



Cytotoxicity, Antibacterial and Antioxidant Activities of the Tissue Extracts of Marine Gastropod *Hemifusus Pugilinus* (Born, 1778)

Karthiga Arumugasamy and Ravi Cyril*

Department of Zoology, Thiagarajar College, Madurai, Tamil Nadu, India

ABSTRACT

The objective of the present study is to examine the antibacterial, antioxidant and anticancer activity of acetone and methanol tissue extracts of the marine gastropod *Hemifusus pugilinus*. Solvent extracts of *H. pugilinus* were tested for their inhibitory action against *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus* and *Streptococcus sp.* by agar well diffusion method. Maximum zone of inhibition was exerted by methanol extract against *S. typhi* (21.36 ± 1.44 mm) at $150 \mu\text{g/mL}$ concentration. Antioxidant potential of extracts of *H. pugilinus* was evaluated using free radicals of 1,1-diphenyl-2-picrylhydrazyl, nitric oxide, superoxide anion and hydrogen peroxide. Methanol extract exhibited more efficient radical scavenging activity with IC_{50} concentration of $74.73 \mu\text{g/mL}$ towards nitric oxide. The cytotoxicity of the tissue extract was tested against MDA-MB-231 breast cancer cell line by MTT assay. Minimum cancer cell viability ($20.08 \pm 2.83\%$) was observed in methanol extract at $100 \mu\text{g/mL}$ concentration. The tissue extracts exhibited statistically significant ($p < 0.05$) activity in all the bioassays. However, the standard drug exerted better activity than the tissue extracts. Preliminary qualitative analysis revealed that the crude extract has alkaloids, phenol, flavonoid, terpenoid, glycoside, sterols and fatty acids. From the present study, it has been concluded that tissue extract of *H. pugilinus* possess drug like bioactive compounds.

Keywords: Marine mollusk; Agar well diffusion; Free radical scavenging; Cytotoxicity; Bioactive compounds

INTRODUCTION

Several pathogenic bacteria developed resistance against the commonly used antibiotics and there is urgent need to discover safe drug leads to combat the antibiotic resistance [1,2]. Marine natural products have provided novel chemical scaffolds to pharmaceutical industry and researchers are inspired by the diversity of secondary metabolites harbored in marine organisms [3,4]. Mollusca, the second largest phylum in the animal kingdom play a significant ecological role and very recently, received attention for deriving drugs [5]. In molluscs, the physical defense system has not well developed hence utilize the immunologically active antimicrobial peptides and bioactive compounds for their immune response. Haemolymph, digestive gland, egg mass and mucus membrane are some of the tissue parts of molluscs that have the potent immune molecules, but the mode of action of these chemical groups are still unknown [6]. Few studies have emphasized the therapeutic value of mollusc [7] and only narrow range (<1%) of secondary metabolites have been explored for bioactivity profiling [8].

The bioactive compounds isolated from molluscs exhibit anti-inflammatory and antitumor activities [9] which prevent free radical oxidation process that causes cell damage, cancer and degenerative diseases [10]. In recent years, pharmaceutical industry encourages lead compounds from marine organisms thus paved the way for more collaborative efforts between academia and pharmaceutical industry to translate the natural products into clinical trials [3]. However, the biologically active products from marine molluscs are largely unexplored in India [11]. The organism chosen for the present study *H. pugilinus* is abundant in south Indian coast and consumed for their delicacy and nutritive value [12,13]. Biomedical screening of solvent extract of marine molluscs would provide

valuable base for new drug leads and ultimately used for treatment of chronic diseases [1]. Hence, the present study has been planned to investigate the broad spectrum biological activities such as antibacterial, antioxidant and cytotoxicity potential of tissue extracts of *H. pugilinus*.

EXPERIMENTAL SECTION

Collection of Mollusca and Extraction

Live *H. pugilinus* was collected from Thondi coastal area (lat. 9° 44'10"N; long. 79° 10'12"E), Tamil Nadu, India, during the early morning hours (6.00-8.00 am) with the help of local fishermen. The collected samples were brought to the laboratory in sterile containers and washed thoroughly with running tap water followed by distilled water until the removal of sand debris. After that, the hard shells were broken using a hammer, the obtained soft tissue body was shade dried for 120 h. 50 g dried material was infused with 150 mL acetone taken in a conical flask and kept in a shaker for three days. The acquired acetone extract was collected, filtered through Whatman No.1 filter paper, concentrated under vacuum on rotary evaporator and stored in refrigerator at 4°C for further analysis. Similar methodology was followed for methanol extract preparation also.

