure 2) were identified by spiking tests. Further identification was referred to chromatograms published by Apers *et al.* (2004) and Peñalvo *et al.* (2004). The highest peak of the chromatogram (Figure 2) with retention time of 2.6 ± 0.1 min was identified as daidzein.

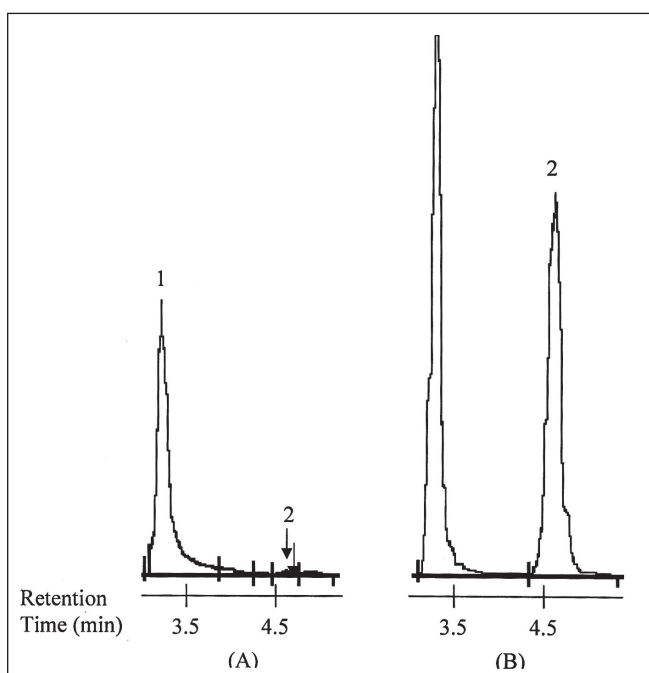


Figure 2. (A) The peak of daidzein (1) and genistein (2) in *Mangifera* fruit; (B) The peak of daidzein (1) and genistein (2).

Table 2. Isoflavones content (mg/100 g edible portion) and recovery (%) in the three *Mangifera* species at optimised condition

Sample	Daidzein		Genistein		Total isoflavone
	Mean	% Recovery	Mean	% Recovery	
Bacang	6.23 ± 3.37 ^a	95	0.58 ± 0.59 ^a	107	6.81
Kuini	9.94 ± 4.47 ^b	95	1.66 ± 0.85 ^b	91	11.6
Bambangan	8.49 ± 5.16 ^b	103	0.53 ± 0.74 ^a	102	9.02

Values are expressed as dry weight basic. The mean isoflavones are shown as average mean of individual isoflavone in each *Mangifera* species from two different locations. The total isoflavone was derived from the addition of daidzein and genistein values. The data are presented as mean ± standard deviation, n = 4 for each type of isoflavone in each *Mangifera* species.

Mean value followed by different letters in the same column differs significantly (p < 0.05).

The smaller peak (peak 2), beside the peak labelled as peak 1 was identified as genistein, with retention time of 3.4 ± 0.1 min. Moreover, the peak of genistein was found between the peaks of other unidentified compounds in different types of *Mangifera* fruits.

The recovery of isoflavones (daidzein and genistein) in the three *Mangifera* species was in the range of 91-107% (Table 2), that is, in the acceptable range (80-110%) (AOAC,1993). The percentage of recovery might have been affected by inaccuracy of standards preparation or high temperature applied during extraction.

Isoflavone content

Three different extraction times were applied in the extraction of isoflavones. The means of daidzein, genistein and total isoflavone per edible portion (mg/100 g) in the fruits of three *Mangifera* species at optimised condition are tabulated in Table 2; while the means of daidzein and genistein in the *Mangifera* fruits determined by three different extraction times are presented in Table 3. The results showed that 'bacang' had daidzein and genistein content in the range of 2.8-8.0 mg/100 g and 0.30-0.8 mg/100 g respectively. A range of daidzein (3.5-8.7 mg/100 g) and genistein (0.2-0.6 mg/100 g) content were found in 'bambangan', while 'kuini' also had a range of daidzein (3.9-10.5 mg/

100 g) and genistein (0.5-1.7 mg/100 g) content.

The mean concentrations of daidzein and genistein of the *Mangifera* species were significantly different among different extraction times (p<0.05). At 60 min extraction, the genistein content of 'Bacang Kg Masjid' was found to be the lowest compared to 30 and 90 min. Moreover, the isoflavone content increased as extraction time was increased. However, the optimum extraction time for the studied fruits was at 90 min.

