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ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF ETHANOLIC EXTRACT FROM OPHIOCOMA ERINACEUS AND OPHIOMASTRIX ANNULOSA

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ABSTRACT

In high-throughput search for bioactive compounds under resource-limited settings from brittle star, the ethanolic extracts of the test samples were screened for antioxidant and antibacterial activity. Antioxidants due to their scavenging activity are useful for the management of many diseases. DPPH stable free radical method is a sensitive way to determine the antioxidant activity of *O.erinaceus a*nd *O.annulosa* extracts, antibacterial assay was performed against human pathogens namely; *Bacillus sps, Pseudomonas aeruginosa*, *Proteus sps* and *E.coli* using turbidimetric method. The results suggest that *O. erinaceus a*nd *O. annulosa* exhibited broad spectrum of antibacterial activity.

KEYWORDS: Antioxidant, DPPH, Antimicrobial, Ophiocoma erinaceus and Ophiomastrix annulosa.

INTRODUCTION

Natural products obtained from marine sources have provided useful resource having medicinal values. When compared with the natural products obtained from terrestrial sources, marine origin also equally produces mammoth resources of novel compounds with possible pharmaceutical importance. These marine products from the marine invertebrates namely the echinoderms (sea stars) have been evolved over millions of years (Banu *et al* 2017). The products specifically the secondary metabolites which are produced by them are an integral part of their survival tactic.

Phylum Echinodermata which included sea lilies, feather stars, brittle stars, sea stars, sand dollars, sea urchins, sea biscuits and sea cucumbers, is one of the most distinct phyla of the animal kingdom. Most of the approximately 6,500 echinoderms are marine species with none of them living in freshwater (Pechenik, 2005). Organisms that belong to this phylum were already proven to contain antibacterial, antifungal, antiviral, antitumor, anticoagulant, cytotoxic, hemolytic, antithrombotic and even anti-HIV agents (Blunt et al., 2007, Kelly, 2005, Fusetani, 2012 & Caulier et al, 2011).

The secondary metabolites are nothing but the chemical defenses produced by the marine sea star in order to protect them from the predators during attack. These chemical metabolites serve as a sole source of compounds having medicinal value for mankind (Blunt et al., 2007). Collection of these organisms for biochemical or biomedical research to further enrich the knowledge concerning local marine lives is very conducive. A lot of marine organisms remained unstudied and by exploiting them as source of drugs or chemotherapeutic agents is of great value.

This study will give pharmacological knowledge about two brittle stars namely *Ophiocoma erinaceus a*nd *Ophiomastrix annulosa*. Ethanolic solvent extracts from these organisms will be screened and possibly be used as potential sources of antioxidant and antibacterial agents.

MATERIALS AND METHODS

Materials

Brittle stars (*Ophiocoma erinaceus a*nd *Ophiomastrix annulosa*) were gathered in Andaman island, from the shallow up to deeper parts of the sea. Each sample was washed with water to remove dirt and sand. Samples were individually packed in polypropylene bags.

Extraction

Air dried samples were cut into small pieces and soaked in 95% Ethanol (1g: 4mL) for one week. The crude extract was centrifuged and the centrifuged was concentrated under reduced pressure at 40°C using rotary evaporator (Buchi R-124).

