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Free Radical Scavenging Potential of Marine Red Seaweed *Gelidiella calcicola* Resource for Human Society

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Abstract
Objective: Antioxidants are very important substances which acquire the capability to defend the body from injure caused through free radical induced oxidative stress. In this study, *in vitro* free radical scavenging activity of *Gelidiella calcicola* evaluated in a succession of different types of *in vitro* assays. Methods: The evaluation of antioxidant activity properties was determined through DPPH (1,1-diphenyl-2-picrylhydrazyl), ABTS (2,2'-azinobis 3ethylbenzthiazoline-6-sulphonic acid, FRAP (ferric reducing antioxidant power), NO (nitric oxide scavenging assay), superoxide, reducing power, OH (hydroxyl) radical scavenging and H_2O_2 (hydrogen peroxide) radical assay, metal chelating activity as well as phosphor molybdenum assay. Results: Amongst the tested red algae, the utmost antioxidant scavenging activity was recorded in MeOH extract of *Gelidiella calcicola*. Whereas crude MeOH extract of red algae showed good quality antioxidant potential. Conclusion: The present study findings are suggesting that MeOh extracts could be a prospective resource of natural antioxidant that could have enormous consequence as therapeutic agents. The current study findings seem promising to assist additional experiments on the identification and characterization particular compounds which are accountable for comparatively lofty antioxidant activities.

Keywords
Biomolecules, Hydroxil, Polyphenols, Red algae, Superoxide.

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INTRODUCTION
Red algae mainly used for their bioactive substances particularly antioxidant [1], in mariend environment seaweed possess huge taxonomic diversity which is used mainly as food, medical applications, isolation and synthesis of secondary metabolites, biological studies, isolating bioactive molecules form seaweeds [2]. Seaweeds contains organic and inorganic substances used for particularly human society those are polyphenols, carotenoids, terpenoids, tocopherols, alkaloids, ascorbic acids, which is used

for different types of antioxidant activities, *in vitro, in vivo* models [3].

Seaweeds are chlorophyta (green), rhodophyta (red) and phaeophyta (brown) algae also depends on nutrient and chemical compounds [4]. About 6,500 to 10,000 species mostly multicellular, red algae growing and attached hard surfaces, its store their sugars as glycogen, is a long chain glucose molecule with lots of side branchs. In their cell walls rhodophyta contains three important chemicals; agar, gelans (mucusy sugars) and carrageenan. Some red algae, like the coralline, deposits calcium carbonate in cell walls. Variety of species used as food it includes Palmaria palmate, dulse. important source for fibre, nori is used to make sushi and added to soups, salads, appetizers and breads. Seaweeds area food source in East Asia, Japanese, used as medicine; alginates are used in wound dressings, dental moulds production, microbial culture growths, toothpaste, cosmetics and paints, industrial products such as paper coatings, adhesives, dyes, gels, explosives, oil and natural gas. Functional materials; polysaccharides, polyunsaturated fatty acids (PUFA), essential minerals and vitamins, enzymes, antioxidants, and bioactive peptides [5].

The novel drug leads the structures and potential biological activities. *Gelidiella calcicola* is a small creeping alga it grows to at least 30 mm long and are dark reddish brown [6]. *Gelidium pusillum* is common in the British Isles. Based on the literature selected for different free radical scavenging activities like; antioxidant by DPPH, ABTS, ferric reducing antioxidant power (FRAP), nitric oxide, superoxide anion, hydroxyl, hydrogen peroxide, reducing power, metal cheating and phosphomolybdenum assayes.

MATERIALS AND METHODS

Chemicals and reagents

DPPH (Sigma – Aldrich, USA), Methanol (HPLC grade, Merck, India), BHT (Analytical grade, Merck, India), Rutin, Potassiumpersulphate, ABTS (2, 2'–azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), potassium persulphate (5 mM), phosphate buffer - 100mM, (pH 7.4), FRAP reagent, sodium nitroprusside 10 mM, griess reagent sulphanilamide (1%), orthophosphoric acid(2%),N-1-naphthylethylenediamine dihydrochlo ride (0.1%), NBT Solution, NADH, EDTA solution, ferrous ammonium sulphate – EDTA solution, nash reagent, Dimethyl sulphoxide (DMSO), ascorbic acid (0.22%), TCA (17.5%), H_2O_2, phosphate buffer (pH 7.4), phosphate buffer (pH 6.6), TCA 10%, $K_3Fe(CN)_6$, $FeCl_3$, Ferric chloride, Ferrozine, sulphuric acid (0.6 M), sodium phosphate, ammonium molybdate (4 mM). All the other reagents were of analytical grade and obtained from Merck.

