ISSN (Online) 2456 -1304

International Journal of Science, Engineering and Management (IJSEM)
Vol 3, Issue 4, April 2018

Antioxidant Studies of a Colonial Ascidian Didemnum Psammathodes

[1] K.F.Roselin, [2] C. Veerabahu, [3]V.K. Meenaksh
[1] Research Scholar
[1][2] PG & Research Department of Zoology, V.O.Chidambaram College, Tuticorin
[3] Department of Zoology, A.P.C. Mahalaxmi College for Women, Tuticorin

Abstract:- **Oxidative stress is an imbalance between the production of free radicals and the ability of the body to counteract or detoxify their deleterious effects by way of neutralisation by antioxidants. An antioxidant is a molecule that inhibits the oxidation of other molecules and protects the body cells and tissues from damages caused by free radicals and ROS. They are released from the diet that we take and enter the cells through the blood stream and prevent damage to cells by cleaning up. Methods like DPPH, Hydroxyl, ABTS, superoxide, and reducing power was used to evaluate free radical scavenging activities of various concentrations of different extracts of Didemnum psammathodes using ascorbic acid and trolox as standard. The radical scavenging effect was found to increase with increasing concentrations. Ethanol extract (108.26, 112.15) showed highest DPPH, hydroxyl radical scavenging activity whereas, in ABTS and superoxide methanol extract exhibited highest (138.16, 126.81). Methanol and ethanol extracts exhibited higher reducing ability among the solvent tested. The results revealed that the extracts possess potential reducing power and hence antioxidant activity compared to standard.**

Key Terms- Colonial ascidian, Didemnum psammathodes and antioxidant.

INTRODUCTION

Metabolic activities and oxidation are indispensible processes in all living organisms including human beings which release natural by-products called free radicals. Hydrogen peroxide, singlet oxygen, superoxide and hydroxyl radicals, collectively known as reactive oxygen species (ROS) are derived from the metabolism of oxygen in aerobic system [1]. Highly reactive free radicals, which are formed by exogenous chemicals, stress or on the food system, are capable of oxidising bio molecules, cause destructive and irreversible damage to components of a cell [2]. DNA, cell membranes, proteins and other cellular constituents are target site of the degradation process and consequently induce different kinds of inflammatory diseases, muscular dystrophy, cataracts, neurological disorders, cancer and invariably aging process. Free radicals are unstable molecules also generated in order to fend off microbes, on exposure to sun, stress and as a part of routine aging. In addition to this, nearly 5 percent of the oxygen that we inhale is converted into reactive oxygen species (ROS) such as *O2, H2O2 and *OH by univalent reduction of O2. Chemical pollutants in the environment, smoke, vehicle exhaust, radiation also add up to the generation of free radicals. They are electrically charged highly toxic molecules that target and break the cell membranes resulting in serious damage to the cellular components, nucleic acids, proteins and enzymes. This charge by free radicals on cells and tissues is known as oxidative stress which brings about the loss of the structure and function of cells leading to their destruction. In normal cases, the natural antioxidant defence system of the cells efficiently manages this threat. Thus oxidative stress is an imbalance between the production of free radicals and the ability of the body to counteract or detoxify their deleterious effects by way of neutralisation by antioxidants.

REVIEW OF LITERATURE

The bioactive metabolites extracted from seaweed, sponges and their associated bacteria which indicated good antioxidant activity leading to attention in terms of drug discovery [3]. The in vitro antioxidant activity of the methanolic extract of 11 gastropods, 4 bivalves and one echinoderm by DPPH, reducing power and total antioxidant activity which implied that bivalves which formed regular sea food for mankind possess higher antioxidant activity than the other groups investigated [4]. Studies on the in vitro antioxidant activity of methanolic extract of gastropod Babylonia zeylanica of Mudasalodia, Southeast coast of India by superoxide, hydroxyl, DPPH radical, reducing power and metal ion chelating assay which indicated potent antioxidant activity [5]. The antioxidant capacity of marine bacteria associated with sponges Jaspis sp. was analysed and attributed it to the presence of flavonoid, alkaloid and triterpenoid compounds [6]. The antioxidant activity of ethanolic extracts of two species of red sea weeds was attributed to the presence of phenolic compounds and fiber [7]. Studies on the antioxidant activity of the different solvent extracts of the simple ascidian Phallusia nigra of

