NON-ENZYMATIC ANTIOXIDANT FROM APPLE SNAIL 
(*Pomacea maculata*) EXTRACT

KHALIDA KHALIL1, SYARUL NATAQAIN BAHARUM2, SHAZRUL FAZRY3, NIK MARZUKI SIDIK4 and FAREED SAIRI1*

1Department of Biological Sciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia
2Institute of Systems Biology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia
3Tasik Chini Research Center, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia
4Fakulti Industri Asas Tani, Universiti Malaysia Kelantan, Kampus Jeli, 17600 Jeli, Kelantan, Malaysia
*E-mail: fareed@ukm.edu.my

Accepted 6 December 2020, Published online 31 December 2020

ABSTRACT

*Pomacea sp.* is a freshwater gastropod that is capable of withstanding oxidative stress during extreme environmental changes. The snail enzymatic oxidative responses have already been elucidated through biochemical, transcriptomics, and proteomics analysis. However, their non-enzymatic oxidative responses have yet to be elucidated. Therefore, this study aims to characterize the antioxidant activity and identify the non-enzymatic antioxidant compounds from *Pomacea maculata*. To address the aims, a polar and non-polar extraction of snail-whole body extract was conducted using methanol and chloroform, respectively. The antioxidant activity of both extracts was elucidated by Folin Ciocalteau (FC), DPPH, and reducing power assay. LC-MS/MS was then used to profile both extracts. The results demonstrated that the crude methanol extract (CME) contains a higher antioxidant capacity (FC=43.22 ± 3.02 mg GAE/ g extract, DPPH IC50=0.073 mg/mL, and reducing the power of methanol and chloroform extract are 0.361 ± 0.07 and 0.051 ± 0.003 respectively). Profiling of the snail metabolites by LC-MS/MS from both extracts resulted in the identification of uric acid and phenolic compounds. The former was detected at the highest intensity in CME followed by crude chloroform extract (CCE). The phenolic compounds, however, were hypothetically identified as plant metabolites. Therefore, the study suggested that antioxidant activity exhibited by *P. maculata* extracts were due to non-enzymatic compounds such as uric acid and phenolic compounds originated from the animal’s metabolic activity and plants, respectively.

Key words: Apple snail metabolite, non-enzymatic antioxidant, LC-MS/MS, phenolic compound, freshwater mollusc, oxidative stress

INTRODUCTION

Free radicals are generated endogenously by the cell’s mitochondrial activity and cellular processes or exogenously through radiation, heavy metal, pesticides, and drugs (Phaniendra *et al*., 2015). The formation of free radicals is regulated by a series of complex antioxidant defense mechanisms that involve enzymatic and non-enzymatic antioxidants. Excessive free radicals or antioxidant agent deficiency contribute to oxidative stress condition that cause DNA damage, protein oxidation, and lipid peroxidation (Murugan & Parimelazhagan, 2014).

In mollusks, oxidative stress caused by the overproduction of free radicals is associated with environmental stress (extreme temperature, water availability), food limitation, and dormancy (aestivation) (Hayes *et al*., 2012; Giraud-billoud *et al*., 2013). For example, the over a generation of reactive oxygen species (ROS) were caused by reoxygenation during arousal after aestivation (Hermes-Lima & Storey 1995). To minimize the adverse effect of ROS during reoxygenation, pulmonates such as *Theba pisana* depends on non-enzymatic antioxidant (endogenous antioxidant) while *Pomacea canaliculata* deploy both enzyme and non-enzymatic defense mechanisms (Giraud-billoud *et al*., 2013; Bose *et al*., 2016).
In addition to endogenous antioxidant production such as uric acid and glutathione (Bose et al., 2016; Vassilev et al., 2020), novel antioxidant compounds were also found in mollusks. Previous studies had found 6-(diphenylphosphoryl)-3,4-bis(diisopropylamino)-5-pyrrolidino pyridazine and 3,3,4,4-tetracyano-5,6-diphenyl-2-(cyclohexylimino)-2,3,4,5-tetrahydropridine from Turbo bruneus (Tamil Muthu and Selvaraj 2015) and 6-bromo-5-hydroxyindole, 6-bromo-4,7-bruneus hexylimino)-2,3,4,5-tetrahydropyridine from Drupella fragum (Ochi et al., 1998). Antioxidant activity was also demonstrated in extracts from the snail eggs (Dreon et al., 2004), the flesh of Pila ampullacea (Haslianti et al. 2017), Pila virens (Gayathri et al., 2017), and visceral extracts (Pachiayappan et al., 2014).