Antibacterial Assay

Slant cultures of *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus* and *Streptococcus* sp. were procured from Bose clinical laboratory, Madurai, Tamil Nadu, India. The antibacterial activity of the acetone and methanol extracts of *H. pugilinus* was evaluated through agar well diffusion method [14] with some modifications. The bacterial inoculum was prepared from 12 h broth cultures and the suspensions adjusted according to McFarland No. 0.5 standard. Muller-Hinton agar plates were inoculated with $\sim 5 \times 10^8$ CFU mL⁻¹ of respective bacterium and spread with sterile swabs. Five wells of 6 mm diameter were made on the plates using well borer. Three different concentrations (50, 100 and 150 µg/mL) of the extracts were prepared by dissolving in dimethyl sulfoxide (DMSO). The wells were loaded (25 µL) with different concentrations of the extracts, standard drug gentamycin (positive control) and DMSO (negative control) and incubated at 37°C for 24 h. After that, the inhibition zone produced if any was measured (using graph sheet) and expressed in mm. Three replications were made for each category.

Antioxidant Assay

DPPH assay:

Radical scavenging activity of *H. pugilinus* extracts against stable 1-1-diphenyl-2-picryl-hydrazyl (DPPH) was determined as per [15] with slight modification. Any antioxidants that can donate hydrogen will react with DPPH and reduce it. Hence, DPPH was used to evaluate the effectiveness of antioxidants. Freshly prepared 4 mL methanolic solution of DPPH (6.34 µM) was added with 1 mL of solvent extract of different concentrations (25, 50, 75, 100, 125 µg/mL in DMSO). The reaction mixture was incubated at room temperature under dark for 30 minutes and the absorbance was measured at 515 nm using UV-spectrophotometer (Systronics, 2203). The percentage of DPPH reduction (decolourization) was calculated according to the following formula:

$$\% \text{ Inhibition} = ((A_{\text{Control}} - A_{\text{Sample}}) / A_{\text{Control}}) \times 100$$

Where,

A_{control} = absorbance of the blank control (containing all reagents except the extract solution)

A_{sample} = absorbance of the test sample

Nitric oxide scavenging activity:

The nitric oxide (NO) scavenging activity of tissue extract was determined by the method prescribed in [15] with slight modification. Sodium nitroprusside solution was used to generate nitric oxide radicals. The reaction mixture was prepared by the addition of freshly prepared sodium nitroprusside (1 mL of 10 mM) with 1 mL of crude extract of different concentrations (25-125 µg/mL in phosphate buffer). After 150 min incubation at 25°C, 2 mL Griess reagent (0.05% N-(1-naphthyl) ethylene diamine dihydrochloride, 0.5% sulfanilic acid and 2.5% phosphoric acid) was added to the reaction mixture and the absorbance was measured at 546 nm against blank. The control contained all the reaction reagents except sample and ascorbic acid served as standard control. The percentage inhibition of NO scavenging activity of solvent extract was calculated by the formula described in DPPH assay.

Hydrogen peroxide scavenging activity:

Hydrogen peroxide (H₂O₂) scavenging ability of *H. pugilinus* tissue extracts was determined by the method mentioned in [16] with slight modification. Different concentrations of extracts (25-125 µg/mL in DMSO) were

prepared and 1 ml each concentration of the extract was taken in separate reaction tubes and 0.6 ml freshly prepared H₂O₂ solution (4 mM in phosphate buffer 0.1 M, pH 7.4) was added and incubated for 10 min. The absorbance of the solution was read at 230 nm using UV-spectrophotometer (Systronics, 2203) and ascorbic acid served as reference compound. The percentage inhibition of H₂O₂ scavenging activity of solvent extract was calculated by the formula described in DPPH assay.

Superoxide radical scavenging activity:

Superoxide radical scavenging activity of *H. pugilinus* tissue extracts was determined by the method stated in [17]. Superoxide radical has been generated by the addition of sodium hydroxide to air saturated DMSO. The stability of the generated superoxide retains in solution and reduces nitroblue tetrazolium (NBT) into formazan dye at room temperature. 0.1 ml NBT (156 µM) was taken in the reaction tube and the reaction was initiated by the addition of 100 µl of phenazine methosulfate (60 µM in phosphate buffer; pH 7.4) solution. Then, 0.3 mL of the extract at various concentrations (25-125 µg/mL in DMSO) was added. After that, the reaction mixture was incubated at 25°C for 5 min and the absorbance was measured at 560 nm against blank using UV-spectrophotometer (Systronics, 2203). The percentage inhibition of superoxide scavenging activity of solvent extract was calculated by the formula described in DPPH assay.