Collection of algal materials

Marine algae samples were collected from the coastal region of Rameshwaram, Tamil Nadu, India on 5th January 2015. Algae were washed with sea water to remove unwanted extraneous materials and transferred to the laboratory in plastic bag containing sea water to prevent evaporation.

Preparation of sample extract

The *Gelidiella calcicola* 5 g of seaweed powdered was extracted overnight with methanol (100 ml) at room temperature and centrifuged (2800 rpm; 10 mins). The supernatant was collected in a separate bottle after passing through a filter paper and the residue was re-extracted three times under the same conditions as mentioned above. The combined extracts were freeze dried. These extracts were kept at 80 ^{0}C until analysis. The freeze dried extracts were redissolved in methanol and used for the analysis.

DPPH (1, 1-diphenyl-2-picryl hydrazyl) radical scavenging assay

The radical scavenging activity of G. *calcicola* methanol extract against DPPH$^\bullet$ was determined spectrophoto metrically in a dark room by the method [7] DPPH$^\bullet$ is a stable free radical and accepts an electron, or hydrogen radical to become a stable diamagnetic molecule. DPPH$^\bullet$ reacts with an antioxidant compound that can donate hydrogen and gets reduced. The change in colour (from deep violet to blue) was measured. The intensity of the yellow colour developed was depends on the amount and nature of radical scavenger present in the sample. Sample (1 ml) different concentrations was taken, DPPH (1 ml) was added and this made up to three ml with water. The blue colour developed was read at 517 nm and BHT (Butylated hydroxytoluene) was used as a standard. The percentage of DPPH scavenging ability at different concentrations was estimated using the formula [(A_{c1} – A_{s2}/A_{c1}) X 100], where A_{c1} is the absorbance of the control and A_{s2} is the absorbance of the sample [8].

ABTS$^+$ (2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) assay

The total antioxidant activity of the samples was measured through [2,2'-azino-bis (3-ethylbenzthiazo line-6-sulphonicacid)] ABTS$\bullet^+$ radical cation decolorization assay according to the method [9] of ABTS•+ was produced through reacting 7 mM aqueous solution with potassium persulfate (2.4 mM) in the dark for 12-16 h at room temperature. The radical was stable in this form for more than two days when stored in the dark at room temperature. Then, ABTS•+ (2 ml) diluted solution was added to the sample varying concentrations of *G. calcicola* methanol extract. The blank contained water in place of *G. calcicola* methanol extract. After 30 minutes of incubation at room temperature, the absorbance was recorded at 734 nm and compared with standard BHT. The percentage of DPPH scavenging ability at different concentrations was estimated using the formula [(A_{c1} – A_{s2}/A_{c1}) X 100], where A_{c1} is the absorbance of the control and A_{s2} is the absorbance of the sample [10].

Ferric reducing antioxidant power (FRAP) assay

A slightly modified method was used to perform the ferric reducing antioxidant power (FRAP) assay. The FRAP solution was prepared with acetate buffer (300 mM; pH 3.6), 2, 4, 6, -tri(2-pyridyl)- s-triazine (TPTZ) (10 mM) in HCl (40 mM) and $FeCl_3.6H_2O$ (20 mM) (10:1:1). The FRAP reagent (150 µl) was added to the sample (20 µl) into 96-well plates, incubated at room temperature under dark condition for 8 min and then the sample was read at 600 nm. BHT was used as reference standard [11].

Nitric oxide scavenging activity

Nitric oxide scavenging activity was determined according to the method suggested [12]. Sodium nitroprusside 3.0 mL of 10 mM in phosphate buffered saline was mixed with different concentrations of the *G. calcicola* extract and incubated at 25°C for 150 min. incubated solution (0.5 mL) and mixed with Griess reagent (0.5 ml). The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with N-1-naphthylethylenediamine dihydrochloride was measured at 546 nm. BHT was used as reference standard.

