Journal of Research in Medical and Dental Science
2021, Volume 9, Issue 1, Page No: 20-26

Available Online at: www.jrmds.in
eISSN No. 2347-2367: pISSN No. 2347-2545

Extraction of Antimicrobial Peptides (AMPs) from *Portunus sanguinolentus* Herbst, *Perna viridis* Linneaus and *Octopus indicus* Orbigny: Identifying the best Solvent for AMP Recovery and Determining its Anti-*ESKAPE* Activity

Muhammad Musthafa Poyil[1]*, MD Nadeem Bari[1], Sanjay Prasad S[2], Mamoun A Alfaki[1]

[1]Department of Basic Medical Sciences, College of Medicine, Prince Sattam bin Abdulaziz University, Al-Kharj, 11942, Saudi Arabia

[2]Department of Microbiology, CMS College of Science and Commerce, Coimbatore, Tamilnadu, India

ABSTRACT

The indiscriminate overuse and misuse of antibiotics worldwide has resulted in high rates of antimicrobial resistance, raising new human health challenges WHO has reported antimicrobial resistance (AMR) as one of the emerging world crises. High level of drug resistance was reported in ESKAPE pathogens which cause most infections within the hospital environment. Marine organisms' synthesis antimicrobial peptides (AMP) for itself defence against several pathogenic microorganisms. Thus the present study focuses on extraction of AMP from three marine invertebrates Portunus sanguinolentus Herbst (crab), Perna viridis Linneaus (Mussel) and Octopus indicus Orbigny and determining its anti-ESKAPE activity against Escherichia coli, Enterococcus faecalis, Staphylococcus aureus, Klebsiella pneumonia, Pseudomonas aeruginosa and Acinetobacter baumanni. The best solvents among Ethanol, Methanol, Acetone and Petroleum ether for AMP recovery was evaluated. Crude extracts were purified using ion-exchange chromatography. Acetone showed higher AMP recovery on Portunus sanguinolentus Herbst (crab); and petroleum ether showed higher AMP recovery on Perna viridis Linneaus (Mussel) and Octopus indicus Orbigny. Antibacterial activity was found to be higher on purified extracts than the crude extracts. AMP from Mussel (Perna viridis Linneaus) showed broad range and higher anti-ESKAPE activity than the other two species. Therefore, AMP from Perna viridis Linneaus can be further used for development of antibacterial drugs for treatment of MDR pathogenic infections.

Key words: Antimicrobial resistance, Antimicrobial peptides, Marine invertebrates, ESKAPE pathogens, Solvent recovery

HOW TO CITE THIS ARTICLE: Muhammad Musthafa Poyil, Mohammed Nadeem Bari, Sanjay Prasad S, Mamoun A Alfaki, Extraction of Antimicrobial Peptides (AMP) from *Portunus Sanguinolentus* Herbst *Perna Viridis Linneaus* and Octopus Indicus Orbigny Identifying the best Solvent for AMP Recovery and Determining its Anti Eskape Activity, J Res Med Dent Sci, 2021, 9 (1): 20-26.

Corresponding author: Muhammad Musthafa Poyil

e-mail✉: pmusthu@gmail.com

Received: 13/11/2020

Accepted: 08/12/2020

INTRODUCTION

The indiscriminate overuse and misuse of antibiotics worldwide has resulted in high rates of antimicrobial resistance, raising new human health challenges. Therefore, infectious illnesses and a dramatic rise in pathogenic multidrug-resistant (MDR) bacteria, resistant to readily available antibiotics, are facing a worldwide re-emergence, threatening the planet with a return to the pre-antibiotic period. The widespread antibiotic resistance observed is now a major public health issue, with medical researchers warning of a return to the pre antibiotic era, whether it is population or hospital-acquired illness due to Vancomycin Intermediate Staphylococcus aureus (VISA), Vancomycin Resistant *Enterococci* (VRE), Methicillin Resistant *S. aureus* (MRSA) or ESBL (β-lactamase extended spectrum) enzymes that generate Gram-negative bacteria. Therefore, there were no more effective antimicrobials available that can cure nearly all bacterial infections. Occurrence of defense among enteric pathogens to many antibiotics *Pseudomonas aeruginosa*, *Streptococcus pneumonia, S. aureus* and *Mycobacterium tuberculosis* shook this optimism further [1].