Cytotoxicity Assay

Cell culture:

The human breast cancer cell line MDA-MB-231 was maintained in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were grown in humidified incubator at 37°C in the presence of 5% CO₂. The cells were passaged upon reaching 70-80% confluency.

MTT assay:

The proliferation of the cells or cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay [18]. 100 µL cells at a concentration of 1×10^5 cells/well were seeded onto a 96-well plate and maintained in the nutrient medium (DMEM) for 24 h. After that, crude extract of *H. pugilinus* was added to the wells and incubated for 72 h. 20 µL MTT solution (5mg/mL) was added into each well and incubated for 3 h. Later, the solutions were removed from wells and 100 µL DMSO was added to solubilize the formazan crystals. Finally, the plate was read in ELISA plate reader (Bio-tek, Plate reader) at 570 nm.

Qualitative analysis of crude extract of *H. pugilinus*:

The crude extracts were screened for the different class of secondary metabolites following standard protocols [19,20].

Statistical Analysis

All the experiments were performed in triplicates. The values are expressed as mean \pm standard deviation (n=3). All the data were analyzed using one-way ANOVA followed by Tukey test at p<0.050 significance level. Statistical calculations were worked out using Graph Pad Prism software, Version 5.01, (Graph Pad Software, San Diego, CA, USA).

RESULTS

Antibacterial Assay

The obtained yields of acetone and methanol extracts were 510 mg and 940 mg respectively. The solvent extracts of *H. pugilinus* exhibited notable inhibitory action against the tested pathogens. The zone of inhibition produced by the methanol extract (150 µg/mL) against *E. coli*, *S. typhi*, *S. aureus* and *Streptococcus* sp. were 17.39 ± 0.25 , 21.36 ± 1.44 , 16.26 ± 0.60 and 14.80 ± 0.30 mm respectively and for acetone extract it was 14.23 ± 0.45 , 20.06 ± 0.25 , 15.66 ± 0.60 and 11.3 ± 0.52 mm respectively (Figure 1). The acetone extract failed to inhibit the growth of *Streptococcus* sp. at low concentration (50 µg/mL). Among the two extracts, methanol extract displayed persuasive antibacterial activity than acetone extract against all the tested pathogens.

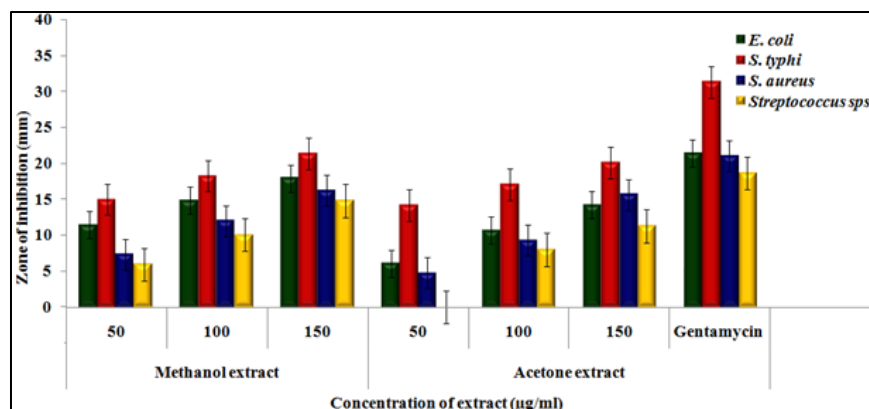


Figure 1: Antibacterial activity of crude extract of *H. pugilinus*, data represented as mean \pm SD (n=3)