Superoxide anion radical scavenging assay

Measurement of superoxide anion scavenging activity based on the method described [13]. The sample (0.1ml) was mixed with NBT (1ml) and NADH (1ml) solution. This mixture was incubated at 25ºC for 5 min. A control was performed with reagent mixture but without the sample. Absorbance was measured spectrophotometrically at 560 nm. BHT was used as reference standard.

hydroxyl radical scavenging activity

The scavenging activity of the G. *calcicola* methanol extract on hydroxyl radical was measured according to the method [14]. Various concentrations (5-50 µg/mL) of *G. calcicola* extracts were added with Ferrous ammonium sulphate - EDTA solution (1.0 ml) of, EDTA solution (0.5ml: 0.018%), and dimethyl sulphoxide (DMSO) (1.0 mL). The reaction was initiated by adding ascorbic acid (0.5 ml) and incubated at 80–90°C for 15 min in a water bath. After incubation the reaction was terminated by the addition of ice - cold TCA (1.0mL). Nash reagent (3 ml) was added and left at room temperature for 15 min. The reaction mixture without sample was used as control. The intensity of the color formed was measured spectrophotometrically at 412 nm against reagent blank. BHT was used as reference standard.

Formula; % HRSA = from [(A_0 - A_1)/A_0] X100, where A_0 is the absorbance of the control and A_1 is the absorbance of the extract/standard.

Hydrogen peroxide scavenging activity

The scavenging capacity of *G. calcicola* methanol extracts on hydrogen peroxide was determined according to the method [15]. Test tubes were prepared with 2.0 ml of various extracts (5-50 µg/mL) and a solution of H_2O_2 (1.2 ml, 40 mM) in phosphate buffer (pH 7.4). A blank solution was prepared in the same way but without H_2O_2. After incubation of the mixture during 10 min, the absorbance was recorded at 230 nm. BHT was used as reference standard. The scavenging activity was calculated using the following formula; % scavenging activity = [(Ac – At)/ Ac] 100, where Ac absorbance of the control.

Reducing ability assay

The reducing power of *G. calcicola* methanol extract was evaluated according to the method [16]. Different amounts of the extracts (5-50 µg/mL) were suspended in distilled water and mixed with phosphate buffer (2.5 ml; 0. 2 M; pH 6.6), and $K_3Fe(CN)_6$ (1%; 2.5 ml). The mixture was incubated at 50°C for 20 min; TCA (10%; 2.5 ml) was added to the mixture and centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml; 0.1%), and the absorbance was measured at 700 nm. Increase in absorbance of the reaction mixture indicated the ability of reducing power. BHT was used as standard

Metal chelating activity

Ferrozine quantitatively chelates with Fe^{2+} to form a red colored complex. But in the presence of other cheating agents, the formation of ferrozine- Fe^{2+} complex is disrupted and hence the intensity of red color also decreases. Significant antioxidant mechanisms as it reduces the concentration of the catalyzing transition metal in the lipid peroxidation [17]. The ferric chloride (1 m1; 2 mM; diluted 20 times) is mixed with different dilutions of the *G. calcicola* methanol extract (1 ml). The reaction is initiated through the addition of ferrozine (1 ml 5 mM; diluted 20 times). The absorbance is measured at 562 nm after 10 minutes. The positive controls that can be used in this assay are EDTA, citric acid. The ability of the sample to chelate ferrous ions canfrom the following equation: chelating Effect (%) = (A0 – A1 / A0) × 100 where; A0 is the absorbance of control, A1 is the absorbance in the presence of sample.

Phosphomolybdenum complex method

In the phosphomolybdenum complex method, the reduction of Mo (VI) to Mo (V) is detected at 695 nm through spectrophotometer due to the formation of green phosphate Mo (V) compounds at acidic pH [16]. For the total antioxidant capacity assay, of methanol extract (0.1 ml) is mixed with reagent solution (1 ml; 0.6 M sulphuric acid, sodium phosphate 28 mM and ammonium molybdate 4 mM) in Eppendorf tube. The tubes are then caped and incubated at 95ºC for 90 min in a thermal block. After incubation, the reaction mixture is cooled to room temperature and the absorbance is measured at 695 nm against reagent blank. BHT may be used as the standard antioxidant.

Statistical analysis

All the assays were carried out in triplicate. Experimental results are expressed as mean ± standard deviation. The results were analyzed using one-way analysis of variance and the group means were compared using Duncan's multiple range tests using SPSS version 16.

RESULTS AND DISCUSSION

DPPH method measures free radical scavenging capacity of antioxidants towards DPPH radical in organic systems and has been used extensively. It may be neutralized through either direct reduction via single electron transfer (SET) or via radical quenching through hydrogen atom transfer (HAT). Upon reduction, the color of the solution fades from purple to yellow and the reaction progress is conveniently monitored by a spectrophotometer [17]. The effect of seaweed extracts and standard on DPPH radical was compared and shown (Fig. 1). The scavenging effect increases with the concentration of standard and samples. At 50 µg/mL concentration, *G. calcicola* possessed scavenging activity 41.22% on DPPH. All the concentration of *G. calcicola* showed higher activity than the standard BHT (37.74%).