A high range of antimicrobial resistance has been discovered as well in *Enterococcus faecium,*

S. aureus, Acinetobacter baumannii, Klebsiella pneumoniae, P. aeruginosa and *Enterobacter spp*.–together referred by the acronym ESKAPE which causes the major class of hospital acquired infections. Enzymatic inactivation, target alteration alteration, alteration of cell permeability by loss of porin or increase of flow pump expression, and biofilm formation provides mechanical protection which provide a broad variety of antimicrobial resistance (AMR) operation used by ESKAPE pathogens. Antimicrobial resistance worldwide is the key concern for these pathogenic species in public systems and is likely to intensify as resistance profiles shift. Antimicrobial resistance (AMR) has been declared an emerging disease in the world by the World Health Organization (WHO) and new antimicrobials should be formulated to combat AMR [2].

More than 70% of the aquatic ecosystem of the planet is associated with such chemical uncertainty that it offers a huge opportunity for new therapeutic agents to be explored. Approximately 106 bacteria and 109 viruses constitute the marine ecosystem and, thus, represent an abundant source of pathogens. Marine Microorganisms thrive close to pathogenic microbes, so they need a strong and efficient immune system for survival in extreme environments. AMPs are the first line of defense against invading microbes. It has been shown that marine AMPs are structurally different from the analogues derived from their terrestrial species, as well as from current novel systems. In addition, the antimicrobial activity of AMPs depends on their initial electrostatic interaction with the bacteria's negatively charged surface; thus, free ions produced by high salt concentrations, typical of some diseases, can effectively reduce interaction and microbicidal activity in the surrounding medium. Marine AMPs have evolved to react to the high salt content in seawater, and this has likely been achieved by replacing arginine with lysine [3].

However, many bioactive compounds from different classes of marine species have been discovered. Tunicates, sponges, sea hares, nudibranchs, bryozoans, sea slugs, and marine microorganisms are considered to be marine animals. Cephalopods include squids, cuttlefishes, octopuses and nautilus. Several bioactive molecules have been obtained from cephalopods; molecules which are linked to their survival or are part of their defence mechanisms [4]. Therefore the present study concentrates on extraction of AMP from three marine invertebrates *Portunus sanguinolentus* Herbst (crab), *Perna viridis Linneaus* (Mussel) and *Octopus indicus* Orbigny and determining its anti-ESKAPE activity against *Escherichia coli*, *Enterococcus faecalis, Staphylococcus aureus, Klebsiella pneumonia, Pseudomonas aeruginosa* and *Acinetobacter baumanni*. The best solvents among Ethanol, Methanol, Acetone and Petroleum ether for AMP recovery was also evaluated.

MATERIALS AND METHODS

Procurement of marine invertibrates and Identification

Live specimens of *Portunus sanguinolentus Herbst, Perna viridis Linneaus* and *Octopus indicus* Orbigny were collected from *Puthiyappa* Fishing, Harbor, Kozhikode-Arabian Sea, West Coast of India. They were identified by the experts at the Calicut Centre of Marine Fisheries Research Institute (CMFRI) and confirmed at Zoological Survey of India, Kozhikode, Kerala (Figures 1-3).

Extraction of Antimicrobial peptides and purification using ion-exchange chromatography

20 g of tissue samples were collected from each of the marine invertebrates. The samples were homogenized (Waring blender, Waring, New Hartford, CT, USA) in 200 ml of four solvents (Ethanol, Methanol, Acetone and Petroleum ether) separately (Figure 4). The resulting homogenates were filtered using nylon cloth to remove large exoskeleton debris and subjected to centrifugation at 20,000 rpm for 30 min at

Figure 1: ***Portunus sanguinolentus*** **Herbst.**

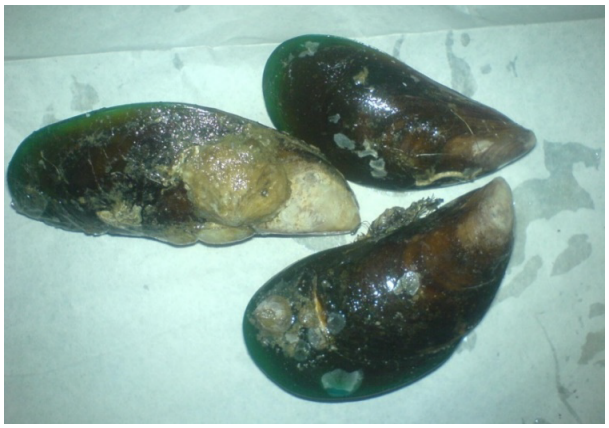

Figure 2: ***Perna viridis Linneaus.***

Figure 3: ***Octopus indicus*** **Orbigny.**

Figure 4: Homogenized tissue samples using solvent.

