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Hemolytic Activities from Ascidian *Polyclinum madrasensis* Sebestian, 1952 and *Phallusia nigra* Savigny, 1816 from Tuticorin Coast of India

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ABSTRACT

Ascidians are marine animals with a great ability to synthesize bioactive substances. This study examined the heamolytic potential of ascidians *Polyclinum madrasensis* and *Phallusia nigra*. Ascidians *P. madrasensis* and *P. nigra* were collected from Tuticorin, Southeast coast of India. The collected ascidians were extracted by methanol at room temperature. The solvents were removed under reduced pressure to give predominantly an aqueous suspension. The protein content of crude *P. madrasensis* showed 680 μg mL^{-1} and crude *P. nigra* showed 543 μg mL^{-} ,[1]respectively. In sodium dodecyl sulfate polyacrylamide gel electrophoresis crude protein of *P. madrasensis* showed 4 bands 20, 27, 37 and 50 kDa and *P. nigra* showed 3 bands 20, 37 and 50 kDa. In the hemolytic assay maximum activity 64 Hemolytic Unit was showed in human "B" erythrocytes by *P. madrasensis* extract and minimum 2 Hemolytic Unit in human 'O' erythrocytes by crude extract of *P. madrasensis*. In ^{1}H-NMR spectrum the spectral signal at δ 8.1 and δ 7.9 indicates the presence of guanidine and thymidine containing alkaloid in *P. madrasensis* and *P. nigra*. Thus results proved that the ascidians have remarkable hemolytic compounds and further studies will fulfill for purification and structural elucidation of the compounds.

Key words: SDS-PAGE, hemolytic assay, haemolytic units, ^{1}H-NMR spectrum

INTRODUCTION

The study of marine organisms as a source of biologically active compounds is considered a very lucrative field, having already led to the discovery of various new pharmacological tools and medicines (Faulkner, 2000). Marine invertebrates, especially ascidians are most prominent sources of new compounds with cytotoxic potential. Ascidians are marine sessile filter feeder animals that belong to the phylum Chordata, class Ascidiacea. Recently, ascidians have increasingly become the target of natural products research (Osinga *et al*., 1998). Ascidians are dominant organisms in many marine communities, having a wide geographic distribution (Teo and Ryland, 1994). Many authors explain this ecological success on the basis of the ability of these animals to synthesize secondary metabolites, which possess an important defensive role against predation (Jimenez *et al*., 2003). The majority of metabolites reported from ascidians are derived from amino acids and it is an important source in drug discovery. In particular, ascidians have given rise to a great array of structurally diverse aromatic amino acid derived metabolites in which the biogenetic

precursor is phenylalanine, tyrosine or both. For an example (2S, 3R)-2-Aminododecan-3-ol 675 was reported as an antifungal component of *Clavelina oblonga* (Brazil) and an Australian *Atriolum robustum* was the source of amino acid derived metabolites 687-691[639]. Tetrahydroisoquinolone alkaloid 'Ecteinascidin 743' from *Ecteinascidia turbinata*, cyclic depsipeptides 'Dehydrodidemnin B and Didemnin B from *Tridemnum solidum*, cyclic peptide 'Vitilevuamide' from *Didemnin cucliferum*, 'Diazonamide' from *Diazona angulata* and a geranyl hydroquinone from the genus *Aplidium* (Prado *et al*., 2004) are a few tunicate compounds in anticancer preclinical or clinical trials (Jain *et al*., 2008). Such potential ascidians need to be explored for the pharmaceutical purpose.

Tuticorin is well known for pearl fishing and fishing centre in the southern peninsular region. Tuticorin port is one of the 12 major ports in India, in particular the second largest in Tamilnadu and has the greatest volume of exports and imports. During cargo handling, ship's hull is very important potential source for the introduction of ascidians (Tamilselvi *et al*., 2011). *P. madrasensis* and *P. nigra* are common persistent biofoulants. Mostly it attached from the cement blocks, rocks, pilings and oyster cages. These biofoulants ascidians were undertaken to investigate the hemolytic effectors in ascidians *Polyclinum madrasensis* and *Phallusia nigra* collected from Southeast coast of Tamil Nadu, India.

