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ORIGINAL ARTICLE

# Exploration of Antioxidant Capacity of Extracts of *Perna viridis*, a Marine Bivalve

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Submitted: 30-07-2019 Revised: 30-08-2019 Published: 28-11-2019

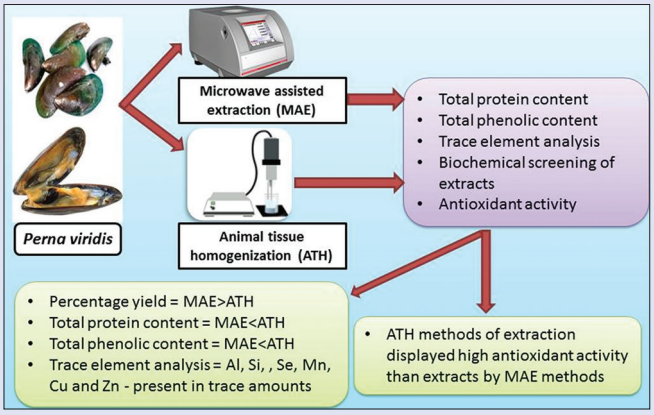
## ABSTRACT

**Background:** The biopotential of Malaysian green mussels (*Perna viridis*) has not been fully explored. The aim of the study is to screen the antioxidant capacity of extracts of Malaysian green mussels. **Materials and Methods:** Mussels were extracted by using solvents such as water, methanol, and ethanol, and methods such as microwave-assisted extraction (MAE) and animal tissue homogenization (ATH) were employed. The percentage yield, total protein content (TPC), total phenolic content, trace element analysis, and biochemical screening of extracts were carried out. The antioxidant capacity of the extracts was assessed by 2,2,-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assay, 2,2′-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS) assay, hydrogen peroxide scavenging assay, and ferric-reducing antioxidant power (FRAP) methods. **Results:** The yield of extracts prepared by MAE methods was higher than the extracts prepared by ATH methods. The total phenolic contents and TPC were higher in the extracts prepared by ATH method than those prepared by MAE methods. Biochemical screening results revealed the presence of alkaloids, phenolic compounds, and saponins. The percentage radical scavenging and inhibitory concentration$_{50}$ ($IC_{50}$) values of all extracts were found to be lesser than the standard. Results showed that extracts by ATH methods displayed high antioxidant activity than extracts by MAE methods. The methanolic extracts of *P. viridis* showed better antioxidant capacity than other extracts in DPPH and ABTS assay methods, whereas the ethanolic extracts displayed high antioxidant activity than other extracts in hydrogen peroxide and FRAP assay methods. The antioxidant activity of the extracts could be attributed to the hydrogen-donating ability of bioactive peptides, phenolic compounds, alkaloids, reducing sugars, and trace elements present in such extracts. **Conclusion:** The results of the present study lay the platform to isolate newer antioxidants from Malaysian green mussels. It is concluded that extensive mechanistic studies are imminent to ascertain the molecular mechanism involved in the antioxidant activity of such extracts.

**Key words:** Animal tissue homogenization, antioxidant capacity and phenolic compounds, microwave-assisted extraction, *Perna viridis*

## SUMMARY

- Mussel extracts contain alkaloids, phenolic compounds, and saponins. The total phenolic content and total protein content were higher in the extracts prepared by animal tissue homogenization (ATH) method
- The methanolic extracts displayed better antioxidant capacity in DDPH and ABTS methods, whereas ethanolic extracts showed high antioxidant activity in hydrogen peroxide and ferric-reducing antioxidant power assays
- The presence of bioactive peptides, phenolic compounds, alkaloids, reducing sugars, and trace elements in the extracts may have contributed to their antioxidant activity
- ATH methods provided extracts with high antioxidant capacity than microwave-assisted extraction methods.




**Abbreviations used:** ABTS: 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), ATH: Animal tissue homogenization, BHT: Butylated hydroxyl toluene, DPPH: 2,2,-Diphenyl-1-picryl-hydrazyl-hydrate, FRAP: Ferric-reducing activity potential assay, AA: Ascorbic acid, GAE: Gallic acid equivalent, $IC_{50}$: Inhibitory concentration$_{50}$, MAE: Microwave-assisted extraction, Min: Minute, mM: Millimolar, MRSP: Mussel-derived radical scavenging peptide, Nm: Nanometer, PMSF: Phenylmethylsulfonyl fluoride, TPTZ: 2,4,6-Tris(2-pyridyl)-s-triazine, Trolox: 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, XRF: X-ray fluorescence.

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**DOI:** 10.4103/pm.pm_301_19

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## INTRODUCTION

Atoms or molecules that possess unpaired electrons are called free radicals and are produced as a result of the normal biochemical reactions in the body. They are extremely reactive, are highly unstable, and are potentially damaging and in excess, they can damage all cellular macromolecules including proteins, carbohydrates, lipids, and nucleic acids.[1] The role of such free radicals in the emergence of various diseases and disorders



**Cite this article as:** Krishnamoorthy V, Chuen LY, Sivayogi V, Kathiresan S, Bahari MB, Raju G, *et al*. Exploration of antioxidant capacity of extracts of *Perna viridis*, a marine bivalve. Phcog Mag 2019;15:S402-9.

such as cancer, cardiovascular disease, diabetes, and neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease, has been well established.[2] The effects of such free radicals are neutralized by antioxidant enzymatic systems and chemical scavengers such as endogenous enzymes and dietary antioxidants. The amount of endogenous antioxidants produced in the body is very much limited, and it is imminent to rely on external (exogenous) sources, primarily from the diet, to fulfill such antioxidants' deficiency.[3] The search for more natural antioxidants from sources other than plants is gaining momentum, and the focus has been laid on marine sources. Foods from various marine sources are consumed in large proportion across the world. The exploration of marine sources for potential antioxidants has not been extensively studied, and the diversified marine environment offers a broad platform for such initiatives.[4,5]