4°C (Figure 5). The supernatant was collected, and the extraction solvents was evaporated to dryness under reduced pressure until residues emerged [5]. The crude extracts were purified by ion-exchange chromatography. Each fraction was collected separately in a test tube and numbered consecutively for further analyses (3 different fractions were collected).

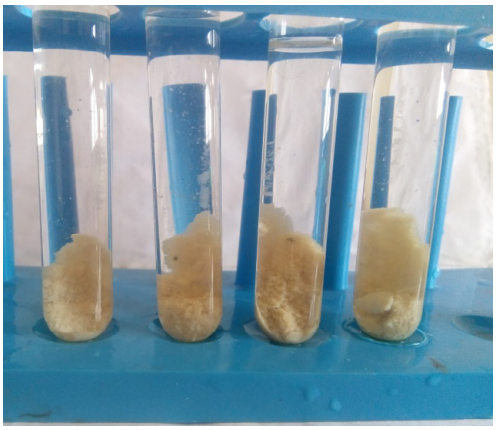

Figure 5: Centrifugation.

Determining the anti-ESKAPE activity of the crude and purified extracts

Anti-ESKAPE operations of crude and purified extracts were performed by standard Kirby-Bauer method. 25 ml of each of the crude and purified extracts were loaded on a sterile filter paper disc of 6 mm diameter and air dried at room temperature. The Muller-Hinton broth was used for the cultivation of *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, Proteus vulgaris* and *Escherichia coli* and *Muller-Hinton* agar were used for plating. The impregnated discs were placed on the inoculated plates and incubated for 24 hrs at 37ºC. The inhibitory zones were measured in mm [6].

RESULTS AND DISCUSSION

Anti-ESKAPE activity of crude extracts from different solvents

All the living organism's synthesis antimicrobial peptides (AMP) for itself defence against several pathogenic microorganisms in the surrounding environment. Three marine invertebrates *Portunus sanguinolentus* Herbst (crab), *Perna viridis Linneaus* (Mussel) and *Octopus indicus* Orbigny were used to extract the AMP against ESKAPE pathogens. Four different solvents (Ethanol, Methanol, Acdetone and Petroleum ether) were used to determine the AMP recovery and to identify the significant solvent for extraction. ESKPAE pathogens used in the study were *Escherichia coli, Enterococcus faecalis, Staphylococcus aureus, Klebsiella pneumonia, Pseudomonas aeruginosa* and *Acinetobacter baumanni*; and the ATCC strain identification number were found to be 13706, 49532, 33592, 13883, 15442 and 19606.

Crude extracts of AMP from crab (*Portunus*

sanguinolentus Herbst) showed inhibitory zones ranging from 8mm to 14 mm. Ethanolic, methanolic, acetone and petroleum ether extracts showed 8 mm, 12 mm, 10 mm and 8 mm against *Escherichia coli*; no inhibitory zones were observed against *Enterococcus faecalis*; 0 mm, 14 mm, 10 mm and 12 mm zones were observed against *Staphylococcus aureus*; 9 mm, 12 mm, 9 mm and 13 mm against *Klebsiella pneumonia*; 7 mm, 11 mm, 8 mm and 12 mm against *Pseudomonas aeruginosa*; and 8 mm, 0 mm, 10 mm and 10 mm against *Acinetobacter baumanni*. Acetone extracts showed higher AMP recovery from crab than the other solvents (Table 1).

Crude extracts of AMP from octopus (*Octopus indicus* Orbigny) showed inhibitory zones ranging from 10mm to 16mm. Ethanolic, methanolic, acetone and petroleum ether extracts showed 0mm, 0mm, 13mm and 12mm against Escherichia coli; no inhibitory zones were observed against *Enterococcus faecalis*; 0mm, 0mm, 14 mm and 14 mm zones were observed against *Staphylococcus aureus*; 0mm, 0mm, 15 mm and 16 mm against *Klebsiella pneumonia*; no inhibitory zones against *Pseudomonas aeruginosa*; and 0 mm, 0 mm, 0 mm and 10 mm against *Acinetobacter baumanni*. Petroleum ether extracts showed higher AMP recovery from octopus than the other solvents (Table 2).