MATERIALS AND METHODS

Specimen collection and identification: Ascidians were collected as common and persistent biofoulants from the cement blocks, pilings and oyster cages of Tuticorin Coast (Lat. 8° 47' 20 and Long. 78° 09' 70"), southeast coast of India by SCUBA diving at the depth ranging from 5 to 8 m between April 2008. The samples were thoroughly washed with sea water and cleaned of sand, mutt and overgrowing organisms at the site collection, and transported to laboratory and collected specimens were identified by the standard study of Kott, (1985, 1989, 1990, 1998, 2002) and Meenakshi (2002).

Extraction: The freshly collected tunicates were soaked in methanol at the site of collection. The methanol extracts were decanted at room temperature and maintained for 5 days. The extracts were filtered through Whatman®No.1 filter papers and the solvents were concentrated by rotary evaporator (VC100A Lark Rotavapor® at 30°C) with reduced the pressure to give a dark brown gummy mass. The resultant residue was stored at 4°C for further analysis.

Protein estimation: The protein content was estimated by the method of Bradford (1976).

Thin layer chromatography (TLC): The extracts were subjected to TLC and grouped into fractions. Samples were analysed by TLC coupled to chemical tests for identification of different secondary metabolites according to MINSAP (1995). For analytical TLC, aluminium sheets (4×5 cm) coated with silica gel 60 F 254, were used. The chromatography was run in a chamber with dichloromethane: Ethyl acetate as medium (9.0:1.0 v/v) as the mobile phase under UV light at 254 nm.

Molecular weight determination-SDS-PAGE: Crude protein of *P. madrasensis* and *P. nigra* was subjected to electrophoresis following the method of Laemmli (1970) in 12% polyacrylamide slab gels.

Hemolytic activity: Crude extracts of *P. madrasensis* and *P. nigra* were assayed on chicken, goat and human erythrocytes (A, B, AB and O blood groups) followed by the method of Prasad and Venkateshwaran (1997), Mortari *et al.* (2005), Mkrtchyan *et al.* (2006), Samanta *et al.* (2008), Thirunavukkarasu *et al.* (2011) and Bragadeeswaran and Thangaraj (2011).

Preliminary characterisation of compound: ^{1}H NMR spectra were performed at Bruker Avance 300 MHz NMR Spectrophotometer operating at 400.24 and 100.614 observations. The crude sample was dissolved in DMSO/$CDCl_3$ as solvents, TMS as internal reference. Spectra were acquired using a 5 mm ^{1}H dual tuned probe. Temperature was maintained at 25°C.

RESULTS AND DISCUSSION

Specimen collection and extraction: The ascidian, *P. madrasensis* Sebestian, 1952 (450 g in wet wt.) and *P. nigra* Savigny 1816 (680 g. in wet wt.) were collected as common and persistent biofoulants from the cement blocks, pilings and oyster cages of Tuticorin coast. Methanol extracts of both *P. madrasensis* and *P. nigra* were concentrated under reduced pressure to give a dark brown gummy mass of 9.65 and 13.50 g, respectively.

Estimation of protein: The amount of protein content in *P. madrasensis* and *P. nigra* showed 680 and 543 μg mL^{-1} of crude respectively. This result clearly indicates the high amount of protein and other biochemical components present in their body. As far as we know, human consumption of ascidians occurs in Japan and Chile. Some ascidians *Halocynthia roretzi*, *Polyclinum* sp. and *Phallusia nigra* are widely enjoyed as food in Japan. The amount of carbohydrate, protein, lipid and minerals such phosphorous and calcium contents in these ascidians previously reported by (Rajesh and Ali, 2008). The protein recorded the maximum level of all the biochemical components in the mantle body (23%) which is followed by lipid, cellulose, carbohydrate and fiber. A total of eight amino acids viz. alanine, arginine, aspartic acid, glycine, leucine, lysine, methionine and tyrosine were found to occur. In addition to the amino acid profile, some B-complex vitamins such as riboflavin and thiamin were also estimated to understand the nutritive value.

