11. REFERENCES


REFERENCES


REFERENCES


Krasinski, R., T. Henryk and L. Przemyslaw, 2009. Antioxidant effect of hyaluronan on polymorphonuclear leukocyte-derived reactive oxygen species is dependent on its molecular weight and concentration and mainly involves the extracellular space *Postepy Hig Med Dosw (online)*, 63: 205–212.


REFERENCES


*REFERENCES*


Zhaohui1, Z.O.U., Q. Wang, Z. Wang, G. Deng, L.I.R. Shu, G.A.O. Meixu1, 
C.H.E.N. Yonghao, F.A.N. Bei1, L.I. Qingseng, Z.H.A.O. Hongwei1, 
2011. Effect of Irradiation on Physico-chemical Properties of Hyaluronic 

Zhou, P.H., S.Q. Liu and H. Peng, 2008. The effect of Hyaluronic acid on IL-1β- 
Induced chondrocyte apoptosis in a rat of osteoarthritis. J Orthop 
Res. 26(12):1643-8.

Isolation, characterization and antioxidant activity of hyaluronic acid from marine bivalve mollusc Amussium pleuronectus (Linnaeus, 1758)

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ABSTRACT

The hyaluronic acid (HA) was isolated from bivalve mollusk Amussium pleuronectus and its antioxidant potential was evaluated. HA was separated from glycosaminoglycans mixture using ion-exchange chromatography (DEAE-cellulose). The net yield of HA was found to be 4.2 mg/g. The presence of HA was confirmed by agarose gel electrophoresis and further analyzed by a standard calorimetric method using Stains All. The disaccharide composition revealed the presence of uronic acid (47.7%) and N-acetyl glucosamine (35.1%). The structure of HA was characterized through FT-IR and 1H NMR spectroscopy. At 1 mg/ml, the scavenging ability of isolated HA towards ABTS, DPPH and hydroxyl radicals was 71.35, 54.42 and 63.42%, respectively. The isolated HA could be used as a potent antioxidant agent.

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1. Introduction

Glycosaminoglycans (GAGs) are a class of natural macromolecules, which is negatively charged, linear heteropolysaccharides classified into several groups on the basis of structure such as hyaluronic acid, heparin sulfate, dermatan sulfate and chondroitin sulfate, exhibiting an attracting interest because of their several applications in the biomedical, veterinary, pharmaceutical and cosmetic field (Linhardt, 2001; Sasisekharan, Raman, & Prabhakar, 2006). Hyaluronic acid (HA) is the most versatile compound also referred as “hyaluronan” (Kakehi, Kinoshita, & Yasueda, 2003) and widely distributed in vertebrates (mainly humans) tissues and body fluids as well as invertebrates (Fraser, Laurent, & Laurent, 1997). It is a linear polymer consists of repeating disaccharide units of N-acetyl-D-glucosamine and D-glucuronic acid linked by β (1, 4) and β (1, 3) glycosidic linkages respectively, and molecular mass in the range from $10^4$ to $10^7$ Da. It is distinct from other GAGs by the non-existence of sulfated groups and lack of covalently linked peptide in their structure (Stern, 2003; Pure & Assoian, 2009). HA is a building block for new biocompatible and eco-friendly polymers that have application in drug delivery, tissue engineering and viscosupplementation (Prestwich, Marecak, Marecak, Vercruyssse, & Ziebell, 1998). Generation of ROS (Reactive oxygen species) plays a key role in human diseases and aging process and it is scavenged by the antioxidants present in the compound. HA has a role in the activation and modulation of the inflammatory response includes its scavenging activity towards ROS, such as hydroxyl radical (-OH). In recent years, several reports described that HA has anti-ageing effect with potential antioxidant properties both in vitro and in vivo (Balogh, Illes, Szekely, Forrai, & Gere, 2003; Campo et al., 2004; Halicka, Mitlitski, Heeter, Balazs, & Darzynkiewicz, 2009). During inflammation,
inhibition of tumor cells and protection of tissue from free radical damage have also been attributed to a mixture of hyaluronic acid fragments (Termeer et al., 2000; Trubucchi et al., 2002; Ghatak, Misra, & Toole, 2002).

So far, many unique compounds of marine origin with different biological activities have been isolated and a number of them were investigated and/or being developed as new pharmaceuticals (Ely, Supriya, & Naik, 2004). Hitherto, only limited number of studies regarding the isolation of HA from bivalve mollusks, so the present study was carried out to isolate and characterize the Hyaluronic acid (non-sulfated) from Amussium pleuronectus and to evaluate its antioxidant potential.

2. Materials and methods

2.1. Chemicals and reagent

Hyaluronic acid from Streptococcus equi sp. (Cat. no-53747) and DEAE-cellulose were purchased from Sigma Chemical Co. (St. Louis, MO). d-Glucuronic acid, Stains all, 1, 2-diaminopropane, 2, 2-azinobis (3-ethylbenzo-thiozoline-6-sulfonic acid) (ABTS), 1, 1-diphenyl, 2-picyrhydrazyl (DPPH) were obtained from Hi Media (Mumbai, India).

2.2. Extraction of glycosaminoglycans

Bivalve A. pleuronectus was collected from the Mudasal oodai landing center along the Parangipettai coast (lat. 11° 29′ N; long. 79° 46′ E) and transferred to the laboratory for further use. The whole body tissue was dissected out and defatted with acetone. After centrifugation at 10,000 g for 10 min, the pellets were dried at 60 °C for 24 h. 5 g of pellet was hydrated (1 g/20 ml) in digestion buffer (100 mM sodium acetate pH 5.5, 5 mM EDTA and cysteine), papain (100 mg/g of tissue) were added and the solution was incubated for 24 h at 60 °C for 6h. After boiling for 10 min, the mixture was centrifuged and the supernatant was subjected to purify in anion exchange column chromatography using DEAE cellulose and barium acetate buffer, pH 5.8. Electrophoresis was carried out in 0.05 M 1, 2-diaminopropane buffer, pH 9.0 with acetic acid at 500 mA for 60 min. After migration, the plate was soaked in 2% Cetyl Pyridinium Chloride solution for 6 h, dried and stained with stains all. The gel was destained with water and the band was documented.

