

## EFFECT OF ETHANOL IN ULTRASONIC ASSISTED EXTRACTION TECHNIQUE ON ANTIOXIDATIVE PROPERTIES OF PASSION FRUIT (*Passiflora edulis*) LEAVES

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### ABSTRACT

The leaves of *Passiflora edulis* (Passion fruit) traditionally used in American countries as a folk medicine to treat both anxiety and nervousness. They are rich in polyphenols which have been reported as natural antioxidants. In this study, five concentrations of ethanol (15%, 30%, 45%, 60% and 75%) were used in the extraction of *P. edulis* leaves extracted using ultrasonic-assisted technique. Ultrasonic-assisted solvent extraction is a method that uses high frequency mechanical vibration to increase the effectiveness of the desire compound to be dissolved into the solvent. The total phenolic content and total flavonoid content were determined in this study. Antioxidant capacity was assessed with FTC, TBA and DPPH. The highest extraction yield was obtained using water extraction. The extract obtained by 75% ethanol showed the highest antioxidant activity by DPPH (2,2- diphenyl-1-picrylhydrazyl) radical scavenging assay ( $85.17 \pm 0.36\%$ ) and Ferric Thiocyanate (FTC) test where the absorbance obtained was  $0.09 \pm 0.00$ . The same extract also exhibited the highest flavonoids content ( $90.94 \pm 0.15$  mg QE/g sample). The extract obtained by 15% ethanol exhibited the highest antioxidant activity in Thiobarbituric acid (TBA) assay ( $56.22 \pm 5.01\%$ ). Apart from that, the highest phenolic content was found in 60% ethanol ( $57.08 \pm 0.29$  mg GAE/g sample). These results indicate that the leaves of *P. edulis* could be a potential source of natural antioxidants which can be used in dietary applications.

**Key words:** Passion fruit leaves, antioxidative activities, ultrasonic-ethanol assisted technique

### INTRODUCTION

*Passiflora edulis*, or commonly known as Passion fruit, is the member of *Passifloraceae* family. It is suitable to be planted commercially in tropical country like Malaysia (Bernacci *et al.*, 2008). Passion fruits are usually consumed freshly or being processed for its juice. Passion fruit leaves are evergreen, alternate and deeply three-lobed when mature. It has been used as folk medicine as sedative and tranquiliser in Brazil (Coleta *et al.*, 2006). The

leaves are reported to contain a bitter principle maracugine, resins, acids and tannin which are exceptionally rich in ascorbic acid (Sunitha & Devaki, 2009). The interest in the bioactive compounds in *P. edulis* has been increasing because of the antioxidant compounds of its leaf extract that can be used in many pharmaceutical preparation and food industry (Gosmann *et al.*, 2014).

Antioxidants are an important component of the food industry. In the past, antioxidants were used primarily to trap free radicals to control oxidation and retard spoilage, but today many are used because of putative health benefits (Finley *et al.*, 2011).

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Phenolic compounds such as flavonoids are secondary metabolites in plants. They are major colouring component of flowering plants and also served as antioxidants compound to combat the oxidative stress in plant by absorbing the most energetic solar wavelengths such as ultraviolet light, inhibit the generation of reactive oxygen species (ROS) and quench the formed ROS (Kumar & Pandey, 2013).

Ultrasonic solvent extraction is a method which uses high frequency mechanical vibration to increase the effectiveness of the desire compound to be dissolved into the solvent. (Luque-Garcia & Luque de Castro, 2003). As an alternative to the conventional extraction methods, such as the Soxhlet extraction, ultrasonic solvent extraction is able to shorten extraction time, decrease solvent consumption, increase extraction yield, and enhance the quality of extracts.

