

Sains Malaysiana 43(8)(2014): 1181–1187

Quantitative HPLC Analysis of Gallic Acid in *Benincasa hispida* Prepared with Different Extraction Techniques

(Analisis Kuantitatif HPLC terhadap Asid Galik dalam *Benincasa hispida* yang
Disediakan dengan Pelbagai Teknik Pengekstrakan)

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ABSTRACT

Ash gourd (*Benincasa hispida*, *Bh*) is traditionally claimed useful in treating asthma, cough, diabetes, haemoptysis and hemorrhages from internal organs, epilepsy, fever and balancing of the body heat. One of the major phenolic acids presented in *Benincasa hispida* is gallic acid, a phenolic compound which is linked with its ability in reducing Type II diabetes. The aim of the present study was to investigate the effect of different extraction techniques on the concentration of gallic acid in *Bh*. The *Bh* extracts were prepared with three different techniques namely; fresh extract (FE), low heating (LH) and drying and heating (DH). The gallic acid has been detected and quantified using high performance liquid chromatography (HPLC) coupled with UV-Vis detector. The amount of gallic acid detected in FE, LH and DH were 0.036, 0.050 and 0.272 mg/100 g, respectively. The limits of detection was 0.75 µg/mL while the limit of quantification and recovery were 2.50 µg/mL and 95.53%, respectively. In summary, HPLC technique coupled with UV detector systems able to quantify gallic acid in *Bh* extracts. The gallic acid were present at higher concentration in *Bh* extracted using drying and heating, followed by low heating and fresh extract methods.

Keywords: *Benincasa hispida*; drying; gallic acid; low heating

ABSTRAK

Secara tradisi, *kundur* (*Benincasa hispida*, *Bh*) telah dipercayai berguna untuk merawat asma, batuk, diabetes, haemoptysis dan pendarahan daripada organ dalaman, keclaruan fungsi otak, demam dan penyeimbangan suhu badan. Salah satu asid fenolik utama yang hadir dalam *Benincasa hispida* ialah asid galik, iaitu sebatian fenolik yang juga dikaitkan dengan keupayaannya mengubati penyakit diabetes Jenis II. Tujuan kajian ini adalah untuk mengkaji kesan pengekstrakan berbeza terhadap kepekatan asid galik dalam *Bh*. Ekstrak *Bh* disediakan dengan tiga jenis teknik pengekstrakan yang berbeza iaitu pengekstrakan segar (FE), pemanasan suhu rendah (LH) dan pengeringan dan pemanasan (DH). Asid galik telah dikesan dan ditentukan menggunakan kromatografi cecair berprestasi tinggi (HPLC) yang dilengkapi dengan pengesan cahaya nampak ultra ungu (UV-Vis). Jumlah asid galik yang dikesan dalam FE, LH dan DH masing-masing adalah 0.036, 0.050 dan 0.272 mg/100 g. Had pengesanan adalah 0.75 µg/mL manakala had penentuan dan peratus perolehan semula masing-masing adalah 2.50 µg/mL and 95.53%. Kesimpulannya, teknik HPLC yang dilengkapi pengesan sistem UV mampu menentukan kandungan asid galik dalam ekstrak *Bh*. Asid galik hadir pada kepekatan paling tinggi dalam *Bh* yang diekstrak menggunakan kaedah pengeringan dan pemanasan, diikuti dengan pemanasan suhu rendah dan pengekstrakan segar.

Kata kunci: Asid galik; *Benincasa hispida*; pemanasan suhu rendah; pengeringan

INTRODUCTION

Frequent consumption of grains, vegetables and fruits is linked with reduced risk of chronic illnesses such as diabetes, cardiovascular disease, cancer, cataract and other illnesses. Fruits are generally low in fat and calories but provide many essential nutrients such as simple sugars, fiber and vitamins, which are essential for optimizing our health. *Benincasa hispida* (*Bh*) commonly known as ash gourd or winter melon is originated from *Cucurbitaceae* family. It has been valued as a healthy vegetable as it provides good source of natural sugars, amino acids, organic acids, mineral elements and vitamins (Zaini et al. 2010). Traditionally, it is useful in soothing asthma, cough, diabetes and hemorrhages from

internal organs, epilepsy, fever and vitiated conditions of pitta. For centuries, *Bh* has been used for several empirical applications in treating some diseases such as dyspepsia, burning sensation, vermifuge, heart disease, diabetes and urinary disease (Anil Kumar 2002; Asolkar & Chakre 2000). It was also reported to effectively healing dropsy, treating diseases related to liver, leucorrhea mineral detoxification, lowering fever and strengthening the function of the bladder in small and large intestines (Lee et al. 2005).