Crude extracts of AMP from Mussel *(Perna viridis Linneaus*) showed inhibitory zones ranging from 10mm to 15 mm. Ethanolic, methanolic, acetone and petroleum ether extracts showed 12 mm, 12 mm, 12 mm and 14 mm against Escherichia coli; no inhibitory zones were observed against *Enterococcus faecalis*; 12 mm, 14 mm, 14 mm and 14 mm zones were observed against *Staphylococcus aureus*; 8 mm, 9 mm, 10 mm and 15 mm against *Klebsiella pneumonia*; no zones against *Pseudomonas aeruginosa*; and 11 mm, 11 mm, 10 mm and 12 mm against *Acinetobacter baumanni*. Petroleum ether extracts showed higher AMP recovery from crab than the other solvents (Table 3).

Anti-ESKAPE activity of purified extracts

Crude extracts were purified using column chromatography technique. Total of three fractions were collected from the purification process. From the three fractions 2nd fraction showed higher AMP concentration than the other two fractions. Therefore, 2nd fraction is used for antibacterial analysis.

Table 1: Anti-ESKAPE activity of Crab (*Portunus sanguinolentus* Herbst) extracts.

S. No	Pathogen	ATCC strain no.	Zone of inhibition (mm) at different solvents			
			Ethanol	Methanol	Acetone	Petroleum ether
1	*E. coli*	13706	8	12	10	8
2	*E. faecalis*	49532	0	0	0	0
3	*S. aureus*	33592	0	14	10	12
4	*K. pneumoniae*	13883	9	12	9	13
5	*P. aeruginosa*	15442	7	11	8	12
6	*A. baumanni*	19606	8	0	10	10

Table 2: Anti-ESKAPE activity of octopus (*Octopus indicus Orbigny*) extracts.

S. No	Pathogen	ATCC strain no.	Zone of inhibition (mm) at different solvents			
			Ethanol	Methanol	Acetone	Petroleum ether
1	*E. coli*	13706	0	0	13	12
2	*E. faecalis*	49532	0	0	0	0
3	*S. aureus*	33592	0	0	14	14
4	*K. pneumoniae*	13883	0	0	15	16
5	*P. aeruginosa*	15442	0	0	0	0
6	*A. baumanni*	19606	0	0	0	10

Table 3: Anti-ESKAPE activity of Mussel (*Perna viridis Linneaus*) extracts.

S. No	Pathogen	ATCC strain no.	Zone of inhibition (mm) at different solvents			
			Ethanol	Methanol	Acetone	Petroleum ether
1	*E. coli*	13706	12	12	12	14
2	*E. faecalis*	49532	0	0	0	0
3	*S. aureus*	33592	12	14	14	14
4	*K. pneumoniae*	13883	8	9	10	15
5	*P. aeruginosa*	15442	0	0	0	0
6	*A. baumanni*	19606	11	11	10	12

Purified extracts of *Portunus sanguinolentus* Herbst showed inhibitory zones higher than the crude extracts. The zone of inhibition against Escherichia coli was found to be 14 mm, no zone against *Enterococcus faecalis*, 12 mm against *Staphylococcus aureus*, 14 mm against *Klebsiella pneumonia*, 10 mm against *Pseudomonas aeruginosa* and 12 mm against *Acinetobacter baumanni* (Table 4).

Purified extracts of *Octopus indicus* Orbigny showed inhibitory zones higher than the crude extracts. The zone of inhibition against *Escherichia coli* was found to be 14 mm, no zone against Enterococcus faecalis, 16 mm against Staphylococcus aureus, 19 mm against Klebsiella pneumonia, no zones against *Pseudomonas aeruginosa* and 12 mm against *Acinetobacter baumanni* (Table 5).

Purified extracts of *Perna viridis Linneaus* showed inhibitory zones higher than the crude extracts. The zone of inhibition against *Escherichia coli* was found to be 20 mm, no zone against Enterococcus faecalis, 15 mm against Staphylococcus aureus, 18 mm against Klebsiella pneumonia, 12 mm against *Pseudomonas aeruginosa* and 14 mm against *Acinetobacter baumanni* (Table 6).

Table 4: Anti-ESKAPE activity of Crab (*Portunus sanguinolentus* Herbst) extracts.