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International Journal of Science, Engineering and Management (IJSEM)
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Thoothukudi coast and revealed that the ethanol extract showed strongest activity which was attributed to the presence of flavonoids and phenols [8]. The antioxidant activities of the organic extracts of 14 sea weeds, 11 sponges, 2 ascidians, 1 holothurian and 1 sea anemone species collected along the Brazilian and Spanish coast and reported that among the marine organisms studied only sponge Ircinia sp. exhibited moderate antioxidant activity [9].

MATERIALS AND METHODS

Collection of animal material

The specimens of colonial ascidian, Didemnum psammathodes were collected from the intertidal rocky shore area of Thoothukudi north break water in the month of July to November. Molluscan shell, calcrete rock fragments attached to the surface of the colony was carefully removed and it was washed several times with sea water. Later it was dried under shade and homogenized to get a coarse powder.

DPPH radical scavenging activity

The study of free radical in the antioxidant component is assayed by DPPH radical scavenging activity [10]. This method is based on the reduction of DPPH in methanol solution in presence of a hydrogen donating antioxidant resulting in the formation of the non-radical form DPPH-H. DPPH stable free radical method is an easy, rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extracts [11]. DPPH in methanol (0.1 mM) was prepared. To 3 ml of various solvent extracts (petroleum ether, benzene, ethyl acetate, methanol and ethanol) of the selected species- Didemnum psammathodes at different concentrations (50, 100, 200, 400, 800 µg/ml), 1 ml of DPPH in methanol was added. After vigorous shaking the mixture was kept undisturbed at room temperature for 30 min. The absorbance was measured at 517 nm in a UV-VIS spectrophotometer. Ascorbic acid was used as the standard. A reagent blank was also run simultaneously.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was measured as per the modified method [12]. 1mM EDTA, Ascorbic acid 10 mM FeCl3, H2O2 and deoxyribose, were prepared in distilled de ionized water as stock solutions. The hydroxyl radical scavenging activity was estimated by serially adding 0.1ml EDTA, 0.01ml of FeCl3, 0.1ml H2O2, 0.36 ml of deoxyribose, 1.0 ml of solvent extracts at different concentration (50, 100, 200, 400, 800 µg/ml), 0.33 ml of phosphate buffer (50 mM, pH 7.9) and 0.1 ml of ascorbic acid. The reaction mixture was incubated for 1h at 37 °C. 1 ml of the incubated mixture was mixed with 1ml of 10% TCA and 1ml of 0.5% TBA (in 0.025 M NaOH containing 0.025% BHA). The pink chromogen developed was measured at 532 nm. Along with the analysis, a reagent blank and standard was also run. Percentage inhibition of deoxyribose degradation is taken as the hydroxyl radical scavenging activity of the extract.

ABTS+ cation radical scavenging activity

Modified method was adopted for ABTS assay [13]. To ABTS solution (7 mM), potassium per sulphate (2.45 mM) was added and the mixture was allowed to stand in the dark at room temperature for 12-16 h in order to produce ABTS radical cation (ABTS+). To 100 µl of extract at different concentration (50, 100, 200,400, 800 µg/ml), 3.9 ml of ABTS+ solution diluted with ethanol was added. The absorbance was measured at 734 nm using a UV-VIS spectrophotometer exactly after 6 min. Trolox was used as standard. The results are expressed as trolox equivalent antioxidant capacity (TEAC). The ABTS+ scavenging assay, which employs a specific absorbance (734 nm) at a wavelength remote from the visible region and requires a short reaction time, can be used as an index that reflects the antioxidant activity of the test samples [14].

