EFFECT OF EXTRACTS OF MARINE SPONGE AXINELLACARTERION HAEMOLYTIC AND HAEMAGGLUTINATION ACTIVITY

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Abstract

Marine sponges are considered to be true "chemical factories" producing hundreds of unique chemical compounds, many of which have been isolated and their structure determined, but their biological roles and activities are still largely unknown. In the present study, extracts were prepared in aqueous and organic solvents and were tested for hemolytic, hem agglutinating and blood coagulation activity. Extracts of Axinellacarteri in acetone and hexane did not cause haemolysis in human erythrocytes while DEAE methanol and DEAE chloroform cause minimum haemolysis. Maximum haemolysis occurred in crude extracts. Extracts of A.carteri showed absence of blood clotting as well as failed to agglutinate human blood. Therefore extracts could be used in surgical procedures to inhibit blood coagulation and further study is required to find the type of lectin present in it. This can help in finding and extracting materials from A. carteri for medicinal purpose and understand their biotechnological perspective.

Keywords: Marine sponges, Axinellacarteri, Haemolytic activity, Blood coagulation, Haemagglutination

1 Introduction

Marine biodiversity is the result of a long evolutionary history and has been evolving and diversifying (Burgess, 2012). This amazing, but understudied, species diversity has provided a huge variety of molecules with biotechnological applications. Many of these compounds, which have already been isolated and studied, belong to marine invertebrates, such as sponges, cnidarians, mollusks, arthropods, echinoderms and tunicates

(Rasjasaet al., 2011). Among these compounds are proteins (Pajicet al.,2002) and secondary metabolites (Kuramotoet al., 2004)with a wide range of biological activities.

Haemolysis is due to red blood cells destruction which results from lysis of membrane lipid bilayer. The haemolysis relates to concentration and potency of extract used and furthermore the chemical composition of each extract is related to the haemolytic activity. Various authors (Cariello and Zanetti, 1979; Makarievaet al., 1983; Hirata and Uemura, 1986; Sepcicet al., 1997; Newbold et al., 1999; Rangel et al.,2001; Kossuga, 2008)have reportedhaemolytic activity using sponge extracts.

The ability of the blood to undergo blood clotting whenever there is an injury to the tissue or blood vessel and then the subsequent dissolution of the clot following repair of the injured tissue is termed haemostasis. Various pharmaceutical compounds find their origins in plant material (Electricwalaet al., 1991). Novel anticoagulants of the plant JatrophacurcasL used as a haemostatic agent, was reported to have pro-coagulant abilities at high concentrations, but anticoagulant activity at low concentrations (Reddy etal., 2011).

Hemagglutinins are proteins that possess a specific affinity for certain sugar molecules and may attach to receptor groups, if the lectin molecules have two active groups. It is characterised by their action on red blood cell membrane causing the red blood cells to clump together (Savage, 2003). Lectins from marine organisms are one of the promising candidates for useful therapeutic agents because carbohydrate structures, such as proteoglycans, glycoproteins, and glycolipids, have been implicated in certain cell types, and their physiological and pathological functions include host-pathogen interactions and cell-cell communication (Ogawa et al., 2011).

The present study was undertaken to evaluate whetherhaem agglutination activity, haemolytic activity and blood coagulation takes place in human erythrocytes when they were treated with the extracts of A.carteri.

2 Materials and methods

The sponge Axinellacarteriwas collected from subtidal areas of the Arabian Sea at KharDanda, Mumbai, India (latitude: 18.79 N and longitude: 72.92 E).

Preparation of Sponge Extracts:

10g each of the dried sponge sample was soaked in 200ml of methanol and 200ml of chloroform:methanol (2:1) and kept standing for 24 hrs(Purushottama et al., 2009). Solvents were removed by squeezing the sponge samples and filtered through Whatman filter paper number 1. The solvents were evaporated at low pressure using BuchiRotavapor R-200 (USA) at 45°C. The resultant compound was finally dried in a vacuum desiccator and labelled as crude

methanol and crude chloroform:methanol extracts. These crude extracts were fractionated using hexane (non-polar) and acetone (semi polar). The crude extracts were also partially purified using DEAE Cellulose column chromatography using 0.2, 0.4, 0.6, 0.8 and 1.0 M NaCl in phosphate buffer.

2.1 Haemolytic activity

Human blood was obtained in EDTA solution (2.7 g in 100 ml of distilled water) as an anticoagulant at 5% of the blood volume and brought to the laboratory. 5ml of blood was collected in a tube containing heparin. The blood was centrifuged at 1500 rpm for three minutes in a laboratory centrifuge. Plasma (supernatant) was discarded and the pellet was washed three times with sterile phosphate buffer saline solution (pH 7.2) by centrifugation at 1500 rpm for 5 min. The cells were suspended in normal saline to 0.5%.

