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Free Radical Scavenging Potential and Total Phenol Content of Two Colonial Ascidian Species, *Eudistoma amplum* and *Polyclinum audum*, from the Coast of Mandapam, Gulf of Mannar, India

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Abstract

Scientific information on antioxidant properties and phenolic content of less widely used ascidians can be useful. Therefore, the assessment of such properties remains an interesting and useful task, particularly for finding new sources for natural antioxidants and nutraceuticals. As knowledge about antioxidant properties and phenolic content of ascidian species is limited, we determined in vitro the total antioxidant activity and the phenolic content of chosen colonial ascidians, *Eudistoma amplum* and *Polyclinum nudum*, collected from Mandapam coast of Gulf of Mannar region, India, using 1,1-diphenyl-2-picrilhydrazyl (DPPH) radical scavenging activity. Antioxidant activity of extracts is expressed as the percentage of DPPH radicals inhibition as ascorbic acid equivalents and IC50 values (mg/ml). The total phenolic content of the extracts is expressed as gallic acid equivalents. The significant linear correlation is confirmed between the values for the total phenolic content and antioxidant activity of the ascidian extracts. The results show that there is a strong correlation between total phenol contents and antioxidant activity of the crude extract of the chosen ascidians. Among the selected ascidian extracts, *P. nudum* displayed higher DPPH radical scavenging activity (91.67%) with an IC_{50} value 20.95 mg/ml and this concentration is close to that of IC_{50} concentration (11.86 mg/ml) of ascorbic acid, whereas *E.amplum* had the maximum scavenging activity (81.67%) with an IC_{50} value 31.34 mg/ml. These findings suggest that phenolic content in the chosen ascidians could be used as an indicator of antioxidant properties. The results of this study encourage investigations on marine ascidian species as sources of antioxidants.

Keywords: Ascidians, Antioxidant activity, phenol content, Mandapam coast

1. Introduction

Natural products play an important role for innovative drugs. Research on natural products has shifted to marine environments as a rich source of biologically active agents for clinical applications. Marine squirts are a point of interest for marine natural product researchers as a source of new bioactive products. An increasing number of studies have been conducted showing the possible antioxidant, anti-inflammatory, antibacterial, antifungal and cytotoxic activity of Ascidian extracts (Matsuno *et al.*, 1985; Rebachuk *et al.*, 1985; Ali *et al.*, 2008; Lee *et al.*, 2010; Loda *et al.*, 2011; Dharan and Prasad, 2013; Camila *et al.*,2014; Agrawal *et al.*, 2016; Priya *et al.*, 2016; Rohmah *et al.*, 2016; Roselin *et al.*, 2018;). Among these bioactive compounds, antioxidants are fascinating because of the role of free radicals in many ailments, including cancer, ageing, and atherosclerosis.

Phenols are organic compounds consisting of a hydroxyl group and an aromatic hydrocarbon ring. Natural polyphenols exist in the form of simple phenol molecules or highly polymerized forms such as tannins. Usually, they turn out to be conjugated with a sugar residue. They can also be conjugated with carboxylic acids and organic acids, amines and lipids or associated with other phenols (Bravo, 1998). According to Harborne (1988), there are 8,000 phenolic structures. They are produced in two different ways, the shikimate route and the acetate route.

Total Phenolic Compounds are powerful donors of hydrogen, making them good antioxidants (Rice-Evans *et al.*, 1995). They are considered secondary metabolites which belong to a large and heterogeneous group of biologically active non-nutrients which serve as active defence factors against various types of stress caused by pathogens or unfavourable environmental conditions. Data describing the formation of TPC in marine organisms are scarce. Still, it is postulated that certain ecological pressures, including low temperatures caused by the winter season can trigger the formation of this compound (Duval *et al.*, 2000).

A free radical is a chemically reactive molecule with an unpaired electron. Free radicals are generated in living systems as part of cellular metabolisms such as respiration, phagocytosis, prostaglandin synthesis and the cytochrome P450 system. In addition, pollution, cigarette smoke, radiation and drugs can also act as external factors that cause free radical production (Freeman and Crapo, 1982). When the covalent bond between two atoms or molecules breaks, the newly formed atoms remain with an unpaired electron, turning them into free radicals. Free radicals turn the surrounding molecule into new free radicals by stealing electrons. Then, these newly formed radicals return to its ground state by stealing electrons from cellular structures or molecules. This leads to chain reactions of free radicals (Halliwell, 1987).

