Pak. j. sci. ind. res. Ser. B: biol. sci. 2020 **63B**(2) 93-99

Effect of Solid to Water Ratio, Time and Temperature on Aqueous Extraction of Gallic Acid from *Labisia pumila var alata* of Malaysia

Noor Adilah Md Salehan[a], Aishath Naila[b], Azilah Ajit[a]* and Ahmad Ziad Sulaiman[c]

[a]Faculty of Chemical Engineering & Natural Resources, Universiti Malaysia Pahang, Lebuharaya Tun Razak, Gambang, Kuantan, Pahang Darul Makmur, Malaysia

[b]The Maldives National University, Central Administration, Rehdebai Hingun, Machangolhi, Male', Maldives

[c]Faculty of Bio-Engineering and Technology, Universiti Malaysia Kelantan (UMK), Campus Jeli, Beg Berkunci No.100, 17600 Jeli, Kelantan Darul Naim, Malaysia

(received May 4, 2017; revised November 7, 2018; accepted November 9, 2018)

Abstract. *Labisa pumila var alata* contains bioactive compounds such as gallic acid that is of use for pharmaceutical and nutraceutical industries. Aqueous extraction is the traditional method that extracts phytochemicals from plant material. The study aimed to find the optimum condition for maximum gallic acid yield from ground *L. pumila* leaves using aqueous extraction. The results revealed that the maxium gallic acid yield obtained was at 1:10 sample to water ratio for 8 h at 50 °C. The maximum yield of gallic acid obtained was 1.025 mg gallic acid per g dried leaves (mg/g). The identification of gallic acid was done on Liquid Chromatograph Mass Spectrometer Quadrupole Time-of-Flight (LCMS-Q-TOF) by comparison to that of reference standard. The morphological structure of the extract that was obtained at optimum condition showed less denaturation of cell wall which indicates that still some gallic acid could be trapped in the sample matrix and other methods need to be employed to release them.

Keywords: *Labisia pumila*, gallic acid, aqueous extraction, phytochemicals

Introduction

Labisia pumila, previously was under family Myrsinaceae, is now placed under family Lamiaceae, genus *Marantodes*, and species *Marantodes pmulium*. L. *pumila* are small woody and leafy plants found in the South East Asian tropical forests (Saeed *et al*., 2018; Chua *et al*., 2012), mainly in the low land and hill forests in Southeast Asia; Vietnam, Laos, Indonesia, Thailand, Cambodia, and Malaysia (Farouk *et al*., 2008). There are eight varieties of *L. pumila* (Sunarno, 2005), but only three of the varieties are widely studied; *L. pumila* var. *pumila*, *L. pumila* var. *alata* and *L. pumila* var. *lanceolata* (Chua *et al*., 2012). *L. pumila* var. *alata* is an important medicinal plant that is predominantly used for women health in Southeast Asia (Nik Hussain and Kadir, 2013). *L. pumila* is unique in that phenol metabolites anf can be used as chemical markers (Karimi and Jaafar, 2011) such as phytochemical, gallic acid (3,4,5-trihydroxy-benzoic acid) (Chua, Lee *et al*., 2012). These metabolites have multiple biological effects including anti-inflammatory and antioxidant activities (Vijayalakshmi and Ravindhran, 2012). Therefore, the aim of this work was to quantify the gallic acid from this plant.

*Author for correspondence; E-mail: azilahajit@ump.edu.my

Traditionally, plant material compounds are extracted using aqueous extraction and still the method is used to obtain baseline data for comparison with other methods of extractions including enzymatic (Gai *et al*., 2013 a), microwave assisted extraction (Gai *et al*., 2013 b) and ultrasound assisted extraction (Gabaldon *et al*., 2007). In 2010 Aiyegoro and Okoh used aqueous extraction method using distilled water as an extract mediam, at 27-30 °C, while shaking the sample medium for 48 h to extract phytochemicals from *Helichrysum longifolium* DC (Family Asteraceae). The extract was then filtered and kept at 40 °C and then dried using a freeze dryer to obtain 30 g of dry extract.

