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Free Radical Scavenging Activity of Methanolic Extract of Seaweed Rhodophyta *Gelidiella calcicola*

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Abstract

Objectives: The aim of the present study was to evaluate the free radical scavenging activity of the compound *Gelidiella Calcicola*. **Material and Methods:** Free radical scavenging was determined by using 1,1-diphenyl-2-picrylhydrazyl (DPPH), ABTS (2,2'-azinobis (3ethylbenzthiazoline-6-sulphonic acid), ferric reducing antioxidant power (FRAP), nitric oxide scavenging assay (NO), reducing power, hydroxy radical scavenging assay, superoxide radical scavenging (SOD), hydrogen peroxide radical assay, metal chelating activity as well as phosphomolypdenum assay. **Results and Conclusions:** The present investigation clearly indicate that the *Gelidiella Calcicola* possesses antioxidant properties and serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants.

Keywords
Gelidiella Calcicola, DPPH, ABTS, FRAP, Antioxidant, Phosphomolypdenum

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INTRODUCTION

Red algae have attracted on the emerging interest mainly for their bioactive substances which have great chances to be used as antioxidant (Nagai and Yukimoto, 2003; Nakai, 2006). The marine environment in which seaweed exists possess great taxonomic diversity and synthesis metabolites with varied structure with interesting biological activities for food material and medical applications (Batista Gonzalez et al., 2009). Seaweeds contain different varieties of inorganic and organic substances which can be used for human health for examples polyphenols, carotenoids and tocopherols, terpenes, ascorbic acid, alkaloid (Chanda et al., 2010). This addition of compounds has demonstrated antioxidant activity in a variety of in vitro studies (Heo et al., 2009).

The red algae, or Rhodophyta rhodon, "rose" and phyton, "plant"), are one of the oldest groups of eukaryotic algae, and also one of the largest, with about 5,000-6,000 species of mostly multicellular, marine algae, including many notable seaweeds. Other references indicate as many as 10,000 species; more detailed counts indicate about 4,000 in about 600 genera (3,738 marine species in 546 genera and 10 orders (plus the unclassifiable); 164 freshwater species in 30 genera in eight orders (Batista Gonzalez et al., 2009).

The red algae form a distinct group characterized by these attributes: eukaryotic cells without flagella and centrioles, using floridean

polysaccharides as food reserves, with phycobili proteins as accessory pigments (giving them their red color), and with chloroplast slacking external endoplasmic reticulum and containing untracked thylakoids. Most red algae are also multicellular, macroscopic, marine, and have sexual reproduction. They often have alternation of generations and may have three generations rather than two. Many of the coralline algae, which secrete calcium carbonate and play a major role in building coral reefs, belong here. Red algae such as dulse (*Palmaria palmata*) and laver (nori/gim) are a traditional part of European and Asian cuisines and are used to make other products such as agar, carrageenans and other food additives (Nagai and Yukimoto, 2003). Figure1.

Fig.1. Red seaweed

MATERIAL AND METHOD

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),

COLLECTION OF ALGAE SAMPLES

Marine algae *Gelidiella Calcicola* were collected from the coastal region of Rameshwaram area on January 5th, 2015. Algae were washed with sea water to remove extraneous materials and brought to the laboratory in plastic bag containing sea water to prevent evaporation.

PREPARATION OF SAMPLE EXTRACT

5 g of G. *calcicola powdered* seaweed was extracted overnight with 100 ml methanol at room temperature and centrifuged at 2800 rpm for 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• was determined spectrophoto metrically in a dark room by the method of Subhashini, et al., (2011). DPPH• is a stable free radical and accepts an electron, or hydrogen radical to become a stable diamagnetic molecule. DPPH• 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.1ml of various concentrations G. *calcicola* methanol extract was taken, 1ml of DPPH was added and this made up to 3ml with water. The blue colour developed was read at 517nm and BHT was used as a standard.

$$\% \text{ Scavenging activity} = \frac{\text{Absorbance of control - Absorbance of sample} \times 100}{\text{Absorbance of control}}$$

2,2'-AZINOBIS-(3-ETHYLBENZOTHIAZOLINE-6-SULFONIC ACID) ($ABTS^{+}$) ASSAY

The total antioxidant activity of the samples was measured by [2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)] $ABTS^{\bullet+}$ radical cation decolorization assay according to the method of Huang et al., (2010). ABTS•+ was produced by reacting 7mM ABTS+ aqueous solution with 2.4mM potassium persulfate in the dark for 12-16 hours at room temperature. The radical was stable in this form for more than two days when stored in the dark at room temperature. Then, 2ml of diluted ABTS•+ 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 734nm and compared with standard BHT. Percentage of inhibition was calculated.

$$\% \text{ Scavenging} = \frac{\text{Control OD - Test OD}}{\text{Control OD}} \times 100$$

FERRIC REDUCING ANTIOXIDANT POWER (FRAP) ASSAY:

The FRAP procedure described by Benzie and Strain was used (Xu et al., 2009). The principle of this method is based on the reduction of a ferric-tripyridyl triazine complex to its ferrous colored form in the presence of antioxidants. Aliquots of 100 µL samples were mixed with 3 ml FRAP reagent and the absorbance of reaction mixture at 593 nm was measured spectrophotometrically after incubation at 37ºC for 10 min. For construction of calibration curve five concentrations of $FeSO_4$ $7H_2O$ (5 to 50 µmol/L) were used and the absorbencies were measured as sample solution. The values were expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1 mM $FeSO_4$.BHT was used as reference standard.