Antioxidant activity

Determination of in vitro antioxidant assay

DPPH radical scavenging assay

Tissue extracts I & II were tested for the scavenging effect on DPPH radical according to the method of Pan *et al*. 0.2 mL of extract solution in ethanol (95%) at different concentrations (0.2, 0.5, 0.8 and 1.2 mg mL-1) was added to 8 mL of 0.004% (w/v) stock solution of DPPH in ethanol (95 %). The scavenging activity on the DPPH radical was determined by measuring the absorbance at 517 nm. As a positive control, synthetic antioxidant Vit C was used. All determinations were performed in triplicate. The DPPH radical scavenging activity (*S%*) was calculated using the following equation: *S%* = ((*A*control – *A*sample)/*A*control)×100, where *A* control is the absorbance of the blank control (containing all reagents except the extract solution) and *A* sample is the absorbance of the test sample.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was determined according to the method of Beara *et al*. with some modification. 2 mL of extract solution (0.2, 0.5, 0.8 and 1.2 mg mL-1), 1.0 mL of *ortho*-phenanthroline (7.5 mmol L-1), 5.0 mL of phosphate buffer (0.2 M, pH 6.6), 1.0 mL of ferrous sulfate (7.5 mmol L-1) and 1.0 mL of H2O2 (0.1 %) were mixed and diluted to 25 mL with distilled water. After incubation at room temperature for 30 min, the absorbance was measured at 510 nm. The scavenging percentage (*P%*) was calculated as *P%* = ((*A* – – *A*1)/(*A*2 – *A*1))×100, where *A*, *A*1 and *A*2 are the absorbance value of the system with all solution including H2O2 and the extract solution, the system without extract solution, and the system without H2O2 and the extract solution, respectively.

Nitric oxide radical scavenging assay

Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction. This assay was done by the method of (Marcocci et al, 1994). 320µl ethanol extract, 360 µl sodium nitroprusside, 216 µl Greiss reagent (1% sulphanilamide, 2% H3PO4 and 0.1% napthylethylenediamine dihydrochlorid) was mixed and incubated at 25ºC for one hour. Lastly 2ml water was added and absorbance was taken at 546nm.

Ferric reducing/antioxidant power (FRAP)

The FRAP method was used to determine the antioxidant activity in easy way according to Benzie. The FRAP reagent was firstly prepared by mixing of 300 mM acetate buffer and pH 3.6 with 10 mM iron reagent (TPTZ) solution which was dissolved in 40 mM and 20 mM for

HCl and FeCl3.6H2O successively. Then, about 1.9 ml of the FRAP solution was kept at 37°C and reacted with 0.1 mg of starfish tissue I & II extracts in light of preparing a good condition for reaction. The mixture was incubated for 4 min, and then the absorbance was measured at 593 nm by using spectrophotometer. In light of calculating the results, the standard curves was prepared by using an appropriate concentration of FeSO4.

ABTS radical scavenging activity

The 2,20-azinobis (3-ethylbenzthiazoline-6-sulphonic acid), commonly called ABTS cation scavenging activity was performed (Delgado-Andrade et al). Briefly, ABTS solution (7 mM) was reacted with potassium persulfate (2.45 mM) solution and kept for overnight in the dark to yield a dark coloured solution containing ABTS radical cations. Prior to use in the assay, the ABTS radical cation was diluted with 50% methanol for an initial absorbance of about 0.70±0.02 at 745 nm, with temperature control set at 30°C. Free radical scavenging activity was assessed by mixing 300 µl of test sample with 3.0 ml of ABTS working standard in a microcuvette. The decrease in absorbance was measured exactly one minute after mixing the solution, then up to 6 min. The percentage inhibition was calculated according to the formula: Scavenging effect (%) [(Control absorbance)/(Control absorbance)] X 100.

Superoxide dismutase (SOD) method

This method might be used for determination of antioxidant activity of a samples, and it was described by McCord and Fridovich. The main purpose of this method that was estimated 5% of a red blood cell after adding 75 mM, 30 mM, and 2 mM from Tris-HCL (pH 8.2), EDTA, and pyrogallol respectively. Then, the absorbance was measured at 420 nm. The percentage of inhibition was calculated depending on that the ability of enzyme to inhibit of oxidation. So, any changes might be happened on the absorbance, it will give a clear picture on the ability of enzyme activity to prevent oxidation.

