International Journal of

Cancer Research

ISSN 1811-9727

Academic
Journals Inc.

www.academicjournals.com

International Journal of Cancer Research 8 (1): 15-26, 2012
ISSN 1811-9727 / DOI: 10.3923/ijcr.2012.15.26
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Antioxidant and Antiproliferative Activities of Marine Algae, *Gracilaria edulis* and *Enteromorpha lingulata*, from Chennai Coast

K. Murugan and V.V. Iyer
Centre for Bio-Separation Technology, VIT University, Vellore 632014, Tamil Nadu, India

Corresponding Author: Vidhya V. Iyer, 612 Technology Tower, Centre for Bio-Separation Technology, VIT University, Vellore 632014, Tamil Nadu, India Tel: +91-0416-220-2721

ABSTRACT

Two species of marine algae, *Gracilaria edulis* and *Enteromorpha lingulata,* from Chennai coast were evaluated for their antioxidant and antiproliferative activities. Both algae were extracted with three solvents: methanol (M), chloroform (C) and ethyl acetate (E). The M, C, E extracts were investigated for 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging, Beta-carotene Bleaching (BCB), total reducing (TRA) and Growth Inhibitory (GI) activities and Total Phenolic Content (TPC). Thin Layer Chromatography (TLC) was used for qualitatively compare DPPH radical-scavenging activity. Except for BCB, *E. lingulata* extracts showed comparable (TRA) or higher (DPPH radical-scavenging) antioxidant activity, TPC and GI in HCT15 cells than the extracts of *G. edulis*. E and C extracts of *E. lingulata* showed greater antioxidant and GI activities in HCT15 cell (no GI in A549) than M extract. Although M extract of *G. edulis* showed slightly greater DPPH radical-scavenging activity than C and E extracts, M showed lower TRA, TPC, BCB and GI in HCT15 cells than E and C extracts. None of the extracts showed GI in A549 cells but the GI trend in HCT15 cells mirrored the one seen for all extracts of both algae for TPC (E>C>M; *E. lingulata*>*G. edulis*). Except for the E extract of *G. edulis* which showed slight pro-oxidant activity in the BCB assay, its C and M extracts showed greater BCB inhibition than all the *E. lingulata* extracts. For all extracts of both algae, DPPH radical-scavenging activity in TLC was associated with the more polar compounds in the extracts.

Key words: Algae, extract, antiproliferative, radical scavenging, antioxidant

INTRODUCTION

Marine algae are a group of aquatic autotrophic organisms that are broadly classified as Chlorophyta (green), Rhodophyta (red seaweeds) or Phaeophyta (brown seaweeds), based on the presence of photosynthetic pigments. Seaweeds are widely included in Japanese and Chinese diet and traditional medicine (Fujiwara-Arasaki *et al*., 1984; Chengkui *et al*., 1984) and have developed biological molecules and approaches which help them survive in their harsh and extreme environment. Seaweed sulfated polysaccharides like fucoidan, carrageenan (red seaweed) or algin (brown seaweed) are rich sources of soluble fibers, which have been reported to perform a varied range of functions such as antioxidant, antimutagenic, anticoagulant and antitumor (De Souza *et al*., 2007; Smit, 2004; Madhusudan *et al*., 2011). Caulerpenyne from the *Caulerpa* spp. (green algae) (Fischel *et al*., 1995) and polysaccharides such as fucoidan and laminarin from brown seaweeds have also been reported to show antitumor activity

(Yamasaki-Miyamoto *et al.*, 2009). Therefore, the objective of the present study was to explore marine algae for new compounds possessing antioxidant and antiproliferative activities, so that these compounds could be used as leads for making more potent, selective and less toxic drugs with better therapeutic indices. *Gracilaria edulis* and *Enteromorpha lingulata* are edible marine algae. However, in Phillipines, three fatal poisoning cases have been reported during 2002-2003 due to ingestion of *G. edulis* and *Acanthophora spicifera* (Yotsu-Yamashita *et al.*, 2004). There are no reports, to date, on the *in vitro* antiproliferative activity of *G. edulis*. To the best of our knowledge, this is the first report on investigation into antioxidant and antiproliferative activities of *Enteromorpha lingulata* and antiproliferative activity of *Gracilaria edulis*.

MATERIALS AND METHODS

Sample collection: *Enteromorpha lingulata* and *Gracilaria edulis* were collected from the Chennai coast of Tamil Nadu, India and identified by Dr. Baluswami, Madras Christian College, Chennai.