Only one research to our knowledge is conducted on extraction of gallic acid from *L. pumila* of Malaysia. The authors conducted aqueous extraction with water at 40 °C, sample to solvent ratio of 1:10 and for 4 h of duration. The maximum yield obtained was 13.42% and found that compared to use of ethanol, ethyl acetate and hexane water as a solvent gave the highest yield (Azrie *et al*., 2014). *L. pumila* var *alata* was extracted for the active compound gallic acid after extraction at 80 °C with sample to solvent ratio of 1:6 at time duration of 3 h with continous shaking. The extract was tested for the effect on post menopausal in Malay women

(Abdul Kadir *et al*., 2012). Choi *et al*. (2010) extracted antioxidant from *L. pumila* at 100 °C with sample to solvent ratio of 1:10 for 4 h (Choi *et al*., 2010). The *L. pumila* was extracted at temperature 80 °C with sample to solvent ratio of 1:10 for 3 h (Zulkarnaini *et al*., 2016). Norhaiza *et al*. (2009) extracted phenolic compound from *L. pumila* var *alata* and *L. pumila* var *pumila* at 20-25 °C with sample to solvent ratio of 1:50 for 1 h (Norhaiza *et al*., 2009) and Al-Wahaibi *et al*. (2008) extracted plant material from *L. pumila* var *alata* at 80 °C with sample to solvent ratio of 1:6 for 3 h (Al-Wahaibi *et al*., 2008). For this research the species of *L. pumila* used was *L. pumila* var. *alata*. Nik Hussain and Kadir (2013) identified gallic acid as a marker compound from *L. pumila* var. *alata* but the authors did not quantify it. This study applied aqueous extraction to quantify total gallic acid yield from *L. pumila* var. *alata* of Malaysia. The conditions selected for the study were based on the published data.

Materials and Methods

Dried *L. pumila* var *alata* (Kacip Fatimah) leaves were purchased from Delima Jelita,Simpang Empat, Alor Setar, Kedah.

All chemicals used in this research is from analytical grade and for HPLC analysis HPLC grade chemicals were used. All the chemicals and reagents were purchased from Sigma-Aldrich (Missouri, USA).

Aqueous extraction of *L. pumila*. *L. pumila* was grounded, sieved (0.15-0.3 mm) using Sieve Shaker (Yuezhou-OHT-SVS-01) and stored at 4 °C until use. Samples were extracted; ground *L. pumila* leaves were immersed in the extraction solvent, water, and the mixture was heated with continuous stirring in a hotplate Magnetic Stirrer-IKA-358120 for 8 h with the volume of infusion set at 300 mL. The mixture was covered with aluminium foil throughout the extraction to minimize the evaporation in order to maintain the sample-to-water ratio. For this method, four different extraction temperature were applied (40, 50, 60, and 80 °C) and also four different sample-to-solvent ratio (*L. pumila* : water) were used; 1: 6, 1:8, 1:10 and 1:12. The time period experimented were 1, 2, 3, 4, 5, 6, 7 and 8 h. The experimental set up is shown in Fig. 1. A 2 mL of samples were taken every hour until 8 h for analysis. The extracted samples were centrifuged (Tabletop Centrifuge Model 4000 from Kubota, Japan) at 5000 rpm for 10 min. The centrifuged samples were kept at 4 °C prior to analysis.

Determination of gallic acid. For determination of gallic acid yield High performance Liquid Chromatography Diode-Array Detection (HPLC-DAD) [HPLC by Agilent Technology 1100 Series, Model G1379A] was used. The samples were diluted by dilution factor of 10 and then were filtered using 0.45 mm nylon syringe filter and transferred into 1.5 mL vial and screw caps with septa.