The phylum *Mollusca* is one of the largest, most diverse, and widely distributed groups in the animal kingdom. It is estimated that at least 50,000 described species and about 30,000 are found in the sea.[6] The class *Bivalvia* is one of the eight classes of molluscs and comprises animals enclosed in two shell valves such as mussels, oysters, scallops, and clams. Although this class contains a relatively small number of species, they elicit substantial interest because they are widely consumed in a large proportion due to their high nutritional value. Bivalves carry a huge scope for exploration of such potent compounds. Green mussel (*Perna viridis* [Family: Mytilidae]) is one of the mollusk species that are widely consumed in Southeast Asian countries due to their high nutritional content and delicacy. The composition and properties of mussels vary with the geographical location.[7] Few studies have reported the anti-inflammatory,[8] bioadhesive,[9] inhibition of osteoclast formation,[10] ameliorative effect,[11] antibacterial activity,[12] biomarker activity[13] of green mussels.

Reports reveal that the biological activity of Malaysian mussels has not been fully explored. The aim of the work is to explore the antioxidant potential of Malaysian green mussel extracts.

## MATERIALS AND METHODS

All chemicals, solvents, and buffer salts were of analytical grade. The chemicals were of the highest quality available (95%–99%) and were used without purification. 6-hydroxyl-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) (Sigma Aldrich, St. Louis, Missouri, USA), Butylated hydroxyl toluene (BHT) (Nacalai Tesque, Inc. Nakagyo-ku, Japan), 2,2,-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) (Friedemann Schmidt, Mainz, Germany), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Nacalai Tesque, Tokyo, Japan), 2,4,6-Tris(2-pridyl)-s-thiazine (TPTZ) (R&M Chemicals, Selangor, Malaysia), aluminum chloride-6-hydrate ($AlCl_3.6H_2O$) (HmbG® Chemicals, Hamburg, Germany), Folin–Ciocalteu phenol (Merck, New Jersey, USA), gallic acid (R&M Chemicals, Malaysia), gelatin (Sigma-Aldrich, Malaysia), iron (III) chloride ($FeCl_3$) (Merck, Malaysia), iron (II) sulfate heptahydrate (Friedemann Schmidt, Mainz, Germany), L(±) – ascorbic acid (AA) (HmbG® Chemicals, Malaysia), and lead (II) acetate (QRëC, Auckland, New zealand) were used for the analysis.

### Collection and extraction

The edible bivalve *Perna viridis* was collected from the local fishing outlets of Pantai Merdeka and Semeling, Kedah, Malaysia. The mussels were identified and authenticated at School of Biological Sciences, Universiti Sains Malaysia, Penang. The mussel samples were collected, grouped according to size, and thoroughly washed with clean distilled water. Their external shell surface was thoroughly cleaned with a brush and water to remove all sand and dirt adhering to the shells. The shells were opened carefully with a plier, and the soft tissues were collected in a beaker and homogenized well.

### Microwave-assisted extraction

About 10 g of the mussel tissues was treated with 10 ml of sterile distilled water and mixed well. The extraction was carried out in a Monowave 450 instrument (Anton Paar, Graz, Germany). The extraction temperature was gradually increased from ambient room temperature until it reaches 110°C within 2 min and then held at that temperature for 15 min, and the stirrer speed was set at 600 rpm. After the process, the samples were cooled down to 55°C and centrifuged at 10,000 rpm for 30 min at 4°C to separate the extract and the residue layer. The extract was then filtered, and the remaining solvent was removed using a rotary evaporator and kept at 4°C until further testing. The procedure was repeated by using methanol and ethanol as solvents to get the respective extracts.[14]

### Animal tissue homogenization

The ratio of green mussel tissues and the selected solvent was fixed at 1:3. About 100 g of the mussel tissue samples was mixed with 300 ml of sterile distilled water, and 1% phenylmethylsulfonyl fluoride protease inhibitor was added and blended in a high- speed blender and homogenized for 5 min. The mixture was subjected to mechanical stirring overnight at room temperature and centrifuged at 10,000 rpm for 30 min at 4°C to separate the extract and residue layer. The extract was filtered using a filter paper, dried, and the extract was kept at 4°C until further testing. The same procedure was repeated with methanol and ethanol as solvents to obtain the respective extracts of green mussels.[15]

### Biochemical screening

The crude extracts of *P. viridis* were subjected to biochemical screening to detect the presence of various biochemical components such as phytosterols, alkaloids, tannins, glycosides, and flavonoids/phenolic compounds. Tests such as Salkowski reaction, Dragendorff's reaction, ferric chloride solution test, Keller–Kiliani test, and Shinoda test were carried out.[16]

#### *Total protein estimation*

Standard solutions of bovine serum albumin at varying concentrations were prepared from the stock solution (1 mg/mL). About 2 ml of Bradford reagent was added to each of the solutions, mixed gently, and maintained at room temperature. The absorbance of the solutions was measured at 595 nm using the blank. The procedure was repeated by using the unknown sample. The amounts of protein in the extracts were estimated using the standard calibration curve and expressed as mg/100 g.[17]

#### *Total phenolic content*

The total phenolic content in extracts was determined by using the Folin–Ciocalteu method as reported. Crude extracts were dissolved in distilled water to prepare solutions with varying concentrations (50–1000 µg/mL). Then, 0.2 mL of the crude extracts was mixed with 0.5 ml of Folin–Ciocalteu reagent, mixed well, and 1 mL of 20% sodium carbonate was added. The mixture was incubated in dark at room temperature for 1 h, and the absorbance was measured at 650 nm. Gallic acid was used as a standard. Calibration curve was generated using authentic samples, and total phenolic content was calculated using the calibration curve. The results were expressed as mg/g gallic acid equivalent (GAE).[18]

### X-ray fluorescence analysis

The extracts *of P. viridis* were subjected to X-ray fluorescence (XRF) analysis in XRF spectrophotometer (Malvern Panalytical, Royston, UK). The elemental composition was determined at a resolution of 145 eV at 10,000 pulses.