At 90 min extraction, 'Kuini' had the highest daidzein and genistein content compared to the other *Mangifera* species studied (Table 3). Paired samples T-test showed that the isoflavone (daidzein and genistein) content in 'bacang', 'kuini' and 'bambangan' was significantly different (p < 0.05) between two different locations (data not shown). 'Bacang Gelok' had significantly higher isoflavone content compared to 'Bacang Kg Masjid'. For 'bambangan', a higher daidzein content was found in 'Bambangan Kenut' than in 'Bambangan Sarawak', but was lower in genistein content. 'Kuini Jelepok' had a isoflavone content that was significantly higher than in 'Kuini Jln Paul'.

Isoflavones have been detected in other fruits such as berries, currants and melons. Liggins *et al.* (2000) reported passion fruit and coconut contained 0.17 and 0.19 mg/

Table 3. Isoflavones content of six types of *Mangifera* species (mg/100 g edible portion)

Sample	Extraction time (min)		
	30	60	90
<i>Daidzein</i>			
B1	2.80 ± 0.077 ^a	3.41 ± 0.002 ^a	4.46 ± 0.025 ^a
B2	3.66 ± 0.013 ^b	5.05 ± 0.015 ^b	8.00 ± 0.036 ^b
K1	3.93 ± 0.002 ^c	6.29 ± 0.005 ^c	10.45 ± 0.029 ^c
K2	4.1 ± 0.003 ^d	5.79 ± 0.004 ^d	9.43 ± 0.003 ^d
Bm1	3.62 ± 0.006 ^{e,b}	5.51 ± 0.001 ^e	8.71 ± 0.001 ^e
Bm2	3.5 ± 0.011 ^f	5.02 ± 0.002 ^f	8.26 ± 0.001 ^f
<i>Genistein</i>			
B1	0.35 ± 0.014 ^a	0.13 ± 0.001 ^a	0.4 ± 0.011 ^a
B2	0.25 ± 0.004 ^b	0.67 ± 0.004 ^b	0.76 ± 0.043 ^b
K1	0.45 ± 0.004 ^c	0.95 ± 0.007 ^c	1.72 ± 0.012 ^c
K2	0.66 ± 0.001 ^d	0.88 ± 0.001 ^d	1.61 ± 0.003 ^d
Bm1	0.3 ± 0.003 ^e	0.61 ± 0.013 ^e	0.42 ± 0.001 ^a
Bm2	0.15 ± 0.005 ^f	0.56 ± 0.001 ^f	0.63 ± 0.001 ^e

Values are expressed as dry weight basic in duplicate analysis. All data are presented as mean ± standard deviation.

Mean value followed by different letters in the same column differs significantly ($p < 0.05$).

B1: Bacang Kg Masjid; B2: Bacang Gelok; K1: Kuini Jelempok; K2: Kuini Jln Pauh; Bm1: Bambang Kenut; Bm2: Bambang Sarawak

kg of isoflavone respectively; while raisin and currant contained about 2.00 mg/kg of isoflavone. Besides, 0.25 mg/kg dry weight of daidzein and 0.21 mg/kg dry weight of genistein were found in mango available in the United Kingdom (Liggins *et al.*, 2000). The present study results showed that the Malaysian *Mangifera* fruits have considerable amounts of high daidzein and genistein content.

In the present study, there was a slight increase in genistein compared to daidzein for 'bacang' when extraction time was increased. Moreover, a wide range of genistein (3.7-30.0 mg/100 g edible portions) and daidzein content (0.4-3.4 mg/100 g edible portions) were found in the studied *Mangifera* fruits. It could be due to low sensitivity and specificity in HPLC-DAD detection. However, acidic mobile phases

usually showed better reproducibility than neutral mobile phases, as reported by MAC-MOD (2004).

The retention capacity of isoflavones in the studied *Mangifera* fruits varied. The variability of isoflavone content in the food matrix was reported to be influenced by several ecological factors, such as plant species, strain, crop year and geographical locations (Joffe, 2003). The present finding is in line with the findings by Prabhakaran & Perera (2006) where an increase in the extraction time resulted in a decrease in genistein content, similar to that observed for 'Bambangan Kenut'. Heat treatment significantly increased the isoflavone aglycone contents. However, application of novel techniques in isoflavone extraction has not been studied extensively.

CONCLUSIONS

The best condition for extraction of isoflavones in *Mangifera* fruits was at 80 °C for 90 min using acidified ethanol as the extraction solvent. Zorbax Eclipse RP C18 was the best separation column for isoflavones analysis. *Mangifera* fruits contain isoflavones that may serve as health promoting compounds in our diet. 'Kuini' possessed the highest daidzein and genistein content among the *Mangifera* species studied. It was also found that the isoflavone content of *Mangifera* fruits harvested from different locations varied considerably. Thus studies on isoflavones need to specify the geographical source of the fruit.

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