Antioxidant Assays

The DPPH scavenging activity of solvent extract at different concentrations has been illustrated in Figure 2. The methanol extract exhibited highest DPPH radical scavenging activity ($77.87 \pm 1.62\%$) at $125 \mu\text{g/mL}$ whereas, $69.27 \pm 0.94\%$ inhibition was observed in the acetone extract at the same concentration. The IC_{50} concentration of DPPH radical scavenging activity of acetone and methanol extract was 95.16 and $79.76 \mu\text{g/mL}$ respectively. The methanol extracts exerted better nitric oxide radical scavenging activity ($75.85 \pm 0.33\%$) than the acetone extract ($72.37 \pm 0.74\%$). The nitric oxide scavenging activity profiles of both the solvent extracts are depicted. The IC_{50} concentration of nitric oxide radical scavenging activity of acetone and methanol extracts were 78.91 and $74.73 \mu\text{g/mL}$ respectively. The hydrogen peroxide scavenging activity of methanol extract ($71.56 \pm 2.16\%$) was higher than acetone extract ($67.24 \pm 1.45\%$). The IC_{50} concentration of hydrogen peroxide radical scavenging activity of acetone and methanol extract was 95.39 and $87.72 \mu\text{g/mL}$ respectively. Further, methanol extract also exerted potent super oxide scavenging activity ($77 \pm 0.51\%$) than acetone extract ($72.45 \pm 1.06\%$). The hydrogen peroxide and super oxide scavenging activity of solvent extracts at different concentrations are depicted in Figure 2. In general, methanol extract demonstrated superior scavenging effect although less potent when compared to the control ascorbic acid. The IC_{50} concentration of super oxide anion radical scavenging activity of acetone and methanol extract was 83.36 and $79.10 \mu\text{g/mL}$ respectively (Table 1). The antioxidant potential of tissue extract was statistically significant by Tukey test at $p < 0.050$ significance level.

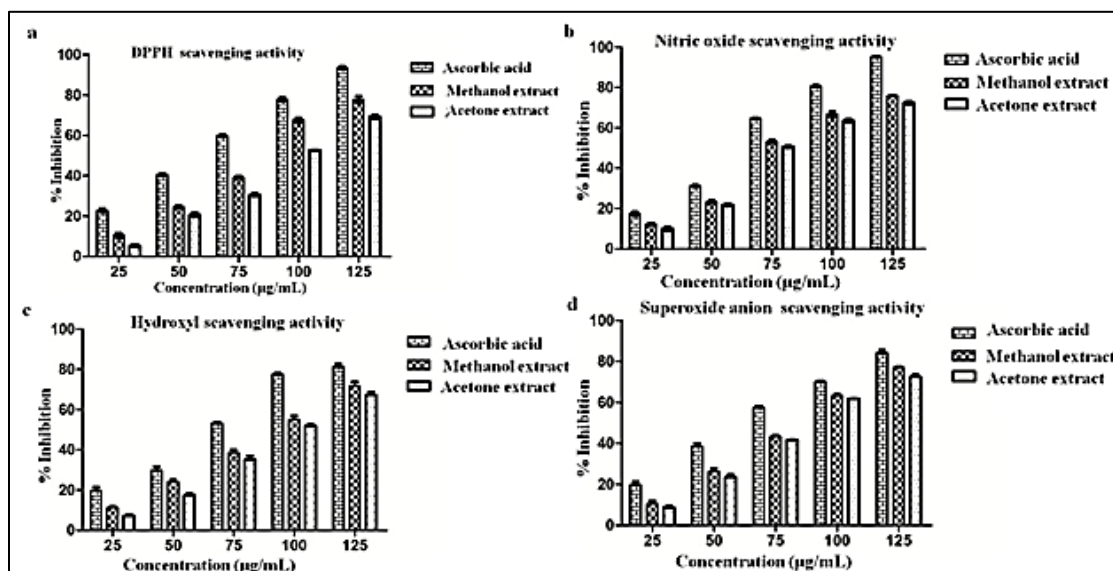


Figure 2: Antioxidant potential of tissue extracts of *H. pugilinus* against various free radicals. Data represented as mean \pm SD (n=3) (a) DPPH radical scavenging activity (P value=0.003) (b) nitric oxide scavenging activity (P value= 0.0001) (c) hydroxyl scavenging activity (P value= 0.0001) (d) superoxide anion scavenging activity (P value= 0.0001), all the data analyzed using one-way ANOVA followed by Tukey test at $P < 0.050$ significance level

Table 1: IC₅₀ concentration of solvent extract of *H. pugilinus* for various anti-oxidant assays

Antioxidant assay	IC ₅₀ Concentration (µg/mL)		
	Positive control (Ascorbic acid)	Methanol extract	Acetone extract
DPPH radical	55.59	79.76	95.16
Nitric oxide radical	60.86	74.73	78.91
Hydroxyl radical	65.74	87.72	95.39
Superoxide radical	60.91	79.1	83.36

#Data represented as Mean ± SD (n=3); statistical significance level at p<0.050 by Tukey test

Cytotoxicity Assay

In MTT assay, acetone and methanol extract of *H. pugilinus* exhibited antiproliferative effect on breast cancer cell line MDA-MB-231. The percentage cell viability of extract treated group was compared with control (100% viability) to calculate the cytotoxic effect. Among the acetone and methanol extract treated group, methanol extract exhibited minimum cell viability ($20.08 \pm 2.83\%$) and significantly suppressed the cell growth (Figures 3 and 4). The results revealed that the tissue extract of *H. pugilinus* has the potential to decrease the viability of cancer cell population (Figure 5).

