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In vitro reactive oxygen and nitrogen speceis scavengeing activity of *ulva reticulate* extract

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Abstract
Antioxidant activity of *Ulva reticulata* extract was carried out for proving its utility in free radical mediated diseases including diabetic, cardiovascular, cancer etc. The seaweed extract was screened for *in vitro* antioxidant activity by oxygen radical scavenging such as DPPH, total antioxidant assay, superoxide, hydroxyl radical and Nitric oxide scavenging activity at different concentrations. Throughout the study seaweed extract showed marked antioxidant activity. The antioxidant activity of the seaweed extract may be due to the phytochemicals present in it. The antioxidant activity was found to be concentration dependent and may be attributed to the presence of bioflavonoids in *Ulva reticulata* extract. Overall, the seaweed extract is a source of natural antioxidants which might be helpful in preventing the progress of various oxidative stress mediated diseases including aging.

Keywords: antioxidant activity, *ulva reticulata*, reactive oxygen species, radical scavenging

Introduction
In living organisms, various reactive oxygen and nitrogen species (ROS/RNS) e.g., superoxide anions ($O_2^{\bullet-}$), hydroxyl radicals ($^{\bullet}OH$), nitric oxide radicals ($NO^{\bullet}$) and non-radical compounds, can be formed by different mechanisms. It is unavoidable one because they are continuously produced by the body's normal use of oxygen. Such species are considered to be important causative factors in the development of diseases such as diabetes, stroke, arteriosclerosis, cancer, and cardiovascular diseases and the aging process (Velavan, 2011; Alma *et al*, 2003) [1, 2]. Prior administration of antioxidant provides a close relationship between free radical scavenging activity (FRSA) and the involvement of endocrinological responses, which help to reverse the effect (Wiseman and Halliwell, 1996) [3].
The recent evidences suggesting the involvement of oxidative stress in the pathogenesis of various disorders and diseases has attracted much attention of the scientists and general public to the role of natural antioxidants in the maintenance of human health and prevention and treatment of diseases (Niki, 2010) [4]. Plant and its products are rich sources of a phytochemicals and have been found to possess a variety of biological activities including antioxidant potential (Velavan *et al* 2007, 2015) [5, 6]. The majority of the active antioxidant constituents are flavonoids, isoflavones, flavones, anthrocyanins, coumarins, lignans, catechins, and isocatechins. In addition to the above compounds found in natural foods, vitamins C and E, beta-carotene and tocopherol are known to possess antioxidant potential (Prior, 2003) [7]. With this background and abundant source of unique active components harbored in plants the green algae *Ulva reticulata* was chosen for the study. *Ulva reticulata* is commonly known as ribbon sea lettuce, and is distributed in western Indian Ocean and south India, coastal lines of Tamil Nadu. The green algae have long been used as food and as a traditional medical agent to treat various infections and diseases (Kim *et al* 2007) [8]. Therefore, the present study was designed to investigate the phytochemical analysis and antioxidant activity of *Ulva reticulate* through the free radical scavenging such as DPPH, total antioxidant assay, superoxide, hydroxyl radical and Nitric oxide scavenging activity.

Materials and methods
Chemicals
Nitro blue tetrazolium (NBT), ethylene diamine tetra acetic acid (EDTA), sodium nitroprusside (SNP), trichloro acetic acid (TCA), thio barbituric acid (TBA), potassium hexa cyano ferrate [$K_3Fe(CN)_6$], and L-ascorbic acid were purchased from Sisco Research Laboratories Pvt. Ltd., India. All other chemicals and solvents used were of analytical grade available commercially.

Plant materials
The collected Fresh edible green seaweed *Ulva reticulata* was cleaned well with sea water to remove all the extraneous matter such as epiphytes, particles of sand, pebbles and shells and brought to the laboratory in air tight container then washed thoroughly with tap water followed by distilled water several times. Washed seaweed was blotted on blotting paper and spread at room temperature in shade to dry. The dried seaweed was ground to a fine powder using tissue blender and then the powdered sample was stored in refrigerator for further use.