Pomacea maculata is regarded as the largest freshwater snail that originated from South America (Hayes et al., 2012). The snail was initially introduced to Asian countries as a food source for humans due to its abundance of flesh and high protein content (Haslianti et al., 2017). However, poor demand from the local market caused the snail to be disposed of irresponsibly. Subsequently, the snail established itself as a pest and invaded local wetlands to feed on native plants (Burlakova et al., 2009; Baker et al., 2010). To date, snails are regarded as highly invasive pests that threaten the agriculture industry and local wetlands (Salleh et al., 2012). In Malaysia, apple snails have infested almost 20,000 ha of rice-growing areas and have threatened the livelihoods of farmers. In 2010, the costs associated with apple snail damage were estimated at RM82 million (the US$28 million) (Hussain et al., 2017). Endogenous and exogenous antioxidants are imperative to snail survival and elucidating their presence in snails may provide a novel solution to facilitate pest management. Although regulation of enzymatic antioxidant has been described by Giraud-billoud et al. (2013), non-enzymatic antioxidant has yet to be reported intensively. Hence, this study was conducted to evaluate the non-enzymatic antioxidant in P. maculata extracted with methanol (polar) and chloroform (non-polar).

MATERIALS AND METHODS

Standards and reagent

Ascorbic acid and gallic acid standards were purchased from Sigma (St. Louis, MO, USA). 2,2-diphenyl-1-picrylhydrazyl (DPPH) was supplied by Alfa Aesar (Ward Hill, MA, USA). Methanol and chloroform were obtained from Univar (Ajax Finechem, Australia) and Merck (Darmstadt, Germany), respectively.

Sampling and sample preparation

Apple snail was collected in November 2017 from Selangor, Malaysia (2° 53’ 0” North, 101° 46’ 0” East) and identified as Pomacea maculata based on their pink egg, 90° open-ended corner of the shell and a rounded shell form (Hayes et al., 2012). The captured snails were then washed with sterilized distilled water. The whole body was removed from the shell and frozen using liquid nitrogen. The samples were then kept at -80°C until extraction.

Crude extracts preparation

Preparation of the crude extracts was performed in methanol/chloroform according to Overy et al., (2005) with modification. Briefly, the whole body of P. maculata (without shell) was frozen using liquid nitrogen, weighed, and then powdered using mortar and pestle. Then, a cold extraction medium comprising of water/chloroform/methanol (8:20:47, by volume), was added to a cooled tube with the powdered sample and left on ice for 30 min. The samples were then centrifuged at 15,000 x g for 4 min to form separate layers. The upper layer (polar extract) that consists of aqueous metabolites were separated and the lower layer (non-polar extract) was subjected to a secondary extraction by adding 4 mL of water per gram of sample. After that, both upper and lower layer extracts were concentrated using a rotary evaporator and dried using a speed vacuum at 37°C, and kept at room temperature.

Folin Ciocalteu assay

The measurement of each extracts by Folin Ciocalteu assay (Singh et al., 2016) were evaluated as the total reducing capacity (Magalhaes et al., 2008). The crude extract from both methanol and chloroform extraction was diluted to 1.0, 0.5, and 0.25 mg/mL with water. A reaction mixture consisting of 30 µL of diluted extracts and 150 µL Folin Ciocalteu reagent (1:11 v/v in water) was prepared in triplicates. The reaction mixture was incubated for 5 min at 25°C. After 5 min, 120 µL of 75 g/L sodium bicarbonate was added to the reaction mixture and incubated at 25°C for 90 min. After that, the absorption was measured at 725 nM using a microplate reader. A standard curve based on Gallic acid concentration (250 - 7.85 µg/mL) was prepared and the total reducing capacity was determined and expressed as milligram gallic acid per gram of extract.

