

Full Length Research Paper

# Investigation of antimicrobial and plasma coagulation property of some molluscan ink extracts: Gastropods and cephalopods

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The ink secretion of molluscan species was identified as one of the novel sources of bioactive compounds. Aqueous ink extracts of *Dollabella auricularia* inhibited the growth of Gram-positive and Gram-negative bacteria. In addition to *D. auricularia*, *Octopus vulgaris* and *Sepia aculeate* ink samples showed antifungal activity. The antimicrobial activities of the samples were not due to the presence of protease activity. All tested ink samples were not shown the property of agglutinating chicken erythrocytes. The *D. auricularia* ink fluid was haemotoxic which shows 100% lyses of chicken RBC with the minimum concentration of protein 4.4 µg/ml. In plasma coagulation assay *S. aculeate* ink showed procoagulant property and it coagulated chicken plasma within 180 s; all the remaining samples took more than 5 min for clot formation. Taken together, all these data suggest that, the presence of a number of factors in the ink secretion of mollusk and some of which are proteins in the range from 62 to 249 kDa which are all playing specific roles in the chemical defense mechanism of inking mollusk.

**Key words:** Anti-microbial activity, aqueous extract, molluscan ink, procoagulant, protease activity.

## INTRODUCTION

In nature, animals are provided with their own protective response against their predators, likewise marine mollusks are protected by their shells, but many of them are not fully protected by shells. Chemical defenses are used extensively by both shelled and shell-less mollusks. Among the chemical defenses, a class of defense including sea hares, squid, octopus and cuttlefish, have a striking defensive behavior-releasing ink when attacked. The ink of cephalopods functions as anti predatory visual stimuli either as like distracting "smoke screens" or as decoys. Very few have proposed that the ink of cephalopods contain compounds that are capable of disrupting predator's chemical senses but evidences are not fully recorded (Caldwell, 2005). The defensive secretions of inking mollusks: sea hares and cephalopods

secretions contain millimolar levels of free amino acids (FAA) and ammonium, among the total FAA highest concentration found were taurine, aspartic acid, glutamic acid, alanine and lysine. Fishes and Crustaceans are the major predators of these mollusks and are having specific receptor systems for these FAA (Derby et al., 2007).

Most of the studies concerning antimicrobial activity includes specific compartments like egg masses, hemolymph or whole body extracts of mollusk (Haug et al., 2003). Mollusks not only exhibit the anti-microbial activity, it constitutes many classes of bioactive compounds which includes antitumor, antileukemic and antiviral activities have been reported world-wide (Pettit et al., 1987; Kamiya et al., 1989; Premanand et al., 1997; Rajaganapathy et al., 2000). The sea hare species *Aplysia* and *Dollabella* have been reported to contain some biologically active substances, including antibacterial compounds (Faulkner et al., 1973), haemagglutinins (Melo et al., 2000), toxic compounds (Kato and Scheruer, 1974), cytotoxic compounds

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(Yamazaki et al., 1990) and substances for chemical defenses (Yamamura et al., 1977). Most of these substances are derived from their algal diets of the sea hares (Kinnel, 1977). The presence of antimicrobial activity has been reported in the digestive gland, albumin gland, egg masses and purple fluid of the Nudibranch (Minale and Riccio, 1976; Kamiya et al., 1989; Yamazaki, 1993). In case of cephalopods, the antimicrobial activity has been reported in the mucous and cuttle bone of the giant snail (Iguchi, 1982; Rajaganapathi, 2001). Other than antimicrobial activity, a novel agglutinin which is responsible for hemagglutination was isolated from the sea hare *A. kurodai*, *A. juliana*, *A. dactylomela*. Apart from ink as a possible first line of defense, sea hares possess a toxic opaline-gland secretion (Ando, 1952) and it has a broad spectrum of algal-derived toxins in the skin and digestive gland (Carefoot, 1987).