In food industry, oxidation deterioration of lipids in food is a major concern because it causes rancid odours and flavours, with a consequent decrease in nutritional quality and safety. Passion fruit leaf should be received much attention for its potential health benefits of high bioactive content (Relw & Espig, 1991), however, most of the study about the passion fruit plant is concentrated on the fruit while there is less literature about the leaves of the plant (Gosmann *et al.*, 2014). There is a need in exploring an alternative extraction to shorten extraction time, decrease solvent consumption, increase extraction yield, and enhance the quality of extracts because the conventional solvent extraction requires higher cost, longer time but low in efficiency (Luque-Garcia & Luque de Castro, 2003). Despite the choice of the method, the solvent used provides the most obvious means of influencing the qualitative composition of the extract. This study is designed to determine the effect of the concentration of ethanol on the yield of passion fruit leaf extract and to measure the effect of concentration of ethanol on the antioxidant activity, total phenolic content and total flavonoids content of the passion fruit leaf extract. This research is hope to be able to explain the effect of the concentration of ethanol on the extraction yield and the antioxidant properties of passion fruit leaf which is extracted by ultrasonic assisted solvent extraction.

## MATERIALS AND METHODS

### Passion fruit leaves

Passion fruit (*P. edulis*) leaves were obtained from Gopeng, Perak, Malaysia in September 2016. All samples which free from disease were harvested and stored at -18°C.

### Chemicals and reagents

The chemical grade materials such as linoleic acid, DPPH and  $\alpha$ -tocopherol were purchased from Sigma-Aldrich, USA. Potassium acetate, sodium carbonate, trichloroacetic acid (TCA), thiobarbituric acid (TBA), sodium chloride, gallic acid, phosphate buffer (pH 7.0), ammonium thiocyanate, Folin-Ciocalteu reagent and aluminium chloride were obtained from Merck. Absolute ethanol, methanol and ferrous chloride were from Hamburg Chemicals.

### Preparation of plant materials

After harvested, passion fruit leaves were washed and kept immediately in a freezer (-18°C) until use. After taken out from the freezer, the passion fruit leaves were dried in 60°C for 24 hrs then stored at 4°C prior to further analysis.

### Ultrasonic-assisted solvent extraction

Ultrasound-assisted extraction was carried out using the method from Kong *et al.* (2015) with some modifications. It was performed at 30°C by ultrasonic cleaner (KH-400KDB, Hechuang Ultrasonic Equipment Co., KunShan, China). *P. edulis* leaves powder (40 g) was put in a glass vial (1000 mL) and six different concentration of ethanol (0%, 15%, 30%, 45%, 60% and 75%) were added before the vial was placed in the ultrasonic water bath (JEIO Tech, Korea) at 40 kHz, 30°C for 1 h. The extract was then filtered and concentrated in a rotary evaporator (BUCHI Rotavapor R-215, Switzerland) at 40°C until all the solvent was removed.

### 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay

Antioxidant activity of passion fruit leaves extract was determined by the 2,2-diphenyl-2-picrylhydrazyl (DPPH) method of Zhang and Hamazu (2004) with some modifications. The extracted *P. edulis* was adjusted to 6mg/ml by dissolving 18mg sample in 3ml methanol. An aliquot of 4.5 ml of 0.025% DPPH radical in methanol was added to a test tube with 3ml sample, at 6 mg/ml. BHT,  $\alpha$ -tocopherol and ascorbic acid were used as positive controls whereas pure methanol was used as negative control. The reaction mixture was mixed using vortex and let to stand at room temperature in the dark for 30 min, and measured at 517nm.

### Ferric thiocyanate (FTC) method

The FTC method as described by Zainol *et al.* (2003) was used. A mixture of 4.0 mg of passion fruit leaves extract in 4ml of absolute ethanol, 4.1 ml of 2.52% linoleic acid in absolute ethanol, 8.0 ml of 0.05 M phosphate buffer (pH 7.0), and 3.9 ml of water were placed in an amber glass bottle and

then incubated in a dark oven at 40°C for an ammonium thiocyanate. Precisely 3 min after the addition of 0.1 ml of 0.02 M ferrous chloride in 3.5% HCl to the reaction mixture, the absorbance of control reached its maximum. Butylated hydroxyl toluene (BHT), ascorbic acid and  $\alpha$ -tocopherol were used as positive controls, while a mixture without any passion fruit leaves samples were used as the negative control.

