



Antibacterial and antioxidant activities of the extracts from marine snail *Hemifusus colosseus* (Lamarck, 1816)

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Abstract. This study aims to evaluate the antibacterial and antioxidant activities of the extracts from marine snail *Hemifusus colosseus*. Methanol and water were employed for the extraction process. Crude extracts were evaluated for antibacterial potency through inhibition of bacterial growth by disc diffusion method. The antioxidant potentiality of the extracts was estimated on total phenolic content, free radical scavenging activity, and ferric reducing antioxidant power. The extracts of *Hemifusus colosseus* showed promising antimicrobial and antioxidant bioactive. The methanolic extract possessed a superior antimicrobial ability with inhibitory performance on both Gram-negative (*Escherichia coli*, *Salmonella typhi*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*) and Gram-positive (*Staphylococcus aureus*) bacteria, whilst, the water extract was only effective against *S. typhi* and *V. parahaemolyticus*. Compared to water extract, methanolic extract had significantly higher amounts of total phenolic compounds, antioxidant potentiality in free radical scavenging, and reducing power activity. This study revealed novel antimicrobe and antioxidant properties in *H. colosseus* that could possibly be used as promising natural products in Vietnam.

Key Words: antibacterial and antioxidant activities, extracts, *Hemifusus colosseus*, natural products.

Introduction. In medicine, the current trend is to research and develop drugs from medicinal natural organisms. The oceans cover more than 70% of the Earth's surface and possess a high level of biodiversity, including numerous species that can benefit the medical industry (Kim & Wijesekara 2010). In recent years, marine resources have shown high potential for biological activities and attracted worldwide researchers (Abad & Bermejo 2001; Datta et al 2015). The Earth's raw materials for pharmacy production have apparently been exhausted, however, marine organisms offer extremely diverse and abundant medicinal sources that still need to be exploited and used. It is very interesting to note that marine organisms, which contain high concentrations of steroid, terpenoid, acid amin, alkaloid, phenol compounds, and others, are exploited more for medical purposes (Datta et al 2015). Due to their biosecurity, fewer side effects, and lack of drug resistance by strains of bacteria, these compounds have very important roles for pharmacology (Seyyednejad & Motamedi 2010). Few species of marine organisms have been thoroughly investigated for alternative production for chemical synthesis from their natural products, which are impressively important sources (Cos et al 2006).

Many marine invertebrates have been studied pharmacologically and contributed to the development of the medical industry for treating human diseases (Senthilkumar & Kim 2013). In addition to its high nutritional value, the snail *Hemifusus colosseus* (Lamarck, 1816) is an organism whose extraction contains some bioactive compounds such as dextromethorphan, fucosterol, cholesta-5,22-diene-3ol, ergosta-5-22-dien-3ol, stigmast-5-en-3-ol, and others (Thu et al 2020).

In this study, extracts from *H. colosseus* from Thua Thien Hue coast, Central Vietnam were examined for antibacterial and antioxidant activities. Antibacterial activity was determined against a number of pathogenic bacteria from both aquatic animals and humans, and antioxidant activity was evaluated through free-radical scavenged DPPH

activity and ferric reducing antioxidant power (FRAP). As a result, the present research contributes to the scientific basis for the use of marine medicinal resources in the pharmaceutical and medical industries. The research also provides useful scientific information for the conservation and proper exploitation of valuable marine species for foods and medicine.

Material and Method

Sample collection and extraction. Live specimens of marine snails *H. colosseus* were collected in October, 2021 from coastal water of Thua Thien Hue province, Central Vietnam (16°33'58.7" N 107°38'01.9" E), and immediately transported back in icebox to the laboratory at the Department of Fisheries, University of Agriculture and Forestry, Hue University, Vietnam. After breaking the shell, the soft bodies of the snails were removed and washed with distilled water, and stored at -30°C until further use. Samples (OK1 and OK2) for DNA extraction were preserved in absolute alcohol in Eppendorf and stored at -30°C. Bioactive compounds from the tissue were analysed according to the method of Ky et al (2019). The fresh tissues (100 g in wet weight) were cut into small pieces and homogenised by blending. The homogenates were separately extracted with water and methanol solvents at a ratio of 1:4 (g:mL) at 4°C for 24 h. The extracts were filtered through Whatman No. 1 filter paper, followed by a complete concentration of the solvents in vacuo on a rotary evaporator. Dried crude extracts were employed for the next experiments.