2.3. Fractionation of GAGs

The crude GAGs extracted from the tissue was dissolved in 10 ml of 0.05 M NaCl. After centrifugation at 10,000 g for 10 min, the supernatant was subjected to purify in anion exchange column chromatography using DEAE cellulose and the column was equilibrated with the same elution buffer. Glycosaminoglycans were eluted with a linear gradient of NaCl (0.05–1.2 M) at the flow rate of 1 ml/min and 2 ml for each fraction was collected. Active fractions were identified by uronic acid estimation (Bitter & Muir, 1962) and the collected fractions were precipitated with ethanol, at 4 °C. After centrifugation at 10,000 g for 10 min, the pellet was dried at 60 °C and solubilized in distilled water for further characterization.

2.4. Agarose gel electrophoresis (AGE)

Agarose gel electrophoresis using barium acetate/1,2-diaminopropane was followed by Volpi and Maccari (2003) with slight modifications. 0.5% agarose gel was prepared in 0.04 M barium acetate buffer, pH 5.8. Electrophoresis was carried out in 0.05 M 1, 2-diaminopropane buffer, pH 9.0 with acetic acid at 500 mA for 60 min. After migration, the plate was soaked in 2% Cetyl Pyridinium Chloride solution for 6 h, dried and stained with stains all. The gel was destained with water and the band was documented.

2.5. Spectroscopic investigation of the hyaluronic acid

Briefly different concentrations of HA were prepared in deionised water ranging from 5 to 25 μg/ml. The dye was prepared by dissolving 5 mg stains all in 43 ml of water and 7 ml of methanol. The absorbance spectra of several solutions of both the dye and hyaluronic acid with different concentrations were scanned between 350 and 750 nm (Fangnola, Pagani, Maffioletti, Tavazzi, & Papagni, 2009).

2.6. Determination of hyaluronic acid and disaccharide composition

The microtitre plate method was used for quantification of hyaluronic acid and uronic acid as described earlier by Cesaretti, Luppi, Maccari, and Volpi (2003). A serial dilution of standard and sample of 50 μl (1 mg/ml) was placed in a 96 well plate. Subsequently, 200 μl of 25 mM sodium tetaborate in sulphuric acid solution was added. Then the plate was heated at 100 °C for 10 min in an oven. After cooling at room temperature for 15 min, 50 μl of 0.125% carbazole in absolute ethanol was carefully added. After heating at 100 °C for 10 min in an oven and cooling at room temperature for 15 min, the plate was read in a micro plate reader at 550 nm. The hexosamine was determined following the method of Wagner (1979).

2.7. FT-IR analysis

Dry powder of HA was subjected to IR spectroscopy (Shimadzu, Japan) to find the presence of different amino, carboxyl and hydroxyl group. One part of the sample was mixed with ninety nine parts of dried KBr and then compressed to prepare a salt disc (3 mm diameter). The absorption was read between 500 and 4000 cm⁻¹.

2.8. 1H NMR spectroscopy

1H NMR spectra were obtained using a Bruker Advance 400 nuclear magnetic spectrometer operated at 400 MHz. The samples were pre-lyophilized three times with D₂O and finally prepared by dissolving 5 mg in 0.6 ml of D₂O at high level of deuterium. All chemical shifts were given in ppm.

2.9. Antioxidant activity

2.9.1. Total antioxidant activity

The antioxidant activity by ABTS assay for HA was determined (Arnao, Cano, & Acosta, 2001). The stock solutions
consist of 7.4 mM of ABTS\(^{2+}\) solution and 2.6 mM potassium per sulfate solution. The working solution was prepared by mixing the two stock solutions in equal ratio and store in dark at room temperature. 1 ml of ABTS solution was mixed with 60 ml methanol and read the absorbance of 1.170±02 units at 734 nm. HA (150 µl) was allowed to react with 2850 µl of ABTS solution for 2 h in a dark condition. Then, the absorbance was read at 734 nm.

2.9.2. DPPH radical scavenging activity
The DPPH radical scavenging effects of HA were measured by using the method as described earlier (Quiao et al., 2009). Briefly, 2 ml of 0.16 mM DPPH solution (in methanol) was added to the test tube containing 2 ml aliquots of samples in different concentrations of 0.2–1 mg/ml. The mixture was vortexed for 1 min and kept at room temperature for 30 min in the dark. The absorbance of all the sample solutions was read at 517 nm. The scavenging percentage was calculated by the following standard equation.

\[
\text{Scavenging percentage} = \left( \frac{A_{control} - A_{sample}}{A_{control}} \right) \times 100
\]

2.9.3. Hydroxyl radical scavenging activity
Hydroxyl radical scavenging activity of HA was followed as reported earlier (Halliwell & Gutteridge, 1989). Hydroxyl radical was generated by the Fe\(^{3+}\)-ascorbate-EDTA-\(\mathrm{H}_2\mathrm{O}_2\) system (the Fenton reaction). The reaction mixture contained a volume of 1 ml, 2-deoxy-2-ribose (2.8 mM); KH\(_2\)PO\(_4\)-KOH buffer (20 mM, pH 7.4); FeCl\(_3\) (100 mM); EDTA (100 mM); \(\mathrm{H}_2\mathrm{O}_2\) (1.0 mM); ascorbic acid (100 mM) and various concentrations (0.2–1 mg/ml) of the test sample or reference compound. After incubation for 1 h at 37 °C, 0.5 ml of the reaction mixture was added to 1 ml 2.8% TCA, then 1 ml 1% aqueous TBA was added and the mixture was incubated at 90 °C for 15 min to develop the colour. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution.

3. Statistical analysis
Results of antioxidant activity were expressed as mean±SD. One way ANOVA followed by DMRT was used to analyze data, using SPSS windows version 16.0. Values with \(P<0.05\) were considered statistically significant. All the experiment was carried out in six replicates.

4. Results and discussion
In the present study, the hyaluronic acid (HA) was separated from Glycosaminoglycans (GAGs) mixture of a bivalve molusc A. plueronectus. The presence of hyaluronic acid was revealed through the uronic acid estimation detected at 550 nm by calorimetric method. Among all the fractions, 7–15, 17–26 and 28–33 (I–III) showed more uronic acid content Fig. 1.