#### Thiobarbituric acid (TBA) test

The test was conducted according to the method of Kikuzaki & Nakatani (1993). The same samples prepared for FTC method were used. To 2.0 ml of the sample solution, 1.0 ml of 20% aqueous trichloroacetic acid (TCA) and 2.0 ml of 0.67% aqueous thiobarbituric acid (TBA) solution were added. The final sample concentration was 0.02% w/v. The mixture was placed in a boiling water bath for 10 min. After cooling to 25°C, it was then centrifuged at 3000 rpm for 20 min. Absorbance of the supernatant was measured at 531 nm. Antioxidant activity was recorded based on the absorbance of the final day of the FTC assay. TBA method described antioxidant activity by percent inhibition:

$$\% \text{ Inhibition} = \frac{(\text{Abs control} - \text{Abs sample})}{(\text{Abs control}) \times 100}$$

#### Determination of total phenolic compound

The total phenolic content of passion fruit leaves extracts were evaluated using a method described by Kim *et al.* (2003). The sample with a concentration of 1mg/ml methanol was added to 4.5 ml of deionized distilled water and 0.5 ml of Folic-Ciocalteu's reagent was added to the solution. Samples were maintained at room temperature for 5 min followed by the addition of 5 ml of 7% sodium carbonate and 2 ml of deionized distilled water. Next, the samples were incubated for 90 min at 23°C with intermittent shaking. The absorbance was measured by spectrophotometer at 750 nm. The total phenolic content was expressed as mg of gallic acid equivalents (GAEs) per gram of sample.

#### Determination of total flavonoids content

The flavonoids content of passion fruit leaves extracts was measured based on methods described by Ebrahimzadeh *et al.* (2008). Briefly, 50 mg/10ml methanol of sample was mixed with 1.5 ml of methanol and then 0.1 ml of 10% aluminium chloride was added, followed by 0.1 ml of potassium acetate and 2.8 ml of distilled water. The mixtures were incubated at room temperature for 30 min. The absorbance was measured by a spectrophotometer at 415 nm. The result was expressed as mg QE per g

sample in five different concentrations (12.5, 25, 50, 80 and 100 mg/L).

#### Data analysis

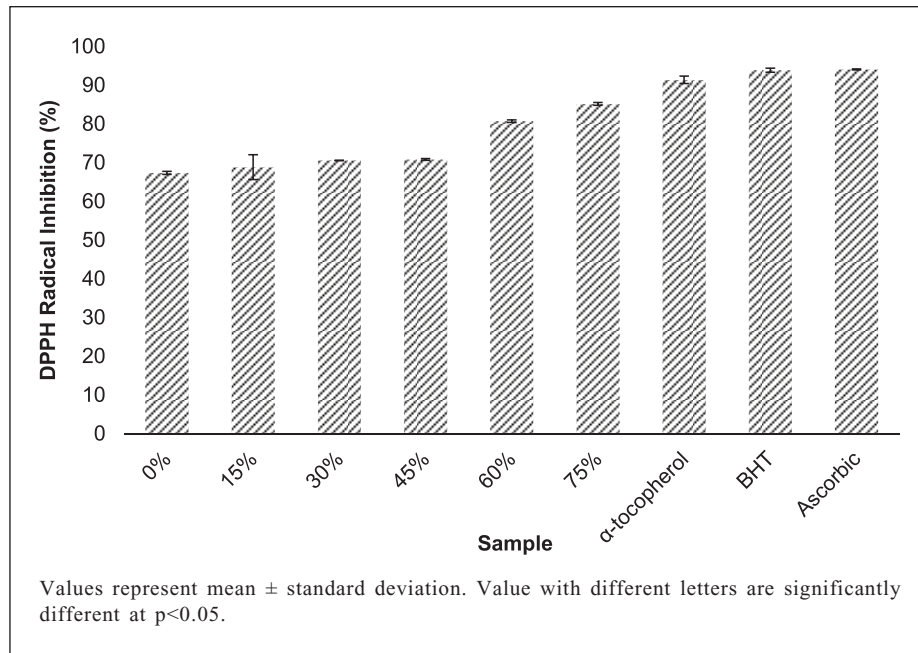
Data were presented as mean  $\pm$  standard deviation of triplicate. Minitab 14 Statistical Software was used to analyse the data using two way analysis of variance (ANOVA). Multiple comparisons was carried out by Tukey's post-hoc comparison. If  $p < 0.05$ , the difference was considered as significant.