NITRIC OXIDE SCAVENGING ACTIVITY:

Nitric oxide scavenging activity was determined according to the method suggested by Sakat et al., (2010). Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacted with oxygen to produce nitrite ions, which can be estimated by using the Griess reagent. Scavenging of nitric-oxide act against oxygen, leading to reduced production in brief, 3.0 mL of 10 mM sodium nitroprusside in phosphate buffered saline was mixed with different concentrations of the G. *calcicola* extract and incubated at 25°C for 150 min. 0.5 mL of the incubated solution was taken and mixed with 0.5 ml of Griess reagent. 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 by Yen et al., (1999). 0.1ml of sample solution was mixed with 1ml of NBT and 1ml of NADH solution. This mixture was incubated at 25ºC for 5 minutes. A control was performed with reagent mixture but without the sample. Absorbance was measured spectrophotometrically at 560nm. 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 of Chou et al., (2009). Various concentrations (5-50 µg/mL) of G. *calcicola* extracts were added with 1.0ml of Ferrous ammonium sulphate - EDTA solution, 0.5ml of EDTA solution (0.018%), and 1.0mL of dimethyl sulphoxide (DMSO). The reaction was initiated by adding 0.5ml of ascorbic acid and incubated at 80–90°C for 15 min in a water bath. After incubation the reaction was terminated by the addition of 1.0mL of ice - cold TCA. 3 ml of Nash reagent was added and left at room temperature for 15min. 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 [(A0 - A1)/A0] X100, where A0 is the absorbance of the control and A1 is the absorbance of the extract/standard.

SCAVENGING ACTIVITY AGAINST HYDROGEN PEROXIDE

The scavenging capacity of G. *calcicola* methanol extracts on hydrogen peroxide was determined according to the method of Naskar et al., (2010). 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 of Delpour, (2009). Different amounts of the extracts (5-50 µg/mL) were suspended in distilled water and mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6), and 2.5 ml of 1% $K_3Fe(CN)_6$. The mixture was incubated at 50°C for 20 min; 2.5 ml of 10% TCA 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. The chelating activity of a compound to compete with ferrozine for the ferrous ions can be evidenced by the reduction in the color. Metal chelating activity is one of the significant antioxidant mechanisms as it reduces the concentration of the catalyzing transition metal in the lipid peroxidation. (Smirnoff 1989). In this assay, 1 m1 of ferric chloride (2 mM; diluted 20 times) is mixed with different dilutions of the G. *calcicola* methanol extract (1 ml). The reaction is initiated by the addition of 1 ml of ferrozine (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 can from 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 by spectrophotometer due to the formation of green phosphate Mo (V) compounds at acidic pH. (Sahaa et al., 2008). For the total antioxidant capacity assay, 0.1 ml of G. *calcicola* methanol extract is mixed with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in eppendorff tube. The tubes are then caped and incubated at 95ºC for 90 minutes 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 the radical-scavenging capacity of antioxidants toward DPPH radical in organic systems and has been used extensively as a prescreening method for new antioxidants from natural resources due to its stability, simplicity, rapidity and reproducibility. The DPPH radical in fact may be neutralized by either direct reduction via single electron transfer (SET) or by radical quenching via 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 (Huang, 2005; Baskaran et al., 2014). The effect of seaweed extracts and standard on DPPH radical was compared and shown in Figure 2. The scavenging effect increases with the concentration of standard and samples. At 50 mg/mL concentration, G. *calcicola* possessed 41.22% scavenging activity on DPPH. All the concentration of G. *calcicola* showed higher activity than the standard BHT (37.74%).

The ABTS scavenging activity was determined by differential extraction methods. This has chain breaking antioxidant property (Subashini and Prasanth, 2014). The percentage efficiency of ABTS scavenged by seaweed extract was found to increase with increasing concentration. (Figure3). The IC_{50} values of $ABTS^+$ radical scavenging activity of G. *calcicola* extracts of experimental alga was 50 mg/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 by radical quenching (HAT) (Ou et al., 2002). The reducing activity of the green algae G. *calcicola* as determined by reducing power assay varied as seen in Figure4. The antioxidant activity of the G. *calcicola* extract determined by reducing power assay was as followed: The reducing powers were found to be higher in G. *calcicola* extract. At concentration of 50 mg/mL of G. *calcicola* 50% of FRAP generated by 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 (Moncada et al.,1991). 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 in Figure5.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 mg/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 oxidized intermediates of lipid peroxidation processes, allowing them to act as primary and secondary antioxidants (Chanda et al., 2009). Such as antioxidants react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride (Fe^{3+}) to form ferrous complexes (Fe^{2+}) that have a maximum absorbance at 700 nm (Ferreira, 2007; Jayanthi et al., 2011). Percentage scavenging activities of hydroxyl radical examined at different concentrations of G. *calcicola* were revealed in Figure6. G. *calcicola* were exhibited a maximum hydroxyl radical scavenging activity of (42.63%) at 50 mg/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 (Sarikurkcetal, 2010; Towatana and Phromkunthong, 2009). 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 in Figure7. G. *calcicola* were exhibited a maximum Superoxide Anion scavenging activity of (40.95%) at 50 mg/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 (Aruoma, 1999). The scavenging effect of OH was investigated using the Fenton reaction and the results shown as the 50% inhibition rate in Figure 8.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) (Ma et al., 2011). Thus, removal of H_2O_2 is very essential to protect the biological system in general, and food components. It was reported that extracts of some brown seaweeds registered more than (90%) H_2O_2 scavenging activity (Heo et al., 2005), 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 (Gupta and Abu-Ghannam, 2011). Percentage scavenging activity of hydroxyl radical examined at different concentrations of G. *calcicola* was revealed in Figure9. G. *calcicola* were exhibited a maximum hydroxyl radical scavenging activity of (44.64%) at 50 mg/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 (Halliwell, 2011). 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 (Gordon,1990) Figure 10. 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 (Prieto et al., 1990). 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 95ºC. 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, Figure11 shows 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. *calcicole.*