The bioactive compounds involved in sensory disruption and phagomimicry include free amino acids (FAA) and ammonium, which are extraordinarily concentrated in ink and opaline of the sea hares (Kicklighter et al., 2005) and various simple amines and paralyzing proteins were found in the cephalopods (Walker and Masuda, 1990; Cariello and Zantli, 1977). In the past few decades, mining of bioactive compounds from marine sources are considered promising because of its rich species diversity. So the present study focuses the antibacterial, antifungal, protease, hamagglutination and plasma coagulation activity of sea hare, octopus, cuttlefish and squid ink extracts.

## MATERIALS AND METHODS

### Collection of ink samples

Squid (*Loligo duvalucelii*), octopus (*Octopus vulgaris*) and cuttlefish (*Sepia aculeate*) were collected from annankoil landing centre near Portonovo, ink glands were removed and extracted with water. The sea hare (*Dollabella auricularia*) specimens were collected from the region of Gulf of Mannar (Thondi). The ink fluid was obtained by disturbing the animals and extracted with water. All aqueous ink samples were centrifuged at 15,000 g for 15 min and the supernatant was taken and stored in -20°C for further use.

### Estimation of protein concentration

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as a standard. The protein concentration of aqueous extracts of sea hare, squid, octopus, cuttlefish ink samples were 44, 108, 144 and 420 µg/ml respectively.

### Microorganisms and inoculum preparation

The microorganisms used in the study were molds (*Fusarium* sp and *Aspergillus fumigatus*) and two strains of Gram positive bacteria *Pseudomonas aeruginosa* and *Staphylococcus aureus*; five strains of Gram-negative bacteria *Vibrio cholerae*, *Salmonella*

*paratyphi*, *Shigella boydii*, *Shigella dysenteriae*, *Klebsiella pneumoniae*. All microorganisms were clinical isolates, obtained from the Department of Microbiology, Annamalai University, Annamalai Nagar, TamilNadu, India.

Nutrient broth and Sabouraud Dextrose Agar (SDA) were used for growing and diluting the microorganism suspensions. Bacterial strains were inoculated and grown to exponential phase in nutrient broth at 37°C for 18 h and adjusted to a final density of 108 CFU/ml by diluting fresh cultures by comparing with McFarland density. *Fusarium* sp and *A. fumigatus* were aseptically inoculated on Petri plates containing sterile SDA medium. Petri plates were incubated at 28°C for 48 h and the colonies were aseptically sub-cultured on SDA slants. The mold colonies from SDA slants was suspended in sterilized saline and compared with McFarland solution. The final concentration was adjusted to  $2 \times 10^7$  cells/ml.

### Microbial sensitivity test

Inhibition of bacterial growth by the ink samples was determined as described by Bauer et al. (1966). Sterile swabs were immersed in the microbial suspensions (108 cells/ml) 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 the ink samples and placed over the agar in the plates. Erythromycin disc (Himedia, India) and chloramphenicol (Himedia, India) were used as positive control the plates were incubated overnight at 37°C and then examined for zone of growth inhibition around each disc.