## RESULTS AND DISCUSSION

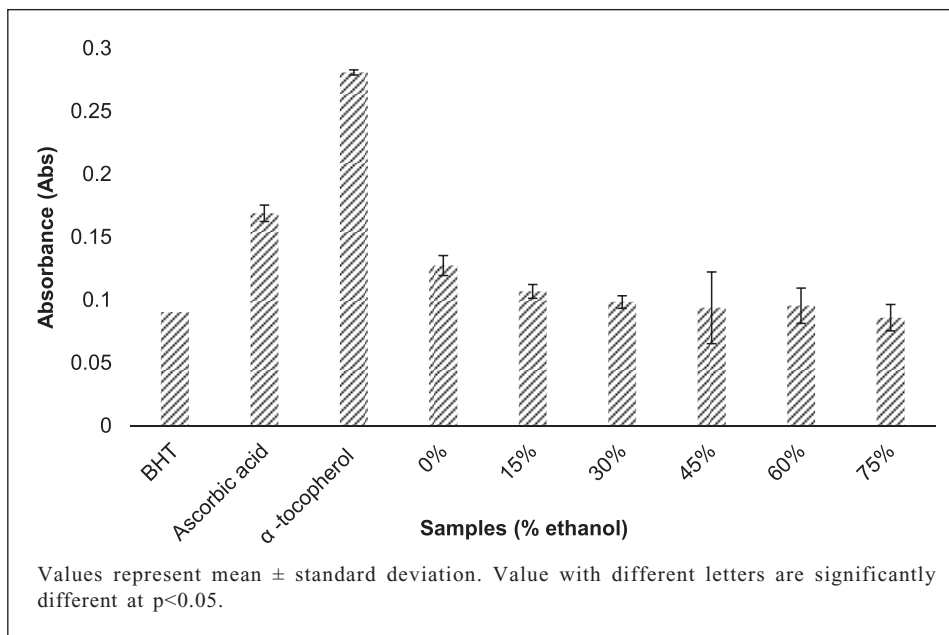
In this research, it was aimed to determine the effect of the concentration of ethanol and ultrasonic extraction technique on the yield of passion fruit leaf extract and the effect of concentration of ethanol on the antioxidant activity, total phenolic content and total flavonoids content of the passion fruit leaf extract.

#### 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

Figure 1 shows that there was a trend indicating the higher the concentration of the ethanol used as the solvent in the extraction, the higher the percentage of inhibition of DPPH radical and they decreased in the following order: 75% ethanol > 60% ethanol > 45% ethanol > 30% ethanol > 15% ethanol > 0% ethanol. Among all the samples, 75% ethanol (85.17  $\pm$  0.36%) was significantly higher ( $p < 0.05$ ) than 60% ethanol (80.72  $\pm$  0.34%). Moreover, 60% ethanol was significantly higher ( $p < 0.05$ ) than the rest of the samples. No significant difference ( $p > 0.05$ ) were recorded between 0%, 15%, 30% and 45% ethanol in DPPH radical inhibition. This result might be related to the solubility of the flavonoids and the polarity of the solvent. The solubility of the flavonoids of the *P. edulis* leaves increased when the polarity of the solvent decreased by increasing the percentage of ethanol. Other than that, Figure 1 also shows that there was no significant difference ( $p > 0.05$ ) between standards: BHT (93.87  $\pm$  0.52%), ascorbic acid (94.05  $\pm$  0.13%) and  $\alpha$ -tocopherol (91.37  $\pm$  0.97%), yet the DPPH inhibition activities of the standards were significantly higher ( $p < 0.05$ ) than all samples except for  $\alpha$ -tocopherol and 75% ethanol (85.17  $\pm$  0.36%). Coleta *et al.* (2006) had identified the major flavonoids in *P. edulis* leaves, mainly flavone such as orientin 2''-rhamnoside and luteolin 7-O-(2-rhamnosyl)glucoside, which had some hydrophobic characteristic (Verstraeten *et al.*, 2003). This result was in accordance with the research done by Diem *et al.* (2013) on the *Moringa oleifera* leaves which



**Fig. 1.** Percentage of DPPH inhibition by each samples extracted at different ethanol concentration using ultrasonic extraction technique.