Further, all fractions were analyzed by agarose gel electrophoresis and stained with Stains-all, in which unique blue bands were prominently detected for the peak I and II only (Fig. 2a and b). The fractions I and II were pooled together and further confirmed with the standard HA again in the agarose gel. The strong blue colour band shows the presence of isolated HA from A. plueronectus (Fig. 3). The amount of HA presents in the A. plueronectus (4.2 mg/g) was lower than the HA from Mytilus galloprovincialis (6.2 mg/g) (Volpi & Maccari, 2003). At the same time the isolated HA was much higher than the horny layer of guinea pig (0.25 mg/g) (Miyamoto & Nagase, 1984) and from the liver of Aetobatus narinari (0.81 mg/g) (Giji, Arumugam, Abirami, & Balasubramanian, 2013). The net yield may vary due to species variation and extraction method. The disaccharides, including uronic acid and N-acetyl glucosamine contents were estimated as 47.7% and 35.1%, respectively. Similarly, the uronic acid and N-acetyl glucosamine composition of HA from the cortex of fresh kindey in pigs, sheep and dogs were found to be 42.7 & 39.2%, 36.8 & 35.4% and 40.8 & 37.3%, respectively (Dicker & Franklin, 1966).
4.1. Spectroscopic investigation of hyaluronic acid

HA is an anionic compound which binds with Stains-All, a cationic dye and forms a complex. The absorbance at 520 nm showed a peak for free dye alone, whereas the spectral peak for Stains-All with standard HA or sample exhibited their absorbance at 640 nm and the peak intensity was observed in a dose-dependent manner. Generally, the typical magenta colour of the free dye turns cyan or blue colour after the addition of sample with the dye. Hence, Stains-All interacts with all GAGs which includes sulfated GAGs at 450 nm and non sulfated GAGs at 650 nm (Homer, Denbow, Whiley, & Beighton, 1993). The isolated HA interacts with Stains-All and spectrum was read at 640 nm to confirm the non sulfated GAGs hyaluronic acid (Fig. 4a and b).

4.2. FT-IR

The functional group of HA from A. plueronectus was studied in FT-IR spectroscopy and compared with HA from S. equi sp (Fig. 5a and b). The transmittance of the isolated HA shows 14 characteristic peaks from 4000 to 500 cm$^{-1}$. A sharp peak was observed in the region of 3437.15 cm$^{-1}$, it is due to the hydrogen bonded O–H stretching and N–H stretching vibration of the N-acetyl side chain. The peaks at 1612.49 and 1359.82 cm$^{-1}$ assigned to amide I group of C=O carboxyl and aromatic primary amine CN stretching respectively. The peak at 2929.87 cm$^{-1}$ was due to the methyl C–H stretch responsible for glucuronic acid. The peak at 1029.99 cm$^{-1}$ was observed for the primary alcohol C–O stretch. Alkyne C–H bonds were responsible for 667.37 cm$^{-1}$ stretch of A. plueronectus HA. Further, the aryl S–S stretch band was found at 464.84 cm$^{-1}$. The FTIR results confirmed that the maximum HA functional peaks are presented in A. plueronectus as similar to standard hyaluronic acid. In addition to that the structural difference between HA and Chondrotin sulphate is detected by the presence of –O–SO$^3_-$ side group’s absorbance at 1240 cm$^{-1}$ (Servaty, Schiller, Binder, & Arnold, 2001); therefore, the above absorbance was not ascertained in the above spectrum of HA from A. plueronectus.

4.3. NMR

Proton NMR spectra of most organic compounds is characterized by chemical shifts in the range +14 to −4 ppm and by spin–spin coupling between protons. The peak observed at 5.442 ppm strongly indicates the presence of glycosidic linkages in the isolated HA (Fig. 6). Peak responsible for the presence of N-acetyl glucosamine was recorded between 1.6 and 1.7 ppm. Earlier signals were observed at similar chemical shifts for HA isolated from a vertebrate source (Giji et al., 2013). Further the presence of reducing ends and glycosidic linkages in acid hydrolysed HA (Tommeraas & Melander, 2008) significantly correlates with our results. The presences of methylene groups were identified in the shielded region between 0.999 and 1.769 which agrees with the earlier reports on proton NMR spectra of HA isolated from bacterial source (Saranraj, Sivakumar, Sivasubramanian, & Geetha, 2011).
4.4. Antioxidant activity

The antioxidant potential of HA was measured in terms of their efficiency in scavenging free radicals generated by DPPH, ABTS and H2O2. The ABTS assay was based on the generation of a blue/green ABTS⁺ which is applicable to both hydrophilic and lipophilic antioxidant systems. The isolated HA shows the minimum activity (32.56%) and maximum activity (71.35%) when compared with the standard ascorbic acid (98.04%) (P<0.05) (Fig. 7). DPPH is a stable free radical, when its exposure to the radical scavengers, the absorbance decreases significantly and this assay is applicable to hydrophobic systems (Kim, Lee, Lee, & Lee, 2002). The DPPH radical scavenging activity of A. pleuronectes HA shows minimum activity (19.77%) at 0.2 mg/ml and maximum activity (54.42%) at 1 mg/ml when compared with the ascorbic acid (P<0.05) (Fig. 8). The DPPH radical scavenging activities were 53.63%, 59.38% and 50.23% for HA, low molecular weight HA-1 and HA-2 at 1600 µg/ml, respectively (Ke, Sun, Qiao, Wang, & Zeng, 2011).

Apart from these, the hydroxyl radical is one of the most reactive free radicals, and it has created in a cascading process of reduction of molecular oxygen causing damage to DNA, lipids and proteins (Raja & Pugalendi, 2010). The isolated HA was examined for its ability to act as OH⁻ radical scavenging agent. Ferric EDTA was incubated with H2O2 and ascorbic acid at pH 7.4, hydroxyl radicals were formed in free solution and were detected by their ability to degrade 2-deoxy-2-ribose into fragment that on heating with TBA and form a pink chromogen (Halliwell & Gutteridge, 1987; Aruoma & Laughton, 1989). The isolated HA exhibited scavenging effect of hydroxyl radical, which may inhibit the lipid damage. When sample HA and ascorbic acid was added to the reaction mixture, they removed hydroxyl radical and prohibited the degradation of 2-deoxy-2-ribose. The isolated HA showed maximum (63.42%) and minimum (23.77%) antioxidant activity against hydroxyl radicals, which shows the hydroxyl radical scavenging potential (Fig. 9). Previously, the hydroxyl radical scavenging activities of low molecular weight HA-1 and HA-2 were reported as 91.71% and 75.24% at 1200 µg/ml, respectively (Ke et al., 2011). The crude and purified capsule polysaccharides from S. equi showed the hydroxyl radical scavenging effect of 63 and 76.6% at 1120 µg/ml (Ke et al., 2009). In addition, the prominent antioxidant activity of HA was also reported from rooster comb (Balogh et al., 2003), chicken crest (Rosa, Hoelzelii, Viera,
Barreto, & Beirão, 2008) and squid eye (Qjong et al., 2012). Thus, the maximum antioxidant activity (71.35%) was found in ABTS scavenging activity than the hydroxyl radical (63.42%) and DPPH (54.42%) scavenging activity. Therefore, the isolated HA may utilize for natural antioxidant agent.