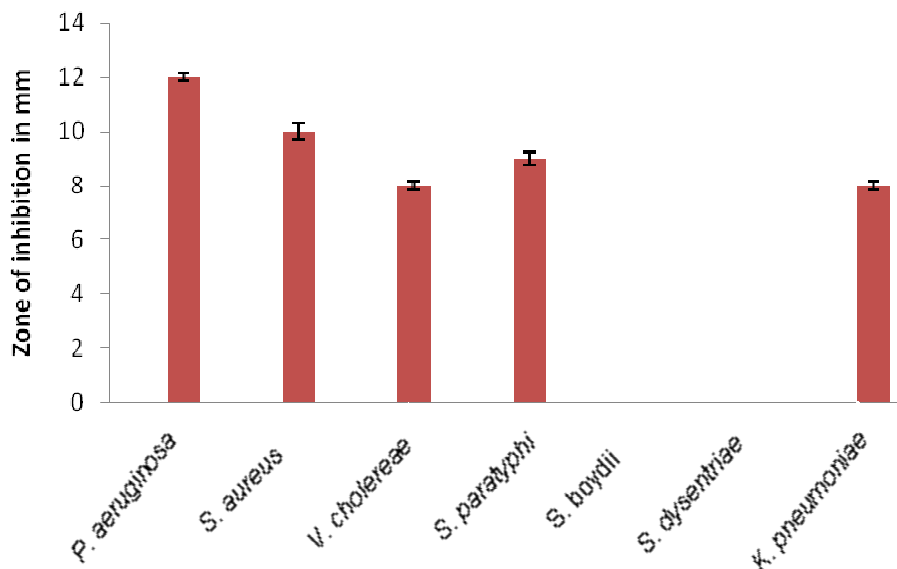
Growth inhibition of Mold strains by the ink samples was determined as described by Roberts and Selitrennikoff 1990. Briefly, agar assay plates were prepared by autoclaving potato dextrose agar (PDA). Sterile swabs were immersed in the microbial suspensions and evenly applied to Petri dishes containing PDA. Sterile whatman No 1 filter paper discs (6 mm in diameter) were fully imbibed with 30 µl of the ink samples and placed over the agar surface of the plates. Nystatin (Himedia, India) was used as positive control. Plates were incubated at 37°C for 48 to 72 h and examined as described by the antibacterial assay.

### Protease assay

Zymographic method was used to assay the protease activity of the samples. Casein, gelatin were used as substrate and the method was adapted from Heussen and Dowdle (1980). Briefly, 2 mg/ml of casein and gelatin (Himedia, India) was incorporated as a substrate in the 10% of resolving gel with a 5% stacking gel without substrate. Ink samples were mixed with the non-reducing sample buffer and loaded in the gel. The gel was run at 20 mA at 4°C. After electrophoresis, the gel was washed twice with Triton X-100 to remove the SDS for 20 min, then incubated in incubation buffer containing 20 mM Tris-HCl, 0.4 mM calcium chloride pH 7.4 at 37°C for 16 h and stained with Coomassie blue. The clear area in the gel indicates the region of enzyme activity.

### Coagulation assay

The effect of sea hare, octopus, cuttlefish and squid ink upon coagulation was accessed using the recalcification time assay (Hougie, 1963) adopted for the SpectraMaxPro microplate reader. In a final volume of 150 µl, 50 µl of citrated chicken plasma was incubated with different amount of ink samples in 90 µl of 20 mM Tris-HCl buffer, pH 7.4. After 5 min at 37°C, 10 µl of 150 mM CaCl<sub>2</sub> was added and clot formation was monitored at 37°C for 20 min in the Spectra Max system at 650 nm. To access calcium independent coagulation activity, EDTA was added instead of CaCl<sub>2</sub>, to a final



**Figure 1.** Antibacterial activity of sea hare ink against *P. aeruginosa*, *S. aureus*, *V. cholerae*, *S. paratyphi*, *S. boydii*, *S. dysenteriae*, *K. pneumoniae*.

concentration of 10 mM.

#### Erythrocyte agglutination assays

The hemagglutinating activity was assayed according to vasconcelos et al. (1994). Briefly 1: 2 serial dilutions of ink in Tris-Cl buffer pH 7.4 was mixed in small glass tubes with 0.25 ml of a 2% suspension of erythrocytes. The extent of agglutination was monitored visually, after the tubes had been left at 37°C for 30 min and subsequently at room temperature for a further 30 min. The results were reported as the number of hemagglutination units (HU) per mg of fluid protein able to induce visible erythrocyte agglutination. One HU was defined as the minimum protein concentration required producing visible agglutination.