Table 1. Gallic acid yield extracted from aqueous extraction (AE)

Time	Aqueous extraction (AE) gallic acid concentration (mg/g)	
1	0.6221	±0.0241
2	0.7162	±0.0357
3	0.7834	±0.0201
4	0.8249	±0.0134
5	0.8829	±0.0270
6	0.9198	±0.0124
7	1.0056	±0.0057
8	1.0251	±0.0569

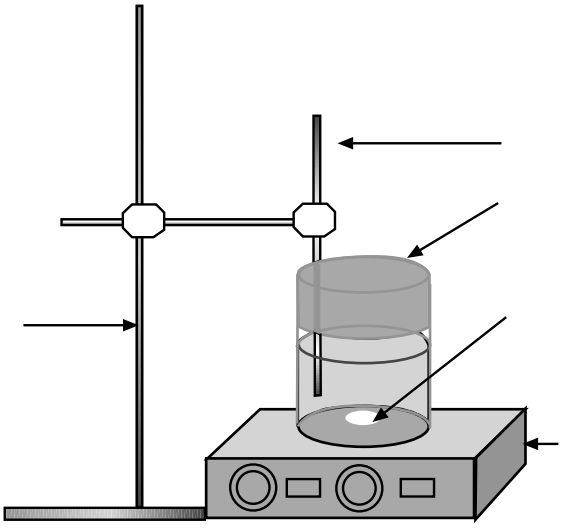

Fig. 1. Schematic diagram of the conventional water extraction setup.

A standard calibration curve of gallic acid in the range 5-80 mg/L was used to quantify gallic acid (Fig. 2 a). The standard solution of 5, 10, 20, 40 and 80 mg/L of gallic acid were prepared. Next mobile phase was prepared and filtered using 0.25 mm nylon filter and then degassed in ultrasonic bath to remove all bubble inside the solution.

The amount of gallic acid (CAS Number 149-91-7) yield from the extract was measured by HPLC-DAD. Reverse-phase C-18 column (4.6 mm × 250 mm ,5 µm particle size or equivalent) and mobile phase; acetonitrile (CAS Number 75-05-8) (A) and 3% phosphoric acid (CAS Number 7664-38-2) buffer (B), with isocratic elution system (A/B = 10/90) at the flow rate of 1 mL/min and at wavelength of 270 nm were used to quantify the gallic acid. The run time was 10 min and the retention time gallic acid (standard) was 4.657 min (4.606 min in the extract).

Identification of *L. pumila* extract metabolites. For identification of gallic acid, Liquid Chromatograph Mass Spectrometer Quadrupole Time-of-Flight (LCMS-Q-TOF) was used. The samples were sent to Integrative Pharmacogenomics Institute (IPROMISE), University Teknologi MARA (Puncak Alam, Selangor Malaysia) for analysis.

Identification of secondary metabolites in *L. pumila* extracts. Gallic acid metabolite was identified and quantified using LCMS-Q-TOF analysis and developed databases system (Plant Metabolic Network(PMN) and Metabolite database (METLIN) in aqueous extract of *L. pumila*. Figure 6 shows the mass spectral characteristics and identification of phenolics at 0 Volt (Fig. 6 a) and 10 Volt (Fig. 6 b) collision energy.

The mass spectrum obtained from the analysis was compared with mass spectrum of gallic acid which was available in Metlin Database, the pattern of spectrum was similar as shown in Fig. 6 a-b. Which were the fragment structure of gallic acid at 0 Volt and 10 Volt collision energy, respectively. Therefore, the mass spectrum confirms that the gallic acid compound is present in the *L. pumila* extract. Moreover, some of the important metabolites were also identified by their retention time and mass to charge ratio (m/z) (Table 2). The secondary metabolites identified from the *L. pumila* extract were gallic acid, syringic acid, vanillic acid, protocatehuic acid and salicyclic acid (Table 2).