## Antioxidant capacity assays

Varying concentrations of *P. viridis* extracts were prepared from the stock solutions of *P. viridis* extract of 1 mg/ml.

### *2,2,-Diphenyl-1-picryl-hydrazyl-hydrate radical (DPPH) scavenging capacity assay*

The stock solution of DPPH (0.24%) was prepared by dissolving 24 mg of DPPH in 100 ml of methanol and stored at 20°C. The working solution of DPPH radical avenging capacity assay was prepared by diluting the stock solution with methanol to obtain an absorbance of about 0.98 (±0.02) at 517 nm. A 3 ml aliquot of this solution was mixed with 100 µl of *P. viridis* extracts at various concentrations (50–1000 µg/ml); the extracts were shaken well and incubated in dark for 15 min at room temperature, and the absorbance was taken at 517 nm. A blank was also prepared as mentioned above without any sample. AA and BHT were used as positive controls, and the study was carried out in triplicates. The radical scavenging activity is calculated by the following equation.[18,19]

$$\text{DPPH radical scavenging activity}(\%) = \frac{(\text{Abs}_{\text{standard}} - \text{Abs}_{\text{test}})}{(\text{Abs}_{\text{standard}})} \times 100$$

### *2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicalscavenging activity*

The ABTS cation scavenging activity was performed as reported.[18,20] Trolox and butylated hydroxytoluene were used as positive controls and methanol was used as a negative control. The ABTS stock solution (7 mM) was mixed with potassium persulfate (2.45 mM) solution and kept overnight in dark to yield a dark-colored solution containing ABTS radical cations. The ABTS solution was diluted with 50% methanol for an initial absorbance of about 0.70 ± 0.02 at 745 nm at 30°C. About 300 µl of the test extracts at varying concentrations was mixed with 3 ml of ABTS working standard, and decrease in absorbance was measured every minute. The study was carried out in triplicates. The percentage inhibition was calculated by using the following equation.

$$\text{ABTS radical reducing activity}(\%) = \frac{(\text{Abs}_{\text{standard}} - \text{Abs}_{\text{test}})}{(\text{Abs}_{\text{standard}})} \times 100$$

The antioxidant capacity of the test samples was expressed as inhibitory concentration$_{50\%}$ ($IC_{50\%}$) (antiradical activity), the concentration necessary for 50% reduction of ABTS.[21]

### *Hydrogen peroxide scavenging activity*

Hydrogen peroxide solution (2 mM) was prepared in 50 mM phosphate buffer (pH 7.4). Aliquots (0.1 ml) of *P. viridis* extracts with varying concentrations (50–1000 µg/ml) were transferred into the test tubes and their volumes were made up to 0.4 ml with 50 mM phosphate buffer (pH 7.4). After addition of 0.6 ml hydrogen peroxide solution, tubes were vortexed, and absorbance of the hydrogen peroxide at 230 nm was determined after 10 min, against a blank.[22] The percentage of scavenging of the extracts and standard compounds was calculated using the following equation:

$$\text{Hydrogen peroxide scavenging activity} = \frac{(\text{Abs}_{\text{standard}} - \text{Abs}_{\text{test}})}{(\text{Abs}_{\text{standard}})} \times 100$$

### *Ferric-reducing activity potential assay*

The reducing power assay was carried out as per the reported method.[23] 300 mM of sodium acetate buffer (pH 3.6), 10 mM of TPTZ solution in 40 mM HCl, and 20 mM of iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) solutions were prepared and used as stock solutions. The ferric-reducing antioxidant power (FRAP) reagent was freshly prepared by mixing 25 ml sodium acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml $FeCl_3.6H_2O$ solution. The crude mussel extracts were dissolved in distilled water to prepare varying concentrations of extracts (50, 100, 200, 400, 600, 800, and 1000 µg/ml). Briefly, 0.5 ml of the extracts was mixed with 4.5 ml of FRAP reagent and incubated at 37°C for 30 min in dark condition. After incubation, the absorbance was measured at 595 nm using distilled water as the blank (negative control). Similarly, a calibration curve with varying concentrations of iron (III) sulfate heptahydrate ($FeSO_4.7H_2O$) was prepared and plotted. After obtaining the absorbance values, the negative control value was subtracted from the sample absorbance values, and a linear curve was constructed at various concentrations of the extracts. The regression value ($R^2$) was used to calculate the FRAP value units, and results were expressed in µM Fe (II)/g.[23]

## Statistical analysis

Data were expressed as mean ± standard deviation from three separate observations. For *in vitro* antioxidant assays, one-way ANOVA test followed by Tukey's test ($P<0.05$) was used to analyze the differences among $IC_{50}$ of various fractions for different antioxidant assays. Data on biochemical investigations of *in vivo* experiments were analyzed by one-way ANOVA, and the group means were compared by Dunnett's multiple range test. $P<0.05$ was considered statistically significant.

# RESULTS

## Extraction yield, total phenolic content, and total protein content

The percentage yield, total protein content (TPC), and total phenolic content of *P. viridis* extracts were compared [Table 1]. The extraction yield of *P. viridis* extracts varied from 1.3% to 5.7%, with aqueous extracts showing high percentage yield as compared to methanolic and ethanolic extracts, and this pattern was similar in both the methods used for extraction.