Extract preparation
20gm powdered sea weed was soaked in 50ml of Absolute alcohol overnight and then filtered through Whatmann filter paper No.41 along with 2gm sodium sulfate to remove the sediments and traces of water in the filtrate. Before filtering,

the filter paper along with sodium sulphate is wetted with absolute alcohol. The filtrate is then concentrated by bubbling nitrogen gas into the solution and reduce the volume to 1ml.The extract contains both polar and non-polar phyto components. Doses such as 20, 40, 60 and 80µg/ml were chosen for *in vitro* antioxidant activity.

In vitro antioxidant activity

DPPH ASSAY

The scavenging ability of the natural antioxidants in the *Ulva reticulata* extract towards the stable free radical DPPH was measured by the method of Shimada *et al*. (1992) [9]. Briefly, a 2 ml aliquot of DPPH methanol solution (25µg/ml) was added to 0.5 ml sample solution at different concentrations. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 min. Then the absorbance was measured at 517nm in a spectrophotometer. L-Ascorbic acid was used as the standard.

Radical scavenging activity (%)

$$= 100 - \frac{A_C - AS}{A_C} X\ 100$$

Where A_C = control is the absorbance of the control and A_S = sample is the absorbance of reaction mixture (in the presence of sample). All tests were run in triplicates (n = 3), and the average values were calculated.

Determination of total antioxidant capacity

The antioxidant activity of the *Ulva reticulata* extract was evaluated by the phosphomolybdenum method according to the procedure of Prieto *et al*. (1999) [10]. The assay is based on the reduction of Mo (VI)–Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. 0.3 ml extract was combined with 3ml of reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer against blank after cooling to room temperature. Methanol (0.3 ml) in the place of extract is used as the blank. The antioxidant activity is expressed as the number of equivalents of ascorbic acid.

Superoxide anion scavenging activity assay

The scavenging activity of the *Ulva reticulata* towards superoxide anion radicals was measured by the method of Liu *et al*. (1997) [11]. Superoxide anions were generated in a non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH, and oxygen. It was assayed by the reduction of nitroblue tetrazolium (NBT). In these experiments the superoxide anion was generated in 3 ml of Tris-HCl buffer (100mM, pH 7.4) containing 0.75 ml of NBT (300 µM) solution, 0.75 ml of NADH (936 µM) solution and 0.3 ml of different concentrations of the extract. The reaction was initiated by adding 0.75 ml of PMS (120 µM) to the mixture. After 5 min of incubation at room temperature, the absorbance at 560 nm was measured in spectrophotometer. The superoxide anion scavenging activity was calculated according to the following equation:

% Inhibition = $[(A_0-A_1) / A_0 \times 100]$,

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

Hydroxyl radical scavenging activity assay

The scavenging activity for hydroxyl radicals was measured with Fenton reaction by the method of Yu *et al*. (2004) [12]. Reaction mixture contained 60µl of 1.0mM $FeCl_3$, 90µl of 1mM 1,10- phenanthroline, 2.4 ml of 0.2M phosphate buffer (Ph 7.8), 150µl of 0.17M H_2O_2, and 1.5 ml of extract at various concentrations. Added H_2O_2 start the reaction. After incubation at room temperature for 5 min, the absorbance of the mixture at 560nm was measured with a spectrophotometer. The hydroxyl radical scavenging activity was calculated according to the following equation:

% Inhibition = $((A_0-A_1) / A_0 \times 100)$

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

Nitric oxide scavenging activity assay

Nitric oxide radical scavenging activity was determined according to the method reported by Garrat (1964) [13]. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be determined by the use of the Griess Illosvoy reaction. 2 ml of 10mM sodium nitroprusside in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of extract at various concentrations and the mixture incubated at 25ºC for 150 min. From the incubated mixture 0.5 ml was taken out and added into 1.0 ml sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. Finally, 1.0 ml Naphthyl ethylenediamine dihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 min. The absorbance at 540 nm was measured with a spectrophotometer. The nitric oxide scavenging activity was calculated according to the following equation:

% Inhibition = $((A_0-A_1) / A_0 \times 100)$

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

Statistical analysis

Tests were carried out in triplicate for 3–5 separate experiments. The amount of extract needed to inhibit free radicals concentration by 50%, IC_{50}, was graphically estimated using a nonlinear regression algorithm.