Superoxide radical scavenging activity

Modified method was adopted to measure the superoxide anion scavenging activity [15]. To 3 ml of Tris-HCl buffer (16 mM, pH 8.0), containing 0.5 ml of NBT (0.3 mM), 0.5 ml NADH (0.936 mM),1 ml extract at different concentration (50, 100, 200,400, 800 µg/ml) and 0.5 ml Tris-HCl buffer (16 mM, pH 8.0) were added in order to generate superoxide anion radicals. Initiation of the reaction was done by adding 0.5 ml PMS solution (0.12 mM). The mixture was incubated at 25°C for 5 minutes. The absorbance was measured at 560 nm against a reagent blank and standard ascorbic acid.

Ferric reducing power

Determination of the reducing power of the extract was done by the method [16]. 1ml of extract at different concentration (50, 100, 200,400, 800 µg/ml) was mixed with 5 ml sodium phosphate buffer (0.2 M, pH 6.6) and 5 ml potassium ferricyanide (1%). The mixture was incubated at 50 °C for 20 minutes and 5 ml trichloro acetic acid (10%) was added. It was centrifuged in a refrigerator centrifuge at 5 °C and 980 g for 10 minutes. 5 ml of supernatant was diluted with 5 ml of distilled water and ferric chloride. The absorbance was read at 700 nm. A reagent blank and standard ascorbic acid was used.

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Statistical analysis

All experiments were carried out in triplicate and the results are expressed as mean of these three independent analysis ± standard deviation (n=3). The free radical scavenging activity was calculated using the formula,

% scavenging activity = $\{(A_{Control}-A_{Test})/(A_{Control})\times100\}$ where $A_{Control}$ is the absorbance of control, A_{Test} is the absorbance of the extracts.

RESULTS AND DISCUSSION

Screening of the antioxidant activity of the various solvent extracts of the two colonial ascidians Didemnum psammathodes by DPPH, Hydroxyl, Superoxide, ABTS+ cation radical scavenging and Ferric reducing power indicated the following results.

DPPH radical scavenging activity

The results of DPPH radical scavenging activity of different concentrations of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of Didemnum psammathodes and standard ascorbic acid is given in Fig. 1. DPPH radical scavenging activity was higher in ethanol followed by methanol compared to standard ascorbic acid. The radical scavenging effect was found to increase with increasing concentrations. Didemnum psammathodes extracts and standard showed maximum percentage inhibition in the following order - ethanol (108.26), methanol (103.26), petroleum ether (92.84), ethyl acetate (91.15), benzene (81.13) and standard (96.16) with IC50 values of 39.40, 36.13, 29.13, 30.24, 26.12 and 32.92 µg/ml respectively. Methanol and ethanol extract showed the highest scavenging and the lowest was noted with benzene. The activity was dependent on the concentration of the extract. Upon reduction by hydrogen or electron donation, DPPH which is nitrogen centered free radical changes its colour from violet to yellow. Those substances which are able to perform this reaction are considered as antioxidants and radical scavengers [17]. Studies on ascidians like Styela clava, Ecteinascidia venui, Didemnum psammathodes, Eudistoma viride also exhibits similar observations of antioxidant activity by DPPH method [18]-[23].

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of Didemnum psammathodes is given in Fig. 2. Hydroxyl scavenging activity was higher in ethanol compared to standard ascorbic acid. The hydroxyl radical scavenging effect was found to increase with increasing concentrations. Didemnum psammathodes extracts and standard showed maximum percentage inhibition in the following order - ethanol (112.15), methanol (98.65), ethyl acetate (91.26), petroleum ether (84.15), benzene (73.92) and standard (94.56), with IC50 values of 31.46, 30.22, 28.13, 24.86, 21.13 and 29.96 respectively. Ethanol extract showed the highest radical scavenging and there was a proportionate increase with increase in the concentration of the extract. The lowest activity was noted in benzene. The hydroxyl radical generated in the body by fenton reaction is highly reactive and potent among the reactive oxygen species which can react with all bio macromolecules in the living cells, often inducing severe oxidative damage to proteins, DNA, PUFA and other nucleic acids causing aging, cancer and several other diseases [24], [25].

