Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 26(6): 737 – 749 (2022)
www.ejabf.journals.ekb.eg

Antibacterial Potential of Tridacna sp. and Pearl Oyster Aqueous Extracts on Food Spoilage *Salmonella enterica*

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ARTICLE INFO

Article History:
Received: Nov. 20, 2022
Accepted: Dec. 4, 2022
Online: Dec. 21, 2022

Keywords:
Bivalve,
Marine shellfish,
Bioactive metabolites,
Giant clams,
Pearl oysters,
Antioxidant activity

ABSTRACT

A high intake of synthetic food preservatives leads to a variety of health problems and diseases. Antibacterial compounds have been discovered in marine invertebrates. As a result, four marine bivalve molluscs species, three giant clams, *Tridacna maxima*, *Tridacna gigas*, *Tridacna squamosa*, and (pearl oysters) *Pinctada margritifera* were collected from the Red sea and assayed for antibacterial activity against the reference food spoilage pathogenic strain *Salmonella enterica*. Among the four bivalves, *T. squamosa* aqueous extract demonstrated a significant antibacterial effect on *S. enterica*, showing a MIC of 12.5mg/ ml. Phenolic and flavonoid total content of *T. squamosa* water extract were 18.5±1.63mg/ g and 2.67±1.48mg/ g, respectively. The antioxidant activity of *T. squamosa* water extract was demonstrated by a DPPH radical scavenging IC_{50} of 58.61±1.02 µg/ml. HPLC analysis confirmed the presence of polyphenol components, including gallic acid, chlorogenic acid, and methyl gallate. The IC_{50} value of *T. squamosa* aqueous extract for growth inhibition on normal PBMCs cells was 199.2±2.24µg/ ml, determining its safety. In addition, it might be employed as innovative dietary supplements and effective natural food preservatives instead of chemical ones.

INTRODUCTION

With the spread of diseases and the detrimental impact of synthetic medicines, it is critical to investigate traditional medicine (**Abd El Hafez *et al.*, 2022**, **El Feky *et al.*, 2022**). Finding a natural substance that may be used as medicine or as a supporting agent to treat various illnesses becomes a major challenge. As a result, searching for a novel, potent natural component produced from marine species is recommended (**Abd El Hafez *et al.*, 2020, 2021**). Marine molluscs are a valuable source of biologically active compounds demonstrating anticancer, antileukemic, antibacterial, anti-inflammatory, and antiviral activities (**Khan AN, 2014**). Marine molluscs have gained its importance as an alternative resource to traditional fisheries in various parts of the world over the last few decades. Molluscs were valued not just as a nutritious diet in various ancient cultures, but also in a variety of traditional cures (**Herbert *et al.*, 2003**).

Many molluscs, such as mussels, oysters, and clams are commercially valuable conventional aquaculture organisms as well as having a critical ecological functions in many ecosystems (**Hamed *et al.*, 2010; Ri *et al.*, 2022**). In recent years, there has been a surge in research into the therapeutic properties and nutritional content of bivalves. Molluscs' high nutrient content was reported to have a significant role in strengthening our immune system (**Khan & Liu, 2019**). Bioactivities and nutritional factors of some molluscans retain after pre-treatments and cooking (**Kim & Pallela, 2012**). Bivalves are low-cost proteins and vitamin sources (**Chakraborty & Joy, 2020**). Nutraceuticals and functional foods have become more common in consumers' daily meals to protect from rising lifestyle illnesses. As a result, dietary supplements as part of everyday diets may be able to prevent diseases, leading to a reduction in the need for synthetic drugs with serious side effects (**Intelligence, 2019**).

Marine molluscs have been identified as untapped sources of a variety of antibacterial compounds (**Chakraborty *et al.*, 2016**). Although their pharmaceutical and biomedical properties were not widely recognized, bivalves held the highest position in the ranking of total edible molluscs. Previous research found that bivalves contained a variety of compounds with diverse bioactive properties (**Chakraborty & Joy, 2020**). Previous and ongoing studies have revealed that marine molluscs are not only a valuable food source, but they also hold great promise for the improvement of antibiotic medications to treat microbial infections. Giant clams (Family: Cardiidae, subfamily: Tridacninae) are Indo-Pacific coral reef molluscs found in the Red Sea and the Pacific ocean, where they exist up to 30m deep (**Neo *et al.*, 2017**).