5. Conclusion

In conclusion, hyaluronic acid was reported from a bivalve mollusks A. pleuronectus with considerable yield. The structural analysis through FT-IR and NMR spectrum confirmed that the non-sulfated GAGs as hyaluronic acid. The significant antioxidant activity provides additional information of non-sulfated GAGs, HA from mollusks should be considered as a natural antioxidant.

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References


Alternative exploration of hyaluronic acid from marine superstore

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ABSTRACT:
Hyaluronic acid (HA) is non-sulphated, linear glycosaminoglycans (GAGs) extensively used in biomedical, cosmetic and nutraceutical field. This type of GAGs has created a more attraction to biologists for exploration of HA from various sources. Consequently, this present study was focused towards the marine mollusk for alternative source target for the isolation of HA from the gastropod- *Hemifusus cochlidium*. The whole body tissues were defatted by acetone and pellet was extracted using digestion buffer followed by proteolytic treatment. Then the crude GAGs were subjected to anion exchange column for purification. Further, HA, D- glucuronic acid and N- acetyl glucosamine content was measured using the calorimetric method. Fourier transform infrared spectroscopy (FTIR) and Nuclear magnetic resonance (H NMR) were used for characterization. The yield from *H. cochlidium* and the presence of functional group in the isolated HA was characterized by (FTIR) and H NMR. In this investigation, the results suggested that marine gastropod could be an alternative source of HA.

KEY WORDS: Marine gastropod, Hyaluronic acid, FTIR, H NMR, polysaccharide.

INTRODUCTION:
Marine environment is an exceptional reservoir of bioactive natural products, many of which exhibit structural or chemical features not scientifically evidence located within terrestrial natural products. It offers a tremendous biodiversity to discover useful therapeutic compounds that largely depend on the species specificity. The therapeutic potential of natural bioactive compounds such as polysaccharides, especially glycosaminoglycans (GAGs), is now well documented, and this activity combined with natural biodiversity will allow the development of a new generation of therapeutics [1]. GAGs have created a new arena of research owing to their several applications in the biomedical, veterinary, pharmaceutical and cosmetic field. GAGs include hyaluronic acid, heparin, heparansulphate, dermatan sulphate, chondroitin sulphate and kertansulphate. The structure and organization of glycosaminoglycans and proteoglycans from tissues of several vertebrates and invertebrates of marine organism have been extensively studied [2].

Hyaluronic acid (HA) is the most versatile compound also referred as “hyaluronan”. Hyaluronan, an anionic linear polysaccharide was formerly known as acid mucopolysaccharide and are now called as glycosaminoglycans [3]. It is a high molecular weight polysaccharide of the order of 10 to 10 KDa [4] with an un-branched backbone composed of alternating sequences of β (1 – 4) glucuronic acid and (1 – 3) -N- acetylglucosamine moieties and widely distributed in vertebrates as well as invertebrates. It is distinct from other GAGs by the devoid of sulfated groups and non - covalently linked peptide in their structure [5,6]. HA is a building block for new biocompatible and eco-friendly polymers that have application in drug delivery, tissue engineering and viscosupplementation [7]. Concurrently, it also has been used in a medical, cosmetics and food industry [8]. So far HA was isolated from various sources include human umbilical cord, skin, rooster combs and bacteria [9]. In that human umbilical cord have the huge amount of HA and its cost expensive due to their source. This attempt of isolation of HA from marine source was carried out to provide with alternative sources and to change the source target for HA isolation.
MATERIALS AND METHOD:
Sample collection
The marine gastropod *H. cochlidium* were collected in the mudsaloodai landing center along the east coast of India. Samples were freshly collected and transferred to the laboratory in ice cold temperature and then the whole body tissues were dissected out from shells using the forceps.

Isolation of GAGs
The HA was isolated by the method of Volpi and Maccari [10]. The 250 gm of whole body tissue was dissected out and defatted with acetone for 24 h at 4°C. The defatted content was centrifuged at 10,000g for 10 min; the pellets were dried at 60°C for 24 h. Subsequently, 2.5 g of pellet was hydrated (1g/20ml) in digestion buffer containing 100mM sodium acetate at pH 5.5, 5mM EDTA, 5mM cysteine and papain 100mg/g of tissue and incubated for 24 h at 60°C with continuous stirring. After boiling for 10 min, the mixture was centrifuged at 5000g for 15 min, and three volumes of ethanol saturated with sodium acetate was added to the supernatant and stored at 4°C for 24 h. The precipitate was recovered by centrifugation at 5000g for 15 min and dried at 60°C for 6 h.

The crude GAGs extracted from the tissue was dissolved in 10 ml of 0.05 M NaCl and centrifuged at 10,000g for 10 min, the supernatant were then subjected to anion exchange chromatography using DEAE cellulose column equilibrated with the same elution buffer. Glycosaminoglycans were eluted with a linear gradient of NaCl (0.05-1.2M) with a flow rate of 1ml/min and the fractions of 2ml were collected. Active fractions were identified by uronic acid estimation [11]. The respective active fractions were pooled with two volumes of ethanol and precipitated at 4°C. After centrifugation at 10,000g for 10 min, the pellet was dried at 60°C.

Determination of analytical composition
The microtitre plate method was used for quantification of Hyaluronic acid and Uronic acid [11, 12]. A serial dilution of standard and sample of 50 μl (1mg/ml) was placed in a 96 well plate. Subsequently, 200μl of 25mM sodium tetraborate in sulphuric acid solution was added. Then the plate was heated at 100°C for 10 min in an oven. After cooling at room temperature for 15 min, 50 μl of 0.125% carbazole in absolute ethanol was carefully added. After heating at 100°C for 10 min in an oven and cooling at room temperature for 15min, the plate was read in a micro plate reader at 550nm. The hexosamine was determined following the method of Wagner [13].

FTIR Analysis
Dry powder of HA was subjected to IR spectroscopy (Shimadzu) to determine the presence of the different amino, carboxyl and hydroxyl group. One part of the sample was mixed with ninety nine parts of dried KB and then compressed to prepare a salt disc (3mm diameter). The absorption was read between 500 and 4000cm⁻¹.

¹H -NMR spectroscopy
¹H NMR spectra were obtained using a BrukerAvance 400 nuclear magnetic spectrometer (Bruker DRX 500 Rheinstetten, Germany) operated at 400 MHz. The samples were pre- lyophilised three times with D₂O and finally prepared by dissolving 5mg in 0.6 ml of D₂O at high level of deuterium. All chemical shifts were given in parts per million (ppm).