Results and discussion

The search for new molecules, nowadays, has taken a slightly different route where the science of ethnobotany and ethnopharmacognosy are being used as guide to lead the chemist towards different sources and classes of compounds (Gurib-Fakim 2006) [14]. Plant derived natural products hold great promise for discovery and development of new pharmaceuticals (Mc Chesney *et al* 2007) [15]. Present study to investigate the phytochemical analysis of ethanoic extract of *Ulva reticulate*. The results of the present study showed that the presence of flavonoids, sterroids, terpenoids,

alkaloids, phenol, anthocyanins, saponin and anthroquinone while tannin, coumarins and emodins were absent.

DPPH Assay

Recently, the use of the DPPH· reaction has been widely diffused among food technologists and researchers, for the evaluation of free radical scavenging activity on extracts from plant, food material or on single compounds. In the DPPH assay, the antioxidant was able to reduce the stable radical DPPH to the yellow colored 1, 1-diphenyl-1, 2-picryl hydrazine. The molecule of 2, 2-diphenyl-1-picryl hydrazine is characterised as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole. The proton transfer reaction of the DPPH· free radical by a scavenger causes a decrease in absorbance at 517 nm, which can be followed by a common spectrophotometer set in the visible region. The effect of antioxidants on DPPH· is thought to be due to their hydrogen donating ability (Sindhu and Abraham 2006) [16]. DPPH radical scavenging activity of the seaweed extract and standard as ascorbic acid are presented in Fig 1. The DPPH radical was widely used to evaluate the free-radical scavenging capacity of antioxidants (Nuutila *et al* 2003) [17]. The half inhibition concentration (IC_{50}) of ascorbic acid and seaweed extract were 41.66µg ml^{-1} and 46.73 µg ml^{-1} respectively**.** The seaweed extract exhibited a significant dose dependent inhibition of DPPH activity. The potential of L-ascorbic acid to scavenge DPPH radical is directly proportional to the concentration. The DPPH assay activity is near to standard as ascorbic acid.

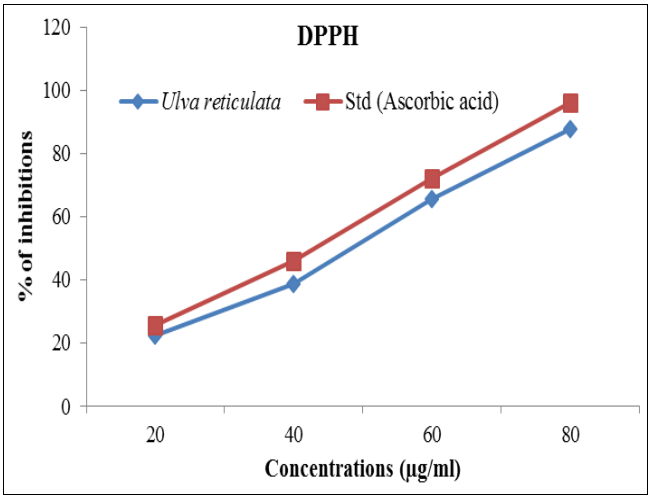

Fig 1: DPPH radical scavenging activity of *Ulva reticulata*

Total antioxidant activity

The phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/ Mo (V) complex with a maximal absorption at 695 nm. The assay is successfully used to quantify vitamin E in seeds and, being simple and independent of other antioxidant measurements commonly employed, it was decided to extend its application to seaweed extract (Prieto *et al* 1999) [10].