Tridacna and *Hippopus* are the two genera of giant clams; however, only Tridacna is reported in the Red Sea (**Fauvelot *et al.*, 2020**). Many organic compounds found in marine molluscs have promising therapeutic properties. We highlighted the potential antibacterial and antioxidant activities of polyphenol compounds of bivalve molluscs in the present study, and hence inferred their utility as innovative dietary supplements and effective natural food preservatives.

MATERIALS AND METHODS

Samples collection and preparation

Four marine bivalve molluscs species; three giant clams, *Tridacna maxima* (Röding, 1798), *Tridacna gigas* (Linnaeus, 1758), *Tridacna squamosa* (Lamarck, 1819), and (pearl oysters) *Pinctada margritifera* (Linnaeus, 1758) were obtained from the rearing tanks at the aquatic laboratory, National Institute of Oceanography and Fisheries (NIOF) at (27°17'7.80"N, 33°46'19.90"E) during summer 2021 (Fig. 1). *T. maxima* (length: 12-20cm and total weight: 135- 1025g), *T. gigas* (length: 30- 40cm and total weight: 2715- 4885g), *T. squamosa* (length: 14- 25cm and total weight: 450-1095 g), and *P. margritifera* pearl oysters (length: 18-21cm and total weight: 385-1025g) were subject to study. All of the collected species were photographed with the underwater camera (Canon G7 X Mark II) (Fig. 2).

Fig.1. A map showing the collection site

The identification was conducted at NIOF by Dr. Elsayed Abd ElAziz Hamed. Specimens were classified based on morphological characteristics of Tridacninae based on the previous published articles (**Newman & Gomez**, **2000; Lieske & Robert, 2004; Militz *et al.*, 2015**). The samples were cleaned up from the sand and stored in an icebox and rapidly transferred to the laboratory and preserved at –20°C.

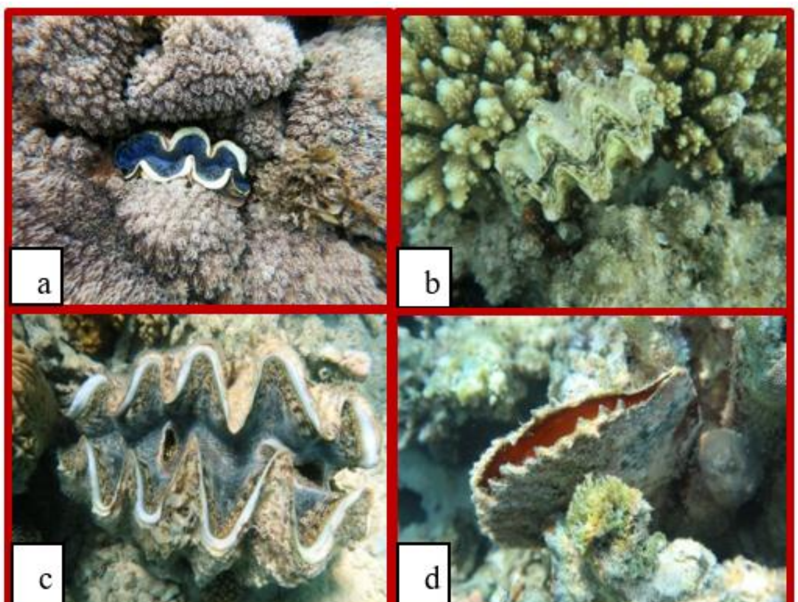

Fig.2. Phtographs of underwater specimens of the giant clams **(a)** *Tridacna maxima*; **(b)** *Tridacna squamosa*; **(c)** *Tridacna gigas*, and **(d)** pearl oysters, *Pinctada margritifera*.

Samples were washed with fresh water; tissues were removed by stripping away the adductor muscle and mantle tissue from shells, dried in shade, cut into small pieces 1-3cm^3 then pulverized in a mixer grinder. Hot water (80°C) was used to extract the compounds from the samples (1:2, w/v) for one hour before separating the filtrate and residue. The extraction has been repeated three times under similar conditions. Lastly, freeze-drying the filtrate yielded powdered bivalve aqueous extracts.