ABTS+ cation radical scavenging activity

ABTS+ cation radical scavenging activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of Didemnum psammathodes are noted in Fig. 3. The methanol and ethanol extract exhibited high antioxidant activity. The cation radical scavenging effect was found to increase with increasing concentrations. Didemnum psammathodes extracts and standard showed maximum percentage inhibition in the following order - methanol (138.16), ethanol (131.16), ethyl acetate (101.65), petroleum ether (98.16), benzene (84.13) and standard (98.27) with IC50 values of 46.94, 42.26, 40.22, 39.16, 34.18 and 31.92 (µg/ml) respectively. In this method the antioxidant activity increases with increasing concentration. The maximum scavenging effect was shown by methanol and ethanol extract and the lowest were noted with benzene. ABTS assay is a simple indirect method for determining the activity of natural antioxidants [26].

Superoxide radical scavenging activity

Different solvent extracts of Didemnum psammathodes were subjected to superoxide radical scavenging assay and the results are noted in Fig. 4. The methanol, ethanol and petroleum ether extracts exhibited high antioxidant activity. The effect was found to increase with increasing concentrations. Didemnum psammathodes extracts and standard showed maximum percentage inhibition in the following order - methanol (126.81), ethanol (118.26), petroleum ether (112.13), ethyl acetate (96.16), benzene (89.26) and standard (106.26) with IC50 values of 40.82, 36.23, 34.55, 31.83, 29.56 and 33.43 respectively. To test whether the extract scavenges superoxide anions or not, NBT assay was carried out. All the solvent extracts of Didemnum psammathodes showed dose dependent superoxide anion scavenging activity. The highest activity was noted for methanol and the lowest activity was observed for benzene. The percentage inhibition of anions was almost same compared to the other radicals studied.

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Similar observation of direct relationship between increase in the % of inhibition and concentration of the extract has been reported by earlier workers [20]-[22] on studies with colonial ascidians and indicated that it may be due to polyphenols with redox properties which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers.

Ferric reducing power

Reducing power of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of Didemnum psammathodes is compiled in Fig. 5. The radical scavenging effect was found to increase with increasing concentrations. Higher reducing power is indicated by higher absorbance. Methanol and ethanol extracts exhibited higher reducing ability among the solvent tested. In comparison with ascorbic acid, the extracts possess potential reducing power and hence antioxidant activity. The reducing power increased with the increase in the concentration. The highest reducing power was observed in the ethanol and methanol extracts compared to the standard and the lowest value was noticed in the benzene and petroleum ether extract of both the species studied. The results show the presence of reductants or electron donors in the extract that can react with free radicals which might have led to the reduction of ferric/ferricyanide complex to ferrous form, thus converting them to stable products and terminate the chain reaction as has been reported on studies with halophilic bacterial bionts [27]. The reducing capacity of the compound serves as a significant indicator of its potential antioxidant activity.

The neutralisation of free radicals or their actions by the antioxidants occurs in different levels. It may act at the stage of prevention, interception and repair. Preventive antioxidants attempt to stop the formation of ROS. This includes SOD that catalyses the dismutation of super oxide to H2O2 and CAT that breaks it down to water [28]. Interception of free radicals is mainly by radical scavenging and at the secondary level peroxyl radicals are affected. The effectors include non enzymatic antioxidants like vitamin C, E, glutathione, thiol compounds, carotenoids and flavonoids. At the repair and reconstitution level, repair enzymes are involved. The present study clearly indicates that the different extracts of Didemnum psammathodes show strong antioxidant activity when compared with standard ascorbic acid or trolox and suggests its safe use as a therapeutic antioxidant.