Free radicals are called reactive oxygen species (ROS) if they involve oxygen. The inner mitochondrial membrane is the site of an electron transfer chain that uses oxygen to produce energy in the form of ATP. During these

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reactions, electron leakage often occurs, which leads to the formation of very destructive ROS. The most common types of reactive oxygen species are superoxide anion (O_2-), hydroxyl radical (OH.), Singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) (Halliwell, 1987).

Free radicals play a useful role in the human body in many ways such as energy production, the destruction of bacteria as a result of phagocytosis, participation in signal transduction in cells, and the synthesis of biologically important compounds (Cadenas, 2004; Edison, 2010). In addition, they have a harmful effect, as they destroy the oxidative status of the body, which leads to a number of health problems. They attack lipids in cell membranes, proteins in tissues or enzymes, carbohydrates and DNA, and cause oxidation, which result in membrane damage, protein modification and DNA damage (Aruoma, 1998; Pietta, 2000; Atoui *et al*., 2005; Pietta, 2000). This oxidative damage is thought to play a causative role in ageing and several related degenerative diseases such as cardiovascular disease, cancer, cataract, decreased immune systems and brain dysfunction (Percival, 1998; Young and Woodside, 2001).

An antioxidant is any substance that protects molecules from oxidative damage by giving its electrons to free radicals. Aerobic organisms have developed antioxidant defense systems to improve the elimination activity of free radicals and reduce damage caused by free radicals. The main antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, and non-enzymatic endogenous antioxidants such as lipoic acid, glutathione, L-arginine, coenzyme Q10, melatonin, uric acid. , bilirubin, protein transfer and metal chelation in humans can neutralize reactive oxygen and reactive nitrogen species (Jinu *et al*., 2015). A large number of exogenous antioxidants in our diet can work in coordination with the endogenous antioxidant system to detoxify free radicals and keep the body healthy.

The cancer development process called carcinogenesis has three stages consisting of initiation, promotion and progression. Initiation involves damage to the DNA, chromosome or epigenome that regulates gene expression. Oxidative stress is the main driving force for initiation. Among phytochemicals, phenolic compounds are subject to great research as they offer a wide variety of bioactivity. For example, phenolic acids, ellagic acid and resveratrol increase the expression of apoptotic genes, thus preventing cell proliferation in prostate cancer (Narayanan *et al.*, 2002). Weng and Yen, (2012) observed that the inhibition of the matrix metalloproteinase mobility of tumor cells when MDA-MB-231 cells were treated with injected intercardial mouse curcumin, that disrupted the basement membrane and extracellular matrix.

There are several methods developed to measure antioxidant activity. These methods differ in terms of test principles and experimental conditions. They are usually based on preventing the accumulation of oxidized products. The production of free radical species is inhibited by the addition of antioxidants, which leads to a reduction in endpoints by eliminating free radicals.

2. Materials and Methods

2.1 Study Animals

For the present study, two colonial ascidians *Eudistoma amplum* and *Polyclinum nudum*, which are commonly available abundantly in Mandapam coast of Gulf of Mannar, southern east coast of India, were collected from the pillars of jetty during low-tide periods by adopting a variety of collection methods such as scrapping, peeling off and dislodging from their surfaces using a sharp knife, scalpel, etc.

2.2 Preparation of animal material

The whole colony of the study animals were used for the present study. The freshly collected samples were cleaned and washed separately with fresh seawater to remove all impurities, and other associated plants and animals. They were dried under shade at room temperature. The dried samples were ground and sieved in order to remove shell particles, and were used for the preparation of crude methanol extracts.

2.3 Preparation of crude extracts

Dried materials were soaked separately in 100% A.R. grade methanol (1g/20 ml) for 10 days at room temperature under constant agitation in an orbital shaker. The extracts were filtered and then dried overnight at room temperature for complete evaporation of the solvent before subsequent extraction. The dried extracts were re-suspended in methanol for 24 hours, and the extracts were collected, filtered with Whatman no.1 paper and concentrated using rotary evaporator (Buchitype model). The extract residues were re-suspended in 20 ml of 100% A.R. grade methanol and transferred to new vials to remove salt precipitates. The methanol-soluble extracts were dried and solubilized in deionized water.

2.4 Free Radical Scavenging Activity by DPPH Method

The free radical scavenging efficiency of the methanol extract of the selected ascidians was measured according to the DPPH method specified by Blois (1958).

2.5 Preparation of standard ascorbic acid

Two milligram of ascorbic acid (Sigma-Aldrich)was dissolved in 2.5ml distilled water to make the concentration of ascorbic acid solution of 800 µg/ml and treated as stock solution. The different concentrations such as 5, 10, 25, 50, 100, 125 and 150 µg/ml were prepared.