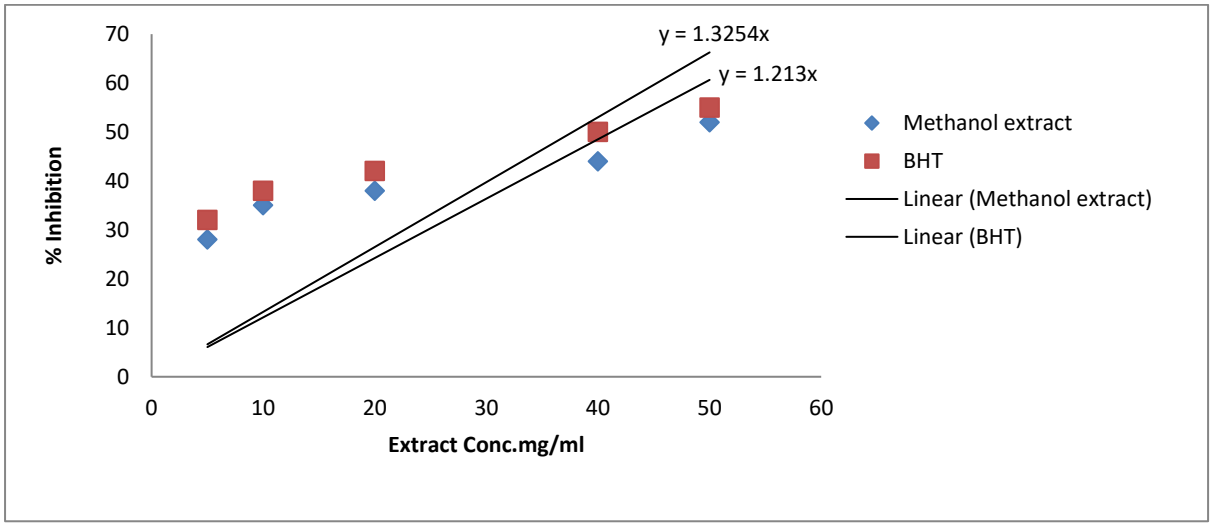

Fig.2. DPPH radical scavenging activity of *Gelidiella Calcicola*
Each value is expressed as mean ± standard deviation (n=3)

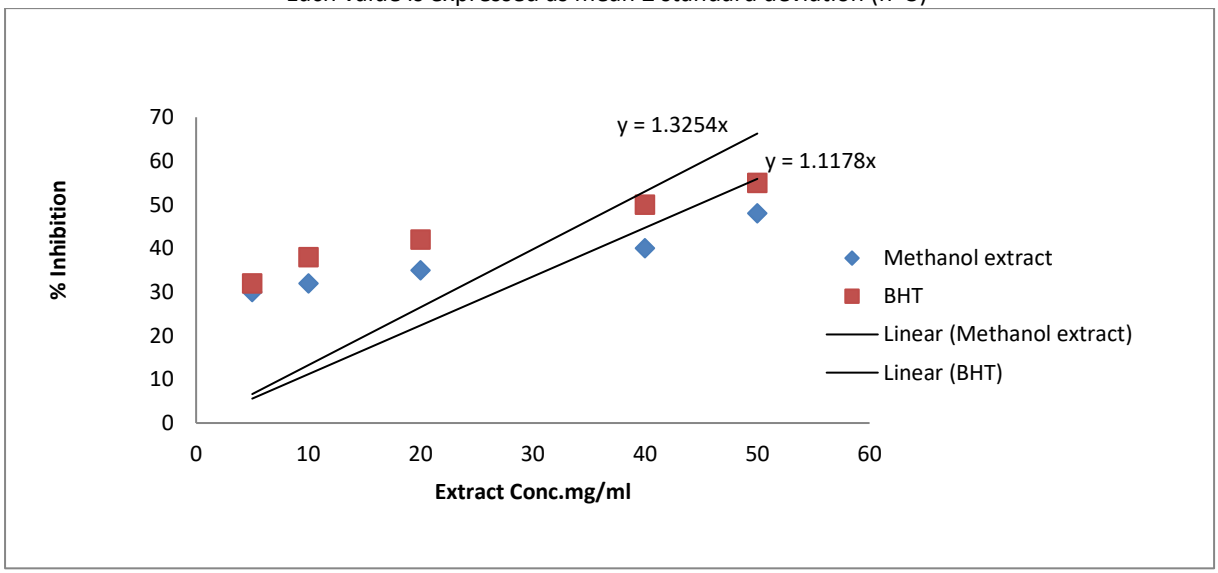

Fig.3. ABTS Radical scavenging ability of *Gelidiella Calcicola*
Each value is expressed as mean ± standard deviation (n=3)