Free radical scavenging assay

(2,2-diphenyl-1-picrylhydrazyl; DPPH)

The crude methanol and chloroform extracts were screened for free radical scavenging activity using DPPH free radical scavenging assay according to Mensor et al. (2001) with slight modification. The samples (15.7, 31.3, 62.5, 125, 250 and 500}
µg/mL) and ascorbic acid (1.57, 3.13, 6.25, 12.5, 25 and 50 µg/mL) were diluted using methanol. The reaction mixture was prepared by adding 125 µL sample of various concentrations and 50 µL of 0.3 mM DPPH solution. A total of 50 µL methanol and 125 µL samples at various concentrations were prepared for blank and 50 µL of 0.3 mM DPPH and 125 µL methanol were used as a control. The mixtures were then incubated in the dark at room temperature for 30 min. Consequently, the absorption was measured at 517 nM wavelength and converted to the percentage of antioxidant activity (AA) using the following formula:

\[
\text{AA}^\% = 100 - \left(\frac{\text{Abs sample} - \text{Abs blank}}{\text{Abs control}}\right) \times 100
\]

Reducing power assay

The reducing power of both extracts was evaluated according to (Singh et al., 2016) with slight modification. Methanol and chloroform extracts were diluted to different concentrations (1 – 0.25 mg/mL) and ascorbic acid concentration (0.125 – 0.008 mg/mL) were prepared in 0.25 mL solution with phosphate buffer (500 µL, 0.2 M, pH 6.6) and potassium ferricyanide [K₃Fe(CN)₆] (500 µL, 10 mg/mL). The mixture was incubated at room temperature (25°C) for 20 min. After incubation, 500 µL of 10% trichloroacetic acid (TCA) solution was added to each tube and the mixture was centrifuged at 8000 rpm for 10 min. A total of 100 µL of clear supernatant was mixed with an equal amount of sterilized distilled water, 20 µL of ferric chloride (0.1% w/v) solution was added and absorbance was recorded at 700 nM. Phosphate buffer was used as a control. The effective concentration providing 0.5 of absorbance (EC₅₀) was calculated from the graph of absorbance at 700 nM against extract concentration.

LC-MS/MS profiling

A total of 0.1 g of ground P. maculata (whole body without shell) was used to prepare CME and CCE according to (Overy et al., 2005) while the profiling of both extracts were performed on Mamat et al. (2018). Briefly, chromatography separation was performed on the Thermo Scientific C18 column (AcclaimTM Polar Advantage II, 3 x 150 mM, 3 µM particle size) on an UltiMate 3000 UHPLC system (Dionex). Gradient elution was performed at 0.4 mL/min at 40°C using water + 0.1% Formic Acid (A) and 100% acetonitrile (B). One microliter of the sample was injected into the column and the run was performed for 22 min. The gradient started at 5% B (0-3 min); 80% B (3-10 min); 80% B (10-15 min) and 5% B (15-22 min). High-resolution mass spectrometry was carried out using a MicroTOF QIII Bruker Daltonic with an ESI positive ionization based on the following settings: – capillary voltage: 4500 V; nebulizer pressure: 1.2 bar; drying gas: 8 L/min at 200°C. The mass range recorded was 50 – 1000 m/z.

Data analysis of CME and CCE metabolite profile

The molecular ions mass data provided by the TOF analyzer were processed by Compass Data Analysis software version 4.2 (Bruker Daltonik GmbH). The m/z values of compounds were referred to the METLIN database to putatively identify each compound present in both profiles.

Statistical analysis

Results were presented as mean ± standard deviation from triplicates (n=3) and statistical significance between the two solvents was analysed using t-test with p-value less than 0.05 using GraphPad Prism 6.

RESULTS

In this study, the presence of total reducing capacity in both methanol and chloroform crude extracts of P. maculata has confirmed in the Folin Ciocalteu assay. As summarised in Table 1, crude methanol extract (CME) from P. maculata displayed higher total reducing capacity than crude chloroform extract (CCE) with a value of 43.37 ± 3.02 mg GAE/g extract and 13.62 ± 1.92 mg GAE/g extract, respectively.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Antioxidant Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Folin Ciocalteau Assay</td>
</tr>
<tr>
<td></td>
<td>(mg GAE/g extract)</td>
</tr>
<tr>
<td>Methanol Extract</td>
<td>43.37 ± 3.02</td>
</tr>
<tr>
<td>Chloroform Extract</td>
<td>13.62 ± 1.92</td>
</tr>
<tr>
<td>Ascorbic Acid</td>
<td>–</td>
</tr>
</tbody>
</table>

Table 1. The antioxidant activity of crude methanol and chloroform extracts of P. maculata measured by Folin Ciocalteu and DPPH
Table 2. The DPPH scavenging activity of crude methanol and chloroform extracts of different mollusks