The total phenolic content of *P. viridis* extracts was estimated by using Folin–Ciocalteu reagent, and it was solvent dependent and expressed as mg/g GAE. The total phenolic content and TPC are displayed in Figure 1a and b. The phenolic content in various extracts of *P. viridis* widely ranged from 5.53 ± 8.19 to 13.5 ± 5.82 mg/g GAE. The ethanolic extracts exhibited the highest phenolic content than other extracts in both the methods used for extraction [Figure 1].

The protein content of all the extracts varied from 166.53 ± 4.53 to 278.42 ± 6.78 mg/100 g, with the methanolic extracts exhibiting high content as compared to other extracts in both the methods used for extraction. The total phenolic content and TPC were higher in all extracts prepared by animal tissue homogenization (ATH) method as compared to extracts by microwave-assisted extraction (MAE) method [Figure 1].

## Biochemical screening

Results of biochemical screening data of *P. viridis* extracts are shown in Table 2. The elemental analysis data [Table 3] of *P. viridis* extracts revealed the presence of elements such as sodium, potassium, chlorine, sulfur, and phosphorus in high amount in all the extracts. It was also noticed that elements such as aluminum, silicon, selenium, manganese, copper, and zinc were also present in trace amounts. The extracts

**Table 1:** The yield of *Perna viridis* extracts

| Parameters | Extraction method | | | | | |
|---|---|---|---|---|---|---|
| | MAE | | | ATH | | |
| | Water | Methanol | Ethanol | Water | Methanol | Ethanol |
| Percentage yield | 5.7 | 5.5 | 4.5 | 2.9 | 1.9 | 1.3 |

MAE: Microwave-assisted extraction; ATH: Animal tissue homogenization

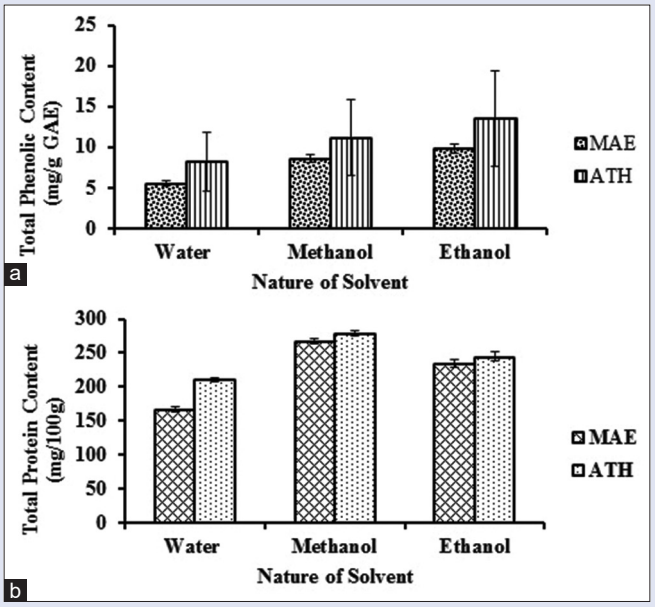


**Figure 1:** (a) Total phenolic content and total protein content of *Perna viridis* extracts. Each value represents mean ± standard deviation ($n = 3$). (b) Total protein content of *Perna viridis* extracts. Each value represents mean ± standard deviation ($n = 3$)

prepared by MAE method did not provide any data in the elemental analysis.

## Antioxidant capacity

The DPPH scavenging capacity of *P. viridis* extracts by MAE and ATH methods is shown in Figure 2. The scavenging activity increased with the concentration of extracts. The high radical scavenging ability and low $IC_{50}$ values [Table 4] of methanolic extracts of *P. viridis* reflected their high antioxidant potential as compared to ethanolic and aqueous extracts. The $IC_{50}$ values of all extracts were lower than the corresponding values of standards (AA and BHT). The antioxidant capacity of extracts could be ranked as follows: AA > BHT > methanolic > ethanolic > aqueous extracts, irrespective of the method used for extraction, the antioxidant potential of *P. viridis* extracts was found to be low ($P < 0.05$) than that of the standards. The results revealed that extracts prepared by ATH method displayed better antioxidant capacity as compared to extracts by MAE method.

The ABTS radical scavenging activities of *P. viridis* extracts prepared by ATH and MAE methods are shown in Figure 3. The extracts scavenged ABTS radical in a concentration-dependent manner (50–1000 µg/mL), and the scavenging activity of methanolic extracts was higher than ethanolic and aqueous extracts. The reducing activity of extracts was lower than the activity of standards used in estimation. The $IC_{50}$ values of methanolic extracts were lower than the corresponding values of ethanolic and aqueous extracts, thus supporting the ABTS radical

**Table 2:** Biochemical screening data of *Perna viridis* extracts

| Biochemical analysis | MAE | | | ATH | | |
|---|---|---|---|---|---|---|
| | Water | Methanol | Ethanol | Water | Methanol | Ethanol |
| Carbohydrates | – | – | – | + | + | + |
| Reducing sugars | – | – | – | + | + | + |
| Monosaccharides | – | – | – | – | – | – |
| Tannins | – | – | – | – | – | – |
| Flavonoids | | | | | | |
| Lead acetate solution | – | – | – | – | – | – |
| Sodium hydroxide | – | – | – | – | – | – |
| Phenols | + | + | + | + | + | + |
| Alkaloids | | | | | | |
| Dragendorff's | + | + | + | – | – | – |
| Wagner's | – | – | – | + | + | + |
| Saponin | + | + | + | + | + | + |
| Proteins | + | + | + | + | + | + |
| Steroids | – | – | – | + | + | + |

+Present; –Absent. MAE: Microwave-assisted extraction; ATH: Animal tissue homogenization