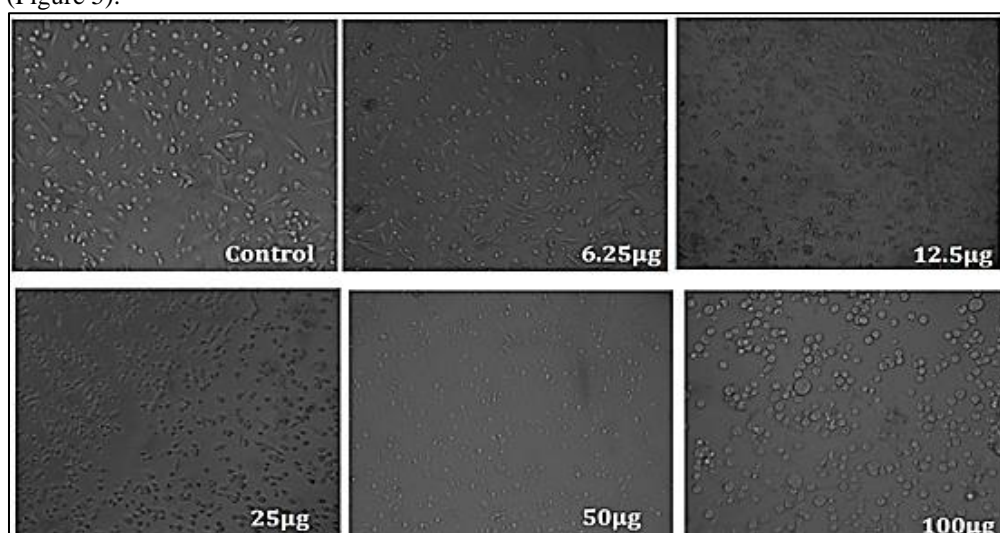


Figure 3: Cytotoxicity of acetone extract of *H. pugilinus* against MDA-MB-231 cell line at different concentrations

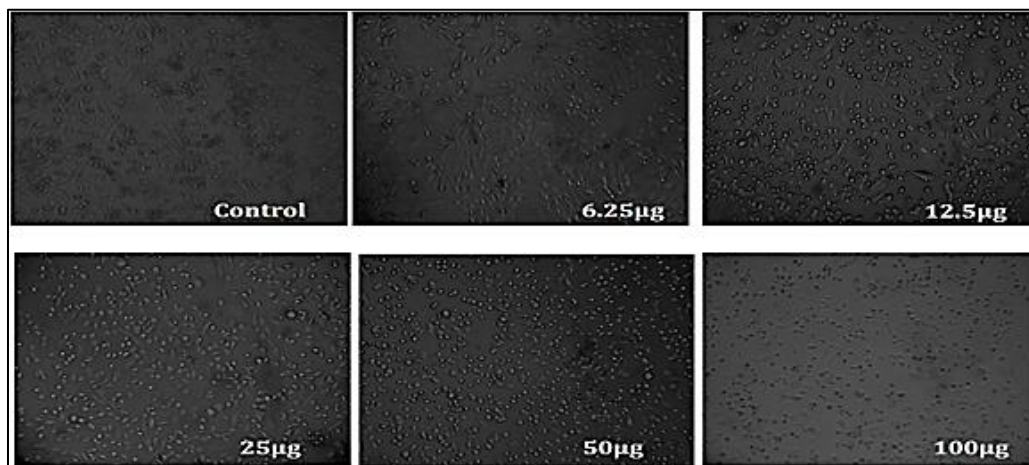


Figure 4: Cytotoxicity of methanol extract of *H. pugilinus* against MDA-MB-231 cell line at different concentrations