**Fig. 2.** Antioxidant activities of *P. edulis* leaves on 6<sup>th</sup> day as measured by FTC method.

showed higher DPPH inhibition activity in 80% ethanol ( $85.2 \pm 1.7$ ) than that of pure ethanol ( $70.9 \pm 2.2$ ).

#### Ferric thiocyanate (FTC) method

Figure 2 shows the absorbance of the all the samples which had been stored in 40°C water bath and oxidized for the period of six days. The absorbance of the blank increased rapidly from day

0 to day 5 and then remained constant until day 6. The reason of the rapid rising of the absorbance of the blank sample might be because of the absence of antioxidant compound in the sample which allowed the lipid oxidation to occur faster. The absorbance of other samples did not increase as much as the blank. Most of the absorbance of the samples increases until Day 5 and then either remain constant or experience a little decline on Day 5 and

6. This might be because of the presence of antioxidant in the samples which suppressed the oxidation of lipid. The result of this study was in accordance to the study by Zainol *et al.* (2003) and Malik *et al.* (2017) which stated that in FTC assay, the blank of the sample increases rapidly before it decreased after day 6.

The decline in absorbance indicated the decrease in the amount of primary products of lipid oxidation because these primary products had been broken down into secondary products which would not be detected in FTC assay (McClements, 2005). Malik *et al.* (2017) also stated that the lower absorbance of samples compared to the blank which meant that there was higher occurrence of antioxidant activity in samples. However, the  $\alpha$ -tocopherol in the study of Malik *et al.* (2017) was lower than blank until day 7 which was not the same as the result of this study. The reason behind the oxidation of  $\alpha$ -tocopherol of current research might be because of the sensitivity of  $\alpha$ -tocopherol to light and heat which induced it to be oxidized on day 3 and it might be turned into a prooxidant. Jorge *et al.* (1996) and Verleyen *et al.* (2002) showed that  $\alpha$ -tocopherol was degraded faster in less unsaturated oils.

Figure 2 shows that the result on the 6th day,  $\alpha$ -tocopherol ( $0.28 \pm 0.01$ ) had the highest absorbance which was significantly different ( $p < 0.05$ ) than other samples. The absorbance of ascorbic acid ( $0.17 \pm 0.002$ ) was significantly higher ( $p < 0.05$ ) than all samples except for 0% ethanol ( $0.13 \pm 0.01$ ) and 15% ethanol ( $0.11 \pm 0.01$ ). The absorbance of 0% ethanol was the highest while the absorbance of 75% ethanol ( $0.09 \pm 0.001$ ) was the lowest. The absorbance of 75% ethanol and

BHT were not significantly different ( $p > 0.05$ ), which showed the antioxidant activity of the 75% ethanol extract towards lipid oxidation was comparable with that of BHT. This result could be explained by the presence of linoleic acid in the samples was polyunsaturated fatty acid which was susceptible to oxidation. During the early stages of autoxidation of lipid, the positions of double bonds in unsaturated fatty acids were changed and hydroperoxides (primary product) were produced (Pegg, 2001). The results also showed that lesser polar extracts exerted greater antioxidant activity. This result was in accordance with Correia *et al.* (2010) who stated that methanol extract of *Cordia multispicata* Cham. leaves and *Tournefortia bicolor* Sw leaves had lower antioxidant activity in FTC assay compared to that of ethanol extract which was less polar than methanol. This difference might be explained by the fact that many of the active compounds, probably flavonoids and other phenolic compounds containing hydroxyl groups were dissolved more easily in less polar solvents (Correia *et al.*, 2010).