S. No	Pathogen	ATCC strain no.	Zone of inhibition (mm)
1	*E. coli*	13706	14
2	*E. faecalis*	49532	0
3	*S. aureus*	33592	12
4	*K. pneumoniae*	13883	14
5	*P. aeruginosa*	15442	10
6	*A. baumanni*	19606	12

Table 5: Anti-ESKAPE activity of octopus (*Octopus indicus Orbigny*) extracts.

S. No	Pathogen	ATCC strain no.	Zone of inhibition (mm)
1	*E. coli*	13706	14
2	*E. faecalis*	49532	0
3	*S. aureus*	33592	16
4	*K. pneumoniae*	13883	19
5	*P. aeruginosa*	15442	0
6	*A. baumanni*	19606	12

Table 6: Anti-ESKAPE activity of Mussel (*Perna viridis Linneaus*) extracts.

S. No	Pathogen	ATCC strain no.	Zone of inhibition (mm)
1	*E. coli*	13706	20
2	*E. faecalis*	49532	0
3	*S. aureus*	33592	15
4	*K. pneumoniae*	13883	18
5	*P. aeruginosa*	15442	12
6	*A. baumanni*	19606	14

DISCUSSION

Nearly 80% of the world's biota is aquatic life and is the source of specific natural products used as food,fragrances,pigments,insecticides,medicinal products, etc. Around 10,000 pharmacologically bioactive chemicals, such as tunicates, sponges, soft corals, sea hares, nudibranches, bryozoans, sea slugs and other marine species, have been extracted from marine invertebrates. There are antibiotic, anti-parasitic, antiviral and anti-cancer activities in secondary metabolites derived from the number of marine animals. The first natural product of marine origin reported in literature is the Tyrian purple, an ancient dye pigment. Many mollusks produce mucus in the mantle cavity, e.g. Muricidal gastropods (rock snails) which defend against microbial infection by developing larvae [7]. In addition to the large variety of bioactive metabolites known to occur in the *Helicona Petrosia* and *Discodema* sponge genera, which serve as potent anti-cancer and anti-inflammatory agents. In neurophysiological and neuropharmacological research, marine toxins such as tetrodoxin, soxitoxin, ciguatoxin and brachiotoxin act as unique sodium channel blockers [8].

Marine molluscs are a good source of bioactive metabolites for marine invertebrates. There is anti-tumor, anti-leukemic, antibacterial and antiviral properties of bioactive compounds isolated from several groups of molluscs. Inherent immune mechanisms affecting both humoral and cellular responses depend solely on marine invertebrates. Antimicrobial agents found in marine invertebrates are distinguished by humoral immunity in the cells and plasma of the blood. Cellular immunity is dependent on cell defence reactions in marine invertebrates, including encapsulation, nodule formation, and phagocytosis. Hemocytes (blood cells) of motile cells that phagocytize microbes and secrete soluble antimicrobial and cytotoxic substances into hemolymph (circulatory fluid of invertebrates comparable to blood) [9] mediate the cellular portion of marine invertebrate immunity.

In all invertebrates, peptides are essential biochemical constituents and have gained great attention due to their potential bioactive and functional properties. Various peptides derived from marine proteins have been described in

this context as having antimicrobial activity [10]. This study focuses on the detection and isolation of antimicrobial peptides (AMP) from three marine invertebrates: *Portunus sanguinolentus* Herbst (crab), *Perna viridis Linneaus* (mussel) and *Octopus indicus* Orbigny. Experts at the Calicut Centre of Marine Fisheries Research Institute (CMFRI) identified the samples and confirmed them at the Zoological Survey of India, Kozhikode, Kerala.

Owing to its low concentration in specific tissues and the presence of proteases and other non-peptidic molecules, AMP extraction from tissue samples is a difficult step in peptide analysis. Peptide analysis using different methods for isolation, purification, and characterization can be highly time-consuming without sufficient sample preparations. The initial recovery of the peptide allows the sample to be homogenised first. Homogenization utilises the use of organic solvents for peptide recovery. There is no single solvent that can remove a full collection of peptides [11] because of unknown deproteinization abilities and inherent limitations. Since several solvents may have different effects on each tissue type, four different solvents (ethanol, methanol, acetone, and petroleum ether) have been used and the significant effects of antibacterial analysis against ESKAPE pathogens have been determined. *Escherichia coli, Enterococcus faecalis, Staphylococcus aureus, Klebsiella pneumonia, Pseudomonas aeruginosa* and *Acinetobacter baumanni* were the ESKAPE pathogens used in the study, and the ATCC strain identification number was found to be 13706, 49532, 33592, 13883, 15442 and 19606. Acetone showed higher AMP recovery on *Portunus sanguinolentus* Herbst (crab); and petroleum ether showed higher AMP recovery on *Perna viridis Linneaus* (Mussel) and *Octopus indicus* Orbigny.