Table 2. Mass spectral characteristics and identity of some important metabolites present in *Labisia pumila* extract

RT (min)	Compound	$[M-H]^-$ (m/z)
1.146	Gallic acid	169.014
1.486	Syringic acid	197.0457
1.764	Vanillic acid	167.0348
2.686	Protocatehuic acid	153.019
5.283	Salicylic acid	137.0242

Gallic acid from the extract was identified at retention time of 1.146 min and mass to charge ratio of 169.014 $[M-H]^-$ (m/z).

Morphological study of *L. pumila* extract. For the morphological study of the extracted samples Field Emission Scanning Electron Microscope (FE-SEM) [JEOL, Tokyo, Japan] was used. The samples were sent for analysis to Central Laboratory, Universiti Malaysia Pahang (Gambang, Pahang Malaysia).

Statistical analysis. Each experimental factor was conducted in triplicate and reported the average with its standard deviation. The analysis was done using analysis of variance (ANOVA).

Results and Discussion

Calibration curve and quantification of gallic acid. The gallic acid was quantified using equation 1.

$$\text{Sample A (mg of gallic acid / g of dry sample)} = \frac{\text{(Concentration got from HPLC analysis, ppm)} \times \text{(volume of solvent during extraction, l)} \times \text{(dilution factor}}{\text{mass of sample use during extraction, g}} \quad \text{(Equation 1)}$$

Figure 2 a shows the calibration curve of gallic acid in the range of 5-80 mg/L concentrations with excellent regression. The graph was used to quantify the gallic acid concentration. Figure 2 b and c shows the chromatograms of gallic acid standard and the extract of the sample, respectively.

Effect of sample to water ratio on the gallic acid yield. The effect of sample-to-water ratio on the gallic acid yield from *L. pumila* were studied using the water to sample ratios of 1:6, 1:8, 1:10 and 1:12 (g/mL) at 60 °C for 8 h (Fig. 3 a-b). The amount of gallic acid yield from *L. pumila* continued to increase upto 1:10 and afterwards gradual decrease was observed. The optimum gallic acid yield was 0.9530 ± 0.0377 mg/g at sample-to-water ratio of 1:10. This finding is in line with the work of Choi *et al*. (2010) and Zulkarnaini *et al*. (2016). These authors used the sample-to-water ratio of 1:10 to extract active compounds from *L. pumila*.

Effect of temperature on the gallic acid yield. The effect of extraction temperature on the gallic acid yield from *L. pumila* was studied using four different temperature; 40, 50, 60 and 80 °C for 8 h with sample to water ratio of 1:10 (Fig. 4 a-b). The increasing pattern was observed for temperature between 40 to 50 °C and afterwards gradual decrease was observed in the yield of gallic acid. The optimum gallic acid yield was

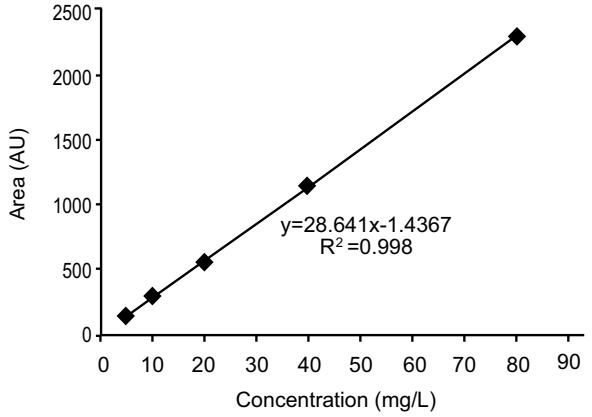

Fig. 2a. Calibration curve of gallic acid.