DNA extraction and species identification. The genomic DNA was extracted from 100 mg of tissue sample by CTAB-based method according to Adamkewicz & Harasewych (1996). The concentration and purity of the extracted DNA samples was checked on 1.5% agarose gel and by a spectrophotometer. PCR reaction was performed with a total volume of 50 µL consisting of an initial denaturing phase at 95°C for 1 minute, followed by 27 amplification cycles (95°C for 15s, 45°C for 15s, and a final step at 72°C for 10s). PCR products were checked and evaluated by electrophoresis on 2% agarose gel and detected by imaging system (UV Transilluminator). Sequencing samples were sent to 1st BASE, the Gemini, Singapore Science Park II, Singapore for DNA Barcoding, using sequence analysis tool available in BOLD (www.barcodinglife.org) to find distance summary as well as for the construction of Neighbour-Joining tree.

Microorganisms preparation for antimicrobial assay. Antimicrobial activities of the extracts were performed on both Gram-negative bacteria (*Escherichia coli*, *Salmonella typhi*, *Vibrio parahaemolyticus*, and *Vibrio alginolyticus*) and Gram-positive bacteria (*Staphylococcus aureus*). All experimental bacterial strains were obtained from the Drug, Cosmetic and Food Quality Control Centre of Thua Thien Hue province (HueQC), Vietnam. The selective growth media including Chrome Agar (Himedia, India) for *Vibrio* spp. and Mac Conkey (Merck, Germany) for the other bacterial strains were prepared and sterilized in an autoclave at 121°C for 15 min. These bacterial strains were individually incubated in the media at 37°C for 24 h for reconfirmation via their specific colonies. To obtain active cultures for subsequent experiments, a loop full of culture from the stock cultures was transferred to test tubes of Tryptic soybean broth (Merck, Germany) for bacterial enrichment. Standard strain broths were cultured with a shaker at 120 rpm for 24h at 37°C until cell density matched 0.5 McFarland standards (1.5×10^8 CFU mL⁻¹) for bacterial activity test.

Antimicrobial activities of extracts. The antimicrobial potency of the crude extracts was evaluated by agar well diffusion method on Mueller Hinton Agar (MHA - Himedia, India) as described by Kuppusamy & Ulagesan (2016). For each test, the Petri dishes MHA were swabbed with 100 µL of bacterial broth cultures and maintained within 15 min in a laminar chamber to obtain an ultimate absorption. Wells were made in agar plates using a sterile cork borer of 6 mm. Then 30 µL dissolved extracts (100 mg crude extract: 1 mL methanol) was added into wells on the inoculated MHA dishes. Methanol (Merck, Germany) and chloramphenicol (Biobasic, Canada) were used as negative and positive

control, respectively. The plates were allowed to stand for 30 min prior to incubation at 37°C for 24 h. Clear growth inhibition zones (IZ) around the discs indicated the appearance of antimicrobial activity. The diameters of the inhibition zones against the test organisms were recorded in millimetres (mm) (Laith et al 2017).

Determination of total phenolic contents. The total soluble phenolic contents in the extracts were determined by the Folin-Ciocalteu reagent method according to Vaghasiya et al (2011) with a slight modification. A volume of 1 mL of snail extract ($1000 \mu\text{g mL}^{-1}$) was mixed with 2.5 mL of Folin-Ciocalteu reagent (10% in distilled water) and 2 mL of Na_2CO_3 (2%). After incubation for 15 min at room temperature, the absorbance was measured at 750 nm using UV-Visible Spectrophotometer (Zuzi 4111RS, Rumani). Gallic acid was used as standard solutions ($0\text{-}100 \text{ mg L}^{-1}$ in 50% methanol). Total phenolic contents were calculated using the standard calibration curve of gallic acid and then expressed as gallic acid equivalents (GAE mg g^{-1}):

$$\text{Absorbance}_{750\text{nm}} = 0.0066 \times [\text{Phenols } (\mu\text{g mL}^{-1})] + 0.005 \quad (R^2 = 0.9996)$$