2.6 Preparation of stock solution of ascidian extracts

Two ascidian methanol extracts were separately mixed with 95% methanol to make a stock solution (10 mg/10 ml). The different concentrations such as 5, 10, 25, 50, 100, 125 and 150 µg/ml were prepared.

2.7 Free radical scavenging assay

One hundred microliter of each concentration was mixed with 1400 µl of DPPH (1.5x10-4 M) dissolved in 95% methanol. The reaction tubes were incubated for 30 minutes in the dark at room temperature. For reference, the absorbance against methanol was measured at 517 nm using the Shimadzu UV visible recording spectrophotometer. Ascorbic acid was used as a standard. To eliminate the absorbance effect of the extracts, the absorbance of the blank solutions of the sample consisting of 100 µl of each concentration at 517 nm plus 1400 µl

distilled water was also measured. DPPH uptake was measured against methanol and recorded for relevant results in each experiment. The radical scavenging activity (RSA%) was calculated using the following equation:
Radical Scavenging Activity (% RSA) = Abs (blank) – [Abs (sample) – Abs (sample blank)] / Abs (blank) X 100
The results are expressed in terms of Ascorbic acid Equivalents and used as standard, and IC_{50} value corresponding to amount of antioxidant required to decrease the initial DPPH radical concentration by 50% was also calculated by the method of Sharma, (2009).

2.8 Determination of Total Phenolic Content

The total phenolic content of the methanol extracts of ascidians was measured according to the Folin-Ciocalteu method proposed by McDonald *et al.* (2001) using gallic acid as standard.
Gallic acid standards were prepared by dissolving gallic acid (Sigma-Aldrich) in distilled water. Different concentrations from 25 to 150 µg / ml were prepared from the stock solution. Ascidian extracts were dissolved in distilled water to prepare 1 mg/ml concentration.
100 microlitre of each standard sample/gallic acid was mixed with 800 µl 1M Na_2CO_3 and 1 ml 1:9 diluted Folin-iocalteu reagent. Mixtures were incubated for 15 minutes at room temperature and absorbance measured at 765 nm. Samples were analyzed in duplicate. To eliminate the effect of the sample on absorbance, reagent samples (100 µl ascidian sample + 1800 µl distilled water) were measured and subtracted from the absorbance values of the samples. The total phenol content of ascidian extracts was calculated based on the standard curve of gallic acid as shown in the equation and expressed as mg gallic acid equivalent (GAE) / g extract.

3. Results and Discussion

The ability to scavenge free radicals with hydrogen donation is a known mechanism for antioxidation. In addition, DPPH has the advantage of not being influenced by some side reactions of polyphenols such as chelation of metal ions and inhibition of enzymes. A freshly prepared DPPH solution shows a dark purple colour at 517 nm, which gradually disappears in the presence of a good hydrogen donor.
Many antioxidant compounds naturally present in plant sources have been described as free radical scavengers (Aryal *et al* 2019). In this study, the in vitro antioxidant activity of two marine ascidians showed potential free radical scavenging activities expressed in IC50 values. The antioxidant activity of two colonial ascidians such as *E.amplum* and *P.nudum* was tested by DPPH scavenging test with concentrations ranging from 5 to 150 µg/ml based on the reduction of the maximum absorption of the DPPH radicals in the presence of an antioxidant compound. Ascorbic acid was chosen as the standard antioxidant. The percentage inhibition of *E. amplum* extract varied between 11.37 and 81.67, and the IC50 value was 31.34 µg/ml whereas the percentage range of inhibition of *P. nudum* was between 21.33 and 91.67, and the IC50 value was 20.95 µg/ml (Table 1).
The decrease in absorbance of DPPH in the presence of different concentrations of two colonial ascidians and the standard showed the analogous scavenging capacity of the DPPH radical. The inhibitory effect of these extracts was dose-dependent in the range of concentrations tested. The inhibitory effect increased with increasing concentration. However, *P.nudum* extract was found to be a stronger scavenger than that of *E.amplum*. The activity of ascidian extracts was comparable to the positive control, ascorbic acid ($p < 0.05$).
Similar observations of the direct correlation between the increase in the inhibition rate and the concentration of the extract were reported by Sankaravadivu *et al.* (2016), Shanmuga*et al.* (2016a,b) and Roselin *et al.* (2018) in studies on colonial ascidians and indicated that polyphenols with redox properties allow them to act as reducing agents, hydrogen donors and individual oxygen quenchers.
The concentration which caused the 50% loss of DPPH (colour) activity of the selected ascidian extracts and the IC_{50} standard, was calculated from the graphs shown in Fig. 1 to 3. The percentage efficiency of DPPH scavenging of ascorbic acid at different concentrations is shown in Table 3. The IC_{50} range for both *Polyclinum nudum* and *P. amplum* extracts is similar to that of ascorbic acid..The result revealed that *P. nudum* had higher free radical scavenging activity than *E. amplum*, but there is no significant difference between methanolic extract of *E. amplum* and *P. nudum* ($p < 0.05$) in the DPPH radical scavenging activity test (Table 2).
An antioxidant concentration required to reduce the initial concentration of DDPH by 50% is normally used to measure the antioxidant activity of the crude extract (Sanchez *et al.*, 1998; DuToit *et al.*, 2001). The IC_{50} value of the *Polyclinum nudum* extract was found to be 20.95 mg/ml compared to 11.86 mg/ml of standard vitamin C. The results of this study showed that the crude methanolic extract of *P. nudum* contains secondary natural product with significant antioxidant activity
Total phenolic contents are effective hydrogen donors, which make them good antioxidants (Rice-Evans *et al.*, 1995). These are considered secondary metabolites belonging to a large and heterogeneous biologically inactive nutrient group that acts as active defense factors against various types of stress caused by pathogens or adverse environmental conditions. There are few data