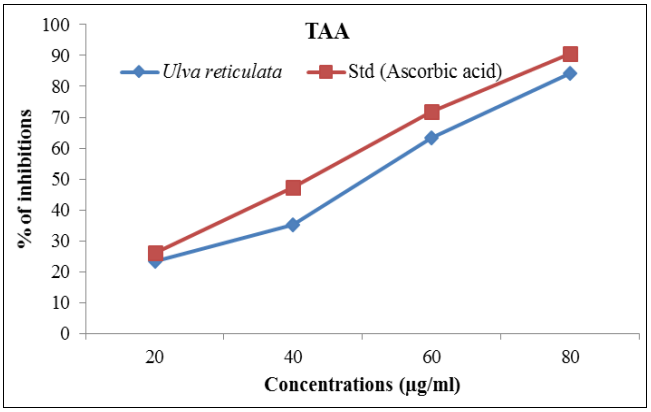

Fig 2: Total antioxidant assay of *Ulva reticulata*

Moreover, it is a quantitative one, since the antioxidant activity is expressed as the number of equivalents of ascorbic acid. The yield of the ethanol extract of *Ulva reticulata* and its total antioxidant capacity are given in Fig 2. Total antioxidant capacity of the sea weed is expressed as the number of equivalents of ascorbic acid. The study reveals that the antioxidant activity of the extract was in the increasing trend with the increasing concentration of the sea weed extract. The half inhibition concentration (IC_{50}) of ascorbic acid and seaweed extract were 41.78µg ml^{-1} and 48.54 µg ml^{-1} respectively**.**

Superoxide anion radical scavenging activity

Superoxide is biologically important since it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals, which are very harmful to the cellular components in a biological system (Korycka-Dahl and Richardson 1978) [18]. The superoxide anion radical scavenging activities of the extract from *Ulva reticulata* assayed by the PMS-NADH system were shown in Fig 3. The superoxide scavenging activity was increased markedly with the increase in concentrations. The half inhibition concentration (IC_{50}) of the extract and ascorbic acid were 49.74 µg ml^{-1} and 42.00 µg ml^{-1} respectively**.** These results suggested that *Ulva reticulata* had notably superior superoxide radical scavenging effects.

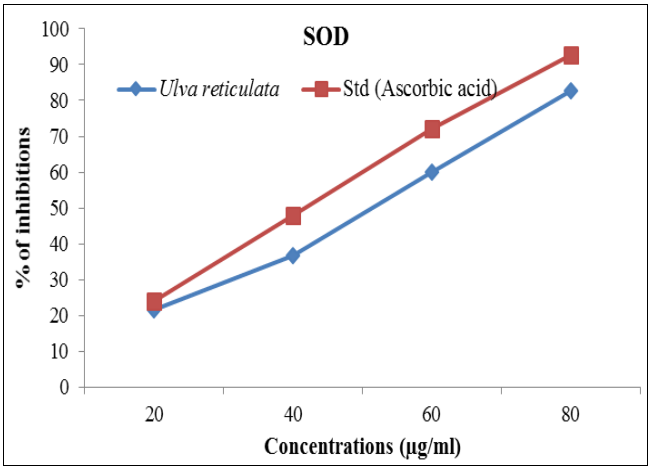

Fig 3: Super oxide scavenging activity of *Ulva reticulata*

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of ethanolic extract of *Ulva reticulata* was measured by 1, 10 phenanthroline-Fe^{2+} complex oxidation method. Fe^{2+} was formed when ferrous sulphate added to hydrogen peroxide. This formed ferrous ion reacts with 1, 10 phenanthroline and forms 1, 10 phenanthroline Fe^{2+} complex which acts as indicator in oxidation reduction reaction. Simultaneously the hydroxyl radical formed from the H_2O_2-Fe^{2+} reaction mixture oxidize Phenanthroline - Fe^{2+} into Phenanthroline – Fe^{3+} complex. Presence of free radical scavenger in the extract reduces the oxidation reaction accompanied with reduction in the absorbance which can be measured quantitatively at 560 nm (Olabinri *et al* 2010) [19]. Hydroxyl radical scavenging activity of *Ulva reticulata* increased with increasing dosage (Fig. 4). The half inhibition concentration (IC_{50}) of *Ulva reticulata* was 47.30 µg/ml^{-1} and ascorbic acid was 41.56 µg/ml$^{-1.}$