Estimation of antioxidant activity

Free radical scavenging activity assay. The radical scavenging potentiality of crude extracts was identified as per the description of Borquaye et al (2016) based on a bleaching of purple-coloured methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). Briefly, different concentrations of $40\text{-}200 \mu\text{g mL}^{-1}$ of each extract was mixed into 10 mL of 0.1 mM DPPH solution in methanol. After incubation in the dark at room temperature for 30 min, the absorbance of the mixture was determined at 517 nm using UV-Visible Spectrophotometer (Zuzi 4111RS, Rumani). Methanol and ascorbic acid were utilized as a blank and positive control, respectively. The DPPH radical scavenging activity (%) was calculated as the following equation:

$$\text{DPPH radical scavenging (\%)} = [(A_0 - A)/A_0] \times 100\%$$

where: A_0 is the absorbance of blank (DPPH solution without sample) and A is the absorbance of the test sample (DPPH solution plus test sample).

Ferric reducing antioxidant power assay. Determination of the ferric reducing antioxidant power (FRAP) was performed in accordance with Kurniawati et al (2017) using ascorbic acid with various concentrations ($20\text{-}200 \text{ mg L}^{-1}$) as the reference standard solution. In brief, 1.0 mL of $1 \mu\text{g mL}^{-1}$ extracts in methanol were mixed with 2.5 mL phosphate buffer, pH 6.6 (Biobasic, Canada) and 2.5 mL of 1% $\text{K}_3\text{Fe}(\text{CN})_6$. The mixture was vortexed for 5 min, incubated at 50°C for 20 minutes prior to addition of 2.5 mL trichloroacetic acid (10%), was then centrifuged at 3,000 rpm for 10 min. The supernatant (2.5 mL) was mixed with 2.5 mL deionized water and freshly prepared 0.5 mL FeCl_3 (0.1%), followed by well blending until colour formation. The absorbance of standard solutions and samples were determined at 700 nm by UV-Visible Spectrophotometer (Zuzi 4111RS, Rumani).

Data analysis. The experiments were conducted in triplicates with completely randomized design (CRD). The two-tailed t -test was used to examine the significant differences between the content (%) of crude extracts, while one-way ANOVA was used to evaluate the significance of differences among the experimental treatments (multi-comparisons Tukey-Kramer HSD post-hoc test). Statistical comparison tests were conducted at a level of significance of $p = 0.05$.

Results

Species identification. PCR amplification using the mitochondrial COI gene primer set designed in the present study produced a clear single band. Our PCR method is correct since the COI gene of the sample is larger than 600 bp. BLAST tool was used to find similar genes on GenBank and showed that Gene COI sequences (Sequence ID: AY885131.1) from Thua Thien Hue coast samples and the samples isolated by Hayes (2003) in Florida match our sequences with 99% accuracy. Neighbour-Joining tree (Figure 1) also clearly separated the species and genera of *H. colosseus*.