Thin layer chromatography (TLC) of crude extracts: Thin layer chromatography on Silica gel coated plates with ethyl acetate: dichloromethane (9: 1 v/v) showed an intensive spot (R_f = 0.2 and 0.3) under UV light at 254 nm (Fig. 1 shows that the organic phase from aqueous extracts of the two ascidians studied were mixtures of compounds). The chemical analysis applied was consistent with the detection of alkaloids. Alkaloids and peptides were clearly detected after exposure of the spots in TLC to ninhydrin reaction.

Molecular weight determination-(SDS-PAGE) of crude extracts: The present investigation the both crudes were tested for demonstrated the molecular weight of protein (Fig. 2). The crude of *P. madrasensis* yielded 4 well defined bands at 20, 27, 37 and 50 kDa where as *P. nigra* contains 3 well defined bands at 20, 37 and 50 kDa. It supports the previous studies i.e., it's clearly indicating that these two simple ascidians have more or less same type of molecular weight proteins. Nair *et al.* (2001) reported the tunicate, *Styela plicata* from Sydney harbour, Australia, analysed for molecular weight of the proteins by SDS-PAGE revealed that fractions from gel filtration experiments that elicited maximum phagocytic and mitogenic activity contained a single protein of approximately 14 kDa. Green *et al.* (2003) demonstrated the molecular weight of protein from

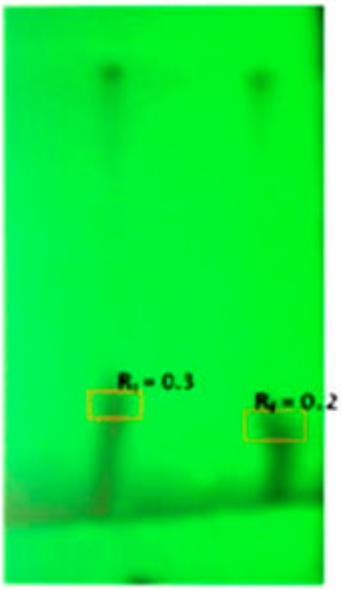

Fig. 1: Showing the TLC of *Polyclinum madrasensis* (R_f value = 0.2) and 2. *Phallusia nigra* (R_f value = 0.3) on Ethylacetate: Dichloromethane (9:1 v/v) solvent system under UV light

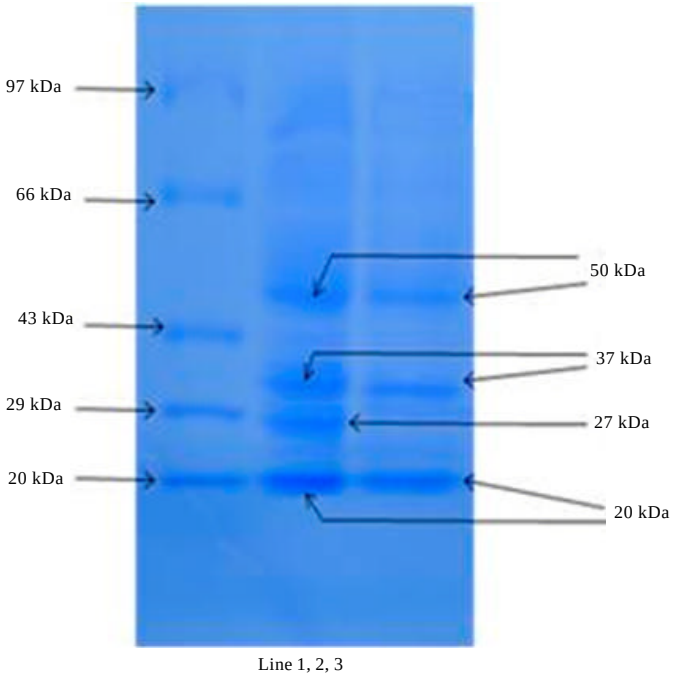

Fig. 2: SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis), Lane 1: Standard Molecular Weight Marker, Lane 2: Crude protein profile of *Polyclinum madrasensis*, Lane 3: Crude protein profile of *Phallusia nigra*

hemolymph (43 kDa) of the solitary ascidian, *Styela plicata* from Australian waters. The endoderm specific alkaline phosphate protein with molecular mass of 86 kDa and 103 kDa were reported by Kumano *et al.* (1996) from the ascidian, *Halocynthia roretzi*, Japan.