**Table 3:** Elemental analysis data of *Perna viridis* extracts

| Element detected (%) | Normal value of elements (%) | Water extract (%) | Methanol extract (%) | Ethanol extract (%) |
|---|---|---|---|---|
| Sodium (Na) | 0.15 | 3.160 | 5.152 | 2.413 |
| Aluminum (Al) | 0.000087 | 0.072 | 0.123 | 0.077 |
| Silicon (Si) | 0.002 | 0.017 | 0.047 | 0.059 |
| Phosphorus (P) | 1.00 | 5.707 | 2.768 | 2.473 |
| Sulfur (S) | 0.25 | 1.303 | 3.950 | 2.853 |
| Chlorine (Cl) | 0.15 | 6.544 | 16.254 | 11.711 |
| Potassium (K) | 0.25 | 1.549 | 6.179 | 5.652 |
| Chromium (Cr) | 0.0000024 | - | - | 0.004 |
| Iron (Fe) | 0.006 | 0.080 | 0.013 | 0.021 |
| Copper (Cu) | 0.0001 | 0.001 | 0.003 | 0.005 |
| Zinc (Zn) | 0.0032 | 0.012 | 0.002 | 0.003 |
| Arsenic (As) | 0.000026 | 0.005 | 0.012 | 0.017 |
| Selenium (Se) | 0.000019 | 0.001 | 0.001 | 0.001 |
| Bromine (Br) | 0.00029 | 0.175 | 0.325 | 0.272 |

scavenging activity. The scavenging ability of the extracts could be ranked as Trolox > BHT > methanolic > ethanolic > aqueous extracts. The radical scavenging order of extracts was not influenced by the method used for extraction. The results also proved that the extracts prepared by ATH method displayed prominent antioxidant capacity than the extracts prepared by MAE method.

The hydrogen peroxide free radical scavenging ability of all *P. viridis* extracts is displayed in Figure 4. The scavenging ability of extracts was lower in comparison to that of standards used in estimation. The radical scavenging ability is directly proportional to concentration of each extract. The ethanolic extracts displayed strong antioxidant potential than methanolic and aqueous extracts, and this pattern was found to be similar in both the methods used for estimation. Based on these findings, all extracts could be ranked as AA > BHT > ethanolic > methanolic > aqueous extracts in terms of scavenging ability. The $IC_{50}$ values of the extracts were found to well correlate with the scavenging ability of the extracts.

In FRAP assay, the yellow color of the test solution changes to green depending on the reducing power of extracts. The presence of the reductants in extracts causes the reduction of the $Fe^{3+}$/ferricyanide complex to the ferrous form, and the amount of ferrous form is monitored by absorbance measurement at 700 nm. The results are represented as ferrous equivalents (FEs) in μM Fe (II)/g). A high FE value indicates high reduction activity potential. The FRAP assay results of all *P. viridis* extracts are shown in Figure 5. The reducing ability of

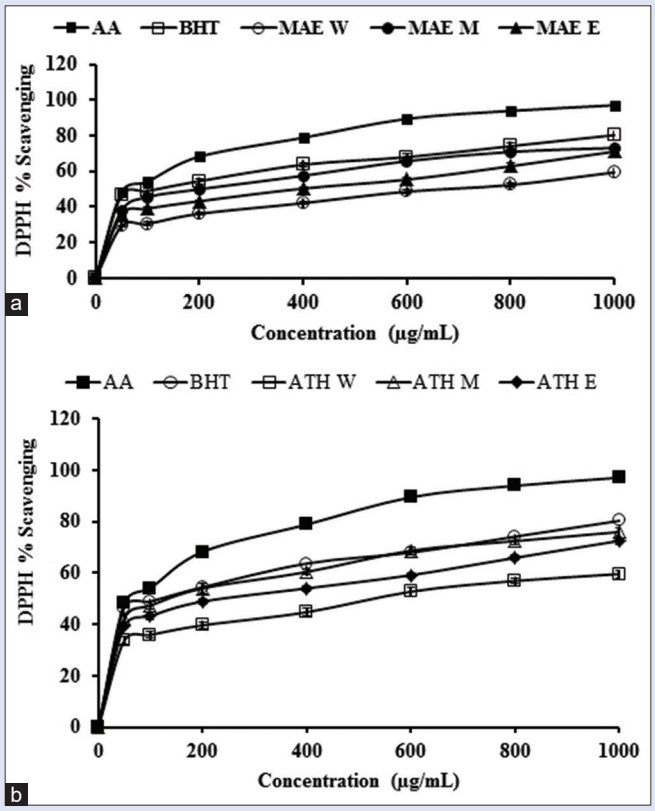


**Figure 2:** (a) 2,2,-Diphenyl-1-picryl-hydrazyl-hydrate radical scavenging activity of MAE extracts of *Perna viridis*. Each value represents mean ± standard deviation ($n$ = 3). (b) 2,2,-Diphenyl-1-picryl-hydrazyl-hydrate radical scavenging activity of ATH extracts of *Perna viridis*. Each value represents mean ± standard deviation ($n$ = 3). MAE: Microwave-assisted extraction; W: Water; M: Methanol; E: Ethanol; AA: Ascorbic acid; BHT: Butylated hydroxyl toluene; ATH: Animal tissue homogenization