Chemicals: Ascorbic acid, beta-carotene, BHA (butylated hydroxyanisole), DPPH (2, 2-diphenyl-1-picrylhydrazyl), gallic acid, linoleic acid, DMEM growth medium and resazurin sodium salt were purchased from Sigma-Aldrich, India. Quercetin was purchased from Fluka, India. All other chemicals were purchased from SRL, India. Bromophenol blue, trichloroacetic acid (TCA) and other cell culture materials were purchased from HiMedia, India. TLC plates (TLC Silica gel 60 F_{254}) were purchased from Merck, India. Cell lines were obtained from the National Centre for Cell Sciences in Pune, India.

Algal extract preparation: The samples were washed thoroughly in fresh water and subsequently shade-dried and powdered (Vinayak *et al.*, 2011). Five grams of the dry powder was extracted twice for 16 h each time, with 50 mL of each solvent (methanol, chloroform or ethyl acetate separately) in a rotary shaker at 37°C. The extracts were filtered and the solvent was removed completely by using a rotary evaporator (Buchi Rotavapor R215, Switzerland). The crude extract was stored at -20°C and reconstituted in methanol for assays.

Antioxidant assays

DPPH radical-scavenging activity: The free radical-scavenging potential of the algal extracts was analyzed according to previously reported methods (Zubia *et al.*, 2009; Butkhup and Samappito, 2011; Jain *et al.*, 2011; Amatya and Tuladhar, 2011; Hanachi *et al.*, 2006). The concentrations of M (methanol), C (chloroform) and E (ethyl acetate) extracts taken for the experiment were 10, 50 and 100 μg mL^{-1}. A methanolic solution of DPPH (200 μL, 20 mg L^{-1}) was added to 22 μL of each extract in a 96 well plate. Ascorbic acid was used as a positive control while 200 μL of DPPH solution plus 22 μL methanol without any extract was used as a control, to calculate the extent of scavenging. The plate was incubated in the dark for 2 h at room temperature and the absorbance measured at 492 nm spectrophotometrically (Fluostar Optima BMG Labtech Gmbh).

% DPPH radical scavenging activity was determined by the formula:

$$\%\ \text{DPPH radical scavenging} = 1\text{-}\frac{A_t}{A_c}\times 100$$

Where:
A_t = Absorbance with extract
A_c = Absorbance of control (200 µL of DPPH, 22 µL of methanol)

Beta-carotene bleaching assay: The antioxidant potential of marine algae was measured by modifying the beta-carotene bleaching assay described earlier (Zubia *et al*., 2009; Duan *et al*., 2006; Amatya and Tuladhar, 2011; Hanachi *et al*., 2006). About 210 µL of a solution of beta-carotene (1 mg mL^{-1}) in chloroform was taken in a round bottom flask containing 5 µL of linoleic acid and 42 µL of Tween-20. The chloroform was removed in a rotary evaporator at 40°C and 10 mL of distilled water was added to form an emulsion with continuous shaking. Approximately 200 µL of the above emulsion was added to 50 µL of extracts (100 µg mL^{-1}) taken in a 96 well microplate. Emulsion without beta-carotene was used as a blank, BHA was used as a positive control and wells containing the beta-carotene emulsion with methanol instead of extract served to calculate the extent of bleaching. The plate was immediately read at 450 nm (0 h) and after 2 h of incubation at 50°C in the dark. Antioxidant activity (AA) was measured by using the formula:

$$AA\% = 1\text{-}\frac{A_{t0}\text{-}A_{t2}}{A_{c0}\text{-}A_{c2}}\times 100$$

Where:
A_{t0} and A_{t2} = Absorbance with extracts measured at 0 and 2 h, respectively
A_{c0} and A_{c2} = Absorbance of control (beta-carotene-containing emulsion and methanol instead of extract) measured at 0 and 2 h, respectively

Total reducing activity: The reducing power of marine algae was determined by using the assay described earlier (Zubia *et al*., 2009; Ashawat *et al*., 2007). To 200 µL of the M, C and E extracts, 200 µL of phosphate buffer (200 mM, pH 6.6) and 200 µL of 1% potassium ferricyanide solution were added. The mixture was incubated at 50°C for 30 min and 200 µL of 10% TCA solution added after the mixture had cooled down. After mixing, 125 µL of the above mixture was transferred to a 96 well plate and 20 µL of 0.1% ferric chloride solution was added. The formation of a Prussian blue complex indicated the reducing power of the samples. The absorbance was measured at 620 nm in a plate reader (Fluostar Optima BMG Labtech GmbH). Ascorbic acid was used as positive control and the reaction mixture with methanol instead of the extract was used as (negative) control. The total reducing activity was determined by using the formula:

$$\%\ \text{Total reducing activity} = 1\text{-}\frac{A_c}{A_t}\times 100$$

Where:
A_c = Absorbance of control (reaction mixture with methanol instead of extract)
A_t = Absorbance with extracts