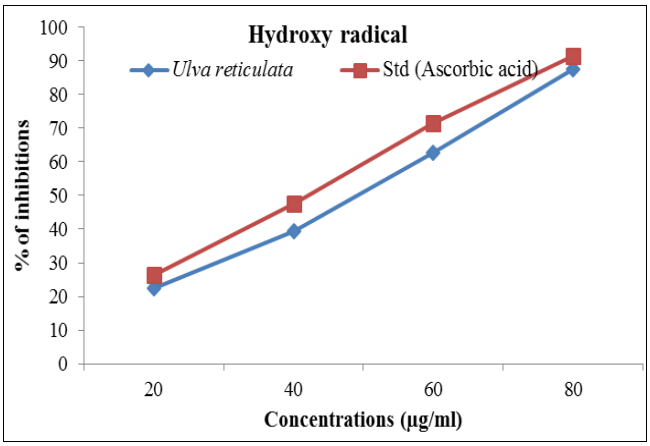

Fig 4: Hydroxyl radical scavenging activity of *Ulva reticulate*

Nitric oxide scavenging activity

Nitric oxide (NO•) released from sodium nitroprusside (SNP) has a strong NO^+ character which can alter the structure and function of many cellular components. The extract of *Ulva reticulata* exhibited good NO• scavenging activity leading to the reduction of the nitrite concentration in the assay medium. The NO• scavenging capacity was concentration dependent with 80µg/ml scavenging most efficiently. The *Ulva reticulata* in SNP solution significantly inhibited the accumulation of nitrite, a stable oxidation product of NO• liberated from SNP in the reaction medium with time compared to the standard ascorbic acid. The toxicity of NO• increases when it reacts with superoxide to form the peroxynitrite anion (•ONOO-), which is a potential strong oxidant that can decompose to produce •OH and NO_2 (Pacher *et al* 2007) [20]. The present study shows that *Ulva reticulata* extract had a potent nitric oxide scavenging activity and the scavenging activity of extract increased with increasing concentrations (Fig. 5). The half inhibition concentration (IC_{50}) of *Ulva reticulata* was 50.45 µg/ml^{-1} and ascorbic acid were 41.25 µg/ml^{-1} respectively.

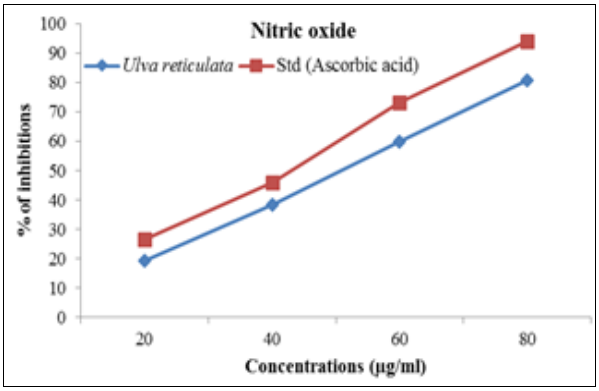

Fig 5: Nitric oxide scavenging activity of *Ulva reticulata*

Conclusion

The results of the present study, clearly indicates that ethanolic extract of *Ulva reticulata* had powerful *in vitro* antioxidant capacity against various antioxidant systems as DPPH, total antioxidant assay, superoxide, hydroxyl radical and Nitric oxide scavenging. The antioxidant activity of *Ulva reticulate* was proven to be concentration dependent and the efficacy of the extract is probably based upon the phytochemicals present in the seaweed. The extract could exhibit antioxidant properties approximately comparable to commercial synthetic antioxidants as ascorbic acid. From the above assays, the possible mechanism of antioxidant activity of *Ulva reticulate predicted* includes reductive ability, metal chelation, hydrogen donating ability and scavenging superoxide and free radicals.

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