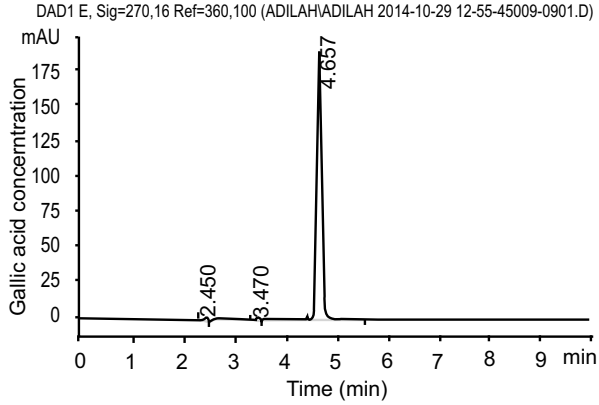

Fig. 2b. Shows one of the standard chromatogram for gallic acids which appears at 4.657 min.

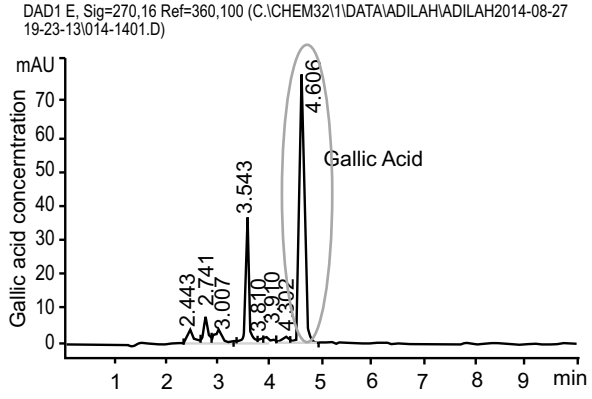

Fig. 2c. The peak of gallic acid from the samples is determined accordingly to the peak that showed in the standard.

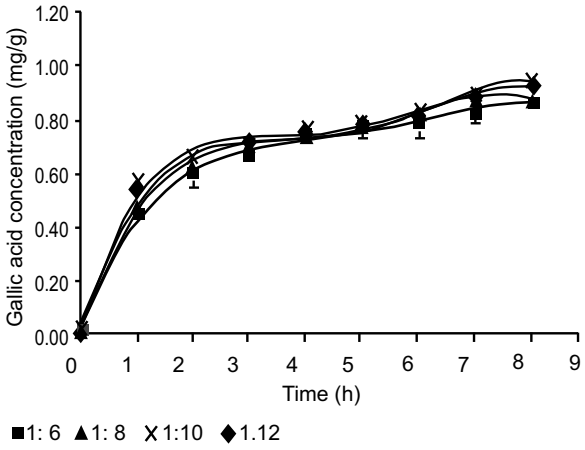

Fig. 3a. Comparison effect of sample-to-water ratio on the average yield of gallic acid from extract (8 h, 60 °C).

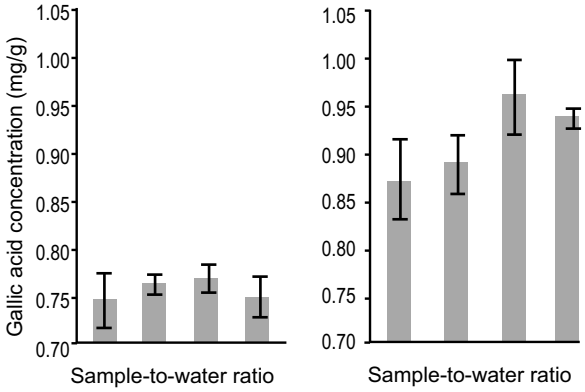

Fig. 3b. Effect of sample-to-water ratio on the average gallic acid yield from *Labisia pumila* extract at 60 °C for (a) 4 h extraction and (b) 8 h extraction at 1:6, 1:8, 1:10 and 1:12 sample-to-solvent ratio.

1.0251±0.0569 mg/g at 50 °C. This finding is in agreement to that of Palma *et al*. (2013) in that according to the authors moderate temperature (less aggressive condition) was more suitable for extraction of natural product to avoid degradation of intracellular constituents. Thus temperature above 50 °C the degrdation of the compounds may have initiated.