Table 1. Antioxidant activity of crude methanol extracts of *Eudistoma amplum*, *Polyclinum nudum* and Ascorbic acid

Conc. (µg/ml)	*Polyclinum amplum*	*Polyclinum nudum*	Ascorbic acid
5	11.67±1.05	21.33±2.35	26.33±2.11
10	20.33±2.24	31.67±2.53	35.33±4.24
25	44.33±3.99	48.33±4.83	66.67±6.00
50	57.67±6.34	69.67±7.66	77.67±8.54
100	70.67±8.48	78.33±9.40	86.33±8.63
125	78.00±6.24	88.33±7.95	90.67±8.16
150	81.67±7.35	91.67±9.17	96.33±10.60
LC_{50}	**31.34**	**20.95**	**11.86**

Table 2. ANOVA between antioxidant activity of two ascidians and the standard

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	301.69	1	301.69	0.39	0.543	* 4.747
Within Grups	9282.12	12	773.51			
Total	9583.816	13				

* Insignificant

describing the formation of TPC in marine organisms, but some environmental stresses are assumed to trigger the formation of this compound (Duval *et al.*, 2000).

Phenolic compounds have been shown to have important antioxidant activities according to their structural properties (Dimitrios, 2006). Therefore, the total phenolic content of the ascidian samples was also determined with the Folin-Ciocalteu reagent method.

The total phenolic content in the analyzed ascidian extracts is expressed in gallic acid equivalent using the standard gallic acid curve as shown in the Fig. 4 (equation of the standard curve: y = 0.002x + 0.032x, r2 = 0.983).

The content of phenolic compounds in *E. amplum* is 46.50 ± 5.11 mg GAE g^{-1}, whereas, it is 48.23 ± 4.34 mg GAE g^{-1} in *P. nudum*. The high antioxidant properties of ascidian extracts correspond to the high total phenol content and the ascidian with low antioxidant activity showed relatively low total phenol content (Table 3).

Phenolic compounds are believed to influence most of the antioxidant activity in many plants and animals. The relationship between total phenolic concentration and antioxidant activity has been widely studied in plants

Table 3. Total polyphenol contents and DPPH radical scavenging activity of chosen ascidians.

Species	Total polyphenol contents (mg GAE/g)	DPPH (IC_{50}-µg/ml)
Eudistoma amplum	46.5 ± 5.11	31.34
Polyclinum nudum	48.23 ± 4.34	20.95

(Klimczak *et al.*, 2007: Jayaprakasha *et al.*, 2008;) and animals (Anand *et al.*, 2006; Bell, 2008; Dhivya *et al.*, 2014).

The phenol contents of *E. amplum* and *P. nudum* were higher than that in *Didemnum perlucidum* (17.2 2 ± 0.6 mg GA/g) and *Polyclinum* sp. (18.2 ± 0.5 mg GA/g) as reported by Biancoa *et al.* (2015). Basically, an increase in the total phenolic content can increase the antioxidant properties. Phenolic compounds act as electron donors and can neutralize undesirable reactions caused by free radicals in the body. Many studies have shown that there is a significant relationship between phenolic content and antioxidant activity in algae extracts (Velioðlu *et al.*, 1998; Siriwardhana *et al.*, 2003; Karawita *et al.*, 2005; Manian *et al.*, 2008).