Hemolytic activity of crude extracts: In the present study the methanolic extracts of *P. madrasensis* and *P. nigra* were assayed on chicken, goat and human erythrocytes (A, B, AB and O blood groups). In this assay *P. madrasensis* and *P. nigra* extracts showed 32 HU, 16 HU against Chicken blood (Fig. 3). In goat blood *P. madrasensis* and *P. nigra* extracts showed 8HU (Fig. 4). In human A blood group, *P. madrasensis* and *P. nigra* extracts showed 16HU (Fig. 5). In human B blood group, *P. madrasensis* and *P. nigra* extracts showed 64 HU, 32 HU (Fig. 6). In human AB

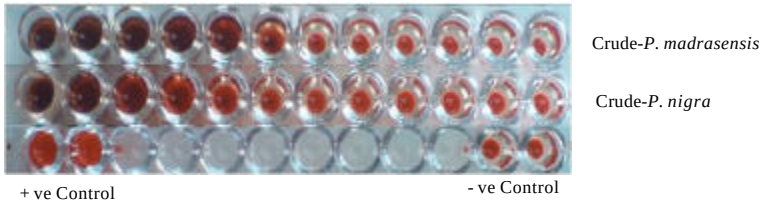

Fig. 3: Heamolytic activity in chicken blood

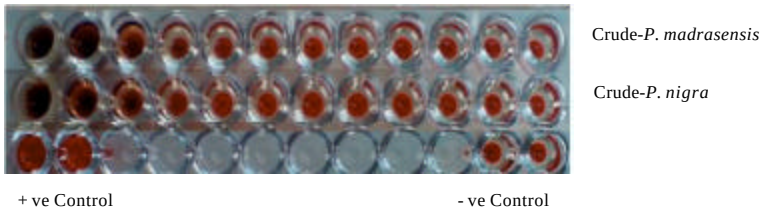

Fig. 4: Heamolytic activity in goat blood

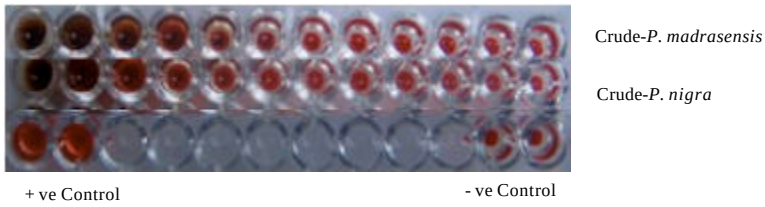

Fig. 5: Heamolytic activity in human "A" blood group

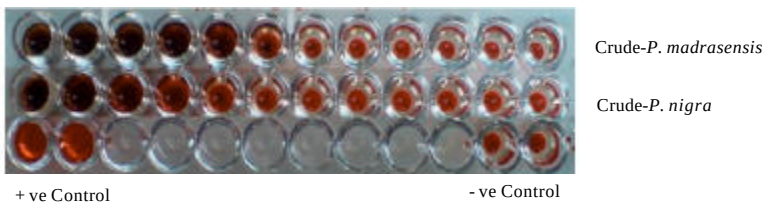

Fig. 6: Heamolytic activity in human "B" blood group

blood group, *P. madrasensis* and *P. nigra* extracts showed 32 HU (Fig. 7). In human O blood group, *P. madrasensis* and *P. nigra* extracts showed 2 HU, 32 HU (Fig. 8). Present study supports the previous reports i.e., the hemolytic activity of tunicate, *Halocynthia aurantium* disrupted 8% and 16% of human erythrocytes respectively (Jang *et al*., 2002). Hemolysis of human and sheep red blood cells has been studied by Al-Hassan *et al*. (1982). Al-Lahham *et al*. (1987) has studied the specific activity of the catfish epidermal factor is 20.6 units mg^{-1} protein, a level somewhat lower than those of most protein hemolytic factors. Lee *et al*. (2001) revealed that hemolytic activity of extracts from the ascidian, *Halocynthia aurantium* showed 21% lysis against human red blood cells. Malarvannan (2002) reported that crude and partially purified protein fractions of fish extract failed to elicit any hemolytic activity on chicken blood. But the crude lipid and partially purified lipid fractions exhibited a potent hemolytic activity in chicken blood i.e., the minimum activity was exhibited by crude lipid of *O. biauritus* and the maximum activity in *P. diacanthus* and *S. tumbil*. Cameron and Endean (1973) found that the hemolytic components in catfish are often elaborated from specilised venom glands. Catfish skin toxin and the venom from their dorsal and pectoral spines have been reported to be hemolytic (Mann and Werntz, 1991). Hemolytic activity has been observed in many of the tissue products of aquatic organisms. The hemolytic activity of monomeric and dimeric cynthacurin compound isolated from the ascidians of Sackcho, South Korea showed 21% of lysis against human red blood cells, an equal concentration of cynthacurin lacked hemolytic activity against other mammalian red blood cells (Lee *et al*., 2001). From the results they concluded that lytic protein substances present in these animals were identified which are found in invertebrates and prochordates may be involved in immunity or prey capture.