Plants are naturally contained bioactive compounds therefore sample preparation is the crucial first step in the analysis of natural products. In fact, it is necessary to extract the desired chemical components from the plant materials

for further separation and characterization (Huie 2002). The extraction and characterization of active compounds from medicinal plants led to the finding of new medicines with high therapeutic value (Colegate & Molyneux 2007). Excitingly, natural compounds contained in plant can act in a synergistic manner within the human body and can provide unique therapeutic properties with minimal or no undesirable side-effects (Kaufman et al. 1998). Dry sample matrix is subject to the Maillard reaction product and this product was reported to have polyphenol that might enhance antioxidant activity of processed foods. Some evidence has suggested that the yield, recovery and type of polyphenolics in an extract are influenced by the type and polarity of extracting solvents, time and temperature of extractions besides physical characteristic of the sample (Naczka & Shahidi 2006). The present study aimed to quantify the present of phenolic acid namely gallic acid in *Benincasa hispida* with three different extraction techniques by HPLC analysis. The HPLC method is developed with the condition that suitable with the samples.

Previous study has shown the presence of triterpenes, catechins, carotenes, tannins, uronic acids and polyphenols compounds in Bh extracts (Gill et al. 2010). Polyphenols are the largest group of phytochemical and many of them have been found in plant-based foods. Phenolic acids are non-flavonoid polyphenolic compounds which can be further divided into two main types, benzoic acid and cinnamic acid derivatives. Gallic acid (3,4,5-trihydroxybenzoic acid) is one type of natural phenolic acid compound widely which exists in plants (Figure 1).

Some research reported that oral treatment with gallic acid (10 and 20 mg/kg) for 21 days significantly decreased the levels of blood glucose, brain lipid peroxidation products and significantly increased the activities of brain enzymatic antioxidants in diabetic rats (Stanely et al. 2011). Gallic acid was reported to induce apoptosis in K562 cells involves death receptor and mitochondrial-mediated pathways by inhibiting BCR/ABL kinase, NF- κ B activity and COX-2 (Chandramohan et al. 2012). In other study, both gallic acid and ellagic acid from *Sanguisorba officinalis* L. were found to be of value as a breast cancer preventive and therapeutic agent by inducing apoptosis and inhibiting angiogenesis (Wang et al. 2012). In addition apoptotic cell death was observed in histological analysis.

The chemical structure of gallic acid (GA) is shown in Figure 1.

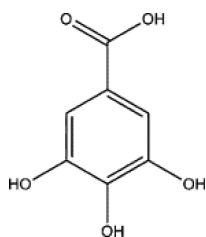


FIGURE 1. Chemical structure of gallic acid (3,4,5-trihydroxybenzoic acid)

The combination of gallic acid with flutamide was found to cause higher toxicity to cancer cells of (AR)(+) LNCaP prostate cancer and normal epithelial cells than either of the compounds alone in Polyherbal Ayurvedic formulation of triphala (Russell et al. 2011). Triphala is a formula of three potent super fruits that have a wide range of activity which known as a cleaning agent and also high in nutritional value as well as often used as a food supplement. Yeh et al. (2011) documented that gallic acid reduced the viability of human promyelocytic leukemia HL-60 cells in a dose- and time-dependent manner. Thus gallic acid as a potential therapeutic agent for treatment of leukemia seems warranted (Yeh et al. 2011). In another aspect of research, gallic acid which was the major bioactive compound presented in *Toona sinensis* leaf extract was seen to be responsible for the anti-neoplastic effect (Chia et al. 2010). Taken together, these findings suggest that gallic acid may be a novel therapeutic agent useful in treating/healing some chronic cancerous cells and potentially applied as anti leukemic, anti inflammatory, antioxidant and anti hyperglycaemic effects.

Benincasa hispida (Bh) is widely used in traditional oriental medicine for treatment of urinary and heart diseases, gastrointestinal and respiratory problems (Aslokar et al. 1992). Aslokar et al. (1992) has explored the preventive and therapeutic efficacy of Bh and chayote juice in rats fed with sweeteners fructose and sucrose on development of these conditions. Juice of vegetables Bh and chayote was observed effective in accelerates normalization of impaired glucose tolerance and oxidative stress induced by fructose and sucrose in rats (Tiwari et al. 2012). Both ash gourd and chayote juice treatment significantly accelerated decrease in oxidative stress level than rats treated as placebo (Tiwari et al. 2012). Recently, the gallic acid equivalent total phenolic content of the Bh plant was found to be 79.94 mg/g dry extract (Harput et al. 2012).