RESULTS AND DISCUSSION:
The whole tissue was defatted using organic solvents, followed by proteolytic digestion the crude GAGs was isolated from marine mollusk species. The crude GAGs was subjected to the anion exchange column (cellulose) and eluted with the 0.05 - 1.2 M NaCl gradient. Further the collected fractions were determined the uronic acid level at 550nm. While evaluating the uronic acid concentration in fractions highest peak indicates the presences of hyaluronic acid. The yield of crude GAGs and HA in gastropod *H.cochladium* (250 mg/g and 1.96 mg/g). Earlier, the net yield of HA from marine bivalve *M. galapovinicialis* was reported as 6.2 mg/gm [10] and *A. pleuroonectus* (4.2 mg/g) [14] which is relatively higher than the present yield. Giji et al [15] reported that HA from the marine stingray liver was found to be 0.81 mg/g which indicates the lower yield when compared to marine gastropod. Likewise, the yield of HA in horn layer of guinea pig was around 0.25 mg/gm [16]. In the present study showed that the yield of *H.cochladium* HA was higher than terrestrial source. But there exists the difference in the net yield of which may be attributed to species variation and extraction methods applied.

The analytical compositional of the HA were quantified through standard methods. The disaccharides, including uronic acid and N-acetyl glucosamine contents were estimated as 47.3%, 32.1% of *H. cochlidium* respectively. Likewise, HA isolated from marine stingray liver and marine bivalve *A. pleuronectus* [14] also showed same range of uronic acid and N-acetyl glucosamine contents [15]. Further Dicker and Franklin [17] have revealed the HA disaccharides composition for cortex of fresh kidney from various sources such as pigs (42.7, 39.2), sheep (36.8, 35.4) and dogs (40.8, 37.3) of similar ranges respectively. IR spectroscopy has been used successfully to characterize glycosaminoglycans. The functional group of HA from mollusc sample of *H. cochlidium* was studied in FT-IR spectroscopy and compared to HA from Human umbilical cord were shown in fig. 1. The Standard HA shows 10 major peaks between the ranges 500 and 4000cm⁻¹. The sharp peak was observed in the region of 3442.94 cm⁻¹, representing to the hydrogen bonded O-H stretching and N-H stretching vibration of the N-acetyl side chain. The peaks at 1633.71 and 1323.17 cm⁻¹ assigned to amide I group of C=O carboxyl and aromatic primary amine CN stretching respectively. The peak at 2924.09 cm⁻¹ was obtained owing to the methyl C-H stretch responsible for glucuronic acid. The peak at 1029.99 cm⁻¹ was observed for the primary alcohol C-O stretch. Alkyne C-H bonds were responsible.
for 667.37 cm⁻¹ stretch. Further, Alkradet et al. [18] reported similar range of bands in the IR for HA digested with HAase which greatly supports our results. The structural difference between HA and Chondrotin sulphate is detected by the presence of –O-SO₃⁻ side groups absorbance at 1240 cm⁻¹ [19]; therefore, the absorbance responsible for the –O-SO₃⁻ group was not ascertained. The FTIR results confirmed that the major functional peaks in the spectra are present in *H. cochlidium* as similar to standard HA.

Fig.1: FTIR spectrum of isolated HA from *H. cochlidium*

Fig.2: FTIR spectrum of standard HA
Proton NMR spectra of most organic compounds were characterized by chemical shifts in the range +14 to -4 ppm and by spin-spin coupling between protons. The peak responsible for the presence of glycosidic linkages at 4.5 ppm and N-acetyl glucosamine at 2 ppm was recorded for H. cochlidium (Fig. 3). The α/β anemic protons of the reducing end of N-acetyl glucosamine peaks were observed at chemical shifts between 5.2-5.3 ppm. Our results significantly agree with with the earlier reports of chemical shifts observed for HA isolated from a marine vertebrate source [15]. Further, the presence of reducing ends and glycosidic linkages in acid hydrolyzed HA [20] considerably concurs with HA from mollusk samples. The presences of methyl groups were identified in the shielded region between 0.999 and 1.769 which concur with the earlier reports on proton NMR spectra of HA isolated from bacterial sources [21].

Fig. 3: H¹NMR spectrum of isolated HA from H. cochlidium

CONCLUSION:
Recently the stipulate for HA in industrial scale has grown remarkably. Therefore, the search for source target for isolation of HA has been focused in marine superstore. Owing to this we have isolated HA from cheaper sources of marine gastropods and characterized its structure using NMR and IR spectroscopy. These results concluded that marine mollusk could serve as an alternative source for HA.

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REFERENCE:
Antagonistic and Cyto-Toxicity Activity of Mollusc Methanol Extracts

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Abstract: Marine mollusc pved a way for the isolation of bioactive compounds. Therefore, the present study was to investigate the Antimicrobial and cytotoxicity activities on 25 mollusc samples. The antibacterial activity was conducted against six human pathogens and anti fungal activity was tested against two fungal strains by the disc diffusion method. Further, in vitro toxicity assays such as hemolytic activity was done in microtiter plates method and Brine shrimp toxicity assay were tested against Artemia mg. The potent antibacterial activities were found against Staphylococcus aureus, Vibrio cholera and Salmonella paratyphi and the high degree of anti fungal activity in Aspergillus fumigatus than Fusarium sp. The hemolytic activity was detected in Meretrix casta, Sepia officinalis, Lophiotoma indica, Octopus vulgaris, Sepia officinalis ink, Ficus ficus, Xancus pyrum, Natica picta, Turrritella attenuata, Architectonica sp. The Brine Shrimp toxicity (LC₅₀) was also found in Comus betilinus, Hemifusus pugilinus, Bursellitalla leachi, Lambis lambis, Ficus ficus, Conus adustus. The results revealed that mollusc samples are a promising source for identifying novel drug lead compounds.

Keywords: Antibacterial, antifungal, marine mollusca, toxicity studies

INTRODUCTION

The marine environment comprises of complex ecosystem with a vast array of organisms and many of these organisms are known to possess bioactive compounds (Lijima et al., 1995). Marine natural products have been explored mostly for their antimicrobial, cytotoxic, antitumor and anti-inflammatory properties (Patterson et al., 2000). The biodiversity of marine organism reveals the way to discovery new bioactive substances. Marine invertebrates provide significant antimicrobial drugs (Barsemir et al., 2006; Bazes et al., 2009; Jayang et al., 2008; Mayer et al., 2007). Among the marine invertebrates the Mollusc constitute many representatives which are widely distributed throughout the world for isolating the biomedical important products (Halstead, 1965). Consequently, reported that mollusc constitute bioactive compounds with promising activities includes antitumor, antileukemic and antiviral (Kamya et al., 1989; Pettit et al., 1987; Arndt et al., 1997; Rajaganapathi et al., 2002).