Antimicrobial activity

Preparation of standard bacterial suspensions

In vitro antibacterial activity was examined for different concentrations of the *O. erinaceus* and *O. annulosa.* The microorganisms investigated were *Bacillus sp, Pseudomonas aeruginosa, Proteus sp* and *E.coli.* All the bacterial strains were maintained at 4°C on nutrient agar slants (1g yeast extract, 1 g beef extract, 0.5 g NaCl, dissolved in 100 ml distilled water). Each time, a fresh stock suspension was prepared; the experimental

conditions were maintained constant so that suspensions with very close viable counts would be obtained.

Antibacterial Assay

The bioassay used was the standard disk diffusion assay adapted from Taylor *et al*. (1995). Test disks were prepared by dipping and saturating sterilized filter paper disks in the test suspensions. Same sized filter paper disks (6 mm diameter) absorbed the same volume of extract. Mueller Hilton agar containing Beef, infusion from [300g/l], Casein hydrolysate [17.5g/l], Starch [1.5g/l] and Agar [17g/l] plates were prepared for doing bioassays against bacteria. The media was prepared by dissolving 19g of Mueller Hilton Agar in 500 ml of distilled water, autoclaved and cooled. The media was poured in the petriplates and kept for 30 minutes for solidification. After 30 minutes the fresh overnight cultures of inoculums (100 µl) of four different culture were spread on to solidified nutrient agar plates using the sterile cotton swab.

All petridishes were sealed with the sterile laboratory parafilm to avoid eventual evaporation of the test samples. The plates were left for 30 minutes at room temperature to allow the diffusion of the samples and then they were incubated at 37°C for 24 hours to allow the maximum growth of the microorganisms. The test materials showing antibacterial activity by inhibiting the growth of the thus shows a clear, distinct zone of inhibition surrounding the discs. After incubation, the diameters of the resulting growth inhibition zones were measured, averaged and the mean values were tabulated.

Statistical analysis

The data were subjected to a one-way analysis of variance (ANOVA) and the significance of the difference between means was determined by Duncan's multiple range test ($P < 0.05$) using statistica (Statsoft Inc., Tulsa, USA). Values expressed are means of three replicate determinations ± standard deviation.

RESULT

In the present study antioxidant and free radical scavenging activities of O. erinaceus and O. annulosa were evaluated. Free radicals are involved in many disorders like neurodegenerative diseases, cancer and AIDS. Antioxidants due to their scavenging activity are useful for the management of various diseases. DPPH stable free radical method is a sensitive way to determine the antioxidant activity of O. erinaceus and O. annulosa extracts.

The results for the free radical scavenging of all the *O. erinaceus* and *O. annulosa* and known antioxidants were presented in Table 1 and Fig 1. The results of one way ANOVA test and post hoc test indicated that there was a significant difference of mean percentage scavenging between all the tested extracts.

The results showed that *O. erinaceus* extract exhibited the highest antioxidant activity the extracts analyzed against the DPPH free radical of 55.37±4.76. Moreover, IC_{50} values were (57.61±1.91) µg/mL, (62.15±2.16) µg /mL, (59.94±3.89) µg /mL, (92.53±1.68) µg /mL and (63.70±2.65) µg /mL for DPPH, HRSA, NO, FRAP, LPO and SOD radical scavenging activities, respectively. Furthermore, *O. annulosa* extract exhibited the highest antioxidant activity the extracts analyzed against the HRSA free radical of 51.21±1.32. IC_{50} values were (91.70±1.09) µg/mL, (09.15±0.94) µg /mL, (25.79±0.93) µg /mL, (163.38±0.81) µg /mL and (145.25±0.93) µg /mL for DPPH, NO, FRAP, LPO and SOD radical scavenging activities, respectively. Moreover, the results are also comparable with the positive control of the vitamin C (Table 2 & Fig 2).

Table 1: IC_{50} values of *O. erinaceus* and vitamin C with various antioxidant activities (µg/ml).