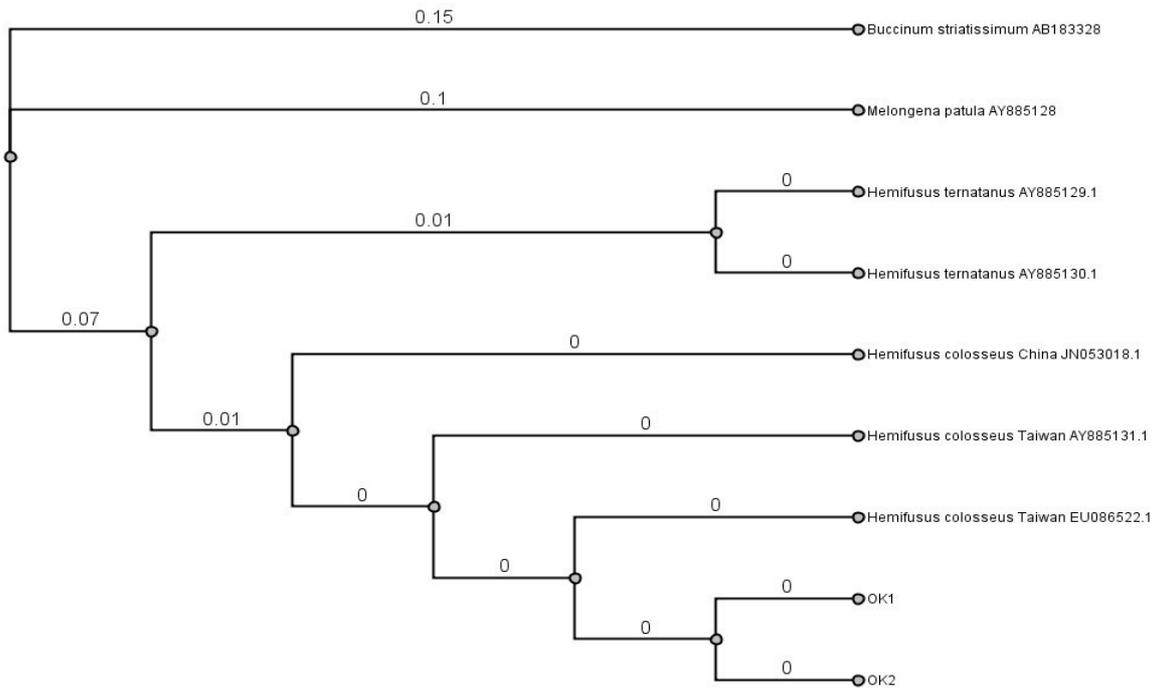


Figure 1. Neighbour-joining tree of *Hemifusus colosseus* (Ok1 and Ok2 are the samples).

Content of crude extracts. The crude extracts of *H. colosseus* from water and methanol solvent showed similar content (Figure 2). For example, water extraction produced $3.35 \pm 0.05\%$ and methanol extraction produced $3.54 \pm 0.02\%$. However, water and methanol did not have significantly different content in crude extracts ($p > 0.05$).

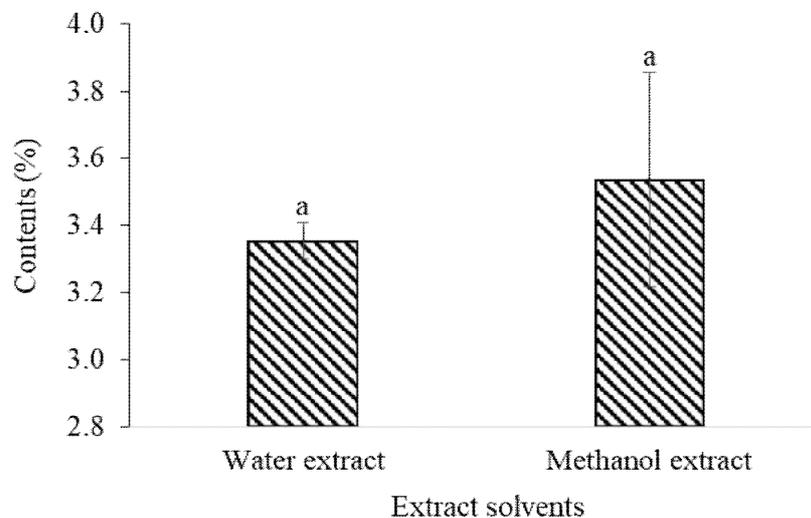


Figure 2. The contents of crude extracts of *Hemifusus colosseus*.

Antimicrobial activity. Antimicrobial activity of extracts from *H. colosseus* showed that crude extract from methanol solvent was able to inhibit the growth of pathogenic bacteria including Gram-negative *E. coli*, *S. typhi*, *V. parahaemolyticus*, *V. alginolyticus*) and Gram-positive *S. aureus*, while extract by water solvent was unable to inhibit *E. coli*, *V. alginolyticus* and *S. aureus* (Table 1).

The greatest antibacterial activity of methanol extract was recorded in *E. coli* (16.33 ± 2.52 mm), followed by *V. parahaemolyticus* (12.67 ± 1.53 mm), *S. typhi* (12.00 ± 2.00 mm), and *V. alginolyticus* (8.67 ± 1.15 mm) ($p < 0.05$). However, a significant lower inhibition zone was observed from water extract against *S. typhi* and *V. parahaemolyticus* as 1.33 ± 0.58 mm and 1.00 ± 0.00 mm, respectively.