HPLC is one of the best equipment for the analysis of phenolic compounds because of its versatility, precision and relatively cost-effective (Escarpa 2000). Reversed phase (RP) HPLC on a C-18 column, with a binary mobile phase containing acidified water and polar organic solvent (acetonitrile or methanol) with UV-visible diode-array detection (DAD), is usually preferred and is a crucial and reliable tool for routine analysis of plant phenolic compounds including gallic acid (Revilla 2000).

The objective of this study was to determine the concentration of gallic acid (GA) in Bh extracted using different methods such as fresh extract (FE), low heating (LH) and drying and boiling (DB). The detection of the GA concentration in these samples was accomplished using HPLC-UV vis systems. In the present study, the quantification of GA in Bh fruit extracts was also validated.

MATERIALS AND METHODS

STANDARD PREPARATION

Gallic acid standard was purchased from Sigma, USA. Other chemicals namely methanol, acetonitrile and potassium

dihydrogen phosphate (KH_2PO_4) were purchased from Merck, Germany. Gallic acid (10 mg) standard was accurately weighed and transferred to 100 mL volumetric flask. The standard was then dissolved in 100 mL methanol-water (70:30, v/v) for preparation of standard stock solution of 100 $\mu\text{g}/\text{mL}$.

SAMPLE PREPARATION

There were three extraction techniques involved in the present study. There were fresh extraction (FE), low heating (LH) and drying and heating (DH).

FRESH EXTRACTION (FE)

The fresh fruit of *Benincasa hispida* (Bh) were purchased from local market at Kota Bharu, a capital city of Kelantan, Malaysia. The fruit was cleaned to remove all unwanted material adhered to the surface of fruit. The cleaned fruit was then manually peeled to separate its seeds, inner pulp and pulp. The pulp was homogenized with food processor (Panasonic MK-5087M) without water addition and the juice was filtered through muslin cloth and centrifuged (2790 g, 10 min). The cleared juice was placed in a specimen bottles and was frozen (-20°C , 12 h) before freeze-drying for further analyses. A vertical freezer with 2 liter space capacity designed by IssinBioBase, Korea was utilized for freeze drying purpose. The condenser temperature was approximately -50°C .

LOW HEATING (LH)

The pulp of Bh was homogenized with food processor and added with distilled water in 1:1 ratio in a beaker to dilute the mixture and able to stir. The mixture was slowly heated ($60\pm 2^\circ\text{C}$) using hot plate for 30 min and continuously stirred. The temperature was administered using laboratory thermometer. The mixture was then cooled at room temperature. After that, the mixture was filtered with muslin cloth and centrifuged (2790 g, 10 min). The cleared juice was placed in a specimen bottles and was frozen (-20°C , 12 h) before freeze-drying for further analyses. A vertical freezer with 2 L space capacity designed by IssinBioBase, Korea was utilized for freeze drying purpose. The condenser temperature was approximately -50°C .

DRYING AND HEATING (DH)

The pulp of Bh was oven-dried in $55\text{--}60^\circ\text{C}$ for two days. After that, the dried pulp were ground and added with distilled water in 1:20 ratio due to the water absorption capability of the dried pulp. The mixture was then slowly heated ($60\pm 2^\circ\text{C}$) using hot plate for 30 min and stirred constantly. The temperature was administered using laboratory thermometer. The mixture was then cooled at room temperature. The mixture was filtered with muslin cloth and centrifuged (2790 g, 10 min). The extract was placed in a specimen bottles and was frozen (-20°C , 12 h) before freeze-drying for further analyses. A vertical freezer

with 2 L space capacity designed by IssinBioBase, Korea was utilized for freeze drying purpose. The condenser temperature was approximately -50°C .

The extraction techniques for DH and LH using distilled water as a solvent for extraction was due to the higher solubility of phenolic compounds in water and heating was involved as it can release bound phenolic due to the breakdown of cellular constituent. In contrast, FE techniques did not involve addition of water and heating treatment to see the differences of phenolic compounds extracted between them.