Antibacterial activity and MIC determination

Agar well diffusion test explained by (**Abd El Hafez *et al.*, 2022**, **Kadaikunnan *et al.*, 2015**) was applied to detect the action of the extracts against the reference pathogenic strain *Salmonella enterica* (EMCC1350) achieved from City of Scientific Research and Technological Applications, by using 100 µl of the inoculums (1×10^8 CFU/ml). Using descending concentrations, the minimum inhibitory concentration (MIC) of bivalve extract toward the reference strain was established. In sterile saline, growing culture suspensions of the reference pathogenic strain were prepared and adapted to a concentration of 10^6 cells/mL. The reference pathogenic strain suspension was inoculated onto a plate of nutrient agar (Oxoid, UK) and were left to dry for 15 min at room temperature. The bivalve extract was serially diluted, and 100 µl of each dilution was independently applied to each well. The plates were incubated for 24 h at 37°C before the MIC values were recorded. For each culture bivalve extract, the test was performed in triplicate (**Hamad *et al.*, 2020**).

Determination of total phenolic contents

The total phenolic content was determined using the Folin-Ciocalteu reagent following **Hamad *et al.*** (**2015**) using gallic acid as a reference. Each sample was evaluated in three replicate.

Determination of total flavonoid contents

The total flavonoid contents were determined using a colorimetric method described by **Sakanaka *et al.* (2005**).

Antioxidant potentials and DPPH radical scavenging activity

The extracts' free radical scavenging activity was determined using the DPPH method described by **Brand-Williams *et al.* (1995**). The percentage of DPPH radical-scavenging activity was calculated using the following equation:

$$DPPH\ radical\ scavenging\ activity\ (\%\ inhibition) = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

HPLC analysis of *Tridacna squamosa* extract

The phenolic profile of *Tridacna squamosa* aqueous extract was screened using an HPLC (Agilent 1260 series). Eclipse C18 column with (4.6 mm × 250 mm i.d., 5 µm) was used and its temperature kept constant at 40 °C. The mobile phase was water (A) and 0.05% trifluoroacetic acid in acetonitrile (B) and the volume of injection was 5 µl. The flow rate was 0.9 ml/min and at 280 nm, the multi-wavelength detector was monitored.

Cytotoxicity assay

In a 96-well tissue culture plate, the IC_{50} of molluscs extract on peripheral blood mononuclear cells (PBMCs) was defined using the neutral red cytotoxicity assay described by (**Panda and Ravindran**, **2013**, **Repetto *et al.*, 2008**). Extract concentrations of 625, 312.5, 156.25, 78.12, 39, and 19.5 µg/ml were obtained using double-fold serial dilutions. The cells in the wells have been individually treated with various concentrations of extract, 150 µl in every well except for the blank (**Hamad *et al.*, 2022**).

The following formula was utilized to determine the percentage of cytotoxicity (inhibition):

$$\% \text{ Inhibition} = \frac{\text{O.D Control} - \text{O.D Treatment}}{\text{O.D Control}}$$

Statistical analysis

All data collected were analyzed using one-way (ANOVA) by SPSS, (n=3) and (p < 0.05) revealed a significant difference. Significant means were compared by Duncan's post hoc multiple comparison test.

RESULTS

Antibacterial activity and MIC determination

Antibacterial activities expressed as inhibition zone diameters of bivalve aqueous extracts against the tested bacterial strain *Salmonella enterica* are shown in Fig. (3) and Table (1). The antibacterial effect of *Tridacna squamosa* (T.sq) aqueous extract was found to be superior to those of other bivalve extracts in the experiment. The *Tridacna squamosa* showed an inhibition zone diameter value of 20±1.25 mm against *Salmonella enterica*.

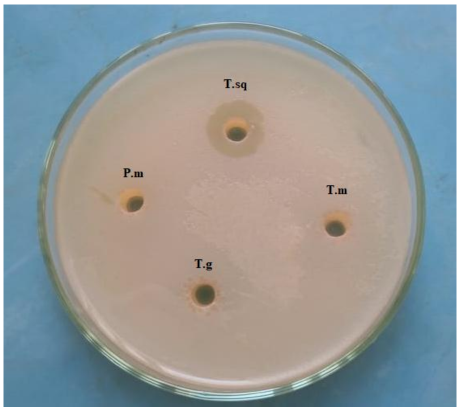

Fig.3. Inhibition zones of bivalve aqueous extracts against *Salmonella enterica*

The minimum inhibitory concentration (MIC) is an essential factor that assesses microorganism resistance and sensitivity to specific substances. The MIC of *Tridacna squamosa* against the bacterial strain *Salmonella enterica* was 12.5 mg/ml, as shown in Table (2).

Table 1. Antibacterial potentials of bivalve extracts against *Salmonella enterica*.

Bivalve aqueous extracts	**Concentration** (mg/ml)	**Inhibition zone diameter** (mm)
Tridacna maxima (T.m)	100	ND
Tridacna gigas (T.g)	100	ND
Tridacna squamosa (T.sq)	100	20 ±1.25
Pinctada margritifera (P.m)	100	ND

-All results are expressed as the means ± standard deviation; n=3; Concentrations of the extract are in mg/mL.