#### Hemolytic assay

Hemolytic activity was assayed on washed chicken erythrocytes as described earlier by Garnier et al. (1995). To 1 ml samples containing various concentration ink protein in 150 mM NaCl, 200 µl of resuspended 2% erythrocytes was added and kept for 30 min at room temperature. The suspensions were centrifuged for 5 min at 3000 rpm. The absorbance of the supernatant was measured at 540 nm to released hemoglobin. A negative control (erythrocyte suspension in 150 mM NaCl) and a positive control erythrocyte suspension in distilled water were prepared to enable the calculation of percentage hemolysis; all assays were carried out in triplicate.

#### SDS-Polyacrylamide gel electrophoresis

Electrophoresis of the crude ink was carried out by the method of Laemmli (1970) on a 1-mm vertical gel consisted of 5% stacking gel mix, and main running gel mix of 12.0% acrylamide. Ink samples containing 2% SDS and 1% 2-mercaptoethanol were incubated at 100°C for 10 min. A few sucrose crystals were dissolved in the samples before being applied (30 µl) to the gel. Electrophoresis

was carried out at 20-mA constant current for 60 min and protein bands were visualized by staining in Coomassie brilliant blue. Standard molecular weight markers from BANGALORE GENEI size ranging from 29 to 205 kDa were used to determine the molecular weight of individual proteins.

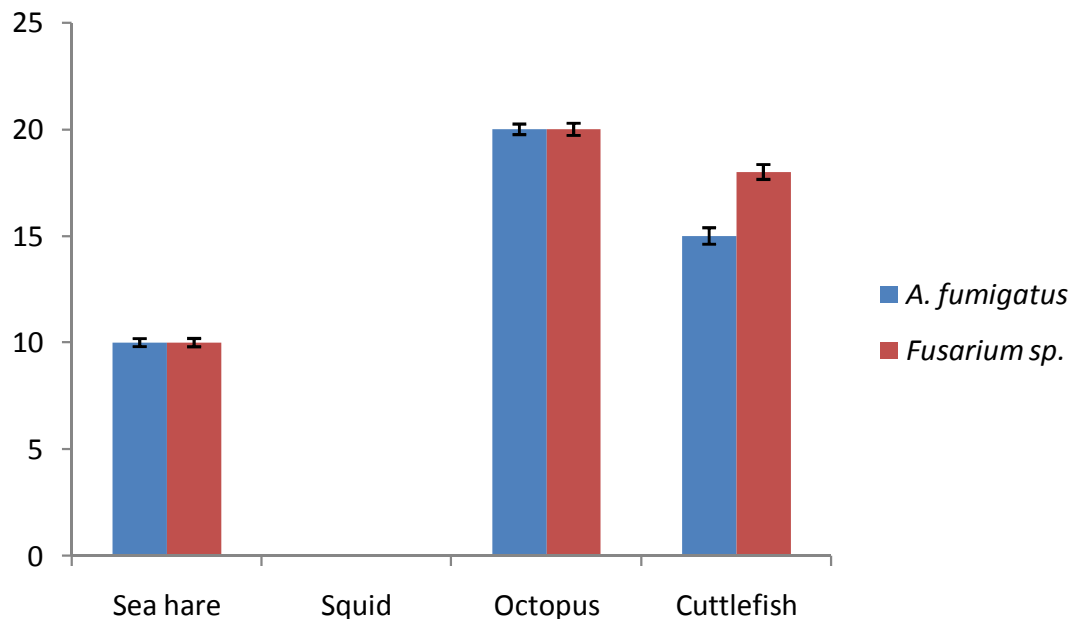
#### Statistical analysis

Results were expressed as mean  $\pm$  SD (n=4). One way ANOVA followed by Duncans multiple range test was used to analyze data, using SPSS windows version 11.5 with  $p < 0.05$  were considered statistically significant.