Effect of time on the yield of gallic acid. Table 1 and Fig. 5 show the effect of extraction time on the amount of yield gallic acid released. As shown in Fig. 5 at 40 °C the highest yield of gallic acid (about 0.9 mg/g) was obtained after 7 h of extraction and afterwards the gallic

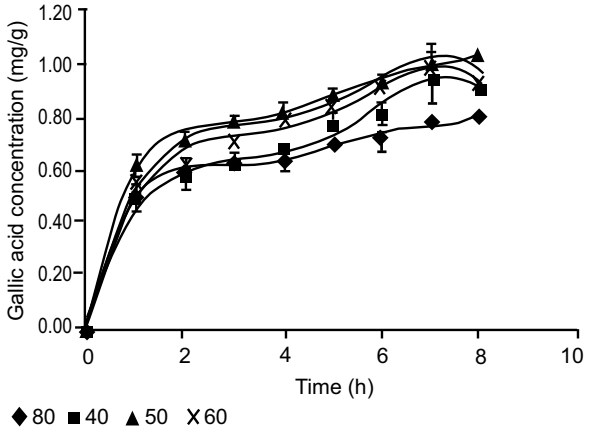

Fig. 4a. Comparison effect of temperature 40, 50, 60 and 80 °C on the average gallic acid yield from extract (1:10 sample-to-water ratio, 8 h).

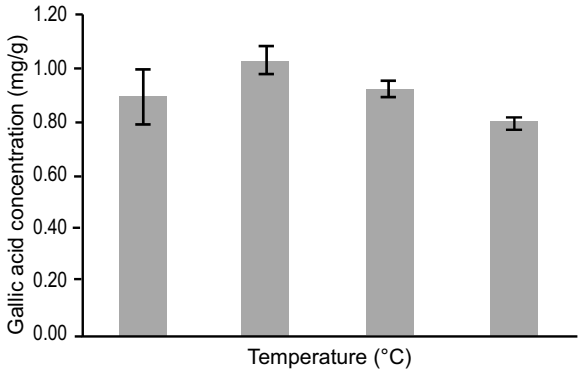

Fig. 4b. Effect of temperature 40, 50, 60 and 80 °C on the average gallic acid yield from extract after 8 h extraction at 1:10 sample-to-water ratio.

acid concentration dropped to 0.8 mg/g at 8 min. Also, at 50 °C the highest gallic acid yield was observed at 7 h of the extraction (about 0.95 mg/g) and still remained almost same on 8 h of extraction. The gallic acid concentration at 60 °C for 7 h was almost same as 40 °C (about 0.9 mg/g). The gallic acid concentration also increased at 80 °C and optimum obtained at 7 h (about 0.75 mg/g) and remained almost the same at 8 h of extraction. Overall description for Fig. 5 is that the gallic acid yield increased as the time increased and the maximum yield in all temperatures were obtained at 8 h. The trend line gradient in Fig. 5 shows that the rate of extraction at temperature 40, 50 and 60 °C was equal at 0.06 mg gallic acid yield per hour. The rate of extraction was slower when the temperature increased to 80 °C at value

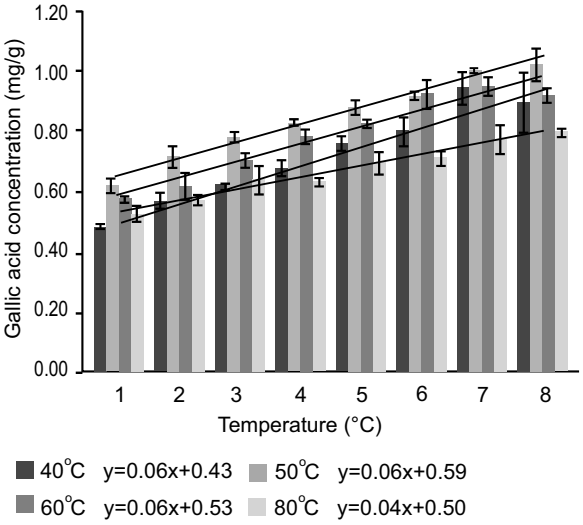

Fig. 5. Effect of time on the average gallic acid extraction rate at temperature 40, 50, 60 and 80 °C from (1:10 sample-to-water ratio).