-Diameter includes 5 mm well diameter.

-ND: Not detected.

Table 2. Minimum Inhibitory Concentration (MIC) of *Tridacna squamosa* against *Salmonella enterica* indicated as a zone of inhibition for each concentration.

	Concentrations of extract (mg/ml)							
	100	**50**	**25**	**12.5**	**6.25**	**3.1**	**1.5**	**MIC**
Tridacna squamosa (T.sq)	20±1.12	15±1.34	11±1.16	8±1.42	ND	ND	ND	12.5

-All results are expressed as the means ± standard deviation; n=3; Concentrations of the extract are in mg/mL.
-ND: Not detected.

Antioxidant activity

Total phenolic contents in *Tridacna squamosa* aqueous extract were 18.5±1.63 mg/g, while total flavonoid contents were 2.67±1.48 mg/g. The DPPH is a significant metric for determining an extract's antioxidant activity. The IC_{50} is the extract concentration needed to scavenge 50% of the DPPH radicals. The IC_{50} values of *Tridacna squamosa* aqueous extract are presented in (Table 3). The better the antioxidant properties, the smaller the IC_{50} value. According to the results, *Tridacna squamosa* aqueous extract demonstrates antioxidant activity with an IC_{50} value of 58.61±1.02 µg/ml. The IC_{50} of L-ascorbic acid as a positive control was 26.36±1.95 µg/ml.

Table 3. Antioxidant activity of *Tridacna squamosa* extract as measured by DPPH assay

Conc. (µg/ml)	**Ascorbic acid**		***Tridacna squamosa* extract**	
	Inhibition (%)	**IC_{50} (µg/ml)**	**Inhibition (%)**	**IC_{50} (µg/ml)**
10	5.11	**26.36±1.95**	8.82	**58.61±1.02**
20	35.19		16.7	
30	56.89		25.66	
40	80		34.53	
50	89.61		42.65	
60	94.72		51.18	
70	97.20		69.71	
80	98.68		82.26	
90	99.34		93.63	
100	99.67		97.34	

Cytotoxicity assay

The IC_{50} value for inhibiting proliferation in normal PBMC cells is given in Table (4). The results indicated that *Tridacna squamosa* aqueous extract was safe, with IC_{50} value of 199.2±2.24 µg/ml, and could be used as novel and effective food preservatives instead of chemical ones.

HPLC analysis

To identify bioactive compounds in *T. squamosa* aqueous extract, high-performance liquid chromatography (HPLC) has been used. The HPLC chromatogram of standards and phenolic compounds of *T. squamosa* aqueous extract are shown in Table (5) and Fig. (4). The HPLC analysis results confirmed the presence of three polyphenolic compounds when compared to the HPLC standard chromatogram. The most abundant polyphenolic component in *T. squamosa* aqueous extract was discovered to be gallic acid, followed by Chlorogenic acid and methyl gallate at varying amounts.

Table 4. The influence of various concentrations of *Tridacna squamosa* aqueous extract on PBMC viability.

Concentration (µg/ml)	Inhibition %	Viability %
625	58	42
312.5	41	59
156.25	32	68
78.12	21	79
39	9	91
19.5	0	100
IC_{50} = 199.2±2.24 µg/ml		

DISCUSSION

Four marine bivalve molluscs species, three giant clams, *Tridacna maxima*, *Tridacna gigas*, *Tridacna squamosa*, and (pearl oysters) *Pinctada margritifera* were collected from the Red sea and examined for antibacterial activity. *Tridacna squamosa* aqueous extract was found to be the most effective antibacterial agent against the food spoilage pathogenic strain *Salmonella enterica*. Furthermore, the total phenolic and flavonoid contents of *T. squamosa* aqueous extract were determined. In addition, antioxidant activity and HPLC analysis were also assessed.