Preliminary characterisation of compound of ascidian extracts: The structure of all compounds was determined mainly from NMR measurements including 15N chemical shifts obtained from 15NH HMBC spectra (Fig. 9, 10.). In the NMR study ^{1}H-NMR spectrum was taken in DMSO D_6. From the spectral signal at δ 7.9 indicates the presence of guanidine and thymidine containing alkaloid, however the compound to be isolated and confirmed. Present study supports

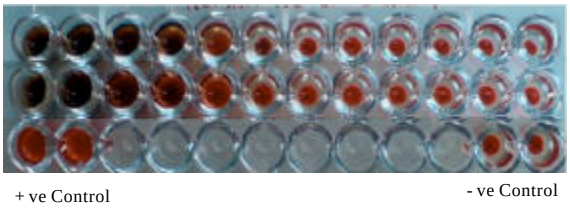

Fig. 7: Heamolytic activity in human "AB" blood group

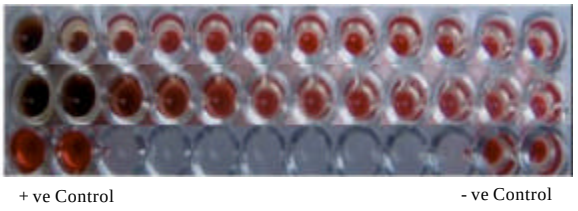

Fig. 8: Heamolytic activity in human "O" blood group

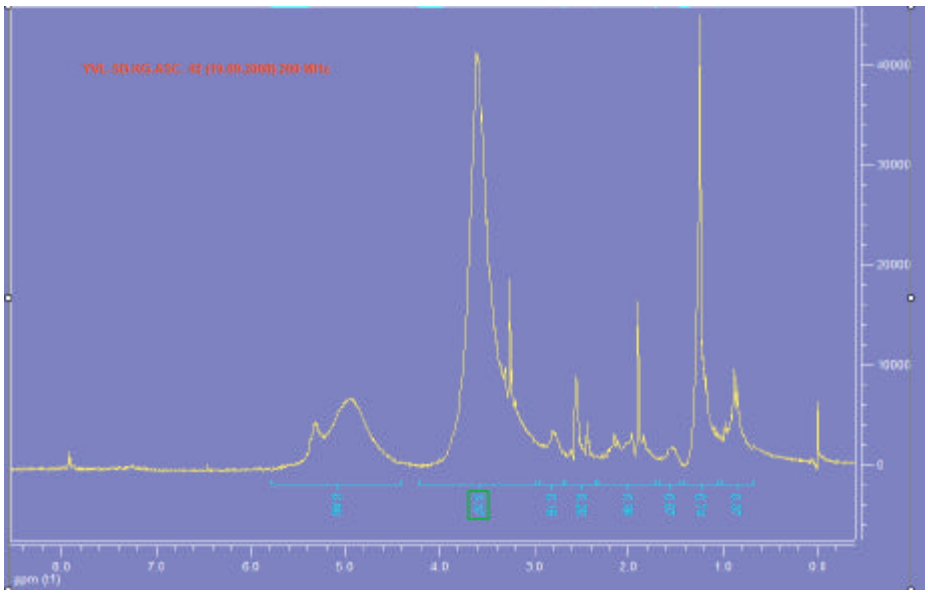

Fig. 9: The ^{1}HNMR study of crude compound of *P. madrasensis* using bruker avance 300 MHz