## RESULTS

#### Antibacterial activity

Among the four ink samples tested for antibacterial activity against two Gram positive bacteria *P. aeruginosa*, *S. aureus* and five Gram negative bacteria *V. cholerae*, *S. paratyphi*, *S. boydii*, *S. desenteriae*, *K. pneumoniae*. Sea hare ink extract (1.32 µg) showed maximum activity against *P. aeruginosa*, *S. aureus* with growth inhibition of 12 and 10 mm respectively. Similar range of inhibition zone was recorded against *V. cholerae*, *S. paratyphi*, *K. pneumoniae* in which the growth of inhibition was found to be 8, 9 and 8 mm respectively (Figure 1). There was no anti-bacterial activity in the ink extracts of squid, octopus, and cuttlefish against the test organisms. Data were reported as the mean  $\pm$  SD, which was carried out in duplicate. All strains were sensitive to the control erythromycin (15 mcg/disc) except *S. boydii* and chloramphenicol (30 mcg/disc) were resistant to *P. aeruginosa* and *S. dysenteriae*.



**Figure 2.** Antifungal activity of sea hare, squid, octopus, and cuttlefish ink against *A. fumigatus* and *Fusarium* spp.

### Antifungal activity

The results of antifungal activity of four ink extracts were shown in Figure 2. In the total of four ink samples tested against two fungal strains, the maximum growth inhibition was exhibited by octopus ink with the inhibition range of 20 mm against *A. fumigatus* and *Fusarium* sp and it was followed by cuttlefish with the maximum inhibition zone of 15 and 18 mm against *A. fumigatus* and *Fusarium* sp. The minimum growth inhibition was recorded by sea hare ink against *Fusarium* sp with the growth inhibition zone of 10 mm. Both strains were resistant to squid ink. Data were reported as the mean  $\pm$  SD which was carried out in duplicate. The antifungal controls Nystatin inhibited the growth of both *Fusarium* and *A. fumigatus*.

### Protease activity

Protease activity from the four ink sample was analyzed using gelatin and casein as substrate in zymographic method. Protease activity was observed in squid ink in both the substrate zymography. Gelatin and Casein zymograms revealed common protease band with the molecular range from 79 to 249 kDa (Figure 3). The remaining ink samples did not show any protease activity in our experimental condition.

### Hemagglutination and hemolytic activity

Molluscan ink extracts did not show hemagglutination

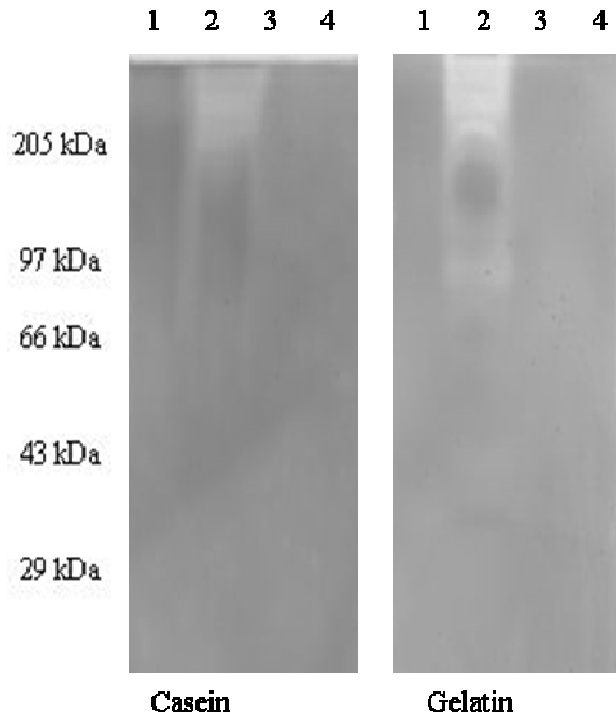
activity on the chicken RBC. The hemolytic property of the ink extracts were tested by using chicken RBC. Among the four inks extracts tested sea hare ink exhibited hemolytic activity. Lyses of erythrocytes by sea hare ink were dose dependent, 2.1  $\mu$ g of ink protein produced 50% hemolysis and 4.4  $\mu$ g produced 100% hemolysis shown in Figure 4. Remaining ink samples of squid, octopus and cuttlefishes did not having the hemolytic property.