Table 5. Phenolic compounds of *Tridacna squamosa* extract identified by HPLC

Phenolic compounds	*Tridacna squamosa* aqueous extract (µg/g)
Gallic acid	252.16
Chlorogenic acid	222.64
Methyl gallate	47.35

Peptides, indole alkaloids, chlorinated acetylenes, and glycol proteins have been reported as antimicrobial substances derived from marine molluscs (**Khan and Liu**, **2019**, **Zhong *et al.***, **2013**). Human bacterial infections, fish pathogens, pathogenic fungi, and the biofilm-forming *Micrococus* sp. are highly sensitive to active constituents derived from two marine molluscan species (*Thais tissoti* and *Babylonia spirata*) (**N. Sri Kumaran**, **2011**). Accordingly, substances extracted from “the giant tun” (*Tonna galea*) have been shown to be potent against *Vibrio cholerae* and *A. hydrophila* (**Santhi *et al.***, **2011**). *Babylonia zeylanica* extracts are said to be effective at inhibiting the growth of *Escherichia coli*, *A. hydrophila*, and *Salmonella typhi* (**Santhi *et al.***, **2013**).

In marine molluscs, antimicrobial peptides play an important role in the innate immune response. These antimicrobial peptides have a mass of <10 kDa and respond quickly to invading pathogenic bacteria in the environment (**Boman**, **1995**). Many experiments were conducted to assess the antibacterial effect of marine molluscs.

Antibacterial effect of green mussel (*Perna virdis*) and edible oyster (*Crassostrea madrasensis*) against ten harmful bacteria was described by (**Annamalai *et al.*, 2007**). (**Chellaram *et al.*, 2004**) investigated oyster (*Pteria chinensis*) antibacterial efficacy against 12 human and 10 fish infections. The widespread presence of antimicrobial compounds, particularly antimicrobial peptides (AMPs), in marine species, such as bivalve mollusks, and their intense antibacterial action may be considered a possible source of new treatment medicines (**Rózańska *et al.*, 2012**).

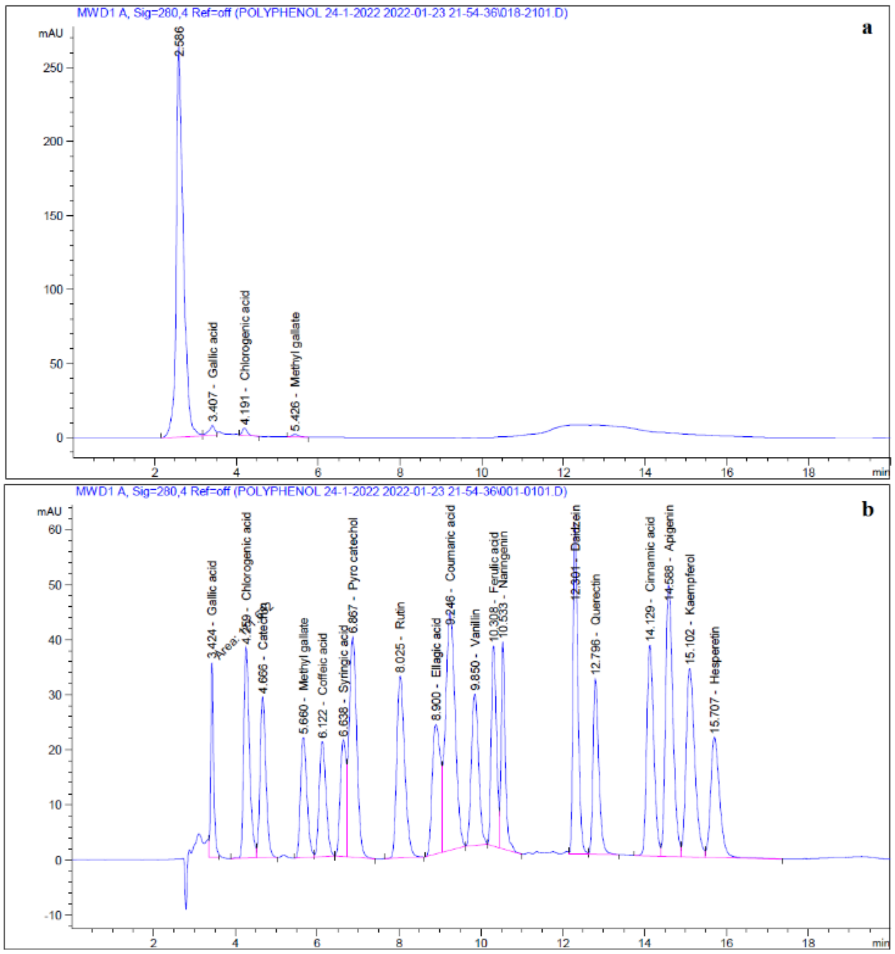

Fig.4. HPLC chromatograms of (a) *Tridacna squamosa* aqueous extract, (b) standards.