Total phenolic content: The total phenolic content present in *G. edulis* and *E. lingulata* extracts was determined by using the Folin-Ciocalteu method described earlier (Masaldan and Iyer, 2011). Gallic acid (1 mg mL^{-1}) in methanol was used as a standard in concentrations ranging from 2 to

10 μg mL^{-1}. To 10 μL of standard/extracts (1 mg mL^{-1}) in a 2 mL tube were added 25 μL of the Folin-Ciocalteu's phenol reagent and 50 μL of 25% sodium carbonate solution. The mixture was vortexed and the volume was made up to 1 mL with distilled water. The tubes were then incubated in the dark for 1 h at room temperature and the absorbance was measured at 725 nm in a spectrophotometer (Beckman Coulter Du 730). Quercetin (1 mg mL^{-1}) in methanol was used as a positive control.

Thin layer chromatography: Thin layer chromatography was used to qualitatively determine the antioxidant activity of selected marine algae by the method described earlier (Masaldan and Iyer, 2011; Jain *et al.*, 2008). Methanolic solutions of M, C and E extracts were spotted on precoated silica gel aluminium sheets. These extracts were resolved separately by using the following three solvent systems listed in the order of increasing polarity: BEA benzene/ethanol/ammonium hydroxide (90:10:1), CEF-chloroform/ethyl acetate/formic acid (5:4:1) and EMW-ethyl acetate/methanol/water (40:5.4:5). The chromatograms obtained were further analyzed with iodine vapor and DPPH solution (0.2% in methanol).

Cell culture: A549 (lung adenocarcinoma) cells were grown in DMEM growth medium and HCT15 (colon adenocarcinoma) cells were grown in RPMI growth medium at 37°C in a 5% CO_2 atmosphere. The medium was supplemented with 10% FBS and penicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}). For experiments, A549 (2500) cells and HCT15 (4000) cells in 200 μL media $well^{-1}$ were seeded in a 96 well plate.

Growth inhibition assay: The antiproliferative potential of the marine algal extracts was studied in the above cell lines by using a resazurin-based assay (Masaldan and Iyer, 2011; Anoopkumar-Dukie *et al.*, 2005). Resazurin is the active component of the blue redox dye, Alamar Blue® which is converted into pink resorufin by the oxido-reductases in the cytoplasm, mitochondria and microsomes of viable cells. Cells were seeded in two 96 well plates-T_0 and T_i, with media blank and untreated control and incubated at 37°C in a 5% CO_2 atmosphere. After 24 h, the T_0 plate was treated with 10% resazurin (1 mg mL^{-1} in 50 mM PBS) for 4 h and the absorbance was measured at 584 nm and 620 nm. At the same time, the T_i plate was treated with 100 μg mL^{-1} of M, C and E extracts (in DMSO) and Doxorubicin (positive control). Medium blank with and without 0.5% DMSO (vehicle blank) as well as untreated (negative) control with and without 0.5% DMSO were also included in the T_i plate. After 48 h, the T_i plate was treated with resazurin like the T_0 plate and the absorbance was measured.

% of growth inhibition (GI) was calculated using the formula:

$$\%GI = \frac{T_i - T_0}{C - T_0} \times 100, \text{ when } T_i \geq T_0$$

$$\%GI = \frac{T_i - T_0}{T_0} \times 100, \text{ when } T_i < T_0$$

Where:

T_0 = $OD_{584\text{-}620}$ of untreated cells after 24 h

T_i = $OD_{584\text{-}620}$ of cells treated with test compound after 48 h and

C = $OD_{584\text{-}620}$ of negative control cells after 48 h (blanks subtracted from all)

Statistical analysis: All experiments were performed in triplicate (n = 3) and expressed as Mean±SEM. Means were compared with SPSS v 16.0 software for Windows (SPSS Inc., Chicago, IL) by using one-way ANOVA followed by the Tukey's post-hoc test. $p<0.05$ indicated statistical significance. Pearson correlation coefficient (r) was calculated ($p<0.05$) to assess the strength of the linear relationship between two variables.