#### Thiobarbituric acid (TBA) test

Figure 3 shows all the absorbance of samples ( $p < 0.05$ ) were not significantly different with one another, which was in agreement with the result in FTC. However, 15% ethanol showed a higher percentage of lipid oxidation inhibition ( $56.22 \pm 5.01\%$ ) which had no significant difference ( $p < 0.05$ ) with BHT ( $25.72 \pm 0.44\%$ ) and  $\alpha$ -tocopherol ( $57.29 \pm 3.01\%$ ). Despite that, all the samples showed a higher lipid oxidation inhibition activity than ascorbic acid ( $35.45 \pm 4.25\%$ ). This might be due to the interaction of the extracted compound with

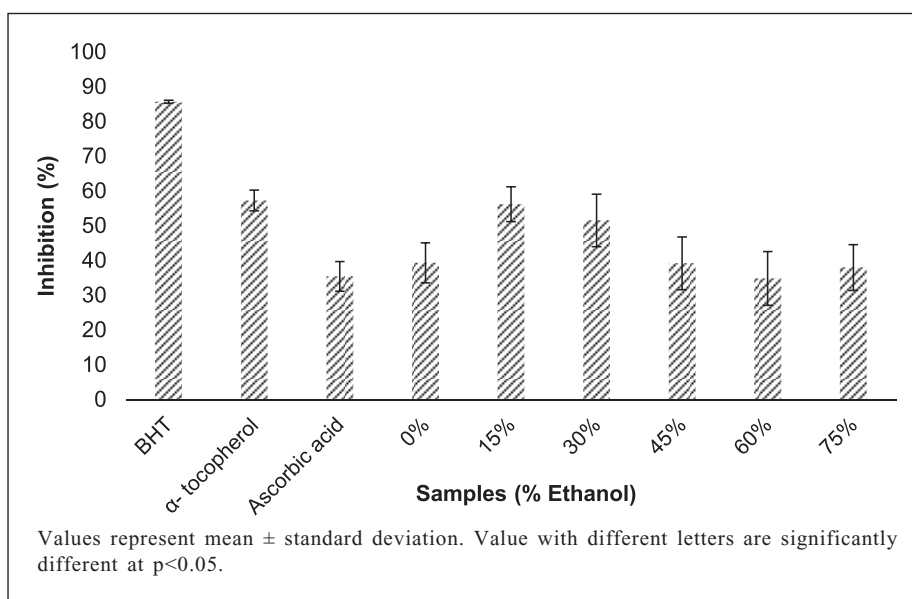


Fig. 3. Antioxidant activities of *P. edulis* leaves as measured by TBA method.

the lipid or primary product of lipid oxidation (e.g. hydroperoxide) and inhibited it to form the secondary product (eg. malonaldehyde) of lipid oxidation. This result was in accordance with the result of the research done by Naczka *et al.* (2003) on wild blueberry leaves which stated that in TBA test, the percentage of inhibition of lipid oxidation of 70% aqueous acetone extract was slightly higher than that of the less polar 95% ethanol. This result was corresponded to the research done by Gülsen and Aysegül (2015) on the *Ribes uva-crispa* leaves which showed that the ethanol extract of the leaves showed higher percentage of lipid oxidation inhibition than the more polar water extract and the less polar n-butanol extract. This shows that the antioxidant compound extracted by the slightly polar solvent might exhibit a better lipid peroxidation inhibition in the second stage compared to polar solvent and non-polar solvent.