A wide variety of anti-microbial factors have been isolated and characterized from the mollusc which includes chlorinated acetylenes (Walker and Faulkner, 1981), terpenes (Ireland and Faulkner, 1978), indole derivates (Bennendorff et al., 2001), glycerol derivates (Gustafson and Andersen, 1985), macrolides (Matsunaga et al., 1986), lysosomes (Nilsen et al., 1999) glycoprotein (Yamazaki, 1993), proteins (Kamiya et al., 1989), peptides (Iijima et al., 2003) and Polysaccharide (Shanmugam et al., 2008). Mollusc has being act as a untapped source for bioactive compounds for discovering a promising lead compounds for the discovery of antimicrobial therapeutics for the pharmaceutical use. Many of this anti-microbial therapeutics should be specific for prokaryotes and non-toxic to euakaryotic cells (Hancock and Rozek, 2002) for the development of lead compounds.

Considering the potential use of mollusc the present study has been made to ascertain the antimicrobial activity of whole body extract of bivalves, gastropod, cephalopods and some of the ink fluid of cephalopods. Here screened antimicrobial activity and the cytotoxic potential of the different Mollusc samples.

MATERIALS AND METHODS

Animal collection and sample preparation: Mollusc specimens (25 species) were freshly collected from eight different stations in the east coast of Tamil Nadu at 0.5-2.0 m depths from the sandy areas. The collected animals were immediately kept into ice box at 4°C, transferred to the laboratory, identified and stored in the deep freezer at -40°C until further use.
The whole body of the animals was cut into small pieces, homogenized with methanol and kept in the deep freezer at -18°C overnight. Samples were centrifuged at 5000 rpm for 15 min and supernatant was collected. The solvent present in the supernatant was evaporated by using rotary evaporator. The condensed samples were lyophilized. The dried crude samples were mixed with Phosphate buffered saline in the concentration of 5 mg mL⁻¹ and used for further analysis.

**Estimation of protein concentration:** Protein concentration was determined by the method of Lowry et al. (1951) by using Bovine Serum Albumin (BSA) as standard.

**Microorganisms and inoculum preparation:** In the present study, the micro organism such as molds (*Fusarium* sp. and *Aspergillus fumigatus*) bacterial strains were used which included two strains of gram positive bacteria *Pseudomonas aeruginosa* and *Staphylococcus aureus*, four strains of gram-negative bacteria *Vibrio cholerae*, *Salmonella paratyphi*, *Shigella dysenteriae*, *Klebsiella pneumoniae*. All micro organisms were obtained from the Department of Microbiology, Annamalai University, India.

Nutrient broth and Sabouraud Dextrose Agar (SDA) was used for growing and diluting the microorganism suspensions. Bacterial strains was inoculated and were grown to exponential phase in nutrient broth at 37°C for 18 h and adjusted to a final density of 10⁶ CFU mL⁻¹ by diluting fresh cultures by comparing with McFarland density. *Fusarium* sp. and *A. fumigatus* was aseptically inoculated on Petri plates containing sterile SDA medium. After inoculation the petri-plates was incubated at 28°C for 48 h and the colonies were aseptically sub cultured on SDA slants. The mold colonies from SDA slants were suspended in sterilized saline and compared with McFarland solution. The final concentration should be adjusted to 2×10⁶ cells mL⁻¹.

**Microbial sensitivity test:** Inhibition of bacterial growth was determined as earlier method by Bauer et al. (1966). Sterile swabs were immersed in the microbial suspensions (10⁶ cells mL⁻¹) and evenly applied to Petri dishes containing Mueller Hinton agar. Sterile whatman No. 1 filter paper discs (6 mm in diameter) were fully incubated with 30 µL of 5 mg mL⁻¹ concentration of samples and disc were allowed to dry at room temperature and placed over the agar in the plates. Erythromycin 15 µg disc⁻¹ and chloramphenicol 30 µg disc⁻¹ were used as positive control the plates were incubated overnight at 37°C and then examined for zone of growth inhibition around each disc.

Growth inhibition of isolated samples against *A. fumigatus* and *Fusarium* sp. was determined as described by Roberts and Selitrennikoff (1990). Briefly, agar assay plates were prepared by autoclaved Potato Dextrose Agar (FDA). Sterile swabs were immersed in the microbial suspensions and uniformly applied to Petri dishes containing PDA. Sterile whatman No 1 filter paper disc (6 mm) were fully absorbed with 30 µL of the samples (5 mg mL⁻¹) and placed over the agar in the plates. Nystatin was used as positive control and it was incubated at 37°C for 48-72 h. Plates were examined as described by the antibacterial assay.

**Hemolytic assay:** The hemolytic activity of the crude samples was assessed by the micro hemolytic method (Paniprasad and Venkateshvaran, 1997). In brief, chicken blood was collected in the tube containing 4% Tri sodium citrate solution in 1:9 ratio from slaughter house and brought to the laboratory. The blood was centrifuged at 5,000 rpm for 5 min, the supernatant was discarded, the pellet was suspended in normal saline (pH 7.4). The mixture was further centrifuged at 5,000 rpm for 5 min, the supernatant was discarded and pellet was resuspended in normal saline (pH 7.4). This procedure was repeated thrice. From these, 1% erythrocyte suspension was prepared by adding 99 mL normal saline to 1 mL of packed RBC (Red Blood Cells).

The micro hemolytic test was performed in 96 well ‘V’ bottom microtiter plate. Normal saline (100 µL) was added in all the wells, crude samples (100 µL) were added in the first well and it was serially diluted up to the last well. RBC suspension of 1% was added into all the wells with appropriate controls were included in the test. RBC suspension of 100 µL was added with distilled water of 100 µL, which served as a positive control and negative control with normal saline (100 µL). The plate was allowed to stand for 3 h at room temperature and the results were recorded. Uniform red color suspension in the wells considered as positive hemolysis and a button formation in the bottom of the wells was considered as lack of hemolysis. Reciprocal of the highest dilution of the crude extract showing the hemolytic pattern was taken as one Hemolytic Unit (HU).