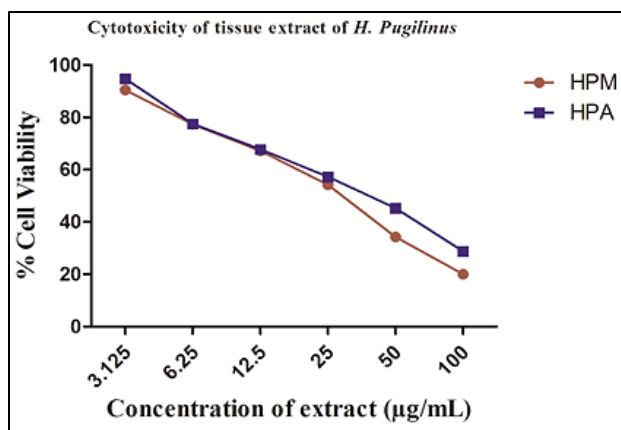


Figure 5: Antiproliferative activity of tissue extracts of *H. pugilinus* on MDA-MB-231 cell lines. HPA; acetone extract of *H. pugilinus*, HPM; methanol extract of *H. pugilinus*, data represented as mean \pm SD (n=3)

Qualitative Analysis

Preliminary qualitative analysis revealed that crude extract of *H. pugilinus* contain the secondary metabolites such as alkaloid, steroidal compounds, phenolic compounds, flavonoids, terpenoids, saponins and glycosides. Anthocyanin and anthraquinones were absent in the crude extracts of *H. pugilinus* (Table 2).

Table 2: Qualitative analysis of crude extract of *H. pugilinus*

Experiment	Observation	Acetone extract	Methanol extract
i)Alkaloids			
a) Mayer's test	Yellow (or) creamy white precipitate	+	+
b) Wagner's test	Brown/reddish	-	-
c) Dragendroff test	Red precipitation	+	+
d) Hager test	Yellow precipitate	+	+
ii)Phenols			
a) Ferric chloride	Bluish green color	+	+
b) Ferric sulphate	Dark violet	-	-
iii)Flavonoids			
a) Ammonium	Yellow color	+	+
b) Aluminium chloride	Yellow color	-	-
c) Alkaline reagent	Yellow color	+	-
d) Lead acetate	Yellow color	+	+
iv)Anthraquinone			
a) Borntrager's test	Pink, red (or) violet layer	-	-
b) Modified borntragers	Rose-pink layer	-	-
v)Anthocyanin			
vi)Terpenoids			
vii)Glycosides			
viii)Sterols			
a) Salkowski's test	Reddish brown	+	+
b) Liebermann-Burchard's test	Brown ring	+	+
xi)Fatty acid			
x)Saponin			

Foot note: "-" absence; "+" Presence

DISCUSSION

In the present study, tissue extracts of the marine gastropod *H. pugilinus* were examined for antibacterial and antioxidant potential which displayed remarkable activity in both the bioassays. In the antibacterial assay performed against *E. coli*, *S. typhi*, *S. aureus* and *Streptococcus* sp., maximum inhibition was exerted by the methanol extract against *S. typhi* (21.36 \pm 1.44). The sRNA, IsrM, sRNA factors found in *Salmonella* play major role in determining the virulence and resistance of the bacteria. During the natural invasion of *Salmonella*, these factors would be over

expressed thereby combat the phagocytosis process and antibiotic toxicity [21]. However, *S. typhi* exhibited less expression of virulence factors in the *in vitro* conditions due to osmolarity, pH and oxygen limitation hence displayed sensitivity pattern against drug treatment [21]. *Streptococcus* sp., exhibited resistance to acetone extract at lower concentration (50 µg/mL). The resistance has been induced by surface secreted proteins such as M proteins, MAC proteins and HA capsule. Detoxification of antibiotics may be caused by SodA, AhpC, GpoA and NoxA enzymes [22]. Further, HtrA, PolA1, PmtA enzymes also involve in enzymatic repair mechanism and protect the strains from antibiotic toxicity [22]. *E. coli* is responsible for various nosocomial infections, of which urinary tract infection is the most predominant. *S. aureus* is the common pathogen associated with the skin infection that subsequently leads to life-threatening septicemia, endocarditis and toxic shock syndrome [23-25]. The pathogens are developing multi drug resistance through various mechanisms such as target modulation, reduced drug concentration and expression of diverse virulence factors of beta-lactamases and metallo-beta-lactamases [25-27]. The activity of crude extract of *H. pugilinus* varied due to several reasons such as permeability barrier provided by the presence of cell wall or presence of enzymes in periplasmic space which are able to reduce the drug activity [28]. The obtained results are also in agreement with the previous reports that methanol extract exhibited high degree of inhibitory activity against various clinical pathogens [29]. Acetone extract of various molluscs also displayed broad spectrum of antibacterial activity against pathogens [5,30]. The solvent extract of *H. pugilinus* exhibited significant inhibition against various bacterial pathogens [12,13] as the tissue extracts are rich in antibacterial lipophilic constituents such as sterol esters, glycerides, free fatty acids, sterol and polar lipids [31,32]. The obtained result affirmed that tissue of *H. pugilinus* has rich source of antimicrobial compounds and could be used for development of new drugs.