<table>
<thead>
<tr>
<th>Samples</th>
<th>Methanol Extract</th>
<th>Chloroform Extract</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. maculata</td>
<td>0.073 IC50 (mg/mL)</td>
<td>78.165 IC50 (mg/mL)</td>
<td>Current study</td>
</tr>
<tr>
<td>P. ampullacea</td>
<td>0.111 IC50 (mg/mL)</td>
<td>0.997 IC50 (mg/mL)</td>
<td>(Haslianti et al., 2017)</td>
</tr>
<tr>
<td>L. littorea</td>
<td>0.78 IC50 (mg/mL)</td>
<td>–</td>
<td>(Borquaye et al., 2016)</td>
</tr>
<tr>
<td>G. paradoxa</td>
<td>0.37 IC50 (mg/mL)</td>
<td>–</td>
<td>(Borquaye et al., 2016)</td>
</tr>
</tbody>
</table>

Fig. 1. Reducing power at 700 nM for CME and CCE of P. maculata. (mean ± SD).

To elucidate the antioxidant compound present in P. maculata, Tandem Mass Spectrometry Analysis (LC-MS/MS) was conducted on both CME and CCE using a positive mode analysis with a non-targeted approach. The extracts were analyzed and the metabolites were identified with the METLIN database. Based on the profile analysis, 260 compounds were detected in CME and 241 compounds in CCE. Twelve compounds were putatively identified as antioxidants in both CME and CCE (Table 3). In both extracts, uric acid was detected and dominantly present in CME. In addition to uric acid, five more antioxidant compounds were putatively identified in CME. The compounds were delphinidin 3,5-di(6-o-malonylglucoside), theaflavin, dalpunin, pelargonidin 3-o-[b-d-glucopyranosyl-(1->2)]-[4-hydroxycinnamoyl-(->6)]-b-d-glucopyranoside][e-] 5-o-(6-o-malonyl-b-d-glucopyranoside] and pelargonidin 3-(6"-p-coumarylglucoside)-5-(6"-acyethylglucoside). On the other hand, gallocatechin-(4alpha->8)-gallocatechin-(4alpha->8)-gallocatechin, epiafzelechin (2R,3R)(-), 3,3'-di-O-galloylpro delphinidin B5, 8-prenylkaempferol3-rhamnosyl-(1->3)-[apiosyl-(1->6)-glucoside] and [3,5,7,4'-tetrahydroxyflavan-(4->8)]2-3,4,5,7,4'-pentahydroxyflavan were identified in CCE. Although both extracts possess a similar numbers of antioxidant compounds, the overall antioxidant activity of CME was higher than CCE based on Folin Ciocalteu, DPPH, and reducing power assay. Differences in antioxidant activity between the CME and CCE extracts may be due to the relative abundance of compounds indicated by the peak intensity. For example, peak intensity values of uric acid in CME were 75129 while in CCE, it was only 1899.
## Table 3. Putatively identified antioxidant compounds from *P. maculata* CME and CCE based on the positive mode of LC-MS/MS