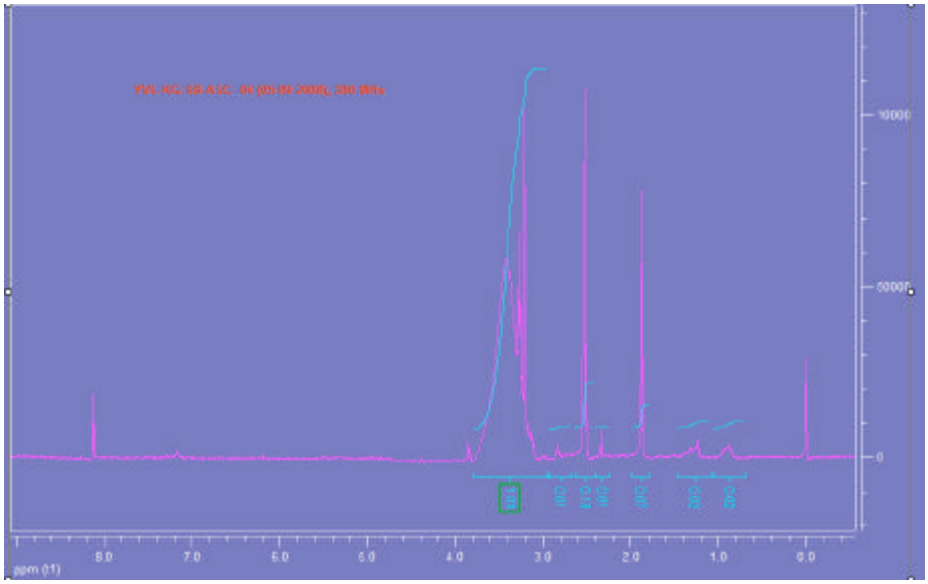

Fig. 10: The ^{1}H NMR study of crude compound using of *P. nigra* bruker avance 300 MHz

the previous reports. i.e., the structures of the pyrrole alkaloids polycitones A and B, originally isolated from a South African collection of the ascidian *Polycitor* sp. and Madagascan collections of *Polycitor africanus* (Rudi *et al*., 2000) have been confirmed by synthesis (Kreipl *et al*., 2002). Two new pyridoacridine alkaloids, kuanoniamines E 689 and F 690 and a putative biosynthetic precursor subarine 691 were isolated from a Singaporean collection of an unidentified

ascidian (Nilar *et al*., 2002). Sebastianines A 683 and B 684 were isolated as biologically active pyridoacridine metabolites from a Brazilian collection of the ascidian *Cystodytes dellechiajei* (Torres *et al*., 2002). The relative stereochemistry of 684 was determined by interpretation of NOESY NMR data. The carbon skeleton of 692 was established by analysis of HMBC and 2D INADEQUATE NMR data, while relative stereochemistry was determined by analysis of ROESY NMR data. The absolute stereochemistries of 694-696 were established by comparison of CD data with those observed for staurosporine which has defined absolute stereochemistry (Funato *et al*., 1994). Less than 10% of bioactive compounds of tunicate are devoid of N_2, but some important antimicrobial aromatic compounds and lactones has been described in this group (Sato *et al*., 1989). This indicates the presence of a kind of defense system possibly based on the biosynthesis of bioactive compounds (Koulman *et al*., 1999). Marine ascidians compounds are simple amino acid derivatives or more complex alkaloids. They often exhibit potent cytotoxic activities so they are considered unusual cytotoxic metabolites. Thus the current studies revealed the presence of potent Hemolytic compounds in marine ascidian which could be used in pharmacological research.

CONCLUSION

Present results bring about that the search for novel bioactive compounds by efficient sourcing method. Screening tactics based on the ecological knowledge of marine organisms are being increasing deployed in the investigation of novel bioactive compounds. However, the result presented here indicated that marine invertebrates show hemolytic properties against human, cow, goat and chicken bloods. Present preliminary results expose that many of these marine organisms produce more or less structurally diverse secondary metabolites which could be of Pharmaceutical interest.

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