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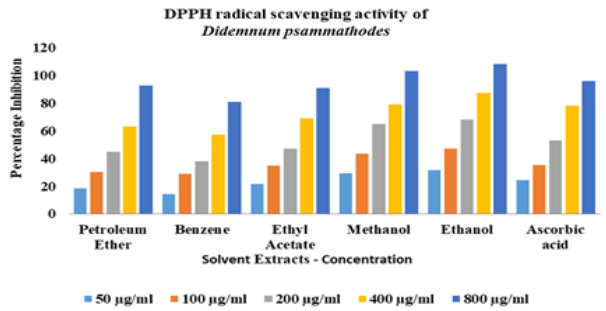

Fig.1 DPPH radical scavenging activity of Didemnum psammathodes

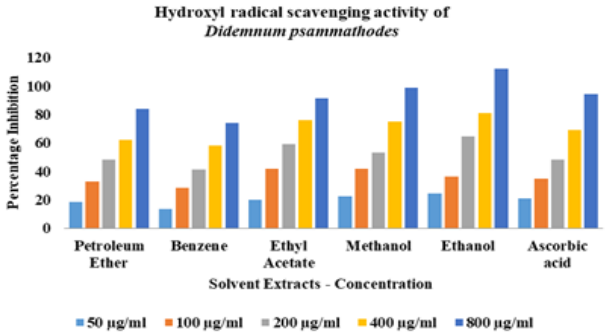

Fig. 2 Hydroxyl radical scavenging activity of Didemnum psammathodes

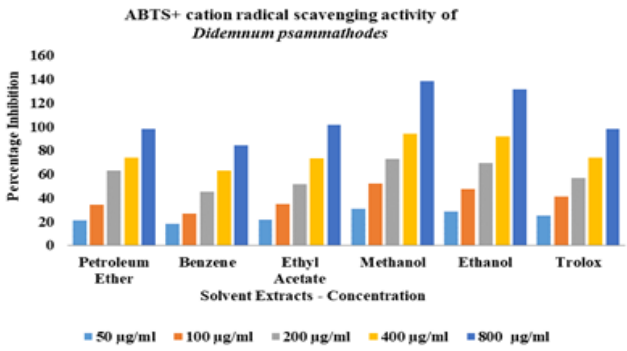

Fig. 3 ABTS+ cation radical scavenging activity of Didemnum psammathodes

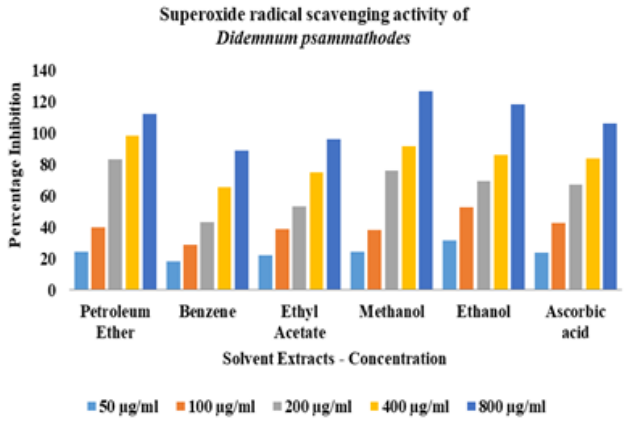

Fig. 4 Superoxide radical scavenging activity of Didemnum psammathodes

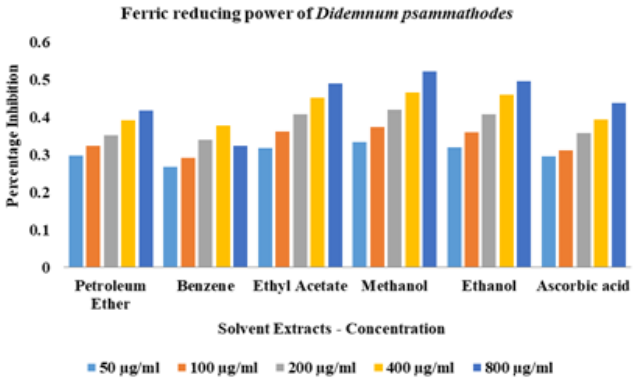

Fig. 5 Ferric reducing power of Didemnum psammathodes