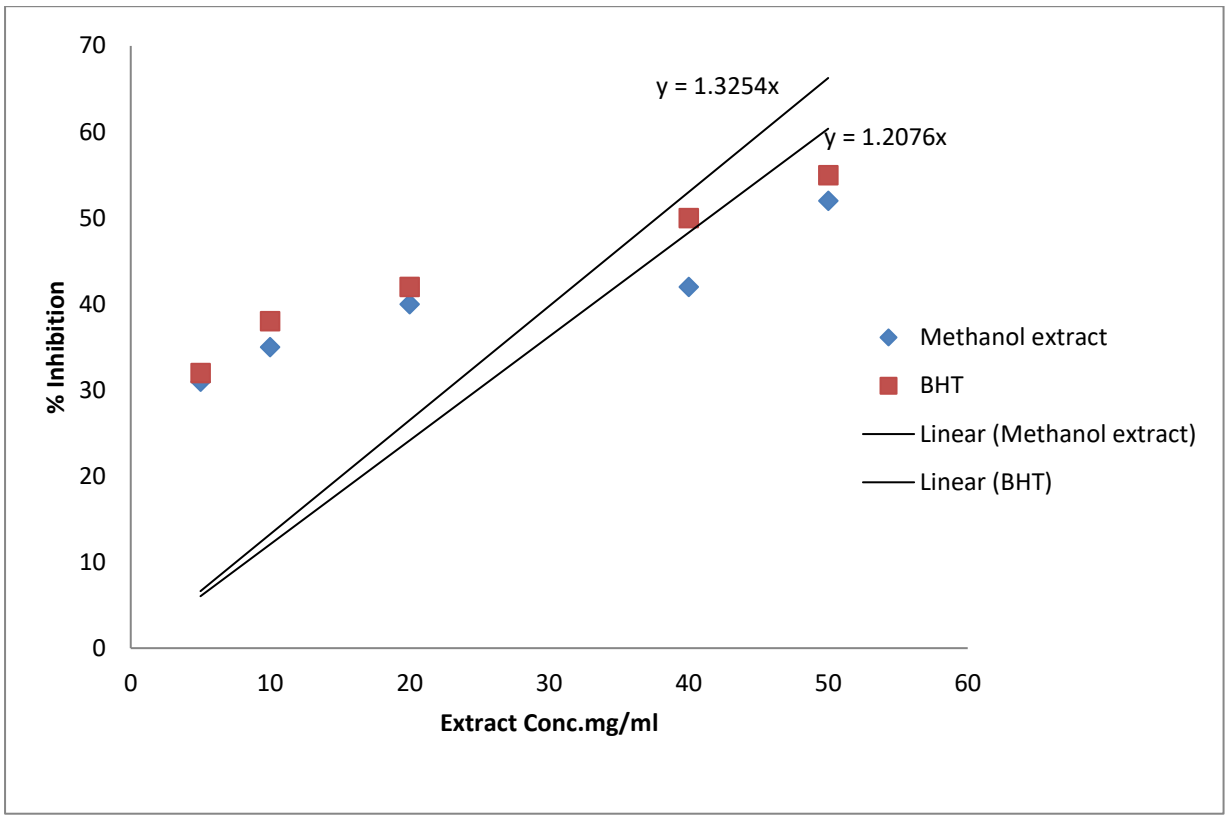

Fig.4. FRAP Radical scavenging ability of *Gelidiella Calcicola*
Each value is expressed as mean ± standard deviation (n=3)

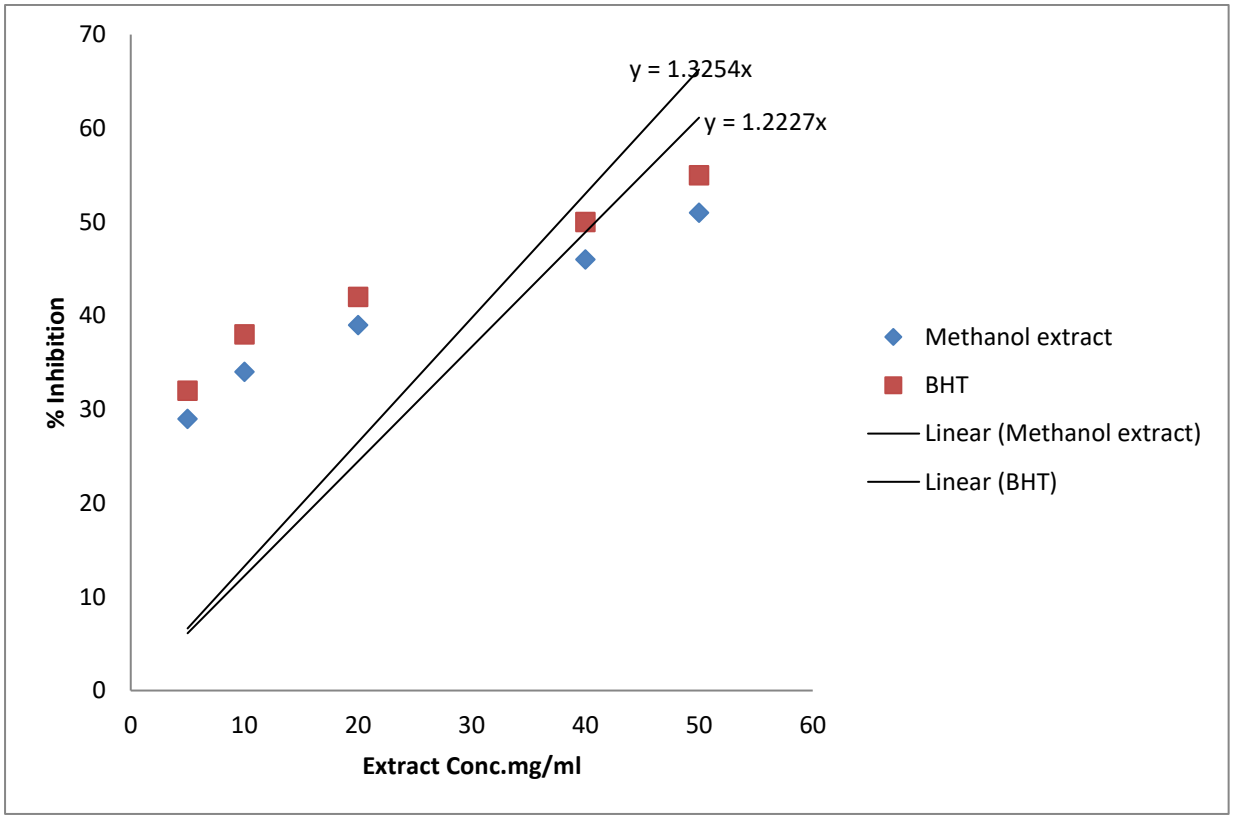

Fig.5. Nitric Oxide Radical scavenging ability of *Gelidiella Calcicola*
Each value is expressed as mean ± standard deviation (n=3)