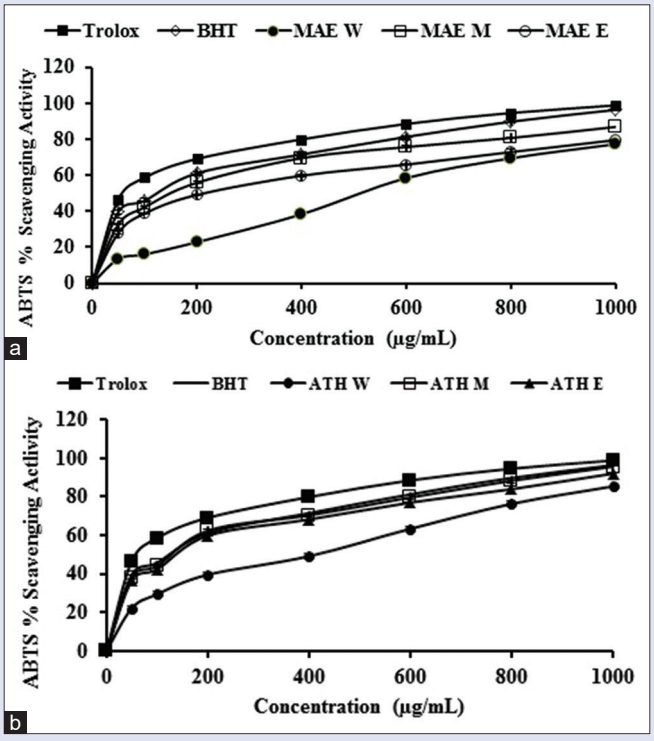


**Figure 3:** (a) 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) reducing ability of MAE extracts of *Perna viridis*. Each value represents mean ± standard deviation ($n$ = 3). (b) ABTS reducing ability of ATH extracts of *P. viridis*. Each value represents mean ± standard deviation ($n$ = 3). MAE: Microwave-assisted extraction; ATH: Animal tissue homogenization; W: Water; M: Methanol; E: Ethanol; AA: Ascorbic acid; BHT: Butylated hydroxyl toluene

**Table 4:** Comparison of inhibitory concentration$_{50\%}$ data of all extracts by different methods

| Sample | $IC_{50\%}$ (μg/ml) | | | | | |
|---|---|---|---|---|---|---|
| | DPPH assay | | ABTS assay | | Hydrogen peroxide scavenging assay | |
| | MAE | ATH | MAE | ATH | MAE | ATH |
| Ascorbic acid | 56.06 | 56.06 | - | - | 395.18 | 395.18 |
| BHT (standard) | 97.77 | 97.77 | 114.59 | 114.59 | 597.73 | 597.73 |
| Trolox | - | - | 75.15 | 75.15 | - | - |
| Methanolic extract | 247.85 | 154.3 | 197.84 | 128.45 | 949.13 | 775.95 |
| Ethanolic extract | 419.97 | 305.05 | 322.64 | 163.91 | 731.13 | 590.43 |
| Aqueous extract | 686.41 | 583.69 | 557.23 | 415.35 | 1343 | 1221.3 |

BHT: Butylated hydroxyl toluene; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); FRAP: Ferric reducing antioxidant power; MAE: Microwave-assisted extraction; ATH: Animal tissue homogenization; $IC_{50\%}$: Inhibitory concentration$_{50\%}$

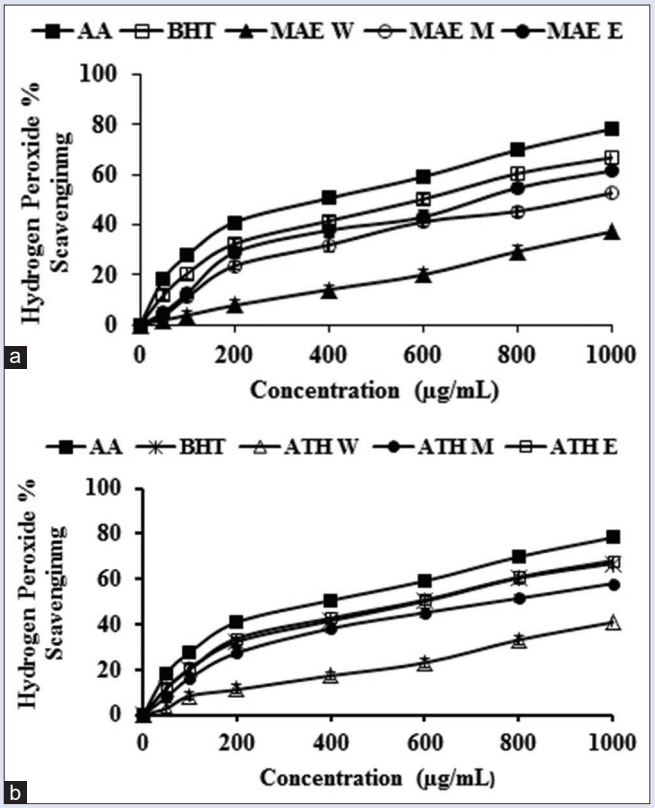


**Figure 4:** (a) Hydrogen peroxide percentage scavenging activity of MAE extracts of *Perna viridis*. Each value represents mean ± standard deviation ($n$ = 3). (b) Hydrogen peroxide percentage scavenging activity of ATH extracts of *Perna viridis*. Each value represents mean ± standard deviation ($n$ = 3). MAE: Microwave-assisted extraction; ATH: Animal tissue homogenization; W: Water; M: Methanol; E: Ethanol; AA: Ascorbic acid; BHT: Butylated hydroxyl toluene

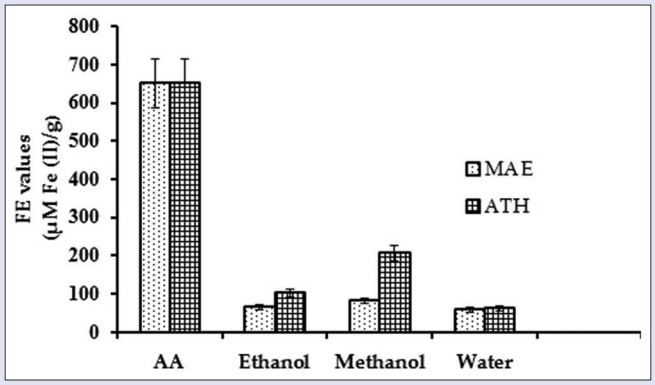


**Figure 5:** Ferric reduction activity potential of *Perna viridis* extracts. Each value represents mean ± standard deviation ($n$ = 3). MAE: Microwave-assisted extraction; ATH: Animal tissue homogenization; AA: Ascorbic acid

*P. viridis* extracts increased with the concentration of extracts, and the FE values were found to be less than the FE values of standards (AA) used in the assay. The reduction activity potential of extracts could be ranked as AA > methanol > ethanol > aqueous extracts, and this ranking order was not affected by the method used for extraction. The extracts prepared by ATH method displayed stronger reduction potential than the extracts prepared by MAE method.