### Plasma coagulation assay

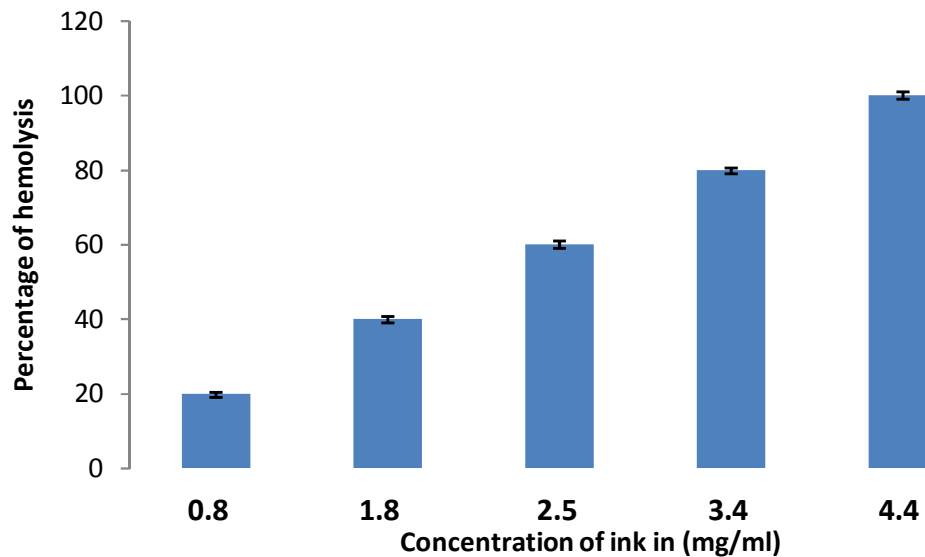
The effect of ink samples on blood coagulation was assessed by the plasma coagulation assay. To analyze the effect of ink samples upon clotting, citrated chicken plasma was incubated with ink samples prior to the induction of coagulation by calcium. From Figure 5, it is clear that the cuttlefish ink showing potent procoagulant effect is capable of shortening the clotting time of human plasma. At the same time, control plasma and other ink samples took more than 5 min to initiate coagulation, after the addition of calcium, plasma incubated with cuttlefish which took around 180 s to start coagulation.

### SDS-PAGE analysis

The electrophoretic profile of the ink samples showed the presence of small to high molecular weight protein, upon these some were distinct (Figure 6). Sea hare ink sample has bands ranging from 84 to 237 kDa, octopus has