**Brine shrimp lethality assay:** The toxic effects of isolated samples were tested against *Artemia nauplii* (Carvalho et al., 1988). Brine shrimp eggs were hatched in dish filled with sea water. Freshly hatched phototrophic larvae (Nauplii) were collected with a pipette and transferred (10 shrimps) to vials filled with sea water (5 mL). The different concentration of sample was added to the vials. In the case of control, a group of vials was filled with sea water. The vials were kept illuminated.
during 24 h of contact with the substances and survivals were counted with the aid of a magnifying glass. This assay was carried out three times with five replicates for each fluid concentration. Further, calculated the LC<sub>50</sub> (mean lethal concentration) values the results were plotted as logit % mortality vs. log concentration. Logit is defined as in (% mortality/%) survival.

RESULTS

Antibacterial activity: The in vitro anti-bacterial activity of mollusc samples was shown in Table 1. Antibacterial activities with a sample concentration of 5 mg mL<sup>-1</sup> were compared, the wide differences were observed depends on various samples. Of the six pathogens V. cholerae and S. paratyphi was the most sensitive than that of S. aureus. All other pathogens were resistant to the samples.

Highest antibacterial activity was found in the C. betulinus (15 mm) against S. aureus. L. cirrhosa and H. pugillus showed the growth inhibition of 13 mm against S. paratyphi. The highest activity was exhibited by L. cirrhosa against V. cholerae. Moderate activity was observed in P. virdis (7 mm), S. officinalis (7.5 mm), T. attenuata (8 mm), Architectonica sp. (8.5 mm) against V. cholerae and S. officinalis (7.5 mm), X. pyrum (8 mm) against S. paratyphi. Apart from these no antibacterial activity was detected in the remaining samples.

All pathogens were sensitive to the positive control Erythromycin (15 μg disc<sup>-1</sup>). Chloramphenicol (30 μg disc<sup>-1</sup>) inhibited all the pathogen except P. aeruginosa and S. dysenteria.

Antifungal activity: The result of anti-fungal activity for the various mollusc samples was shown in Table 2. Most of the samples were inhibited the growth of the fungi A. fumigatus. The growth of A. fumigatus was inhibited with the highest zone of inhibition by B. zealyica (18 mm), S. officinalis (15 mm), L. indica (15 mm), S. urceus (13 mm), B. leachi (13 mm), C. adustus (16 mm), O. vulgaris (14 mm), N. picta (12 mm) and Architectonia sp. (15 mm).

Moderate growth inhibition was detected in C. betulinus (8 mm), H. pugillus (10 mm), M. tributa (10 mm), S. officinalis (9 mm), M. mererix (7 mm) and X. pyrum (8 mm). Least anti fungal activity against A. fumigatus was observed in O. vulgaris (6 mm), F. ficos (4 mm), L. lambris (6 mm). In case of Fusarium sp., the highest activity was exhibited by B. leachi (13 mm), Architectonia sp. (15 mm) and T. attenuata (16 mm). The moderate activity was detected in S. urceus (8 mm), S. listeri (8 mm), C. adustus (9 mm). The antifungal control Nystatin inhibited the growth of both Fusarium and A. fumigatus.

### Table 1: Antibacterial activity of methanol extract of Molluscan samples

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<thead>
<tr>
<th>Samples</th>
<th>Pathogen</th>
<th>I</th>
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<th>III</th>
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<td>Turritella attenuata</td>
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<td>Architectonica sp.</td>
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<td>Aplysia sp.</td>
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### Table 2: Antifungal activity of methanol extract of 25 molluscan samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fusarium sp.</th>
<th>A. fumigatus</th>
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<tr>
<td>Babylonia zeylanica</td>
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<td>Mererix casta</td>
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<tr>
<td>Lambis cirrhosa</td>
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<tr>
<td>Sepia officinalis</td>
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<tr>
<td>Lophiotoma indica</td>
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<tr>
<td>Hemichaeus pugillus</td>
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<tr>
<td>Strombus urceus</td>
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<td>Bursatella leachi</td>
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<td>Octopus vulgaris</td>
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<td>Strombus litori</td>
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<td>Pecis ficos</td>
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<td>+</td>
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<td>+</td>
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<tr>
<td>Xancus pyrum</td>
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<td>Navicula picta</td>
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<td>Architectonica sp.</td>
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<td>Aplysia sp.</td>
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</table>

Pathogens indicates as I: Staphylococcus aureus, II: Vibrio cholerae, III: Vibrio paralytici, IV: Salmonella paratyphi, V: Pseudomonas aeruginosa, VI: Klebsiella pneumonia. Diameters of growth inhibition were used to defined incubation categories: +: Corresponding to the growth of inhibition diameter between 7 mm and 10 mm; ++: Corresponding to the inhibition diameter between 7 mm and 10 mm; +++: Corresponding to the inhibition diameter above 10 mm and (-) means that no inhibition was detected. Assays were carried out in duplicate.
Fig. 1: Hemolytic activity of 10 molluscan samples which exhibited the positive activity out of 25 samples.

Table 3: Brine shrimp toxicity of methanol extract of 25 molluscan samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>LC₅₀ (µg mL⁻¹)</th>
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<td>NT</td>
</tr>
<tr>
<td>Pernazavida</td>
<td>NT</td>
</tr>
<tr>
<td>Morerezia casta</td>
<td>NT</td>
</tr>
<tr>
<td>Conus foricula</td>
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</tr>
<tr>
<td>Conus brutaunus</td>
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<tr>
<td>Lembis chirengi</td>
<td>NT</td>
</tr>
<tr>
<td>Sepia officinalis</td>
<td>NT</td>
</tr>
<tr>
<td>Lophiotoma indica</td>
<td>NT</td>
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<tr>
<td>Hemisaces pugilinus</td>
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<td>Octopus vulgaris</td>
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<tr>
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<td>Murex tribata</td>
<td>NT</td>
</tr>
<tr>
<td>Pictus ficus</td>
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<td>Conus achatas</td>
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<tr>
<td>Sepia officinalis ink.</td>
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<td>Lembis lantibis</td>
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<tr>
<td>Morerezia meretrix</td>
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<tr>
<td>Octopus vulgaris ink.</td>
<td>NT</td>
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<td>Nioxus pyram</td>
<td>NT</td>
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<td>Nioxus picta</td>
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<td>Turritella ocellata</td>
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<td>Architectonica sp.</td>
<td>NT</td>
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<tr>
<td>Aplysia sp.</td>
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</table>

NT: Indicates no toxicity

TOXICITY STUDIES

Hemolytic activity: The hemolytic properties of mollusc samples were tested using chicken blood RBC. Among the 25 samples, 10 samples exhibited hemolytic activity were shown in Fig. 1. F. ficus (7.6 µg) and N. pica (17.8 µg) exhibited highest hemolytic activity in the highest dilution with the hemolytic unit of 2048 HU produced 100% hemolysis. Lysis of erythrocytes was dose dependent S. officinalis (11 µg) exhibited 256 HU units. L. indica (21 µg), S. officinalis ink (7.2 µg) showed 100% hemolysis of 36 HU units. Hemolytic activity with lowest dilution of 16 HU was recorded in Architectonica sp. (4.8 µg), M. casta (9.1 µg), X. Pyrum (8.2 µg), T. attenuata (13.5 µg) and O. vulgaris (18.2 µg), respectively. The remaining samples were not having the hemolytic activity.