#### Determination of total phenolic content

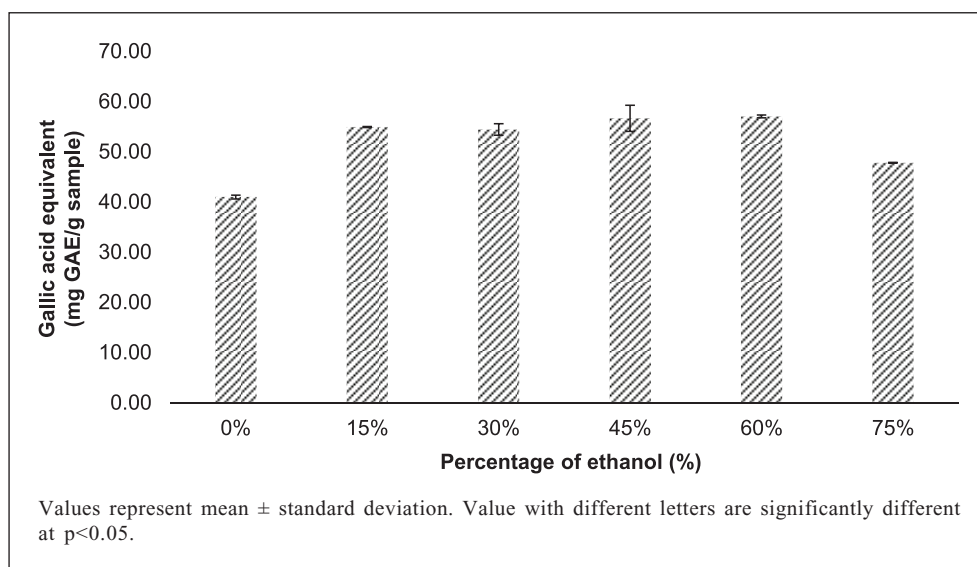
Generally, the total phenolic contents in the examined extracts ranged from 41.02 to 57.08 mg GAE/g sample. Figure 4 shows there was no significance difference ( $p > 0.05$ ) between the 15% to 60% ethanol extracts. The highest concentration of phenols was measured in 60% ethanol extracts ( $57.08 \pm 0.29$  mg/g sample), followed by 45% ethanol extracts ( $56.69 \pm 2.60$  mg/g sample), 30% ethanol extracts ( $54.48 \pm 1.15$  mg/g sample) and 15% ethanol extracts ( $54.96 \pm 0.10$  mg/g sample). 0% ethanol extracts and 75% ethanol extracts were significantly different ( $p < 0.05$ ) from other samples and contained considerably small concentration of phenols which were  $41.02 \pm 0.38$  mg/g sample and  $47.85 \pm 0.10$  mg/g sample, respectively. The result

in this analysis showed that the total phenolic contents in plant extracts of the species *P. edulis* leaves depended on the polarity of solvent used in extraction. This might be because the high solubility of phenols in non-polar solvents resulted in the higher concentration of these compounds presented in the non-polar extracted samples.

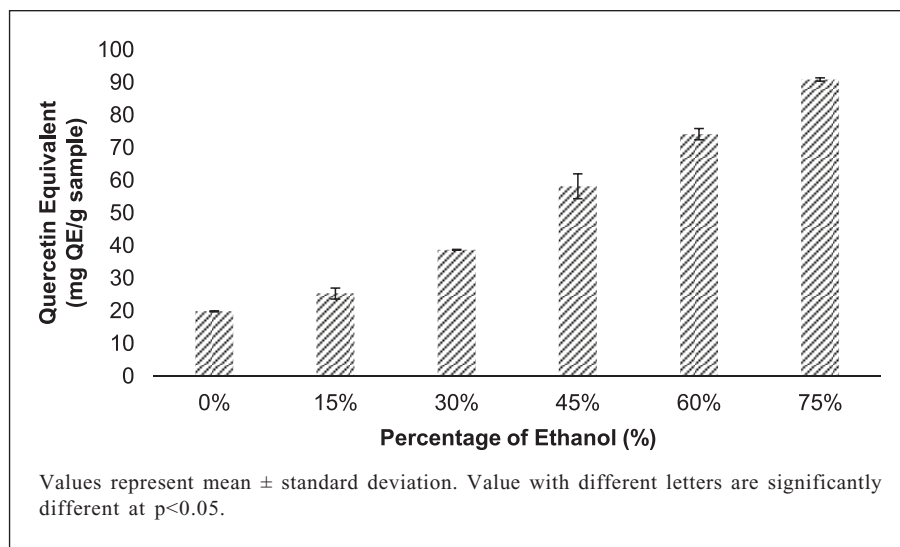
The amount of total phenolic content obtained in this study corresponds with the study done by Ramaiya *et al.* (2014) which stated that the total phenolic content of *P. edulis* leaves was 33.2 g GAE/L of extract. The result of the current research also showed that there was a slight drop in total phenolic content in 75% ethanol extracts compared to 60% ethanol extracts. The fluctuated trend might be caused by the possible complex formation of some phenolic compounds in the aqueous methanol extract. These phenolic complex with higher molecular weight could be extracted easily and possessed higher reducing activity because the complex has more phenol groups (Diem *et al.*, 2013).