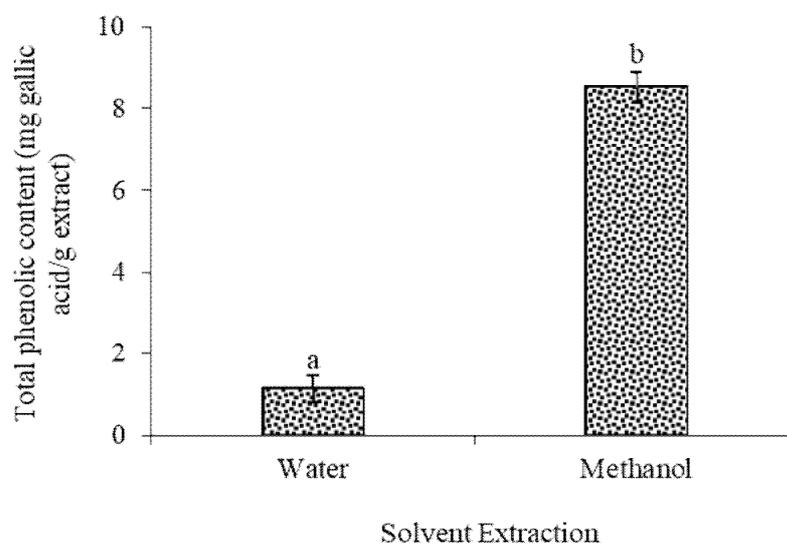
Table 1

Antibacterial activities of the extracts from *Hemifusus colosseus*

Extraction solvents	Inhibition zone (mm)				
	<i>E. coli</i>	<i>S. typhi</i>	<i>V. parahaemolyticus</i>	<i>V. alginolyticus</i>	<i>S. aureus</i>
Water	-	1.33±0.58 ^a	1.00±0.00 ^a	-	-
Methanol	16.33±2.52 ^a	12.00±2.00 ^b	12.67±1.53 ^b	8.67±1.15 ^c	5.33±1.53 ^d

Each value represents as mean±standard deviation (n = 3). Means within a row with different letters ^{a-d} show significant difference by Tukey's test (p < 0.05)

Total phenolic contents. The total phenolic contents for methanol and water solvents were 8.54±0.38 mg gallic acid/g extract and 1.16±0.32 mg gallic acid/g, respectively (Figure 3). The phenolic content of *H. colosseus* was effectively extracted by methanol than by water.

Figure 3. The total phenolic contents of extracts from *Hemifusus colosseus*.**Antioxidant activities**

Free radical scavenging activity. The percentage of DPPH radical scavenging activity of extracts is described in Table 2. At increasing concentrations of 40-200 µg mL⁻¹, the water extract showed the free radical scavenging activity in the range of 3.92±0.19% to 10.63±0.25%. On the other hand, the methanolic extract expressed a much greater DPPH radical scavenging activity, ranging from 18.59±0.37% to 55.09±0.26%.

Table 2

The radical scavenging activity towards DPPH of *Hemifusus colosseus* crude extracts at different concentrations

Extracts concentration (µg mL ⁻¹)	Percentage of DPPH scavenging activity (%)	
	Water extract	Methanolic extract
40	3.92±0.19 ^a	18.59±0.37 ^b
80	6.13±0.43 ^a	23.17±0.26 ^b
120	7.36±0.39 ^a	37.68±0.31 ^b
160	9.81±0.26 ^a	41.72±0.50 ^b
200	10.63±0.25 ^a	55.09±0.26 ^b

Each value represents as mean±standard deviation (n = 3). Means within a row with different letters ^{a-d} show significant difference by Tukey's test (p < 0.05)

Reducing power activity. The ferric reducing antioxidant power (FRAP) of *H. colosseus* crude extracts at concentration of 1,000 µg mL⁻¹ was represented in Figure 4. Both extracts displayed a noteworthy antioxidant activity with significant differences. The

intense absorbance of water and methanolic extracts were 0.018 ± 0.003 and 0.053 ± 0.006 at $OD_{700\text{ nm}}$, respectively ($p < 0.05$).