As a preparation for HPLC analysis, the accurately weighed freeze-dried samples, FE, LH and DH (0.2 g each) were dissolved in extraction solvent (methanol-water (70:30, v/v) and transferred in 10 mL volumetric flask and diluted up to mark to make 20 mg/mL each. After that they were filtered through 0.45 μm membrane filter prior to injection into HPLC UV systems.

ANALYTICAL METHOD

CHROMATOGRAPHIC PARAMETERS

Separation of gallic acid was performed by HPLC with a Gilson UV/Vis 151 Series HPLC system (operated at 280 nm) with UV-VIS detector (operated at 280 nm) and injection valve with 20 μL sample loop. The compound was separated on 125 \times 4 mm, i.d., 5- μm pore size LiChrospher 100 RP-18 column (Merck, Germany). The mobile phase, prepared using 0.01 M potassium dihydrogen phosphate-acetonitrile (85:15, v/v) at pH 3.2, was filtered through a 0.45 μm . The flow rate was 0.75 mL/min. Data were integrated by Trilution LC software and the results were obtained by comparison with standards.

CALIBRATION PLOTS

The gallic acid standard with different concentrations was prepared by dilution of the standard stock solution. The standard response curve for gallic acid was a linear regression fitted to triplicate values obtained at each of five concentrations (6.25 to 50 $\mu\text{g}/\text{mL}$). Twenty μL of each solution was chromatographed and the peak areas were measured. Peak areas against the respective concentration for gallic acid were then plotted to find the linear range of gallic acid.

METHOD VALIDATION

The method was validated for repeatability, linearity and accuracy, precision, selectivity and specificity. Accuracy was assessed by spiking standard of gallic acid in the three different samples and measures the recoveries. Precision was measured by assessment of intra and inter-day relative standard deviation (R.S.D) of peak area and retention time. All validation studies were performed by replicates injection of standard and sample solutions. For intra-day study the concentrations of the gallic acid was

calculated three times on the same day at intervals of 1 h whereas for inter-day study, the concentrations of the gallic acid was calculated on three different days. Selectivity and specificity of the method were assessed by injecting solutions containing the standard. Limit of detection (LOD) and limit of quantification (LOQ) were measured to assess the detection and quantitation limits of the method. They were calculated using the equation $LOD = 3 \sigma/S$; $LOQ = 10 \sigma/S$, where σ is the standard deviation of the response and S is the slope of the calibration curve.