Brine shrimp toxicity: The brine shrimp toxicity test (LC₅₀) results were shown in Table 3. The higher LC₅₀ value indicates the low level of toxicity and vice versa. The highest toxicity was detected in L. lambis 12 µg mL⁻¹, F. ficus 19 µg mL⁻¹, B. leechi 25 µg mL⁻¹, H. pugilinus 30 µg mL⁻¹ and the low level of toxicity were observed from the C. betulinus 42 µg mL⁻¹ and C. achatas 46.25 µg mL⁻¹. No mortality was observed in the other sample.

DISCUSSION

The antibacterial drugs were derived from the marine invertebrates and it serves as potential sources for identification of novel drugs (Bansmir et al., 2006; Bazex et al., 2009; Jayaraj et al., 2008). Molluscs serve as an untapped resource for many bioactive compounds which includes peptide, depsipeptide, sterols, sequiterpene, terpenes, polypropionate, nitrogenous compounds, macrolides, prostaflagnin (Carvalho et al., 1988; Balcazar et al., 2006; Bhant et al., 2006).

The result for in vitro antibacterial activity was present in 8 samples out of 25 samples, affecting the growth of human pathogen S. aureus, V. cholerae and S. paratyphi. The present study reveals the fact that presence of antibacterial factors in the P. virdis (7 mm), C. betulinus (15 mm), L. chirengi (13 mm), S. officinalis (7.5 mm), H. pugilinus (13 mm), X. pyrum (8 mm), T. attenuata (8 mm) and Architectonica sp. (8.5 mm).

The higher percentage of antimicrobial activity derives in the animal diet form sponges, bryozoans and algae which stored near the body surface as a chemical defense mechanism (Maktoob and Ronald, 1997; Carte and Faulkner, 1983). Results of the present study do not indicates that the antimicrobial activity originate from the diet-derived metabolites. This may be due to the breakdown of sponges, bryozoans and algae by the digestive enzyme of mollusk.

The presence of anti bacterial activity was due to the presence of protease enzyme. A previous study reported in the digestive organ of Modiolus modiolus was sensitive to protease treatment, indicating the presence of protein factor (Okuda and Scheuer, 1985). Proteins and glycoprotein with the antibacterial activity have been characterized from the digestive gland of various molluscs (Haug et al., 2004; Iguchi et al., 1982; Kamiya et al., 1986) and in the hemolymph of the various bivalves.
suggesting that this compound is responsible for animal immune system.

The activity detected from present study probably may due to bacterial symbionts living on the surface of the organism. A bacteria associated with the crystalline style of M. edulis contains a heat-sensitive anti bacterial agent (Seiderer et al., 1987). Barbieri et al. (1997) reported the anti bacterial activity was due to the bacterial symbionts Alteromonas and Shewanella in the nidamental gland of Loligo pealei. The presence of antimicrobial activity in mollusce has been reported from the muscus of giant snail (Kubota et al., 1985) from the egg mass and purple fluid of sea hare A. kurodai (Lijima et al., 1995). Antibacterial activity has been reported in unfraccionated plasma from the mussel Geukensia demissa and from the oyster Crassostrea virginica (Anderson and Beaven, 2001).

In the case of antifungal activity, highest zone of inhibition has been identified in the B. zeylanica (18 mm), S. officinalis (15 mm), S. urceus (13 mm), B. leachi (13 mm), L. indica (15 mm), C. adustus (16 mm), O. vulgaris ink (14 mm), N. picta (12 mm), Architectonica sp. (15 mm) against A. fumigatus. The highest antifungal activity may due to the presence of potential antifungal compound. The result of antifungal activity in O. vulgaris ink (14 mm) and S. officinalis ink (9 mm) supports the previous work done by the Lane (1962) who reported the antibiotic effects of the fluid from the ink sac of cephalopods.

The sea hare B. leachi (13 mm) exhibited antifungal activity against Fusarium sp. and A. fumigatus. Lijima et al. (1995) isolated Aplysianin-E, a compound which inhibit the growth of Yeast sp. A powerful antifungal agent Halicendraimides has been extracted from the marine nudibranch mollusc. High degree of antifungal activity was detected in T. attenuata and Architectonica sp. against Fusarium sp. The antifungal activity probably may due to the presence of antifungal peptide. Charlet et al. (1996) characterized an antimicrobial peptide from the M. edulis. Mitta et al. (2000) isolated a novel antifungal peptide from M. galloprovincialis that delays the growth of Neurospora crassa and F. culmorum.

In the toxicity studies out of 25 Mollusc samples 10 samples lyses the washed erythrocyte. The highest hemolytic activity was recorded in F. ficus and N. picta which showed the toxic nature and ensures the presence of hemolytic factor. Lattore (1977) isolated hemolytic factor from the venom of C. textile. It was suggested that the proteolytic enzyme might contribute to the lysis of the red blood cell membrane by digesting integral protein correspondingly and weakening certain protein of the membrane.

In the brine shrimp Nauplii test, high mortality was detected in the L. lambis and F. ficus. In the present study, toxic activities were found in some sample that also showed antimicrobial activities. However, some samples were shown too toxic without showing any antimicrobial activity, some sample showed little or no toxicity with antimicrobial activity. Haug et al. (2004) reported that from a pharmaceutical point of view it is advantageous when antimicrobial drugs have no toxic effect on eukaryotic cells.

CONCLUSION

In conclusion according to the latter point, the sample with antimicrobial activity with no toxicity is considered for isolating a novel antibiotic. In order to identify the chemical nature and evaluate their potential novel drug leads, further purification of the active sample for the isolation of active compounds is necessary.

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REFERENCES