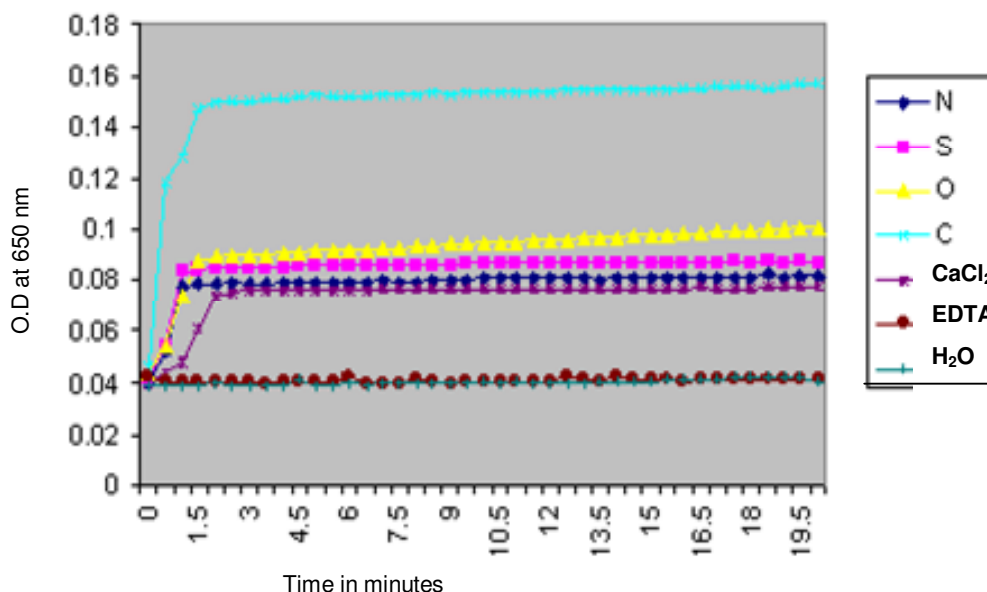


**Figure 3.** Protease activity: Caesinolytic, gelatinolytic activities of the ink samples were determined using the technique of substrate SDS-PAGE 10%. Number on the left indicate the molecular weight markers. Lane, (1) Sea hare (2) Squid (3) Octopus (4) Cuttlefish. Clear area in the gel indicates the region of protease enzyme activity.

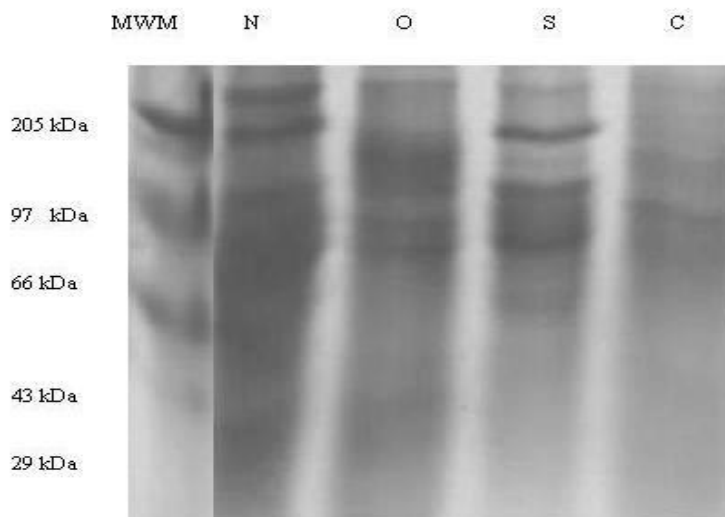


**Figure 4.** Hemolytic activity of sea hare ink on chicken blood erythrocytes. chicken blood erythrocytes were incubated with increasing concentration of ink sample for 30 min. Release of hemoglobin was determined by measuring the absorbance at 540 nm.

prominent bands from 82 to 248 kDa, squid has bands from 62 to 240 and the cuttlefish having 94 to 240 kDa.



**Figure 5.** Plasma coagulation assay: The effect of ink sample in the blood coagulation of chicken plasma was monitored at 650nm in SpectraMax system at 650 nm at 37°C. In the experiment plasma incubated with either sea hare, (♦) octopus, (▲) squid (■) cuttlefish (\*) ink was added prior to the addition of CaCl<sub>2</sub>. (x) Plasma incubated with calcium act as control reaction. (●) plasma incubated with EDTA instead of CaCl<sub>2</sub>. Each values represents mean ± SD of four independent experiments (\*P < 0.05).



**Figure 6.** SDS-PAGE: The four ink samples were subjected to electrophoresis in 10% gel. Lane, (1) Molecular weight marker (2) Sea hare (3) Octopus (4) Squid (5) Cuttlefish.

## DISCUSSION

Marine mollusks are protecting themselves from predators through their unique way, one of them is releasing ink when disturbed. This ink secretion contains a rich array of chemical secretions to escape from

predators. The chemical composition of the ink of all inking mollusk will not be the same. In order to explore the components and its effect on various living systems, we have accessed antimicrobial, protease, hemagglutination, hemolytic, and coagulation assay of the ink samples.