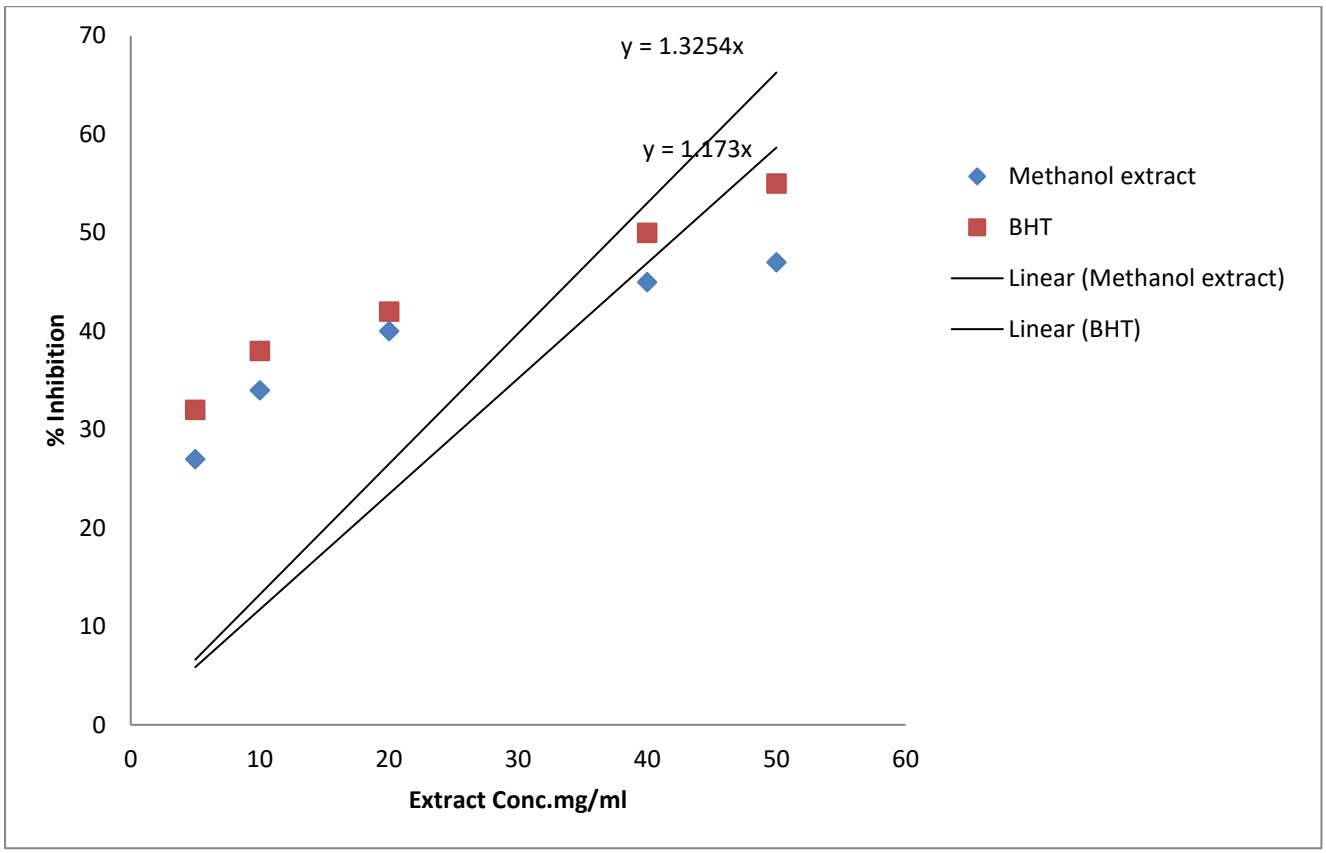

Fig.6. Reducing Power Radical scavenging ability of *Gelidiella Calcicola*

Each value is expressed as mean ± standard deviation (n=3)