#### Determination of total flavonoids content

Figure 5 shows that the higher the concentration of ethanol was used in the extraction, the higher the total flavonoids content of the sample tested in the *P. edulis* leaves. All extracts showed significant difference ( $p < 0.05$ ), except for 0% ethanol and 15% ethanol. The highest concentration of flavonoids was measured in 75% ethanol ( $90.94 \pm 0.15$  mg QE/g sample), followed by 60% ethanol ( $74.22 \pm 1.75$  mg QE/g sample), 45% ethanol ( $58.23 \pm 3.81$  mg QE/g sample), 30% ethanol ( $38.79 \pm 0.08$  mg QE/g sample), 15% ethanol ( $25.38 \pm 1.66$  mg QE/g sample) and the lowest concentration of



**Fig. 4.** Total phenolic content (mg GAE/g sample) of *P. edulis* leaves extracted by different ethanol concentration using ultrasonic extraction technique.



**Fig. 5.** Total flavonoids content (mg QE/g sample) of *P. edulis* leaves extracted by different ethanol concentration using ultrasonic extraction technique.

flavonoids was measured in 0% ethanol ( $19.89 \pm 0.15$  mg QE/g sample). The result showed that the total flavonoids contents in plant extracts of the species *P. edulis* leaves might depend on the polarity of solvent used in extraction. This might be because the flavonoids present in the *P. edulis* leaves are apigenin and luteolin (Dhawan *et al.*, 2004). These flavonoids compounds were insoluble in water but soluble in low polar alcohol such as ethanol (Bin & Weidong, 2010).

#### Correlation between total flavonoids content and antioxidant activity

The high phenolic and flavonoids content was responsible for the bioactivity of the extract of *P. edulis*. The result of Total Phenolic Content (TPC) assay showed that the phenolic content in the sample was high when the concentration of the water or ethanol was not the highest. This was in contrast to the result of DPPH assay which showed that the highest ethanol concentration extract possessed the highest antioxidant activity. High amount phenolic compound extracted might not guarantee the extract possess high antioxidant activity because not all of the phenolic compound possessed high antioxidant activity. Diem *et al.* (2013) stated that the non-phenol compounds and the formation of complex of some phenolic compounds in the extract that were soluble in the solvent might affect the result of TPC. Alkaloids, glycosyl and cyanogenic compounds in *P. edulis* leaves which may form complex with the phenols and affect the result of the analysis. The TFC assay was in accordance to DPPH radical scavenging assay because the amount of flavonoids extracted increased by increasing the concentration of ethanol in the solvent. This showed

that flavonoids might be the major antioxidant compound in *P. edulis* leaves which possessed high radical reducing ability and lipid oxidation inhibition ability. This result was similar with the study carried out by Baba and Malik (2015) which stated that the DPPH radical scavenging activity increased as the flavonoids content in solvent extracted *Vigna sinensis* increased. Moreover, Sun *et al.* (2015) also stated that the strongest antioxidant properties was shown by the propolis extract which found to have highest flavonoids content.

#### CONCLUSION

In this study, phenolic compounds of *P. edulis* leaves were successfully extracted using ultrasonic assisted solvent extraction with ethanol and adequate antioxidant was detected in the analysis carried out by this research. The extraction yield of *P. edulis* leaves extract decreased with increasing ethanol concentration. The highest *P. edulis* yield was found in the 0% ethanol extraction ( $50.38 \pm 3.63\%$ ). *P. edulis* leaves extracted with 75% ethanol possessed the highest antioxidant activity in DPPH radical scavenging assay ( $85.17 \pm 0.36\%$ ). In TBA test, 15% ethanol showed the highest inhibition activity towards the formation of the secondary product in lipid oxidation ( $56.22 \pm 5.01\%$ ). The total phenolic content and total flavonoids content also increased by the increasing of ethanol concentration. The total phenolic content of *P. edulis* extract was found to be in the range of 41.02 to 57.08 mg GAE/g sample, with the highest concentration of phenols was measured in 60% ethanol extracts ( $57.08 \pm 0.29$  mg GAE/g sample).

The total flavonoids content of *P. edulis* leaves extract was found to be in the range of 19.89 to 90.94 mg QE/g sample, with the highest concentration of flavonoids measured in 75% ethanol ( $90.94 \pm 0.15$  mg QE/g sample).

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