In vitro haemolytic activity was performed by spectrophotometer method(Yang et al., 2005). A volume of 0.5ml of the cell suspension was mixed with A. carter fractions of hexane, acetone, DEAE methanol, DEAE chloroform methanol, crude methanol and crude chloroform: methanol of concentration 0.2 mg/ml, 0.4mg/ml, 0.6mg/ml, 0.8 mg/ml, 1.0 mg/ml in phosphate buffer saline. The mixtures were incubated for 30 min at 37^{o}C. The mixtures were centrifuged at 1500 rpm for 10 minutes. The free haemoglobin in the supernatant was measured in UV-Vis spectrophotometer at 540 nm. Phosphate buffer saline and distilled water were used as minimal and maximal haemolytic controls. Each experiment was performed in triplicate at each concentration. The level of percentage haemolysis by the extracts was calculated according to the following formula.

$$\% \text{ Haemolysis} = \frac{\text{Absorbance of sample} - \text{Absorbance of blank}}{\text{Highest Absorbance for positive control}} \times 100$$

2.2 Blood coagulation

Fresh blood samples were collected from pathology laboratory of Wockchardt Hospital, Mumbai. The procedure wasfollowedusing the protocol prescribed in United States Pharmacopoeia(1980). The blood samples were diluted with 8% sodium citrate in the proportion of 1:19 (v/v). It was then centrifuged and plasma component of the blood was separated. The A.carterifractions of hexane, acetone, DEAE methanol, DEAE chloroform methanol, crude methanol and crude chloroform methanol of differentconcentrations (0.2 ml, 0.4 ml, 0.6 ml 0.8ml and 1.0 ml) were added to 1.0 ml plasma along with 0.2 ml of 1% calcium chloride solution. The clot formation was observed and noted in different concentration of various extracts.

2.3 Haemagglutination

Haemagglutination activity was performed by assay of haem agglutination as described by Mebset al.(1985). Fresh blood sample of blood groups A, B AB and O were collected from pathology laboratory of Worckhardt Hospital, Mumbai.

Fresh blood samples were centrifuged and the erythrocytes were separated. 2 % erythrocytesuspensions were prepared in phosphate buffer (pH 7.4). The A. carterifractions of hexane, acetone, DEAE methanol, DEAE chloroform methanol, crude methanol and crude chloroform methanol samples were prepared in different dilutions. From each dilution 1 ml was added to erythrocyte suspension on a blood typing plate and was incubated at 37^{o} C. The plates were left undisturbed for 1 hr at room temperature in order to allow agglutination of the erythrocytes to take place. Positive and negative results were indicated by rough granules and smooth button formation.

3 Results

3.1 Haemolytic activity

The haemolytic activity of different fractions of A.carteri is shown in Table 1. It was observed that there was no haemolytic activity with hexane and acetone fractions, very low activity was observed with DEAE methanol and DEAE chloroform methanol fractions while a high activity was observed with crude methanol and crude chloroform methanol extracts. In the present study the haemolytic activity of A.carteri is expressed as percentage haemolysis Figure 1 and reported as meanof three replicates.

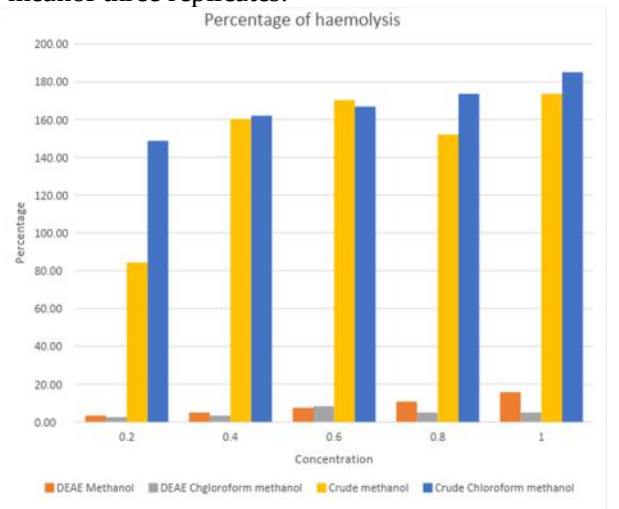

Figure 1: Percentage Haemolytic in different extracts of Axinellacarteri

The lowest activity was observed with DEAE chloroform methanol with a percentage of 2.48%. The haemolytic activity was found to increase with the increase in concentration of fractions. The highest activity was found with crude extracts of chloroform and chloroform methanol. It also showed concentration dependent activity. Maximum activity was observed in chloroform:methanol

with concentration of 1.0mg of about 185.12%. Crude methanol at concentration of 0.2 showed the least activity of 84.30% in crude extracts.

Concentration	**0.2mg/ml**	**0.4 mg/ml**	**0.6 mg/ml**	**0.8 mg/ml**	**1.0 mg/ml**
Hexane	No activity	No activity	No activity	No activity	No activity
Acetone	No activity	No activity	No activity	No activity	No activity
Crude methanol	84.30	160.33	170.25	152.07	173.55
Crude chloroform methanol	148.76	161.98	166.94	173.55	185.12
NaCl M	**0.2M**	**0.4M**	**0.6M**	**0.8M**	**1.0M**
DEAE Methanol	3.31	4.96	7.44	10.74	15.70
DEAE chloroform methanol	2.48	3.31	8.26	4.96	4.96

Table 1: Percentage Haemolytic activity in human erythrocytes in different extracts of A.carteri.