The ABTS scavenging activity was determined through differential extraction methods. This has chain breaking antioxidant property [18]. The percentage efficiency of ABTS scavenged through seaweed extract was found to increase with increasing concentration. (Fig. 2). The IC_{50} values of $ABTS^{+}$ radical scavenging activity of *G. calcicola* extracts of experimental alga was 50 µg/ml (44.76 %) and its IC_{50} values were higher than that of BHT (37.74 %).

The FRAP mechanism is electron transfer rather than mixed SET and HAT; thus, FRAP cannot detect compounds that act through radical quenching (HAT) [19]. The reducing activity of the green algae *G. calcicola* as determined through reducing power assay varied as seen (Fig.3). The antioxidant activity of the G. *calcicola* extract determined through reducing power assay was as followed: The reducing powers were found to be higher in *G. calcicola* extract. At concentration of 50 µg/mL of *G. calcicola* 50% of FRAP generated through incubation was scavenged (41.42%). The IC_{50} value of BHT was 37.74%.

Active oxygen species and free radicals are involved in a variety of pathological events nitric oxide radicals play an important role in inducing inflammatory response and their toxicity multiplies only when they react with O_2- radicals to form peroxynitrite, which damages biomolecules like proteins, lipids and nucleic acids [20]. Nitric oxide is generated when sodium nitroprusside reacts with oxygen to form nitrite. Suppression of (NO) release may be attributed to a direct (NO). Scavenging effect as the seaweed extracts decreased the amount of nitrite generated from the decomposition of sodium nitroprusside in vitro as shown (Fig. 4). The IC_{50} values of the nitric oxide radical assay were compared to the standard antioxidants BHT (50 µg/ml). The IC_{50} values of methanol extracts of brown alga G. *calcicola* was 50 µg/ml (40.92%). It was also found that the IC_{50} value of the algal extracts was lower than that of BHT (37.74%).

Antioxidants with reducing power are those that can act as electron donors and can reduce the oxidised intermediates of lipid peroxidation processes, allowing them to act as primary and secondary antioxidants [21]. Such as antioxidants react with potassium ferricyanide (Fe^{3+}) to form potassium Ferro cyanide (Fe^{2+}), which then reacts with ferric chloride (Fe^{3+}) to form ferrous complexes (Fe^{2+}) that have a maximum absorbance at 700 nm [22, 23]. Percentage scavenging activities of hydroxyl radical examined at different concentrations of *G. calcicola* were revealed (Fig. 5). *G. calcicola* were exhibited a maximum hydroxyl radical scavenging activity of (42.63%) at 50 µg/ml whereas BHT was found to be (37.74%) at 50 mg/ml.

Superoxide anion radicals are formed from cellular oxidation actions in organisms, including in humans. Although it is a relatively weak oxidant, it decomposes to produce stronger oxidative species, such as hydrogen peroxide and hydroxyl radicals, through dismutation and other types of reactions. It is also the source of the free radicals formed *in vivo*. SOA radicals and its derivatives are cell damaging, causing damage to DNA and cell membranes.

Therefore, it is a great important to scavenge SOA radicals [24]. Superoxide is a highly reactive molecule that reacts with various substances produced through metabolic processes. Percentage scavenging activity of superoxide anion examined at a different concentration of *G. calcicola* was revealed (Fig 6). *G. calcicola* were exhibited a maximum Superoxide Anion scavenging activity of (40.95%) at 50 µg/ml whereas BHT (standard) was found to be (37.74%) at 50 mg/ml.

The hydroxyl radical is the most reactive free radical and can be formed from superoxide anions and hydrogen peroxides in the presence of metal ions, such as copper and iron. Hydroxyl radicals can cause damage to nearly all types of biomolecules, including proteins, DNA, polyunsaturated fatty acids, and nucleic acids [25]. The scavenging effect of OH was investigated using the Fenton reaction and the results shown as the 50% inhibition rate (Fig. 7). *G. calcicola* exhibited the inhibition of about (41.28%) but this is lower than the standard BHT (37.74 %).