## DISCUSSION

The effectiveness of an extraction method is assessed by the total yield or the yield of a certain target compound or compounds. The yield from the tissues of *P. viridis* was higher for water as compared to methanol and ethanol. The better yield of aqueous extract may be due to the ability of water to extract high polar ingredients that might be present in mussel tissues. Factors such as solvent, time and temperature of extraction, and chemical nature of sample are the key determinants in any extraction process.[24]

Further, the yield of extracts by MAE method was higher than the yield of extracts by ATH method. It could be related to the nature and volume of the solvent, microwave power, nature of matrix, and time of extraction used in MAE process. The efficiency of MAE process in the extraction of constituents from natural sources has been well reported. These suggestions are well supported by similar findings reported earlier on MAE process.[14,25]

The TPC content was higher in ethanolic extracts than other extracts in both the methods used for extraction. The extracts prepared by ATH method showed high TPC as compared to extracts prepared by MAE method. Although it is assumed that polyphenols are mostly abundant in plant and plant-derived natural products, they are least reported in mollusks and marine organisms. However, green mussels are filter-feeding marine organisms; it was also suggested that phenols and polyphenols may be present due to the algal diet they feed in the marine environment. These findings could be supported by the presence of polyphenols and phenolic compounds in sea cucumber *Cucumaria frondosa* and their antioxidant activity.[16,26,27]

The protein content was higher in methanolic extracts as compared to ethanolic and aqueous extracts in both the methods used for extraction, and it could be linked to the presence of more proteins soluble in methanol than in ethanol and water. Similar to phenolic content, the protein content in extracts prepared by ATH method was higher than that in the extracts prepared by MAE methods. These findings could be owed to the high temperature used by the MAE method which might have denatured the proteins present in mussel tissues and resulted in low protein content. The nature of the matrix determines the conditions used for extraction.[17,24,25]

The biochemical screening results revealed the presence of diverse components such as proteins, phenolics, alkaloids, and saponins in MAE extracts, whereas components such as carbohydrates, reducing sugars, polyphenols, phenolic compound, proteins, alkaloids, and saponins are present in ATH extracts. The presence of such diverse chemical groups can be owed to the dietary sources that the mussels feed on. Green mussels are filter-feeding marine organisms, and they readily assimilate from a diversified marine environment. Alkaloids have been isolated from both classes (gastropods and bivalves) of molluscs. Analogous results were also observed in Indian green mussel's extracts.[16,26-28]

The variation in the presence of chemical constituents in different mussel extracts may be linked to their geographical location, marine environment, quality of sea water, and the conditions used for extractions. The high temperature utilized in MAE might have also affected the composition in the extracts. The presence of these components in various extracts of plants has exhibited significant biological activities such as antimicrobial, antioxidant, and wound healing. Similar findings were reported on variation in the biochemical composition of green mussels.[24,25]

The trace elements play a significant role in the exhibition of various biological activities. The role of elements such as iron, copper, zinc, manganese, and selenium in exerting antioxidant activity has been reported.[29] These trace elements occur naturally within the cytosol and mitochondria of animal tissues, and the animal kingdom was able to

develop a sophisticated antioxidant system that depends on antioxidant nutrients.[30] The trace elements in ATH extracts are present in high amount than the permitted standards. Numerous studies have reported the use of mussels as a biomarker to assess marine pollution based on the amount of these elements in mussels.[31-33] The absence of any elemental analysis data from extracts prepared by MAE method could be related to the harsh conditions used during the extraction process.

Several techniques have been reported to determine the antioxidant capacity *in vitro* in order to allow rapid screening of samples.[34] Free radicals are known to play a definite role in a wide variety of pathological manifestations. Antioxidants fight against free radicals and protect us from various diseases. They exert their action either by scavenging the reactive oxygen species (ROS) or protecting the antioxidant defense mechanisms.[35]

The DPPH method is widely used to test the ability of extracts or compounds to act as free radical scavengers or hydrogen donors. The antioxidant activity is measured by bleaching of DPPH purple-colored solution. The degree of color change depends on the concentration and potency of antioxidants. The higher the decrease in absorbance, the higher the radical scavenging activity. In the present study, the methanolic extracts showed significant percentage radical inhibition than ethanolic and aqueous extracts. It is suggested that the tissue extracts contain phytochemical constituents that are capable of donating hydrogen to a free radical and scavenge it and prevent its potential damage. These findings could be related to the reports on the DPPH radical scavenging activity of extract of *P. viridis*.[19,36]

In ABTS assay, the ABTS chromophore was generated (by treating ABTS with potassium persulfate) and reduced in the presence of hydrogen-donating antioxidant constituents present in extracts. This process induces a color change in ABTS radicals, and it is measured spectrophotometrically at 730 nm. The ABTS radical-reducing ability of extracts could be linked to the electron-donating ability of phenoxide groups, bioactive peptides, alkaloids, and saponins to ABTS free radicals and resulting in its inhibition. It was also observed that the ATH method was more efficient in producing extracts with better percentage radical inhibition and low $IC_{50}$ values as compared to extracts prepared by MAE methods. These findings corroborate well with similar study results reported on Indian mussel extracts.[28,36,37]