Phenolic compounds are key antioxidant and antibacterial agents with numerous benefits for disease prevention and human health. Flavonoids are natural substances with a polyphenolic structure; as a result, they have antibacterial and antioxidant activity and can help prevent illnesses including Alzheimer's, cancer, and atherosclerosis (**Hamad *et al.*, 2015**). TFC's significance comes mostly from its redox characteristics that might account for its antibacterial and antioxidant activities against a variety of microorganisms and free radicals. Furthermore, the action of TFC as an antioxidant agent is closely related to the hydroxyl groups in its structures, which are responsible for scavenging lipid peroxy-radicals, singlet oxygen, superoxide anion, and free radical stabilization (**Hamad *et al.*, 2020**).

The elevated antioxidant potential of *Tridacna squamosa* aqueous extracts might be contributed to the hydrogen donating ability of the extract's phenolic components. Polyphenol antioxidant effects could be related to the hydrogen donating ability of phenoxide ions to free radicals such as DPPH, which could result in chain reaction termination (**Krishnamoorthy *et al.***, **2019**). Most bivalves have the highest antioxidant capacity in gills except for short-necked clams. This may be clarified by the role of the gills, which are subjected to the surroundings and play a significant role in gas exchange, making them the greatest vulnerable tissue to oxidative stress (**Cheng *et al.***, **2019**). (**Tan *et al.***, **2019**) discovered that the antioxidant capacity of the gills of *Chlamys nobilis* was significantly higher than that of the adductor and mantle. Bivalve antioxidant capacity was much higher than that of cuttlefish, shrimp, and gastropods when compared to other invertebrates (**Tan *et al.***, **2021**).

There is currently little known regarding the antioxidant status of molluscs. As a result, the outcomes of this research offer a foundation for the development of powerful antioxidants from *Tridacna squamosa* extracts. By scavenging free radicals and reducing their effects (oxidation) on biological components, antioxidants protect the body from harmful free radicals. Total antioxidant capacity is a broad measure of the total number of antioxidants in organisms that is frequently used to evaluate the antioxidant potential of biological samples (**Tan *et al.***, **2022**).

The current study's findings were consistent with those published by **Mona *et al.*** (**2021**), who discovered that *Cerastoderma glaucum* extracts had little cytotoxicity. Our findings of HPLC analysis agreed with those of **Watanabe *et al.*** (**2012**), who discovered a polyphenolic compound from the edible oyster (*C. gigas*), and determined its antioxidant activity. Furthermore, studies of *C. madrasensis* extract revealed two polyphenolic derivatives possess anti-inflammatory and antioxidant activity (**Chakraborty and Joy**, **2019**).

Polyphenols are essential bivalve components due to their potential health-promoting characteristics. They have the potential to stabilize the unpaired electron and avoid damaging oxidation by scavenging free radicals due to their hydroxyl groups. As a result, the total phenol content of bivalves may directly contribute to their antibacterial and antioxidant effects. Marine molluscs are easily digestible, low in calories, and high in nutrients (**Hamad *et al.***, **2020**). As reported by the Food and Agriculture Organization (FAO), the absorption coefficients of proteins and lipids found in molluscan edible tissue are greater than 0.9, indicating their excellent nutritional value (**Panayotova *et al.***, **2020**). Despite the low lipid content, this category of primary metabolites has so far demonstrated the greatest potential for developing economically relevant functional food and nutritional supplements.

The major polar lipids rich in polyunsaturated fatty acids (PUFA) improve the nutritional value of molluscs. Recent research indicates that bivalves have the potential to be a very good source of antioxidants (phenolic compounds) with antibacterial action (**Khan and Liu**, **2019**, **Panayotova *et al.***, **2020**). The use of molluscan-derived compounds as medicines and functional dietary supplements has recently increased significantly (**Chakraborty and Joy**, **2020**).

CONCLUSION

A limited number of molluscs species have been utilized to extract and identify new bioactive secondary metabolites. Early studies on bivalves' bioactive potential suggested their relevance as a healthy food. The current study provides approximately data on the phytochemical constituents of marine bivalve molluscs *Tridacna squamosa* aqueous extracts for novel antibacterial compounds. Bivalve bioactive compounds are awaiting a significant breakthrough in order to be employed as natural antioxidants in a variety of food products. Moreover, these bivalve extracts have antibacterial activity against bacterial infections, lending credence to their traditional use and implying a future role for these bivalves in combating microbial populations. Molluscs' nutritional and physiological advantages recommended that they could be introduced into our regular diets as natural supplements and preservatives to promote health and immune system functions.

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