H_2O_2 is a non radical compound, and is of potential biological significance because of its ability to penetrate biological membranes. H_2O_2 itself is not very reactive, but it can sometimes be toxic to the cell because it may give rise to hydroxyl radical in the cells (singlet oxygen and HO. radicals) [26]. Thus, removal of H_2O_2 is very essential to protect the biological system in general, and food components, in particular. It was reported that extracts of some brown seaweeds registered more than (90%) H_2O_2 scavenging activity [27], thereby supporting the very fact that brown seaweeds are rich source of natural antioxidant compounds, which can scavenge H_2O_2 radical. Many other species of seaweeds were also reported in literature to possess potential H_2O_2 scavenging activity [28]. Percentage scavenging activity of hydroxyl radical examined at different concentrations of *G. calcicola* was revealed (Fig. 8). *G. calcicola* were exhibited a maximum hydroxyl radical scavenging activity of (44.64%) at 50 µg/ml whereas BHT (standard) was found to be (37.74%) at 50 mg/ml.

Metal chelating ability in terms of ferrous ion chelating capacity is claimed as one of the important mechanisms of antioxidant activity. Ferrous ions are the most powerful prooxidants among various species of transition metals present in food systems. These ions react with hydrogen peroxide via the Fenton reaction and produce dangerous hydroxyl radicals [29]. However, dietary antioxidants (nutrients) having metal chelating ability may act as preventive or secondary antioxidants as they forms-bonds with metal ions and reduce the redox potential thereby stabilizing the oxidized form of the metal ions [30] (Fig. 9). The Metal chelating ability of the G. *calcicola* was increased in a dose-dependent manner, using a concentration ranging from 5-50 mg/mL. The IC_{50} values were 39.84% and 37.74% respectively, for *G. calcicola* and BHT standards, indicating that the scavenging activity of BHT was significantly stronger than that of the *G. calcicola*.

This assay has been routinely used to evaluate the antioxidant capacity of extracts [31]. Various extracts of *G. calcicola* were also used to determine their antioxidant capacities by the formation of green phosphomolybdenum complex. The formation of the complex was measured by the intensity of absorbance in extracts at a concentration of 100 mg /ml at 95 C. The phosphomolybdenum method is based on the reduction of Mo(VI) to Mo(V) by the antioxidant compounds and the formation of green phosphate/ Mo(V) complex with the maximal absorption at 695 nm. Being simple and independent of other antioxidant measurements commonly employed, the assay was extended to plant polyphenols. In phosphomolybdenum assay, the concentration ranges from 5-50 mg/mL, The IC_{50} values were 45.00% and 37.74% respectively, for *G. calcicola* and BHT standards, indicating that the scavenging activity of BHT was significantly stronger than that of the *G. calcicola*.