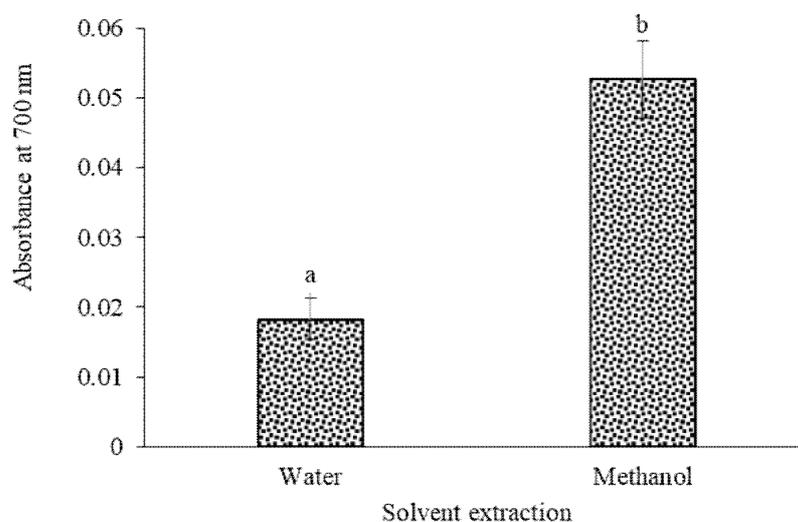


Figure 4. The reducing power activity of *Hemifusus colosseus* crude extracts.

The appearance of antioxidants in the samples leads to a reduction of Fe^{3+} to Fe^{2+} , resulting from electron donation. The amount of Fe^{2+} complex can be estimated by a formation of Perl's Prussian blue at the absorbance of $OD_{700\text{ nm}}$ (Kumar et al 2014). The *H. colosseus* crude extracts from methanol possessed considerable antioxidant activity, as well as superior reducing properties. Furthermore, the observation of free radical scavenging activity, as well as the higher phenolic content, could account for the greater reducing power activity of the methanolic extract in comparison to the water extract.

Discussion. Methanol and water have been effectively used for bioactive extraction in molluscs and crustaceans (Kiran et al 2014). This may be attributed to the fact that bioactive compounds are more soluble in water and methanol, thus preferring highly polar solvents to other solvents such as ethanol and acetone (Zieliński & Kozłowska 2000). However, a greater extraction efficiency by using methanol in comparison to water was also reported by Do et al (2014) for *Limnophila aromatica* and Truong et al (2019) for *Severinia buxifolia*. In the present study, the high extraction efficiency of crude extracts from *H. colosseus* by methanol was consistent with Ky et al (2019), who reported crude extract by methanol solvent from marine snails such as *Turbo chrysostomus*, *Cerithium echinatum*, *Tectus conus*, *Maninella alounia* varied from 2.45 to 3.81%.

Extracts from *H. colosseus* are effective against different types of animal pathogenic bacteria. The result demonstrated that the crude extract showed significant antibacterial activities. According to Zasloff (2002), animals and plants possess broad spectral antimicrobial compounds that fend off a wide range of microorganisms comprising bacteria, fungi, viruses and protozoa. This study observed antibacterial activities of methanol extract was much greater than that of water extract from *H. colosseus*. This can be ascribed to the higher solubility of organic bioactive substances in methanol (organic solvent) than water (non-organic solvent) (Truong et al 2019). Similarly, Ramasamy & Murugan (2005) also reported the highest overall antibacterial screening of methanolic extracts in molluscs (77% gastropods, 21% bivalves, and 2% cephalopods). Similar results were observed by Sugesh et al (2013) for *Hemifusus pugilinus*. Methanol extract showed a strong antimicrobial activity against all assessed pathogenic bacteria. The previous investigation in *Perna viridis* by Annamalai et al (2007) exhibited anti-bactericidal property of extracts with water and methanol solvents after extraction process. In contrast to our findings, the extracts had less inhibitory ability against *E. coli*, *S. typhi*, and *S. aureus*. It might be seen that the differences in extraction