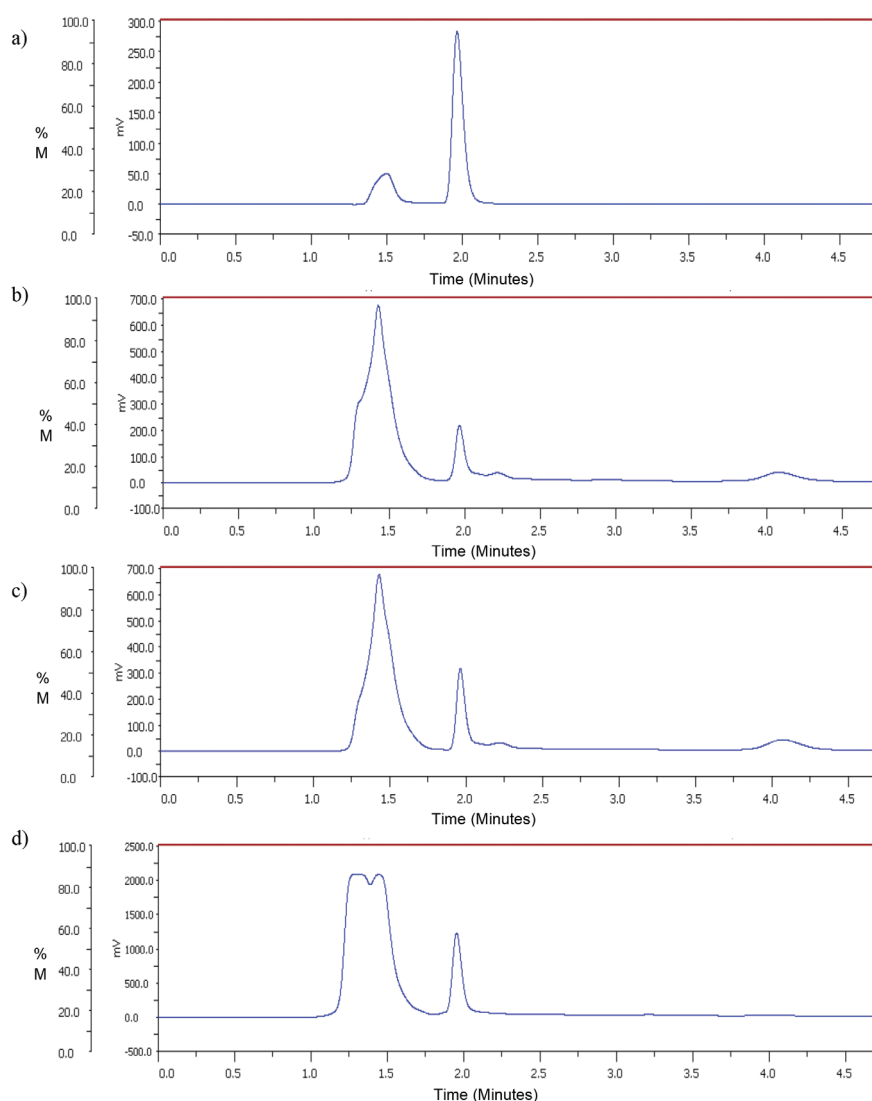
RESULTS AND DISCUSSION

CONCENTRATION OF GALLIC ACID IN BH EXTRACTED WITH DIFFERENT METHODS

Volume fraction of organic solvent and pH of the solution for the mobile phase conditions were successfully

optimized to ensure no interference from solvent and other compounds. It consists of isocratic elution of 15% (v/v) acetonitrile and 85% (v/v) 0.01 M potassium dehydrogen phosphate (KH_2PO_4) in HPLC-grade water at pH3.2. These parameters were found to be suitable mobile phase allowing good separation of gallic acid at flow rate 0.75 mL/min using LiChrospher 100 RP-18 column. Under this system, the concentration of gallic acid of Bh extracted with different extraction techniques was successfully quantified. The chromatograms of gallic acid quantified in *Benincasa hispida* (Bh) extract prepared with three different techniques was shown in Figure 2. The retention time for gallic acid was 1.98 min.

The present result showed that drying and heating (DH) extract of Bh recorded the highest gallic acid concentration at 0.272 mg/100 g followed by low heating (LH) extract (0.050 mg/100 g) and fresh extract (FE) (0.036 mg/100 g) as shown in Table 1. It is due to temperature-



Note: M= mobile phase

FIGURE 2. Chromatograms of (a) gallic acid standard, (b) fresh extract (FE), (c) low heating (LH) and (d) drying and heating (DH) of *Benincasa hispida* extracts

TABLE 1. Concentration of gallic acid recovered from *Benincasa hispida* (Bh) prepared with three different techniques ($n=3$)

Bh extracts	Concentration of gallic acid (mg/100 g)
Fresh extract (FE)	0.036±0.0013
Low heating (LH)	0.050±0.0003
Drying and heating (DH)	0.272±0.0029

imposed during the extraction process. Previous study reported that the amount of the total phenolic contents of bitter melon extracts obtained by the subcritical water extraction increased when the temperature increased (Shotipruk 2008). Other research showed that heated mango kernel was possibly attributable to the degradation of high molecular weight compounds at elevated heating temperatures, releasing the free gallic acid and ellagic acid (Soong 2006).

In the present study, for DB extract, the pulp of Bh fruit has undergone oven-dried at 55-60°C for two days before extraction process taken place. This condition involves the intense heat from boiling water or extracting solvent which is able to release cell wall phenolics or bound phenolics due to the breakdown of cellular constituents causing more polyphenols to be extracted (Toor & Savage 2006). Other study reported by Veronica et al. (2002) showed that heating enhances the concentration of ferulic acid and total free phenolics content of sweet corn.

Other study also reported that drying of pulps and peels of vegetables and fruits may contribute to the successive extraction of the phenolic compounds. At cellular level, the phenolic compounds are located in the vacuoles and are separated from oxidative enzymes in an intact fruit (Shaida et al. 2011). This structure collapses during drying process leading to a release of more phenolic constituents, together with the oxidative enzymes that may destroy the phenolic compounds (Toor & Savage 2006). In addition, drying process will denature these enzymes and conserve the phenolic compounds in the dried samples. Heat treatment applied during Soxhlet extraction may also increase the extraction of phenolic compounds from plant materials.