Parameter	*O. erinaceus* (IC_{50})	Vitamin C (IC_{50})
DPPH radical scavenging	55.37±4.76	2.81±1.26
HRSA radical scavenging	57.61±1.91	42.21±1.50
NO radical scavenging	62.15±2.16	4.91±1.11
FRAP radical Scavenging	59.94±3.89	56.69±1.11
ABT radical scavenging	92.53±1.68	31.79±1.21
SOD radical scavenging	63.70±2.65	24.31±0.71

Values are mean of three replicate determinations (n =3)±standard deviation. Mean values followed by different superscripts in a column are significantly different (P< 0.05).

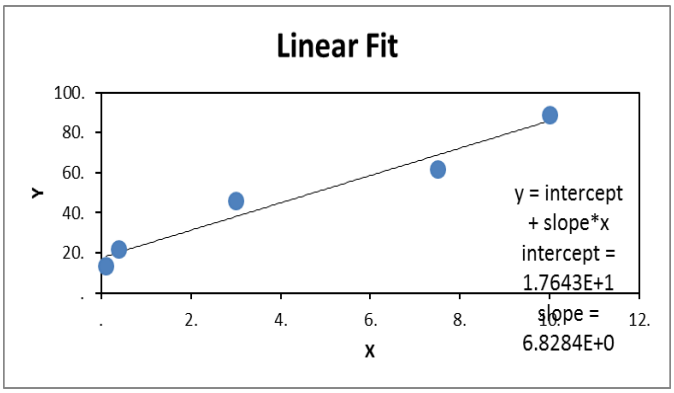

Fig. 1: Inhibition of curve of total antioxidant activity of *O. erinaceus*.

Table 2: IC_{50} values of *O. annulosa* extract and vitamin C with various antioxidant activities (µg/ml).

Parameter	*O. annulosa* (IC_{50})	Vitamin C (IC_{50})
DPPH radical scavenging	91.70±1.09	2.87±1.26
HRSA radical scavenging	51.21±1.32	42.24±1.50
NO radical scavenging	09.15±0.94	4.98±1.28
FRAP radical Scavenging	25.79±0.93	52.69±1.08
ABT radical scavenging	163.38±0.81	28.19±1.26
SOD radical scavenging	145.25±0.93	24.31±0.71

Values are mean of three replicate determinations (n =3)±standard deviation. Mean values followed by different superscripts in a column are significantly different (P< 0.05).

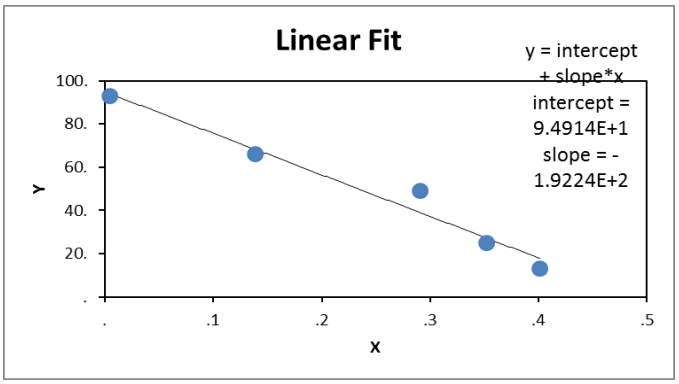

Fig. 2: Inhibition of curve of total antioxidant activity of *O. annulosa*.

The extract of *O. erinaceus* was tested for the antimicrobial sensitivity against human pathogens namely; *Bacillus sps, Pseudomonas aeruginosa*, *Proteus sps* and *E.coli* by well diffusion method. The result of *O. erinaceusc* extract showed maximum zone of inhibition against *Pseudomonas aeruginosa* 4mm in 100 µl followed by Proteus sps at 2.2mm in 100 µl at higher concentrations. However, the extract of *O. annulosa* showed maximum zone of inhibition of 3mm in 100 µl against *bacillus sps* followed *by E.coli* and Proteus sps at 2mm in 100ml respectively (Table 3).

Table 3: Antibacterial activity of *O. erinaceus* and *O. annulosa* against human pathogens.