<table>
<thead>
<tr>
<th>Compound</th>
<th>Group (Class)</th>
<th>RT (min)</th>
<th>m/z</th>
<th>Fragments</th>
<th>Peak Intensity</th>
<th>Known origin</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol Extract</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uric acid</td>
<td>Purine</td>
<td>2.7</td>
<td>169.0345</td>
<td>none</td>
<td>75129</td>
<td><em>Pomacea canaliculata</em></td>
<td>(Giraud-Billoud et al., 2013)</td>
</tr>
<tr>
<td>Dalpanin</td>
<td>Phenolic compound (Flavonoid)</td>
<td>3.3</td>
<td>535.1824</td>
<td>none</td>
<td>1967</td>
<td>Plant <em>Cicer arietinum</em></td>
<td>(Mekky et al., 2015)</td>
</tr>
<tr>
<td>Delphinidin 3,5-d-</td>
<td>-Phenolic compound (phenolic compound)</td>
<td>7.8</td>
<td>800.0168</td>
<td>none</td>
<td>390</td>
<td>Plant <em>Chicoreum intybus</em></td>
<td>(Yannai, 2003)</td>
</tr>
<tr>
<td>Pelargonidin 3-β-[d-glucopyranosyl-(1-&gt;2)-</td>
<td>Phenolic compound (Flavonoid)</td>
<td>7.9</td>
<td>990.1954</td>
<td>none</td>
<td>555</td>
<td>Plant <em>Raphanus sativus</em></td>
<td>(Neveu et al., 2010)</td>
</tr>
<tr>
<td>Pelargonidin 3-β-[d-</td>
<td>Phenolic compound (Flavonoid)</td>
<td>8.4</td>
<td>565.1553</td>
<td>134.0946, 325.0704, 379.0845, 409.1069, 493.1090</td>
<td>4089</td>
<td>Plant <em>Camellia sinensis</em></td>
<td>(Balentine et al., 1997; Takemoto et al., 2016)</td>
</tr>
<tr>
<td>Pelargonidin 3-[d-</td>
<td>Phenolic compound (Flavonoid)</td>
<td>12.7</td>
<td>783.1958</td>
<td>none</td>
<td>653</td>
<td>Plant <em>Hyacinthus orientalis</em></td>
<td>(Harborne &amp; Williams 1998)</td>
</tr>
<tr>
<td>Pelargonidin 3-[d-</td>
<td>Phenolic compound (Flavonoid)</td>
<td>12.7</td>
<td>783.1958</td>
<td>none</td>
<td>653</td>
<td>Plant <em>Hyacinthus orientalis</em></td>
<td>(Harborne &amp; Williams 1998)</td>
</tr>
<tr>
<td>Chloroform Extract</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uric acid</td>
<td>Purine</td>
<td>1.2</td>
<td>169.0403</td>
<td>none</td>
<td>1899</td>
<td><em>Pomacea canaliculata</em></td>
<td>(Giraud-Billoud et al., 2013)</td>
</tr>
<tr>
<td>Gallatechin-(4alpha-&gt;8)-</td>
<td>Phenolic compound (Flavonoid)</td>
<td>8.1</td>
<td>915.2853</td>
<td>none</td>
<td>333</td>
<td>Plant <em>Cistus incanus</em></td>
<td>(Aml-Rúez et al., 2011)</td>
</tr>
<tr>
<td>3,3'-Di-O-galloyl-delphinidin B5</td>
<td>Phenolic compound (Flavonoid)</td>
<td>8.8</td>
<td>915.1696</td>
<td>none</td>
<td>322</td>
<td>Plant <em>Myrica rubra</em></td>
<td>(Yannai, 2003)</td>
</tr>
<tr>
<td>[3,5,7,4'-Tetrahydroxyflavan-(6-&gt;8)]2-3,4,5,7,4'-pentahydroxyflavan</td>
<td>Phenolic compound (Flavonoid)</td>
<td>9.1</td>
<td>835.3548</td>
<td>none</td>
<td>868</td>
<td>Plant <em>Tamanius indica</em></td>
<td>(Yannai 2003)</td>
</tr>
<tr>
<td>Epiautecchin (2R,3R)-</td>
<td>Phenolic compound (Flavonoid)</td>
<td>9.3</td>
<td>275.2779</td>
<td>256.2627, 274.2721, 276.2773, 281.5951</td>
<td>27301</td>
<td>Plant <em>Papaver somniferum</em></td>
<td>(Hwang et al., 2001; Kpegbe et al., 2011; Mekky et al., 2015)</td>
</tr>
<tr>
<td>8-Prenykaempferol 3-Hamnosyl-(1-&gt;3)-(apiosyl-(1-&gt;6))-glucoside</td>
<td>Phenolic compound (Flavonoid)</td>
<td>10.9</td>
<td>809.1536</td>
<td>none</td>
<td>1276</td>
<td>Plant <em>Mosla sieboldii</em></td>
<td>(Nadkam &amp; Hosangadi 1988)</td>
</tr>
</tbody>
</table>
DISCUSSION

The mollusc is exposed to various environmental conditions that can induce fatal oxidative stress that reduces the snail survivability. To reduce the damage caused by oxidative stress, the mollusk depends on both enzymatic and non-enzymatic antioxidant agents (Giraud-Billoud et al., 2013). The study also demonstrated that the mollusk antioxidant capacity (reducing capacity) is due to the presence of phenolic compounds (Murugan & Parimelazhagan 2014) which was also demonstrated by the assay performed in this study (Table 1). Total phenolic or reducing compounds were also studied in Cucumaria frondosa (Zhong et al., 2007), Bursatella leachii (Braga 2014) and Malaysian Sea Cucumber Holothuria leucospilota (Ceesay et al., 2019). In our study, reducing capacity of P. maculata was significantly demonstrated by crude methanol extract (CME) compared to crude chloroform extract (CCE) with p-value less than 0.05. The reducing capacity of P. maculata CME and CCE recorded in this study (43.22±3.02 mg GAE/g extract and 13.62±1.92 mg GAE/g extract, respectively) has also surpassed the reducing capacity from B. leachii, C. frondosa and H. leucospilota of previous studies (7.5±0.1 mg GAE/g extract, 1.00±0.00 mg GAE/g and 4.58±0.002 mg GAE/g, respectively).