3.2 Blood coagulation

The various crude and partially purified extracts like hexane, acetone, DEAE methanol, DEAE chloroform methanol, crude methanol and crude chloroform methanol did not have any effect with the human blood plasma. There was no coagulation observed with the extracts at various concentrations of 0.2 ml, 0.4 ml. 0.6 ml, 0.8ml and 1.0 ml.

3.3 Haemagglutination activity

There was no agglutination activity in the human blood of group A, B, AB, and O against the hexane, acetone, DEAE methanol, DEAE chloroform methanol, crude methanol and crude chloroform methanol extracts of A.carteri.

4 Discussions

In the present study of haemolytic activity on human erythrocytes with the extracts of A.carteri, it was found that the extract in crude methanol and crude chloroform methanol showed high level of haemolysis which exhibited concentration dependent haemolytic activity. This is in agreement with the results reported for haemolytic activity using the venom of Palythoacaribaeorum, a Cniderian (Lazcano-Perez et al., 2018). DEAE methanol and DEAE chloroform showed some activity but it was comparatively very low. The extracts in hexane and acetone did not have any effect on human erythrocytes. Many authors have observed the haemolytic activity of sponge extracts on erythrocytes of human and other animals. The results of haemolytic activity of

sponge Halichondriapanicea on human blood, chick blood and goat blood showed least activity with human erythrocytes compared to other animals(Prahalathanet al.,2009). Studies indicated significant levels of haemolytic activity of the sponge Renierasarai extracts (Sepcić et al.,1997;Cariello and Zanetti, 1979) and moderate haemolytic activity of extracts from Saracotragusmuscarum and Aplysinaaerophoba (Sepcić et al.,1997). Haemolytic activity of halitoxin from sponges of the genus Haliclona(Schmitz et al.,1978),of aqueous extracts from 48 tropical sponge species(Schmitz et al., 1978) and of organic extracts of Geodiacorticostylifer(Rangel et al., 2001)were recorded.

It was observed that there was pronounced haemolysis in crude methanolic and chloroform- methanol extracts of Halichondriapanicea (Purushottamaet al., 2009) with chicken blood and human blood while aqueous extract failed to elicit haemolysis in chicken and human blood which is in agreement with present study.

The present study on the effect of different extracts of sponge A.carteri on human blood plasma showed that there was no coagulation with any of the extracts indicating that A. carteri extracts have no effect on blood clotting. There may not be any compounds in A. carteri which can induce blood clotting as was observed in case of cuttlefish ink (Vennilaet al., 2011). Procoagulant activity observed in extracts may be due to presence of compounds which are calcium dependant prothromb in activator (Guerrero and Arocha-Piñango,1992).Compounds known to suppress the extent of coagulation are the coumarins (Pochet, 2004). Warfarin is a coumarin that has the ability to inhibit vitamin K-dependent clotting factors, such as clotting factors II, VII, IX and X (Rang et al., 2007).Normally surgical procedures require that any anticoagulant therapy should be ceased prior to undergoing the surgery, as the prolonged clotting time increases the risk of bleeding episodes (Cordierand Steenkamp,2012).The extracts of marine sponge Haliclonatubifera were found to exhibit anticoagulant activity(Biegelmeyeret al., 2015).The marine environment plays an important role in the search for anticoagulant products, and the vast majority of these compounds are polysaccharides(Meloet al., 2004; Rocha et al.,2005;Drozdet al., 2006). Nevertheless, only a few anticoagulant products were isolated from marinesponges: A sesquiterpene from Coscinodermamathews i(Kimuraet al., 1998) and a peptide from the Australian sponge Lamellodysideachlore (Carroll et al., 2004).

Agglutination can be used to find the blood type and for estimation of number of virus particle (Wei and Koh, 1978). Haemagglutination activity is generated by protein and the protein found in sponges which show haemagglutination contain lectin. It is however pointed out that the size of such a protein varies and that some types of lectin may not trigger hemagglutination (Prahalathanet al., 2009).

Haemagglutination in twelve species of sponges was observed and found that it was different for different blood types suggesting the presence of lectin like molecules in the sponges (Moura et al.,2015). Since the sponge A.carteriextracts failed to agglutinate human blood, it may be lacking lectins.

Conclusion

Extracts of A.carteriin acetone and hexane did not cause haemolysis in human erythrocytes while DEAE methanol and DEAE chloroform fractions resulted in minimum haemolysis. Maximum haemolysis occurred in crude extracts.Hence extracts of A.carteri can be used for therapeutic purpose with proper extraction and purification.Extracts of A. carteri showed absence of clotting of blood. Therefore, extracts could be used in surgical procedures to inhibit blood coagulation though it is not conclusive and requires further Invivostudies to determine the actual effect.The sponge A.carteriextracts failed to agglutinate human blood indicating thatlectins may be absent or ineffective and further study in this regard is required. This can help in finding and extracting materials from A. carteri for medicinal purpose and understand their biotechnological perspective.

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