The antioxidant activity has been accomplished through various mechanisms such as reductive capacity, radical scavenging activity, and decomposition of peroxides. Free radical oxidation has positive correlation with xenobiotic contamination, reproductive defects and ontogenetic development. Oxidation of free radical has been blocked by several mechanisms such as terminating radical chain reaction, chelating transition metal and stimulating antioxidative enzyme [33]. Based on the IC₅₀ calculation, methanol extract exhibited more efficient radical scavenging activity than acetone extract. Methanol extract of *Littorina littorea* and *Galatea paradoxa* showed potential scavenging activity even at low dosages. Mollusc has adapted themselves in the ever changing environment, oxidative stress and ecological barriers. The defensive chemicals are released to prevent them from free radical scavenging. The methanol extract of molluscs contains phenols and flavonoids that act as potential source of antioxidant agents [1,4]. The qualitative analysis also indicated the presence of phenols and flavonoids in the crude extract of *H. pugilinus*. The feeding habit, environment factors and associated microorganisms of mollusc may contribute to the synthesis of biologically potential secondary metabolites [34].

The morphological feature of cells demonstrated that suppression of cell growth may be caused by apoptosis rather than the inhibition of cell proliferation. The extrinsic apoptosis has been induced by specific trans-membrane receptors such as FAS/CD95 ligand, TNF α and TNFSF10 [35]. The intrinsic apoptosis could be caused by intracellular DNA damage, oxidative stress, cytosolic Ca²⁺ overload and accumulation of unfolded proteins in the endoplasmic reticulum. The process was consequently initiated by cascade signaling of caspases and leads to apoptosis [36]. A previous study reported that crude extract of *Meretrix meretrix* and *Meretrix casta* exhibited potential anticancer activity against hepatoma cell line HepG2. The study revealed that mollusc extract exerted anticancer activity through lactate dehydrogenase (LDH) leakage, depletion of glutathione (GSH), DNA damage and increased the expression of apoptosis stimulating factor such as caspases [37]. In the present study, molecular mechanism of anticancer activity of extract and compounds responsible for cytotoxicity is not clearly known.

The results suggested that methanol could act as efficient solvent to extract the bioactive structurally unique secondary metabolites such as alkaloids, flavonoids and polyphenols [38]. In addition, the mollusc tissue contains proteinaceous metabolites of low molecular weight and short length peptide sequence. In all the biological assays, standard drugs displayed better activity than acetone and methanol extract. In crude extract, the mixture of chemical component may interfere the inhibition mechanism hence the pure fraction of compound could exhibit better activity than crude fraction [4].

CONCLUSION

Acetone and methanol tissue extracts of *H. pugilinus* exerted promising *in vitro* antibacterial, antioxidant activity and cytotoxicity. The analytical chemistry and molecular target identification methods are needed to transform the bioactive compounds into clinically valuable drugs or drug scaffolds.

ACKNOWLEDGEMENT

The authors thank the authorities of Thiagarajar College for the infrastructure provided.