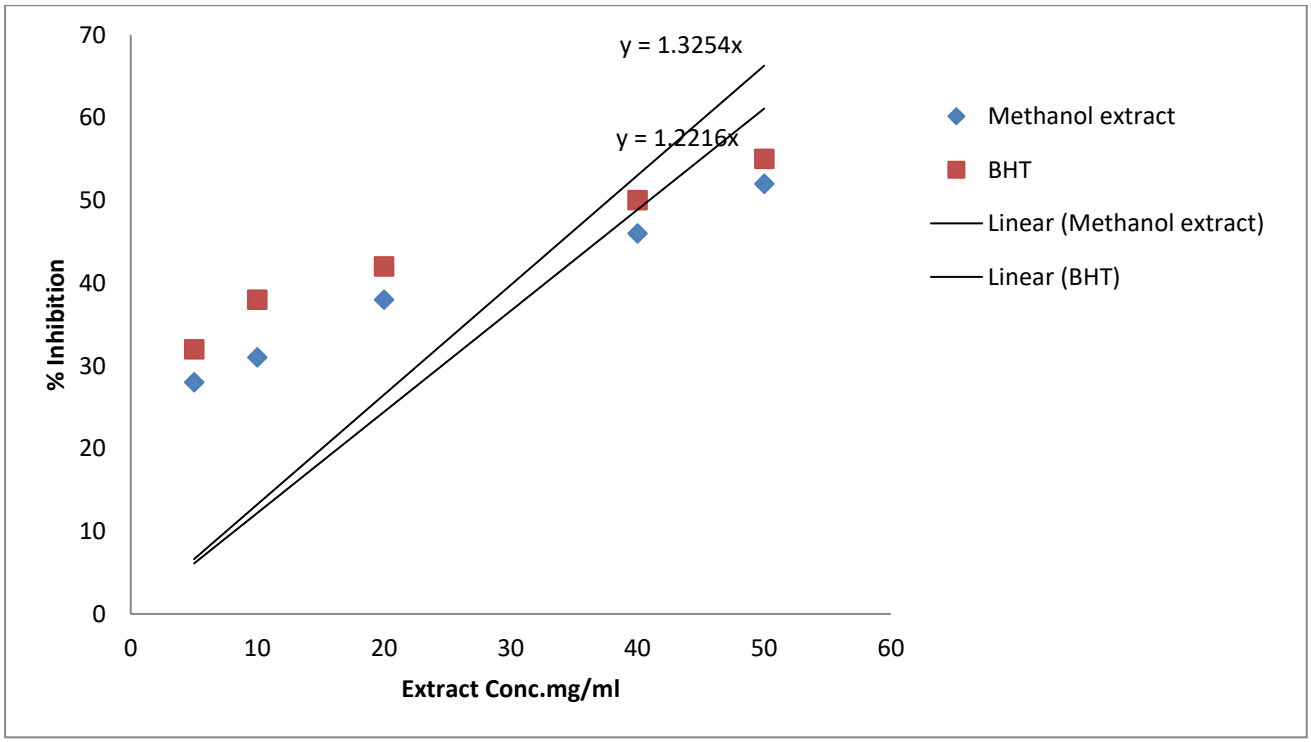

Fig.7. Superoxide Anion Radical scavenging ability of *Gelidiella Calcicola*

Each value is expressed as mean ± standard deviation (n=3)

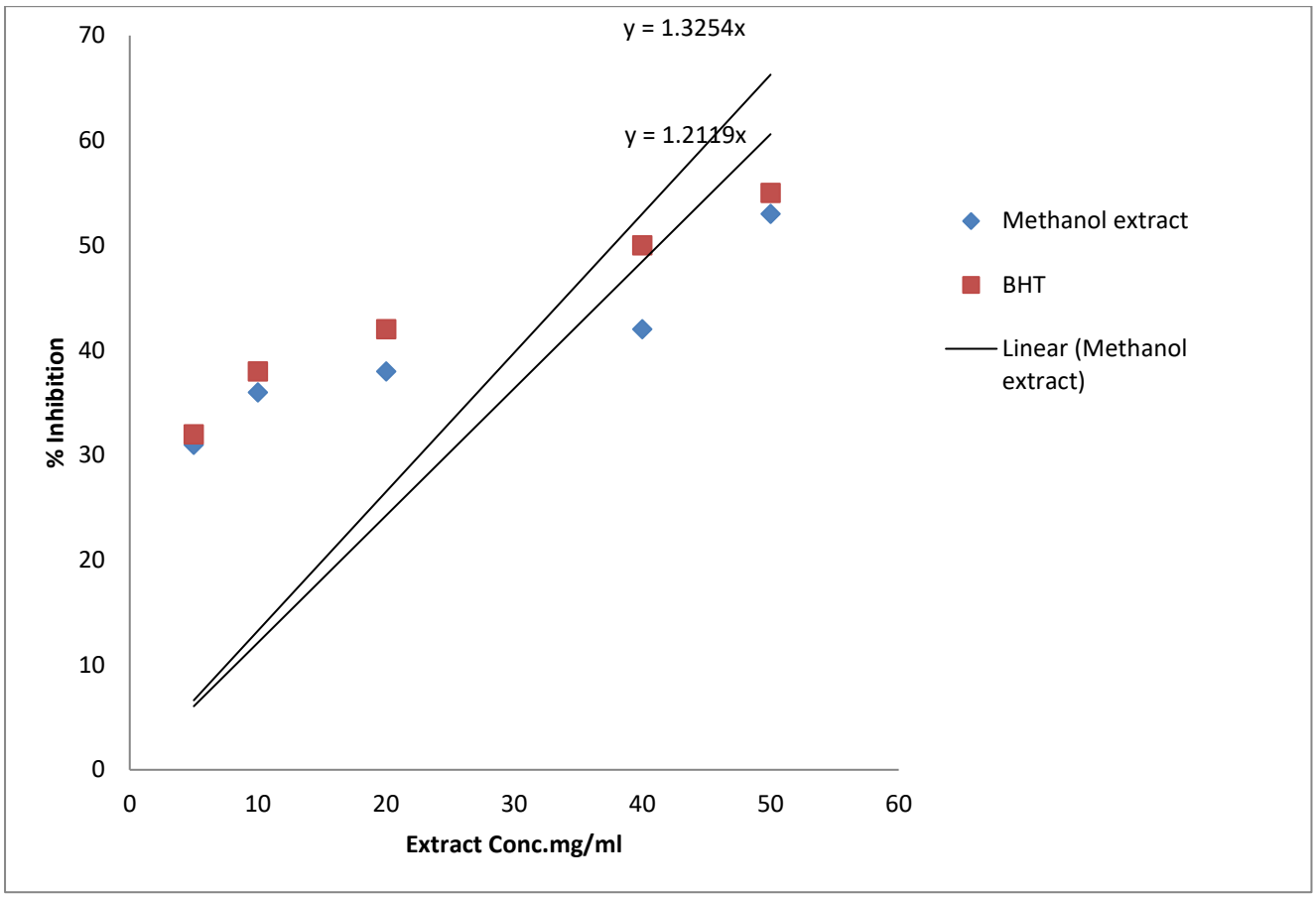

Fig.8. Hydroxy Radical scavenging ability of *Gelidiella Calcicola*
Each value is expressed as mean ± standard deviation (n=3)