0.04 mg gallic acid yield per hour. The latter might be due to the degradation of some active compounds at this temperature as claimed by Palma *et al*. (2013) that the moderate temperature (less aggressive condition) is more suitable for extraction of natural product to avoid degradation of intracellular constituents.

The physical effect on the *L. pumila* extracts. Field emission scanning electron microscopy (FESEM) was carried out on the aqueous extract after the extraction was done at 50 °C, sample to water ratio of 1-10 and for 8 h. FESEM is carried out to study the morphological surface structure of *L. pumila* sample after the extraction. Figure 7 a-b show the FESEM observation of extracted sample at ×3,000 and ×10,000 magnifications, respectively. The morphological surface structure shows that there was not much denature of the cell wall. Therefore, other methods such as enzymatic assisted extraction need to be applied to denature the cell wall to release more gallic acid.

Conclusion

In conclusion aqueous extraction at 50 °C, with sample to water ratio of 1:10, and time duration of 8 h yielded the maximum gallic acid of 1.0251±0.0569 mg/g. The researchers could use these optimum conditions to extract gallic acid from *L. pumila* var *alata* as a baseline data and apply other methods such as enzymatic assisted extraction to extract the gallic acid from the plant.

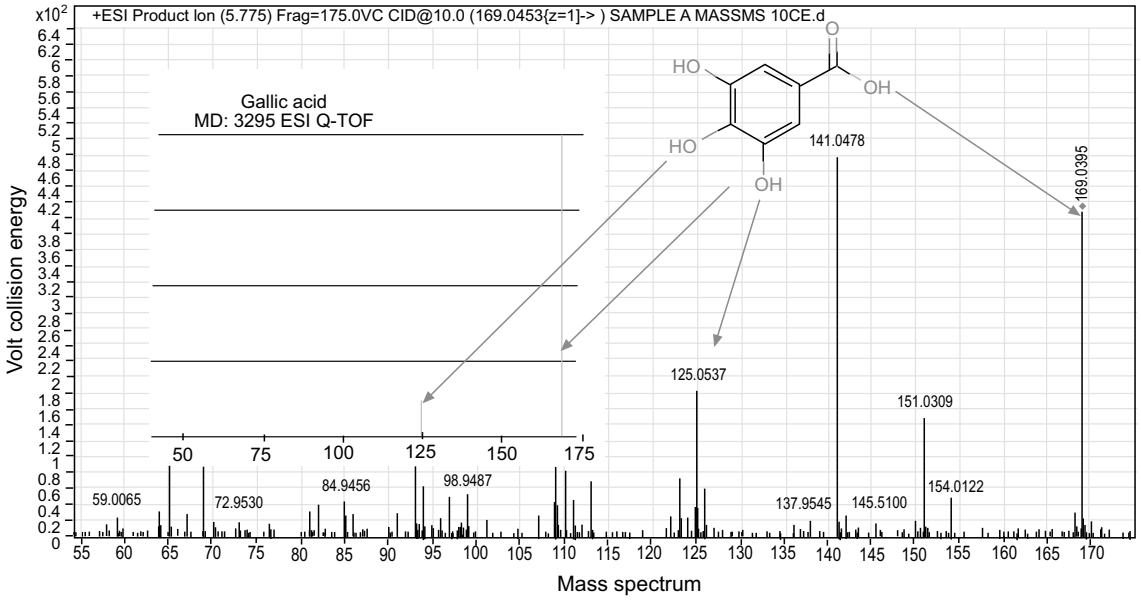

Fig. 6a. Mass spectral characteristics and identity of phenolic analysed by LCMS-QTOF at 0 Volt collision energy.