Several studies suggested that the total phenolic content of different invertebrates was due to their preferences on a selective diet (Mamelona et al., 2007; Zhong et al., 2007; Braga, 2014). For example, B. leachii and C. frondosa were known to consume algae that contained a low concentration of phenolic compounds. However, P. maculata is a ferocious eater and consumes a variety of macrophytes and plant (Hussain 2006; Hayes et al., 2015) which consist of higher phenolic content than algae ranging from 9.02 to 28.39 mg/g dry weight and 0.95 to 10.68 mg/g dry weight; respectively (Li et al., 2007; Boezinová & Vymazal, 2018).

The scavenging activity of CME and CCE were also correlated to phenolic compounds (Singh et al., 2016). Based on current study, CME exhibits higher scavenging activity compared to CCE. This may be due to higher phenolic compounds or reducing compounds that scaveng DPPH radical in CME compared to CCE. Current findings are also supported by previous study done on P. ampullacea. Based on DPPH scavenging activity, the IC50 value of P. ampullacea CME (0.111 mg/ml) is lower than CCE (0.997 mg/ml) (Haslianti et al., 2017). Besides, Borquaye et al. (2016) also performed a DPPH scavenging activity towards Littorina littorea and Galatea paradoxa. In their study, CME of G. paradoxa (0.37 mg/ml) showed higher scavenging activity of DPPH in comparison to L. littorea CME (0.78 mg/ml) (Borquaye et al., 2016). Furthermore, variation in extraction method and time of extraction may also affect the antioxidant content between the studies (Dhanani et al., 2017).

The antioxidant activity of apple snail extract was also investigated by evaluating the extracts reducing power activity. Current study demonstrated that polar extract has higher reducing power activity compared to non-polar extract. Higher reducing power activity by polar extract compared to non-polar extract was in agreement with a study performed by Nazeer and Naqash (2013). In their study, Loligo duvauceli (L) and Donax cuneatus (D) underwent extraction using ethyl acetate (EA) and diethyl ether (EE). The study reported that LEA extract had higher reducing power activity with value of absorbance 0.07 at 0.5 mg/ml compared to DEA (0.05), LEE (0.04) and DEE (0.03) of the same concentration. Regardless of solvents used in both studies, the reducing power activity of our polar (CME, 0.17±0.03) and non-polar (CCE, 0.05±0.003) extracts were comparable to Nazeer and Naqash (2013).

In order to identify the composition of non-enzymatic antioxidant agents in apple snail (P. maculata), LC-MS/MS approach was taken. A total of 12 antioxidant compounds from CME and CCE was detected including the presence of endogenous antioxidant (uric acid) that was more prominent than phenolic compounds in CME (Table 3). It is highly possible that uric acid is the major metabolite that gives CME high antioxidant activity, since uric acid was previously demonstrated to reduce DPPH (Tasaki et al., 2017) and classified as a non-enzymatic antioxidant to combat ROS production after estivation (hypometabolism) in a snail along with reduced glutathione (Giraud-Billoud et al., 2013). During 45 days of estivation, uric acid increased as a consequence of concomitant oxyradical production during uric acid synthesis by xanthine oxidase (Giraud-Billoud et al., 2011). Later, after arousal was induced, uric acid dropped to or near baseline levels within 20 min and remained low up to 24 h after arousal induction (Giraud-billoud et al., 2011). In contrast, the presence of uric acid in the current study was not the result of snail aestivation because the snails were collected when they are still active before they are snap-frezeved using liquid nitrogen.