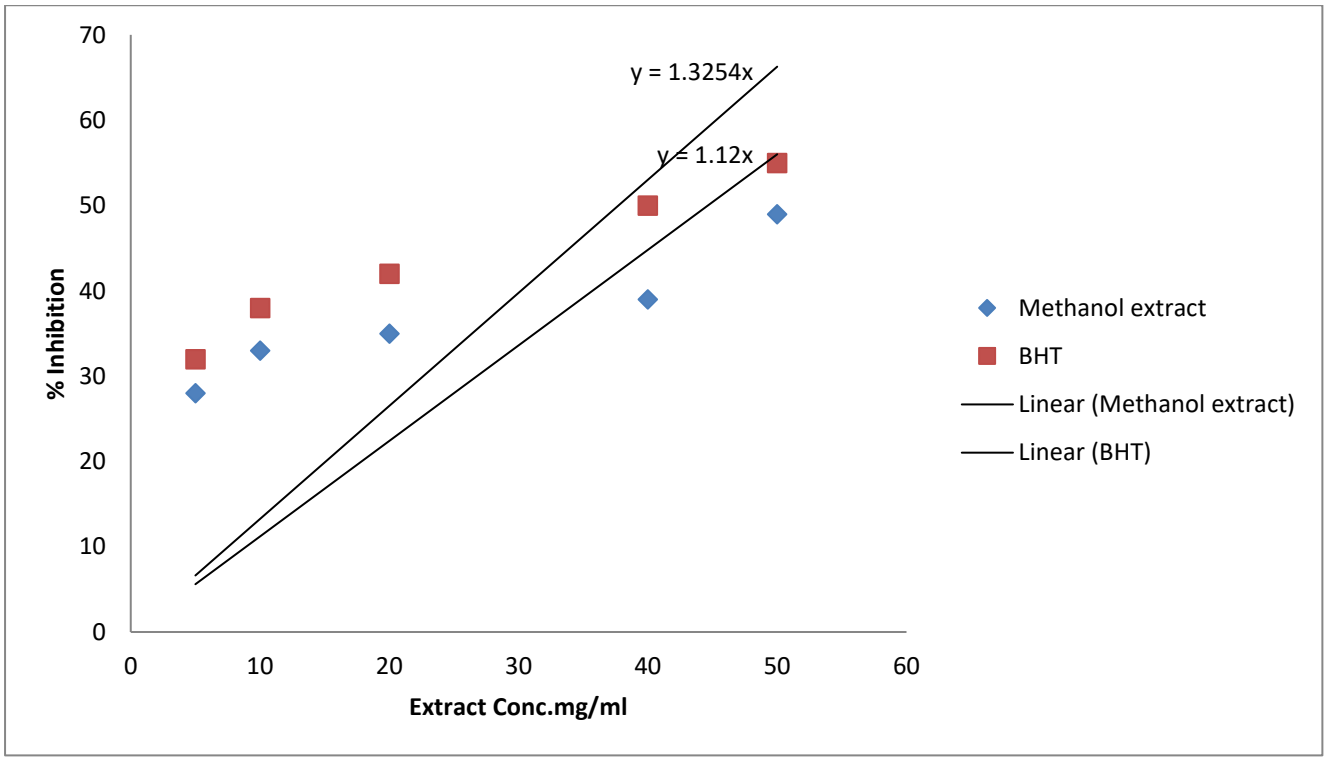

Fig.9. Hydrogen Peroxide Radical scavenging ability of *Gelidiella Calcicola*
Each value is expressed as mean ± standard deviation (n=3)