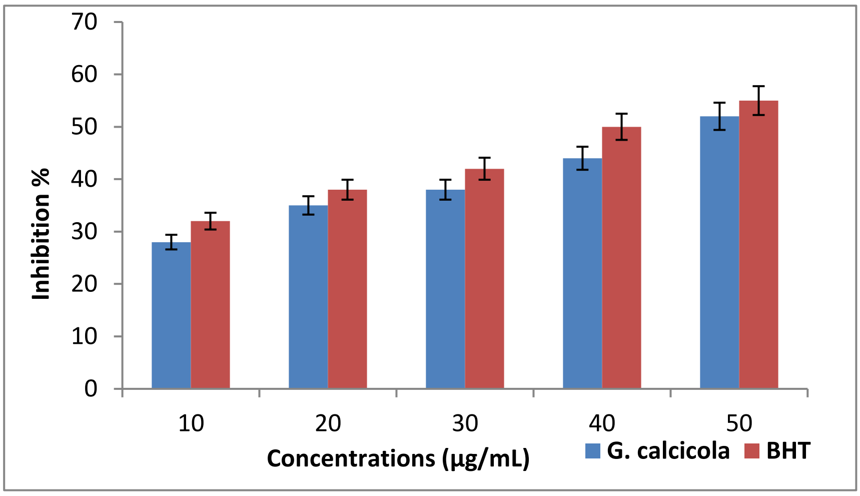

Fig. 1. DPPH radical scavenging activity of *G. calcicola.*

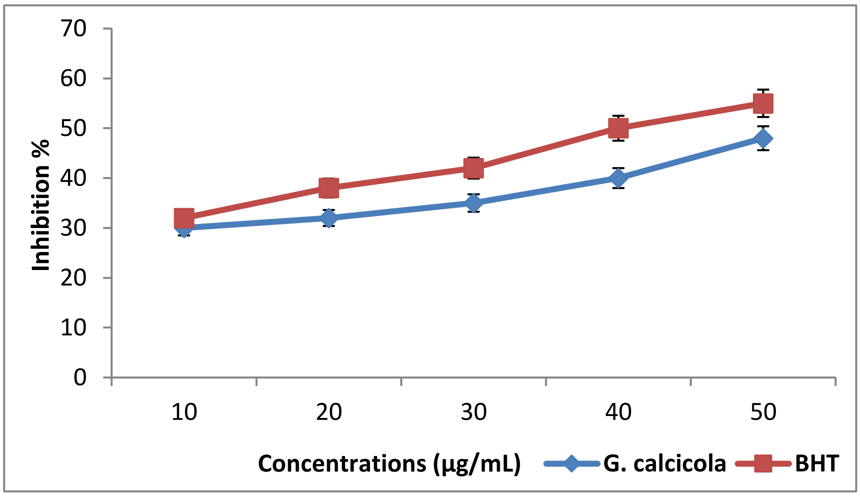

Fig. 2. ABTS scavenging activity of *G. calcicola.*

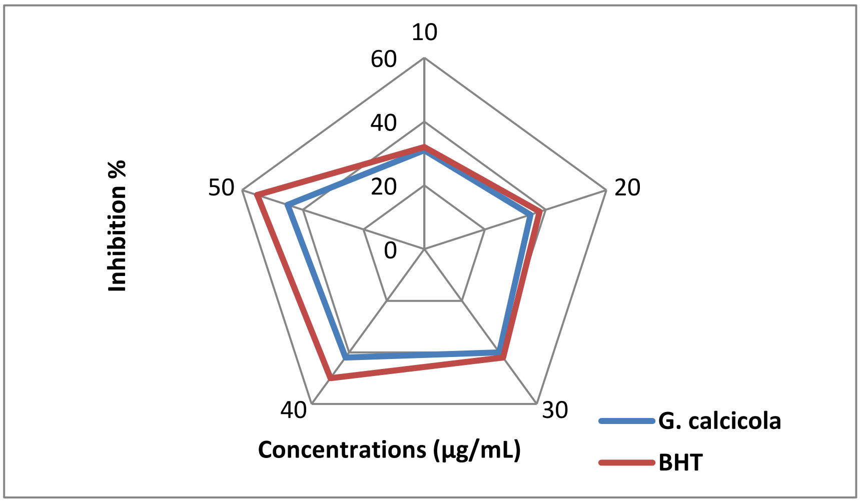

Fig. 3. FRAP radical scavenging activity of *G. calcicola.*

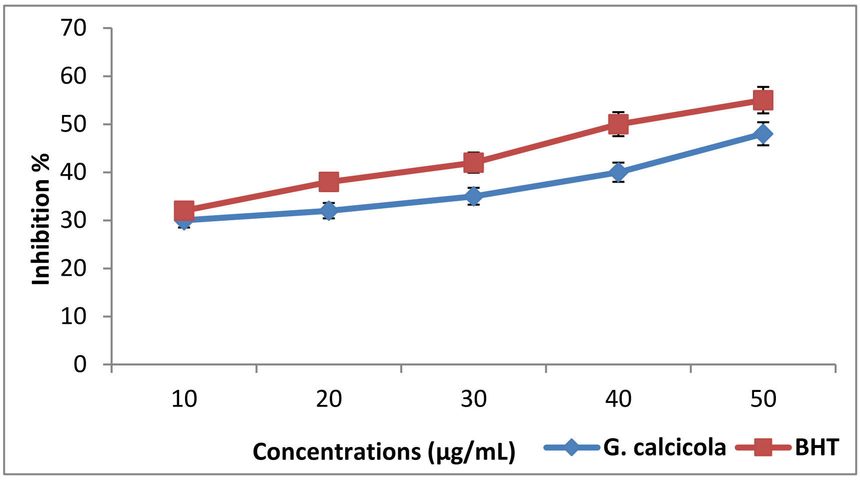

Fig. 4. Nitric oxide scavenging activity of *G. calcicola.*

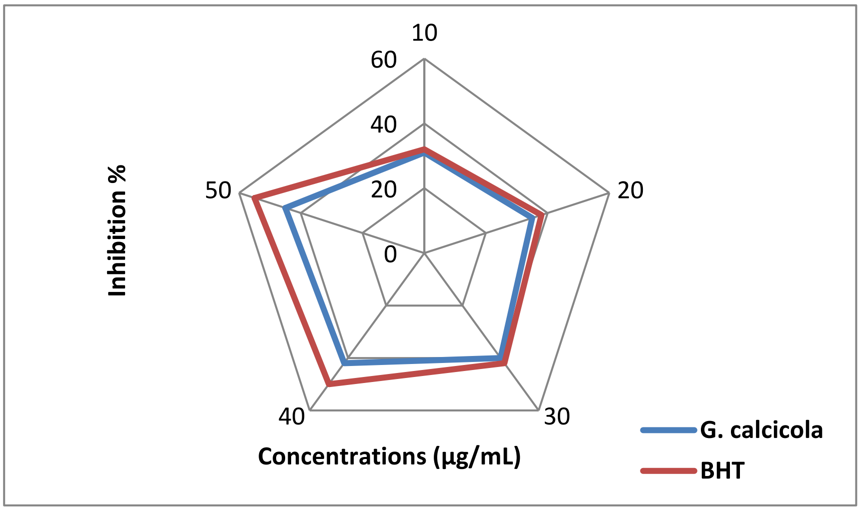

Fig. 5. Reducing power radical scavenging of *G. calcicola.*

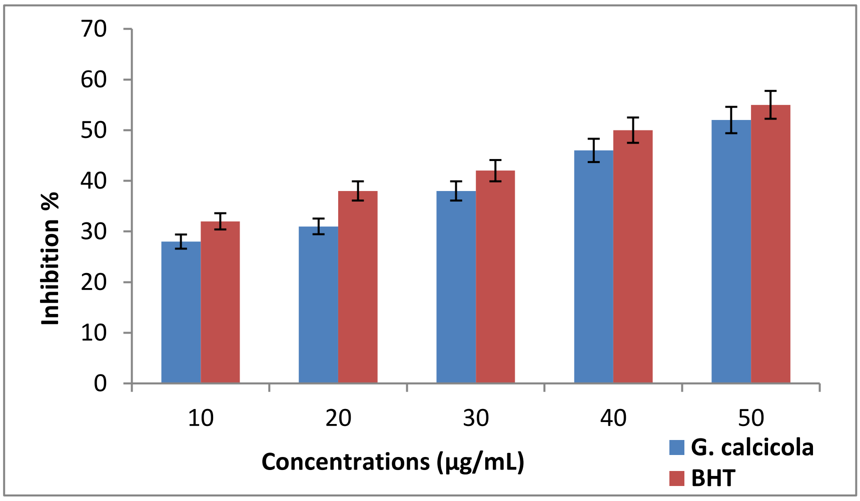