Hydrogen peroxide ($H_2O_2$) free radical is one among the six major reactive oxygen species (ROS), and it plays a major role in the damage of normal cells. The removal of $H_2O_2$ free radical is very vital for antioxidant defense in cell or food systems. The nature of the inhibition of free radicals could follow several potential inhibition pathways, namely, (a) by donating hydrogen and reacting directly with $H_2O_2$, (b) reacting with intermediates formed from enzyme and $H_2O_2$, or (c) inhibiting the horseradish peroxidase from binding $H_2O_2$.[35,38]

The ROS scavenging activity of the extracts could be linked to the presence of bioactive peptides, phenolic compounds, and alkaloids, which might have donated hydrogen and reacted directly with $H_2O_2$ to form $H_2O$. The presence of these chemical constituents in the extracts of *P. viridis* further substantiates this. Studies also have reported the scavenging ability of mussel-derived radical scavenging peptide (MRSP) in mussels.[39,40] The presence of phenolic compounds in ethyl acetate extracts of *P. viridis* can be correlated with the present findings.[28]

The presence of reducing sugars and trace elements in *P. viridis* extracts may responsible for its radical scavenging ability. These findings could be well correlated to similar reports.[16] In FRAP assay the yellow color of the test solution changes to green depending on the reducing power of the extracts. The presence of the reductants in such extracts causes the reduction of the $Fe^{3+}$/ferricyanide complex to the ferrous form, and it is monitored by absorbance measurement at 700 nm. The higher the absorbance, the higher the FRA*P* values. The antioxidant activity of extracts could be ascribed to their hydrogen-donating ability, and this donation process may be from phenolic compounds, bioactive peptides, alkaloids, reducing sugars, and trace elements present in *P. viridis* extracts. These findings were well supported by similar reports on the antioxidant capacity of *P. viridis* extracts.[28,36-38]

The antioxidant capacity of extracts prepared by ATH method displayed strong antioxidant capacity as compared to extracts prepared by MAE method. This pattern was found to be similar in all methods used for assessment. The antioxidant activity of *P. viridis* extracts prepared by MAE method could be attributed to the presence of bioactive peptides present in such extracts. It is stated that proteins present in mussels might have hydrolyzed or denatured at the temperature used in MAE process and resulted in the formation of peptides. These peptides can donate hydrogen and scavenge free radicals effectively. These findings coincide with similar reports on the scavenging ability of MRSP in mussels.[39,40] Further, the presence of alkaloids and saponins in extracts could have also assisted in radical scavenging activity.[16,28,41] These findings correlate with similar studies reported on the scavenging ability of such peptides and carotenoids in exhibiting antioxidant activity.[39,40] Hence, the observed antioxidant activity may be attributed to the presence of these constituents in extracts.

The high antioxidant activity exhibited by *P. viridis* extracts prepared by ATH methods could be owed to the hydrogen-donating ability of phenolic compounds present in such extracts.The antioxidant properties of polyphenols could be ascribed to the hydrogen donating ability of phenoxide ions to free radicals such as DPPH, ABTS, and hydrogen peroxide which could result in termination of chain reaction.[34,35] Further, the presence of micronutrients (such as manganese, chromium, copper, zinc, and selenium) could have contributed to the synergistic effect on the radical scavenging ability of such extracts.[31] The presence of phenolic compounds (containing phenolic hydroxyls) and trace elements in such extracts adds supports to these suggestions.

Collectively, it is suggested that factors such as presence of phenolic compounds and trace elements and mild extraction conditions used in ATH methods could be cited as the possible factors for the exhibition of high radical scavenging and reducing abilities in such extracts. It was also hypothesized that the presence of hydrolyzed bioactive peptides, alkaloids, and harsh extraction conditions used in MAE methods could be owed to the low efficiency of such extracts. These postulations were well supported by similar works on the radical scavenging activity of *P. viridis* on green mussels[15,28,36-38] and black mussels.[19]

The antioxidant capacity ranking order of all extracts in DPPH, ABTS, and FRAP assays is found to differ from that of the hydrogen peroxide radical scavenging method. The variation in ranking order may be associated with the testing conditions and type of method used in the estimation of antioxidant capacity.[21,23] These parameters are the key determinants that affect the antioxidant potential of extracts. Thus, the antioxidant potential could be influenced by the number of factors and cannot be fully evaluated by one single method. Similar variations in results were also reported on the radical scavenging activity of ethyl acetate, methanolic, and aqueous ethanolic extract of *P. viridis*.[28,36-38]

The study results provide a platform to develop potent antioxidants from Malaysian green mussel extracts. Although different postulations have been proposed for the antioxidant capacity of *P. viridis* extracts, extensive studies are essential to investigate the mechanism involved at the molecular level in exhibition of antioxidant capacity.

## CONCLUSION

Malaysian green mussel extracts rich in antioxidants, phenolic content, and proteins. The methanolic extract of green mussel exhibited more antioxidant activity than other extracts in DPPH and ABTS methods and ethanolic extract of green mussel exhibited more antioxidant activity than other extracts in hydrogen peroxide scavenging assay. In all the antioxidant assay methods, the extracts prepared by animal tissue homogenization method exhibited stronger antioxidant activity than extracts prepared by microwave-assisted extraction method.


## Acknowledgements

We would like to thank the Ministry of Higher Education (MOHE), Government of Malaysia, for providing financial assistance to carry out this research.

## Financial support and sponsorship

This research was funded by the Ministry of Higher Education (MOHE), Government of Malaysia, under Fundamental Research Grant Scheme (FRGS/1/2015/WAB09/AIMST/02/1).


## Conflicts of interest

There are no conflicts of interest.

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