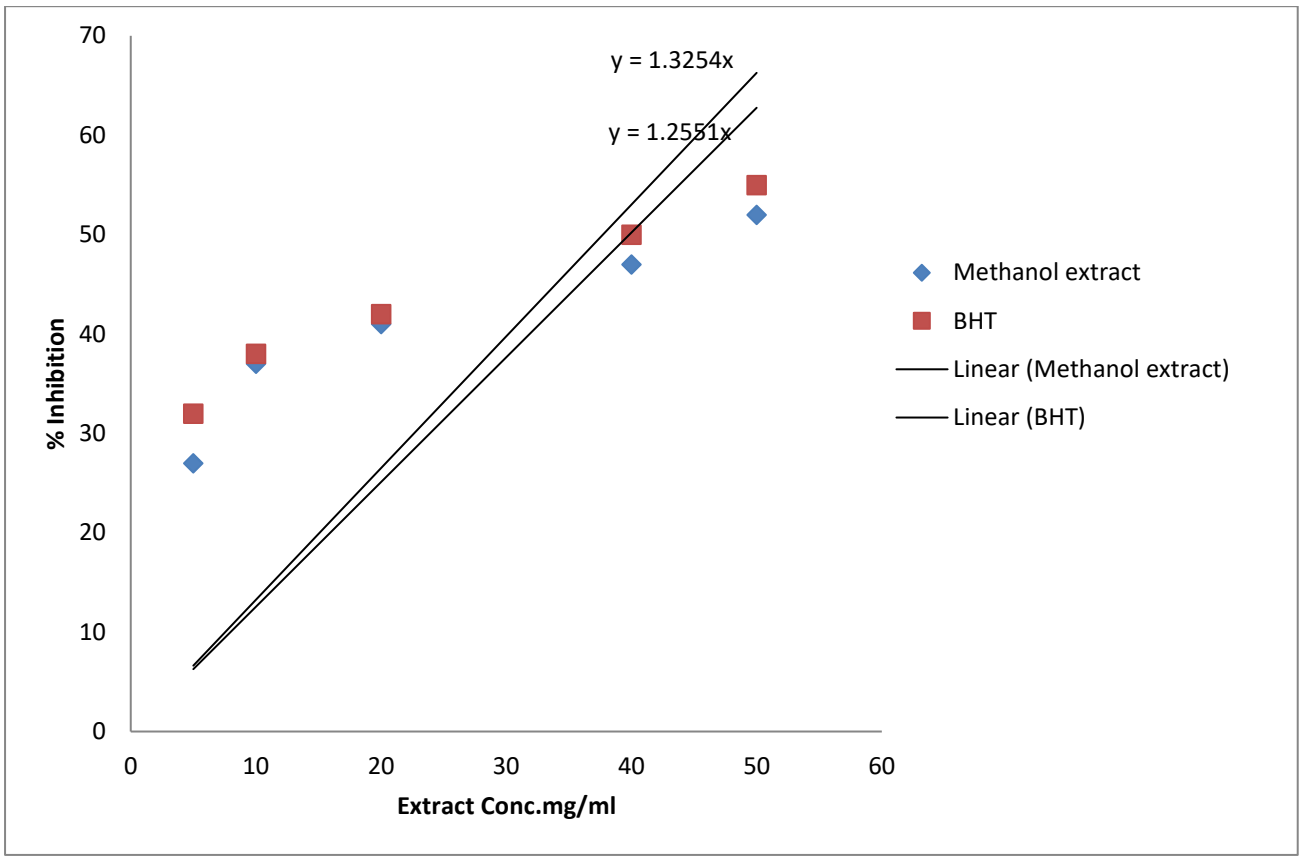

Fig.10. Metal Chelating ability of *Gelidiella Calcicola*
Each value is expressed as mean ± standard deviation (n=3)

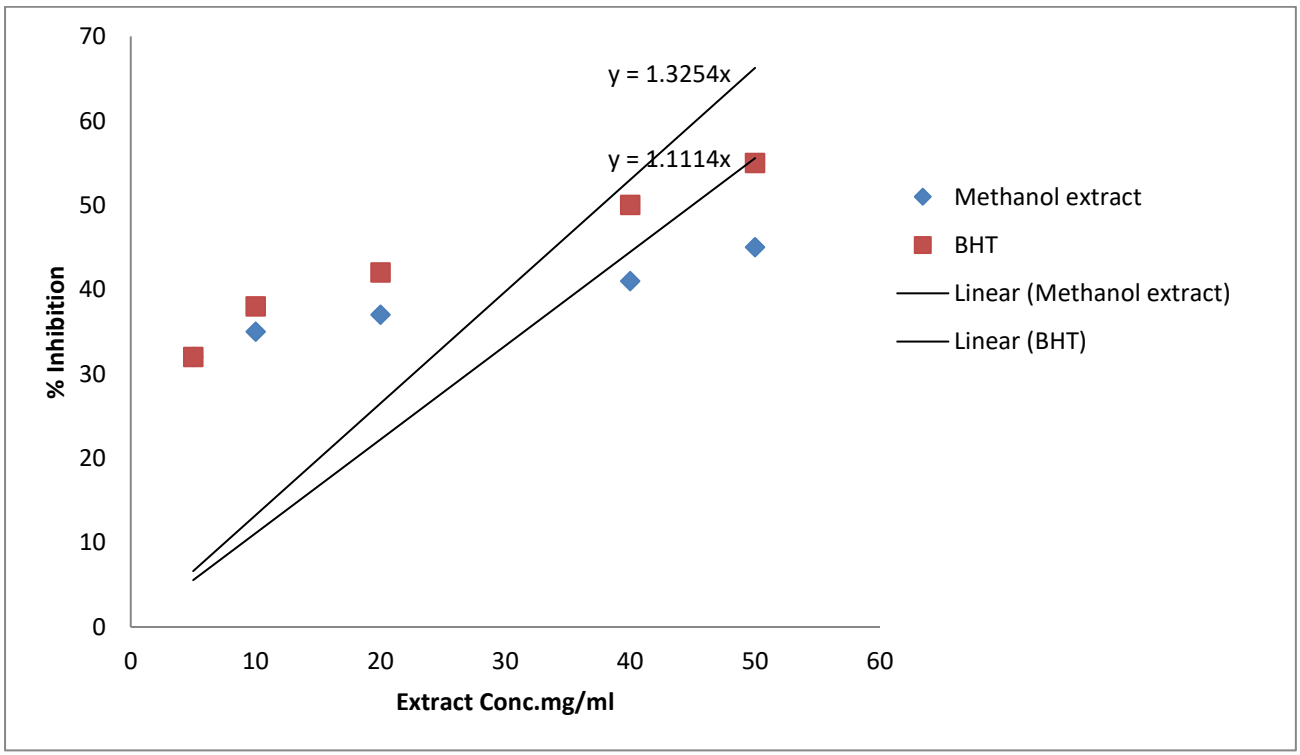

Fig.11. Phosphomolybdenum Radical scavenging ability of *Gelidiella Calcicola*
Each value is expressed as mean ± standard deviation (n=3)

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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