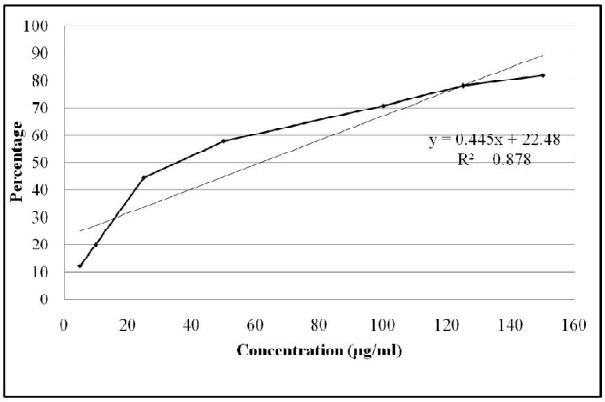

Fig. 1. Calculation of IC50 value for antioxidant activity of methanol extract of *Eudistoma amplum*

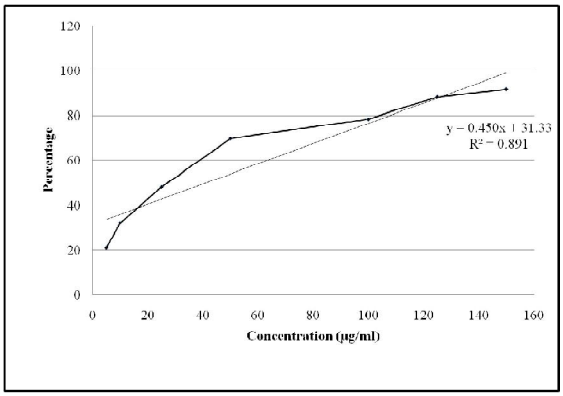

Fig. 2. Calculation of IC50 value for antioxidant activity of methanol extract of *Polyclinum nudum*

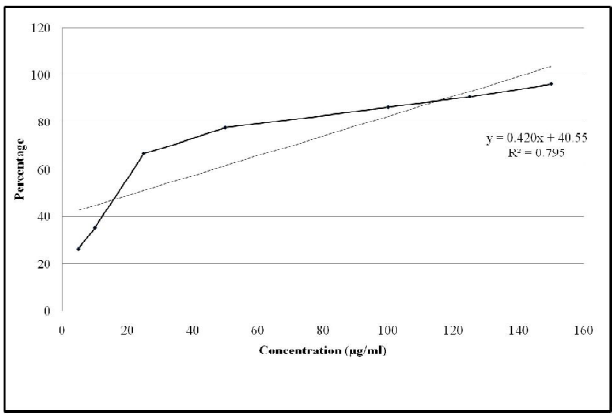

Fig. 3. Calculation of IC50 value for antioxidant activity of Ascorbic acid

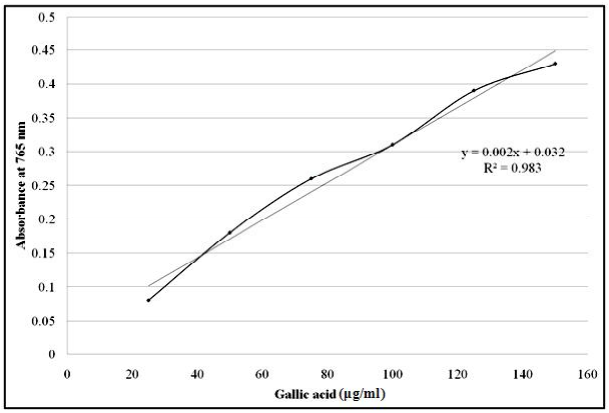

Fig. 4. Gallic acid standard curve. Each point is the mean of triplicate measurements from three different experiments (n=3)

The antioxidant activity of the ethanolic extracts of the two types of grass of the Red Sea has been linked to the presence of phenolic compounds and fibres (Daniel *et al.*, 2014). Studies on the antioxidant activity of different solvent extracts of the simple *Phallusia nigra* of the ascidian on the coast of Thoothukudi have revealed that the ethanol extract hasthe strongest activity attributed to the presence of flavonoids and phenols (Priya *et al.*, 2015). Biancoa *et al*. (2015) also reported the significant correlation between the antioxidant activity and phenolic content in *Didemnum perlucidum* and *Polyclinum* sp.

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