Organism Name	*O. erinaceus* Zone of inhibition [mm]				*O. annulosa* Zone of inhibition [mm]			
Species Name	25µl	50µl	75µl	100µl	25 µl	50 µl	75 µl	100 µl
Bacillus	0.0	0.0	1.0	1.0	1.0	1.0	2.0	3.0
Proteus	1.0	1.5	2.0	2.2	0.0	0.0	2.0	2.0
Psuedomonas	1.0	2.0	3.0	4.0	1.0	1.0	1.5	1.5
E.Coli	0.0	1.0	1.0	1.5	1.0	1.0	2.0	2.0

DISCUSSION

The bioactive compounds generated majorly from marine animals are known as the secondary metabolites. These could be divided into steroids, terpenoids, isoprenoids, nonisoprenoids, quinones, brominated compounds, nitrogen heterocyclics, and nitrogen sulphur heterocyclics. Many of these molecules also represent ancient defence factors. Bioactive substances formed by marine organisms such as protozoans and invertebrates viz. poriferans, cnidarians, annelids, arthropods, molluscs and echinoderms have attracted attention due to their antiviral, antimicrobial, antiprotozoal, antifungal, antihelminthic and anticancer activities (Zapata and Amemiya, 2000). An antioxidant is a compound which counteracts the effect of oxidized and controls the build of free radicals. Due to the presence of different antioxidant components in the samples, it is relatively difficult to measure each antioxidant component separately. Therefore, several assay methods have been developed and applied in recent years to screen and evaluate the total antioxidant activity of such samples (Prabhakar *et al*, 2006; H. Wangensteen *et al*, 2004). These methods target at different mechanisms of the oxidant defense system such as, scavenging active oxygen species and hydroxyl radicals, reduction of lipid peroxyl radicals, inhibition of lipid peroxidation, or chelation of metal ions (Pandithurai and Murugesan, 2014). Antioxidants are tremendously important substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress. Total antioxidant capacity of the extract of *O. erinaceus* and *O. annulosa* was evaluated. The antioxidant potential of the *O. erinaceus* and *O. annulosa* was investigated in the search for new bioactive compounds from natural resources. The results showed that *O. erinaceus* extract exhibited the highest antioxidant activity the extracts analyzed against the DPPH free radical of 55.37±4.76. In recent years, development of multidrug resistance in the pathogenic bacteria and parasites has created major clinical problems in the treatment of infectious diseases (Ravikumar et al., 2010a). The antibacterial activity of the extract of *O. erinaceus* and *O. annulosa* against human pathogen might be due to the presence of major classes such as alkaloid and tannins. Likewise, mixtures of active constituents showed a broad spectrum of biological and pharmacological activities (Robinson, 1967). *O. erinaceus* and *O. annulosa* can be used as a promising multipurpose medicinal source whereas further clinical trial is required to prove its efficacy. This indicated that *O. erinaceus* and *O. annulosa* contained potential antioxidant bioactive compounds, which if properly and extensively studied, could provide many chemically interesting and biologically active drug candidates, including some with potential anti oxidant and antimicrobial properties.

CONCLUSION

Drug discoveries from marine non-chordates have an important research field since decades. Currently, interest in evaluating marine invertebrate products, with the aim of obtaining new potential disease preventive drugs with few side effects, is still growing. In many cases, the bioactive compounds from *O. erinaceus* and *O. annulosa* are difficult to obtain in sufficient amounts and researchers, therefore, have to start mimicry mother nature for preparation of synthetic compounds or drugs. The rich diversity in bioactive compounds from invertebrates have provided molecules that interfere with the prevention of a disease at many different points, which increase the chances of developing selective drugs against specific disease(s). Marine invertebrates have provided many examples of novel secondary metabolites that possess varied chemical status and potent antimalarial, antiinflamatory, anticarcinogenic, antibacterial, antifungal etc. activities from *O. erinaceus* and *O. annulosa.*

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