Crude extracts were purified using column chromatography technique. Total of three fractions were collected from the purification process. From the three fractions 2nd fraction showed higher AMP concentration than the other two fractions. Therefore, 2nd fraction is used for antibacterial analysis. Purified extracts showed higher antibacterial activity than the crude extracts. AMP from Mussel (*Perna viridis Linneaus*) showed broad range and higher anti-ESKAPE activity than the other two species.

CONCLUSION

Live specimens of *Portunus sanguinolentus* Herbst, *Perna viridis Linneaus* and *Octopus indicus Orbigny* were collected from Puthiyappa Fishing, Harbor, Kozhikode-Arabian Sea, West Coast of India. They were identified by the experts at the Calicut Centre of Marine Fisheries Research Institute (CMFRI) and confirmed at Zoological Survey of India, Kozhikode, Kerala. 20 g of tissue samples were collected from each of the marine invertebrates. The samples were homogenized using four solvents (Ethanol, Methanol, Acetone and Petroleum ether) separately and purified using ion-exchange chromatography. The Anti-ESKAPE activity of crude and purified extracts were performed by standard disc diffusion method. ESKPAE pathogens used in the study were *Escherichia coli, Enterococcus faecalis, Staphylococcus aureus, Klebsiella pneumonia, Pseudomonas aeruginosa* and *Acinetobacter baumanni*; and the ATCC strain identification number were found to be 13706, 49532, 33592, 13883, 15442 and 19606. Acetone showed higher AMP recovery on *Portunus sanguinolentus* Herbst (crab); and petroleum ether showed higher AMP recovery on *Perna viridis Linneaus* (Mussel) and *Octopus indicus* Orbigny. Antibacterial activity was found to be higher on purified extracts than the crude extracts. AMP from Mussel (Perna viridis Linneaus) showed broad range and higher anti-ESKAPE activity than the other two species. Therefore, AMP from *Perna viridis Linneaus* can be further used for development of antibacterial drugs for treatment of MDR pathogenic infections.

ACKNOWLEDGEMENT

The authors are grateful to the Prince Sattam bin Abdulaziz University, Al-Kharj, Saudi Arabia for its support and encouragement in conducting the research and publishing this report.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

REFERENCES

1. Reygaert WC. An overview of the antimicrobial resistance mechanisms of bacteria. AIMS Microbiol 2018; 4:482.
2. Neu HC. The crisis in antibiotic resistance. Science

1992; 257:1064-1073.

3. Khan AN SG. Extraction and screening of bioactive compounds with antimicrobial properties from selected species of mollusk and crustacean. J Clin Cell Immunol 2014; 5:1-5.
4. Pushpanathan K, Es B, Sasidharan RS. Characterization of a bioactive protein with antimicrobial property from characterization of a bioactive protein with antimicrobial property from loligo sp . Fishery Technol 2014; 51.
5. Mohtar JA, Yusof F, Ali NMH. Screening of novel acidified solvents for maximal antimicrobial peptide extraction from Zophobas morio fabricius. Adv Environ Biol 2014; 8:803–809.
6. Duval E, Zatylny C, Laurencin M, et al. KKKKPLFGLFFGLF: A cationic peptide designed to exert antibacterial activity. Peptides 2009; 30:1608-1612.
7. Simmons TL, Andrianasolo E, McPhail K, et al. Marine natural products as anticancer drugs. Mol Cancer Ther 2005; 4:333-342.
8. Benkendorff K, McIver CM, Abbott CA. Bioactivity of the murex homeopathic remedy and of extracts from an australian muricid mollusc against human cancer cells. Evidence-Based Complement Altern Med 2011; 2011.
9. Pérez MJ, Falqué E, Domínguez H. Antimicrobial action of compounds from marine seaweed. Mar Drugs 2016; 14:52.
10. Ngo DH, Wijesekara I, Vo TS, et al. Marine food-derived functional ingredients as potential antioxidants in the food industry: An overview. Food Res Int 2011; 44:523-529.
11. Khan AN SG. Extraction and screening of bioactive compounds with antimicrobial properties from selected species of mollusk and crustacean. J Clin Cell Immunol 2014; 5:1–5.