REFERENCE

- [1] AMS Mayer; AD Rodriguez; O Tagliatalata-Scafati; N Fuestani. *Mar Drugs*. **2013**, 11(7), 2510-2573.
- [2] C Ravi; R Muthamil; A Karthiga. *Asian Fish Sci*. **2016**, (29), 43-56.
- [3] KB Glaser; AMS Mayer. *Biochem Pharmacol*. **2009**, 78(5), 440-448.
- [4] LS Borquaye; G Darko; N Oklu; C Anson-Yevu; A Ababio. *Cognet Chem*. **2016**, 2(1), 1-10.
- [5] P Ramasamy; DPK Thampi; G Chelladurai; N Gautham; S Mohanraj; J Mohanraj. *JCLM*. **2013**, 1(3), 181-185.
- [6] SB Bharate; SD Sawant; PP Singh; RA Vishwakarma. *Chem Rev*. **2013**, 113(8), 6761-6815.
- [7] H Giftson; AE Mani; I Jayasanta; S Kailasam; J Patterson. *IJPPR*. **2015**, 4(4), 171-181.
- [8] H Giftson; J Patterson. *IJMR*. **2014**, 5(2), 140-143.
- [9] NV Zhukova. *Mar Drugs*. **2014**, 12(8), 4578-4592.
- [10] C Ravi; A Karthiga; V Venkatesan. *Asian Fish Sci*. **2012**, 25, 158-169.
- [11] G Sarumathi; M Arumugam; S Kumaresan; T Balasubramanian. *Asian Pac J Trop Biomed*. **2012**, 2(10), 759-764.
- [12] AG Babar; A Pande; BG Kulkarni. *Asian Pac J Trop Biomed*. **2012**, 2(2), S1060-S1063.
- [13] S Kanchana; R Vennila; K Rajeshkumar; M Arumugam; T Balasubramanian. *J Biological Sci*. **2014**, 14(1), 60-66.
- [14] AM Shami, K Philip, S Muniandy. *BMC Comple Altern Med*. **2013**, 13(360), 1-11.
- [15] SO Oko; OT Asekun; OB Familoni; AJ Afolayan. *Antioxidants*. **2014**, 3(2), 278-287.
- [16] E Bendary; RR Francis; HMG Ali; MI Sarwat; SE Hady. *AOAS*. **2013**, 58(2), 173-181.
- [17] R Senthil Kumar; B Raj Kapoor; P Perumal. *Asian Pac J Trop Biomed*. **2012**, 2(4), 256-261.
- [18] SUS Najmuddin; MF Romli; M Hamid; NB Alitheen; NM Afizan; NM Nik Rahman. *BMC Comple Altern Med*. **2016**, 16(1), 311, 1-20.
- [19] D Singh; M Mishra; M Gupta; P Singh; A Gupta; R Nema. *Int J Pharm Sci*. **2012**, 2(3), 42-44.
- [20] EK Pranuthi; K Narendra; J Swathi; KM Sowjanya; KVN Rathnakar; SJ Emmanuel; AK Satya. *JPP*. **2014**, 3(1), 57-61.
- [21] H Gong; Y Bai; E Chan; R Wu; E Yang; F Liu; S Lu. *PLoS Pathog*. **2011**, 7(9), e1002120.
- [22] A Henningham; S Dohrmann; V Nizet; JN Cole. *FEMS Microbiol Rev*. **2015**, 39940, 488-508.
- [23] C Selvaraj; J Sivakamavalli; B Vaseeharan; P Singh; SK Singh. *Mol Biosyst*. **2014**, 10(7), 1775-1789.
- [24] Z Heidary; E Bandani; M Eftekhary; A Jafari. *Acta Med Iran*. **2016**, 54(3), 201-210.
- [25] AK Atya; Y Belguesmia; G Chataigne; R Ravallec; A Vachee; S Szunerits; R Boukherroub; D Drider. *Front Microbiol*. **2016**, 7(817), 1-12.
- [26] AC Reis; SR Santos; SC Souza; MG Saldanha; TN Pitanga; RR Oliveira. *Rev Inst Med Trop Sao Paulo*. **2016**, 58-53.
- [27] XA Marialouis; A Santhanam. *J Clin Diagn Res*. **2016**, 10(3), DC05-DC09.
- [28] E Darabpour; MR Ardakani; H Motamedi; MT Ronagh. *Bosn J Basic Med Sci*. **2012**, 12(2), 108-121.
- [29] K Sivasubramanian; S Ravichandran; M Kumaresan. *Asian Pac J Trop Med*. **2011**, 4(4), 310-314.
- [30] M Suresh; S Arularasan; N Srikumaran. *Int J Pharm Pharm Sci*. **2012**, 4(4), 552-556.
- [31] K Benkendorff; D Rudd; BD Nongmaithem; L Liu; F Young; V Edwards; C Avila; CA Abbott. *Mar Drugs*. **2015**, 13(8), 5237-5275.
- [32] U Grienke; J Silke; D Tasdemir. *Food Chem*. **2014**, 142, 48-60.
- [33] MS Ramya; S Ravichandran; R Anbuhezhan. *Int J Nat Product Res*. **2014**, 4(4), 105-109.
- [34] K Benkendorff. *Biol Rev*. **2010**.
- [35] N Abu; MN Akhtar; WY Ho; SK Yeap; NB Alitheen. *Molecules*. **2013**, 18, 10367-10377.
- [36] N Weerapreeyaku; C Junhom; S Barusrux; T Thitimetharoch. *Chin Med*. **2016**, 11(19), 1-10.
- [37] S Sugesh; P Mayavu; S Sharma. *AJPP*. **2014**, 8(34), 832-840.
- [38] NN Azwanida. *Med Aromat Plant*. **2015**, 4(3), 1-6.