Fig. 6. Superoxide anion radicals scavenging of *G. calcicola.*

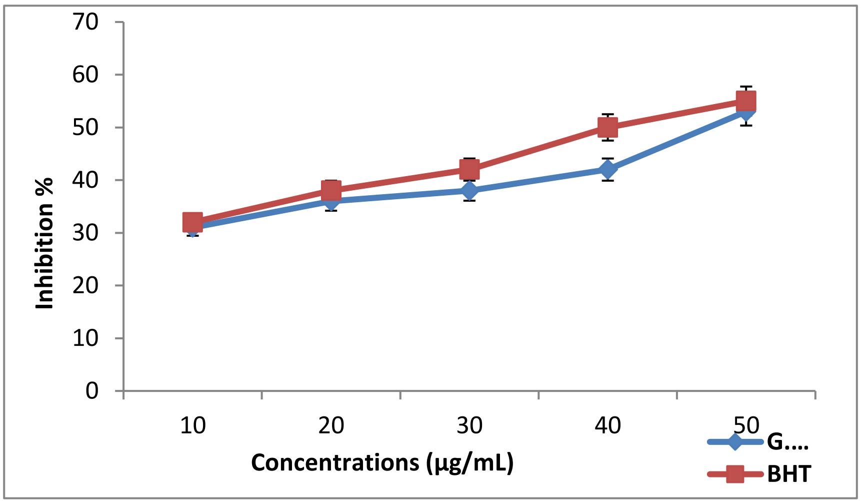

Fig. 7. Hydroxy radicals scavenging of *G. calcicola.*

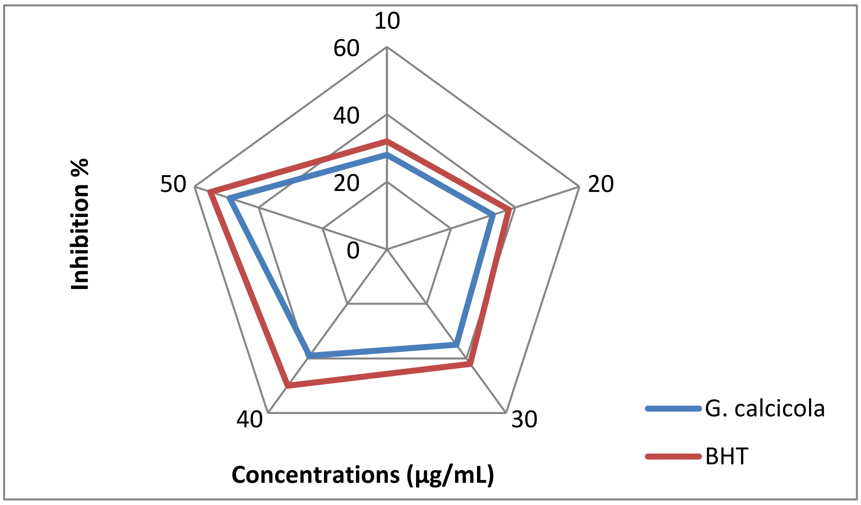

Fig. 8. Hydrogen Peroxide scavenging of *G. calcicola.*

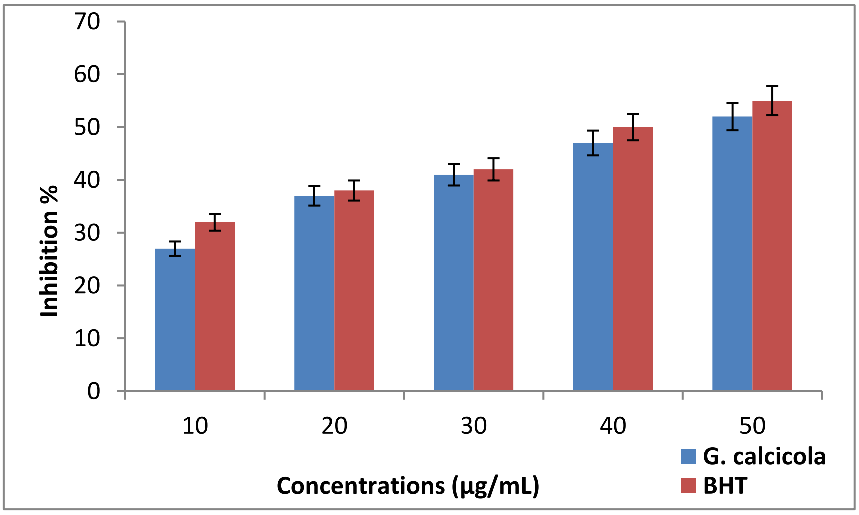

Fig. 9. Metal Chelating scavenging of *G. calcicola.*

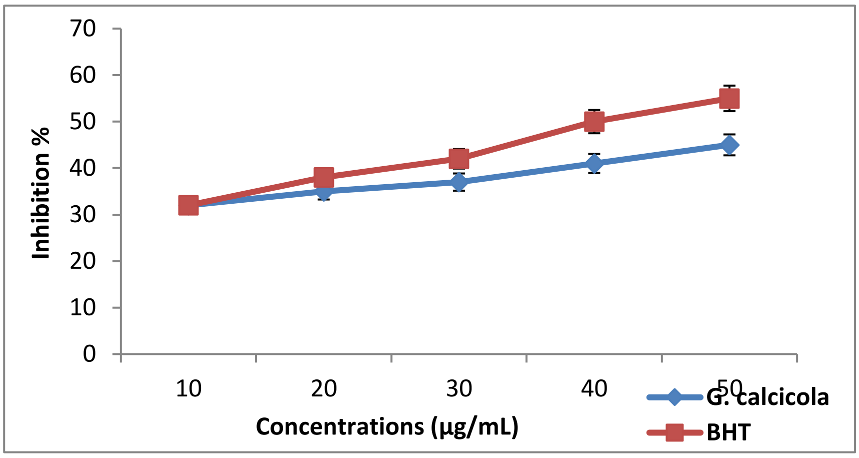

Fig. 10. Phosphomolybdenum assay of *G. calcicola.*

CONCLUSION

The results of the present work indicated that the methanol extract of *G. calcicola* was a fairly active scavenging assay system. The present findings seem promising to facilitate further experiments on the identification and characterization specific of compounds which are responsible for the relatively high antioxidant activities. Importantly, this research may contribute to a rational basis for the application of marine algal extract in possible therapy of diseases associated with oxidative stress and further supported that the antioxidant rich extracts or fractions may be used as a dietary supplement, promoting good health.

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