materials would lead to the self-explanatory availability of extractable components, resulting in the diversified bioactive potential of extracts. On the other hand, antimicrobial properties of marine organisms from a wide range of phyla have been screened for years as promising bioactive potentiality. Anbuselvi et al (2009) found that the maximum antimicrobial inhibition zone of acetone crude extract from *Trochus tentorium* was observed on human pathogen *Streptococcus pneumoniae*. The investigation of Karthikeyan et al (2014) in Sydney rock oyster *Saccostrea glomerata* exhibited an effective inhibition of bacterial, fungal and shrimp white spot syndrome virus (WSSV) growth by crude extracts of hexane, ethyl acetate and methanol on bacterial pathogens, fungal growth. Borquaye et al (2016) figured out that both ethyl acetate and methanol extracts from *Littorina littorea* and *Galatea paradoxa* had some degree of antimicrobial activity, with slightly greater ability for ethyl acetate fractions. The crude extracts by methanol, ethanol and acetone solvents from gastropod *Harpa davidis* showed noticeable anti-bactericidal consequences as reported by Giftson & Patterson (2016). Methanolic extract had the highest inhibitory activity against pathogenic bacteria compared to the other extracts. These findings of the present work indicate a strong bacteria-inhibitory potential of *H. colosseus* against pathogenic bacteria. Phenolic compounds have been so far assumed to be abundant in plant and plant-derived natural products. Nevertheless, phenolics and their derivatives from marine organisms have been highly regarded because of their diverse bioactive properties (Rasmussen & Morrissey 2007). This was supported by studies on microalgae with antioxidant potency (Goiris et al 2012); on bivalve clams with potential of antioxidant, anti-diabetic, anti-inflammatory and anti-hypertensive (Joy et al 2016) and on mussel *P. viridis* with a suggestion of nutraceutical potency (Chakraborty et al 2016). The methanol extract of *H. colosseus* showed a significantly greater total phenolics than those in water extract. Similar results was recorded by Krishnamoorthy et al (2019) for *P. viridis*, who also performed both water and methanol extraction resulting in extraction of total phenolics of 5 mg gallic acid/g extract and 8 mg gallic acid/g extract, respectively.

The *H. colosseus* crude extracts extracted by both water and methanolic solvent displayed remarkable antioxidant activity by radical scavenging activity against DPPH. It was observed that with increasing concentration of crude extract ($p < 0.05$), the DPPH radical scavenging activity was also increased. At all sample concentrations, there was much greater percentage radical scavenging activity against DPPH of methanolic extracts compared to water extracts ($p < 0.05$). The considerable discrepancies may be attributed to levels of total phenol in samples as a higher level of total phenol corresponds to greater DPPH radical scavenging activities. The direct proportion of total phenolics content to DPPH radical scavenging activity was supported by earlier reports on 95 plant species from Jordan (Alali et al 2007); snail *Pila virens* (Gayathri et al 2014) and apple snail *Pomacea maculate* (Khalil et al 2019). The present results of DPPH radical scavenging activity from methanolic extract ($55.09 \pm 0.26\%$) was comparable to previous findings of Pachaiyappan et al (2014) on some Mollusca species such as *Hemifusus conchlidium*, *Rapana rapiformis*, *Babylonia spirata*.

It was discovered that *H. colosseus* crude extracts from methanol possessed significant antioxidant activity as well as superior reducing properties. Likewise, observation of free radical scavenging activity, the higher total phenolic content could be an explanation for the greater reducing power activity of the methanolic extract in comparison to water extract. The reducing power potency of methanolic extract in the present study was better than those of molluscan extracts from *Harpa conoidalis*, *R. rapiformis* and *H. conchlidium*, but similar to that of *B. spirata* as reported by Pachaiyappan et al (2014).

Conclusions. The results achieved in the present study indicate that *Hemifusus colosseus* crude extracts from water and methanol solvents exhibited prospective potency of antipathogenic and antioxidant activities. Among these, the methanolic solvent is preferable to acquire greater bioactive potential for extraction process from *Hemifusus colosseus*. Additionally, the present study suggests that *Hemifusus colosseus* crude extract could be an effective natural antimicrobial and antioxidant source. The results

suggested that these compounds could be highly effective in treating human ailments caused by pathogenic or oxidative factors.

Conflict of interest. The authors declare that there is no conflict of interest.

Ethical approval. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Received: 24 December 2021. Accepted: 19 January 2022. Published online: 29 January 2022.

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How to cite this article:

Nguyen M. T., Tran T. N. A., Nguyen H. V., 2022 Antibacterial and antioxidant activities of the extracts from marine snail *Hemifusus colosseus* (Lamarck, 1816). *AAFL Bioflux* 15(1):251-260.