Lower concentration of gallic acid detected in fresh extract (FE) of Bh fruit may be due to undiffused gallic acid which is stable and intact in its original matrix of phytonutrient compounds. Phenolic compounds including gallic acid can be released from the other complex bioactive compounds from the plant part where they are present by rupturing the plant tissues or by a diffusion process (Escribano-Bailon & Santos-Buelga 2003). Even though fresh extract of Bh recorded lower concentration

of gallic acid, however the other compound like vitamin C still present in the fresh juice of the pulp fruits.

VALIDATION OF THE METHOD

The method was validated for linearity, accuracy, precision, selectivity and specificity. All validation studies were performed by replicate injection of standard and sample solutions.

Standard solutions at five different concentrations were analyzed and calibration plots were constructed by plotting mean areas against respective concentrations. The method was assessed by determining the correlation coefficient and intercept values as shown in Table 2. The linearity range for gallic acid was in the range from 6.25 to 50 µg/mL. The detection limit was 0.75 µg/mL while quantification limit was 2.50 µg/mL.

Recovery study has been conducted to confirm the accuracy of the method developed. Standard solution (10 µg/mL) was added into a three different pre-analyzed sample solutions and recovery of the compound was calculated. The results of accuracy assessments were shown in Table 3. The present accuracy test showed that FE, LH and DH extracts recorded recovery level at 90.62, 96.62 and 99.34%, respectively. It was also shown that the present method enables accurate quantitative analysis of gallic acid in three different Bh extracts. The average of recovery levels for all spiked gallic acid standard in all samples was successfully recovered at 95.53%.

The precisions aspects were assessed by measuring the intra and inter-day of retention time and peak area for gallic acid standard by replicate analysis. It was expressed as the relative standard deviation (R.S.D) (Table 4). The present results showed that intra and inter-day R.S.D for retention time and peak area were both low and the precision is acceptable. The intra-day R.S.D for retention time was 0.11% while the intra-day R.S.D for peak area was 1.60%. Meanwhile, the inter-day R.S.D for retention time was recorded at 0.18 % with the inter-day R.S.D for peak area was 2.23%.

The selectivity of the method for the standards was established by study of the resolution between the

TABLE 2. Validation data from calibration curve of gallic acid

Retention time	Regression equation	Correlation coefficient (R)	Linear range (µg/mL)	Detection limit (µg/mL)	Quantification limit (µg/mL)
1.98	$y=53003x - 44512$	0.9999	6.25-50	0.75	2.50

TABLE 3. Recoveries of gallic acid in the *Benincasa hispida* (Bh) prepared with three different techniques ($n=3$)

Bh extracts	Amount added ($\mu\text{g/mL}$)	Amount found ($\mu\text{g/mL}$)	Recovery (%)	Average of recovery (%)
Fresh extract (FE)	10	9.06	90.62	
Low heating (LH)	10	9.66	96.62	95.53
Drying and heating (DH)	10	9.93	99.34	

TABLE 4. Precision for intra-day and inter-day relative standard deviation of gallic acid ($n=3$)

Intra-day R.S.D. for retention time (%)	Intra-day R.S.D for peak area (%)	Inter-day R.S.D for retention time (%)	Inter-day R.S.D for peak area (%)
0.11	1.60	0.18	2.23

standards peak. Under the chromatographic conditions, the peak of gallic acid is completely separated and no interference. This indicates the method is selective. The specificity was evaluated by comparing the chromatograms obtained from extracts and from the standard as shown in Figure 2. Because of the retention times of standard solution and Bh fruit extracts were identical and no co-eluting peaks from the diluents were observed, the method was specific for quantitative estimation of gallic acid in this plant extract.

CONCLUSION

Determination of gallic acid in *Benincasa hispida* extracts by HPLC-UV represented an excellent technique with high sensitivity, precision and reproducibility. The method gives a good resolution for gallic acid with an isocratic elution. The method was able to detect the occurrence of gallic acid in Bh extracted with three different extracts. Amount of gallic acid detected in fresh extract, low heating and drying and heating were 0.036, 0.050 and 0.272 mg/100 g, respectively. Thus, it is concluded that Bh extracts capable to improve nutritional qualities and functional properties of both processed juices and finished food products. Thus, Bh is another novel fruit/vegetable potentially used as food ingredient as it contains a good source of phenolic acid that can benefit health.

ACKNOWLEDGEMENTS

The authors acknowledged the financial support from Universiti Sains Malaysia under the Short Term Grant No. 304/PPSK/61311083.

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Received: 27 December 2012

Accepted: 6 November 2013