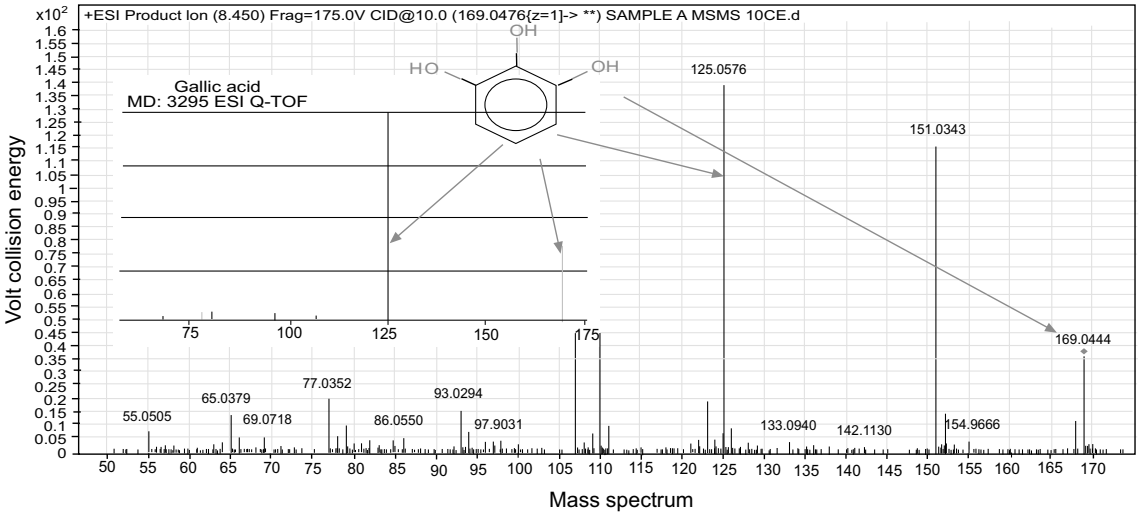

Fig. 6b. Mass spectral characteristics and identity of phenolics analysed by LCMS-QTOF at 10 Volt collision energy.

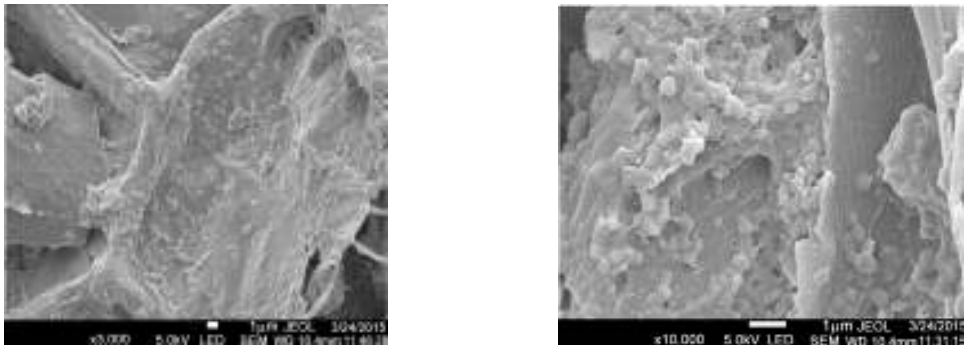

Fig. 7a-b. Mass spectral characteristics and identity of phenolic analysed by LCMS-QTOF at 0 Volt collision energy.

Acknowledgement

This research was funded by Ministry of Agriculture and Agro Based, Malaysia, EPP#1 NKEA National Research Grant Scheme (NRGS) and also by Universiti Malaysia Pahang (UMP; RDU 130703).

Conflict of Interest. The authors declare no conflict of interest.

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