As for phenolic compounds in both CME and CCE, the result of the current study is in agreement with previous studies who suggested that the phenolic content in invertebrates is due to the diet of consuming plants rich in phenolic compounds (Mamelona et al., 2007; Zhong et al., 2007; Braga 2014). The phenolic compound is a large class of plant secondary metabolites, showing the diversity
of structures and act as a defense mechanism including antioxidant properties (Cheynier, 2012). For instance, theaflavin is a phenolic compound isolated from Camellia sinensis. The compounds have been associated with various physiological effects such as anti-obesity, glucose-lowering, anticancer effects as well as antioxidant activity (Leung et al., 2001; Takemoto et al., 2016). A previous study by Leung et al. (2001) on theaflavin had shown that the compound has antioxidant activity by the ability to reduce thiobarbituric acid–reactive substances (TBARS). As for dalpanin, it is an isoflavonoid extracted from Cicer arietinum that possesses antioxidant and estrogenic activities (Mekky et al., 2015).

Pelargonidin and delphinidin are anthocyanins, which is a subgroup of water-soluble flavonoids. Anthocyanin naturally occurs in plants and is responsible for many of the colors observed in nature. Anthocyanin acts as photoprotectants by scavenging free radicals that are produced during photosynthesis (Sui, 2017). In this study, there are two compounds that putatively identified as pelargonidin which are pelargonidin 3-(6″-p-coumarylglucoside)-5-(6"′-acetylglucoside) and pelargonidin 3-o-[b-d-glucopyranosyl-(1->2)-[4-hydroxycinnamoyl-(->6)]b-d-glucopyranoside](e-5-o-(6-o-malonyl-1-d-glucopyranoside). Pelargonidin 3-(6″-p-coumarylglucoside)-5-(6"′-acetylglucoside) is found in red flower of Hyacinthus orientalis meanwhile pelargonidin 3-o-[b-d-glucopyranosyl-(1->2)-[4-hydroxycinnamoyl-(->6)]b-d-glucopyranoside](e-5-o-(6-o-malonyl-1-d-glucopyranoside) is found in Raphanus sativus (Harborne & Williams 1998).

Despite phenolic compound availability, the antioxidant activity of CCE in the respective assays were less than CME. In CCE, epiafzelechin was the most prominent phenolic compound followed by uric acid. However, the epiafzelechin compound was previously isolated using polar solvent (Kpegba et al., 2011). Thus, the compound that was putatively identified in CCE may not be epiafzelechin and did not have antioxidant activity as shown by Kpegba et al. (2011).

Other flavonoid compounds detected in CCE was 8-prenylkaempferol 3-rhamnosyl-(1->3)-[apiosyl-(1->6)-glucoside] and [3,5,7,4′-tetrahydroxyflavan-(4->8)]2,3,4,5,7,4′-pentahydroxyflavan. The former is classified as flavonoid glycoside while the latter belongs to biflavonoids and polyflavonoid subclass (Nassar et al., 2013; Sandesh et al., 2014). As for gallicatechin-(4 alpha->8)-gallocatechin-(4 alpha->8)-gallocatechin and 3,3′-Di-O-Galloylprodelphinidin B5, both compounds were classified as anthocyanidin that can be isolated from Cistus incanus and Myrica rubra, respectively (Harborne & Williams 1998). All compounds were known to display antioxidant activity. Therefore, antioxidant capacity of apple snail extract may not be due to the presence of phenolic compounds only, but also contributed by non-enzymatic products of the snail metabolism.

CONCLUSION

In this study, we conclude that non-enzymatic antioxidant compounds present in P. maculata consist of uric acid and phenolic compounds such as theaflavin and Epiafzelechin. The antioxidant activity was most prominent in polar extract and highly abundant with uric acid, followed by phenolic compounds such as theaflavin, dalpanin, pelargonidin and delphinidin. The presence of phenolic compounds were highly likely accumulated from the snail diet, and play an important role to balance the organism oxidative stress and retain its survival.

ACKNOWLEDGEMENTS

We would like to thank the Department of Biological Sciences and Biotechnology, Faculty of Science and Technology, Analytical Lab of Institute of Biology Systems (INBIOSIS), Centre for Research and Instrumentation Management (CRIM) and Universiti Kebangsaan Malaysia for the research facility and infrastructure. We are also thankful to UKM for the funding of the research (GGPM-2016-013). We also would like to thank Dr. Lim Seng Joe, Aninah Mohammad Hanapiah, Norhabshah Abdul Jalil, Afnani Alwi, and Nurul Shahirah Aziz for their assistance and contribution in this research.

REFERENCES