Both Gram positive bacteria as well as Gram negative bacteria showed susceptibility towards the ink extract of sea hare, which showed the zone of growth inhibition ranging from 9 to 12 mm. The inhibition zone for positive control erythromycin and chloramphenicol was ranging from 15 to 35 mm and the discrepancies between the results could be due to aqueous extracts of test samples might have lower concentration of antibacterial compounds. Kamiya et al. (1984) isolated aplysianin-E, an antibacterial glycoprotein from nudibranch *Aplysia*. Yamazaki et al. (1990) have reported the antibacterial activity of the purple fluid of *Aplysia kurodai*. An antibacterial peptide Dolabellin-A, was identified and characterized from albumin gland of sea hare, *D. auricularia* (Kisugi et al., 1992).

The results from the present work revealed the strongest antifungal activity of octopus ink with the inhibition zone of 20 mm against *Fusarium* sp and *A. fumigatus* respectively. Cuttlefish ink shows the inhibition zone of 18 and 15 mm against *Fusarium* sp and *A. fumigatus* respectively and minimum activity found in the sea hare ink against *Fusarium* sp. The highest activity in octopus probably may be due to the presence of potential antifungal compound. This result supports the previous work done by the Lane (1962) who reported the antibiotic effects of the fluid from the ink sac of the cephalopods. Iijima et al. (1995) successfully isolated the compound Aplysianin-E from *Aplysia* sp, which inhibit the growth of yeast species- *S. cerevisiae*, *Schizosaccharomyces* sp. and *Candida albicans*. The presence of anti-fungal activity in mollusca has been reported in egg mass of the sea hare *A. kurodai* which inhibited the growth of *C. albicans* (Kisugi et al., 1989b). A potential polysaccharide with potent antifungal activity was extracted from the cuttle bone of *S. aculeata* and Brevimana (Shanmugam et al., 2008).

To determine whether the anti-microbial activity was due to protease activity in the sample, we have conducted the protease zymography analysis. Protease are considered antimicrobial proteins because they are involved in the regulatory production of antimicrobial peptides (Aranishi and Nakane, 1997a,b). Zymography analysis revealed the fact that, anti-microbial property of the ink samples were not primarily due to the protease activity indicating that proteases were not responsible for the activity. Notably squid ink having protease activity which was active on broad range of substrates like casein and gelatin but it did not exhibited other activities.

None of the ink samples showed hamagglutination activity against chicken erythrocytes. A different glycoprotein is responsible for agglutination of erythrocytes. These ink samples may not contain agglutinin proteins to agglutinate chicken erythrocytes. Marine opisthobranch mollusks are able to incorporate and accumulate terpenoid toxin from their algal dietary sources (Wagele et al., 2005). Sea hare ink showed hemolytic activity (Figure 4), this activity possibly may contain terpenoid like toxic compound, which may cause

lyses of chicken RBC. The eunicellin based diterpenes isolated from a Japanese *lipophyton* species showed ichthyotoxic and hemolytic activities (Miyamoto et al., 1994).

Test results of plasma coagulation assay showed the presence of blood clotting factors in cuttlefish ink, which shorten the blood clotting time of chicken blood after the addition of  $\text{CaCl}_2$ , indicating that cuttlefish ink has effect on blood clotting. The result emphasized that the cuttlefish ink having compounds which is calcium dependant procoagulant. This is the first time finding that cuttlefish ink having strongest procoagulant effect which takes only 180 s to clot chicken blood whereas other samples took more than 5 min to clot (Figure 5). Procoagulant activity may be due to presence of compounds which are calcium dependant prothrombin activator (Guerrero et al., 1992). The remaining samples showed only moderate clotting effect which is equivalent to positive control. SDS polyacrylamide gel electrophoresis of aqueous extracts of ink samples (Figure 6) showed the presence of proteins with the molecular weight ranging from 62 to 249 kDa. These proteins may be responsible for various biological activities of the ink samples.

From this study, it is clear that molluscan ink sample contains a large number of compounds with various biological and physicochemical properties. These compounds might be one of the factors which are playing a crucial role in the defensive mechanism of the inking mollusk. The ink samples are having properties like antimicrobial, hemolytic, procoagulant etc. These results give additional support that ink secretions are toxic to lower organisms and source of biologically important compounds for biomedical research.

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