RESULTS

ANOVA analysis indicated that the difference between the group means was statistically significant ($p<0.05$). However, except for a high positive correlation observed between the means for GI values in HCT15 and TPC values, none of the other Pearson correlation coefficients were significant for any of the other group mean pairs ($p>0.05$). Tukey's HSD post-hoc test showed that the differences between the following group mean pairs were statistically significant ($p<0.05$): DPPH radical scavenging and BCB assay group means; DPPH radical scavenging and A549 GI group means; A549 GI and TRA group means; A549 GI and BCB group means; A549 GI and HCT15 GI group means; HCT15 GI and BCB group means and BCB and TPC group means.

Antioxidant assays

DPPH radical scavenging activity: The antioxidant potential of marine algae was measured in terms of their ability to scavenge the stable DPPH radical (Fig. 1). *Enteromorpha lingulata* C and E extracts (100 μg mL^{-1}) showed higher activity of 15.85 and 15.61%, respectively. The M extracts of both *E. lingulata* and *G. edulis* (100 μg mL^{-1}) were almost equal in their DPPH radical-scavenging activity (13.59 and 14.84%, respectively). Lower activity was observed in *G. edulis* with 100 μg mL^{-1} C and E showing 9.01 and 8.47%, respectively. Tukey's HSD post-hoc test showed that the differences between the DPPH radical scavenging and BCB assay group means and between the DPPH radical scavenging and A549 GI group means were statistically significant ($p<0.05$).

Thin layer chromatography: The chromatograms showed the presence of organics and DPPH free radical-scavenging molecules when exposed to iodine vapor and DPPH solution, respectively (Fig. 2, 3). All organics appeared as brown spots and the scavenging of the DPPH

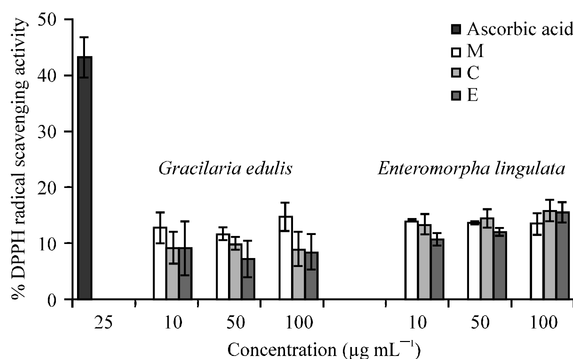

Fig. 1: DPPH radical scavenging activity of the *G. edulis* and *E. lingulata* extracts; M: Methanolic extract, C: Chloroform extract, E: Ethyl acetate extract

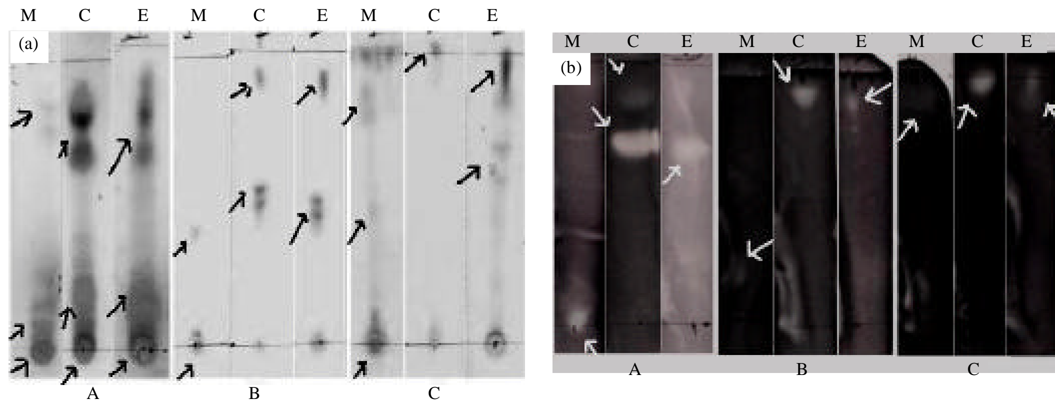

Fig. 2(a-b): TLC analysis of *G. edulis*: methanol (M), chloroform (C) and ethyl acetate (E) extracts using different eluent systems; A: BEA, B: CEF and C: EMW (from left to right) treated with (a) iodine vapor for all organics and (b) DPPH solution for radical scavenging activity

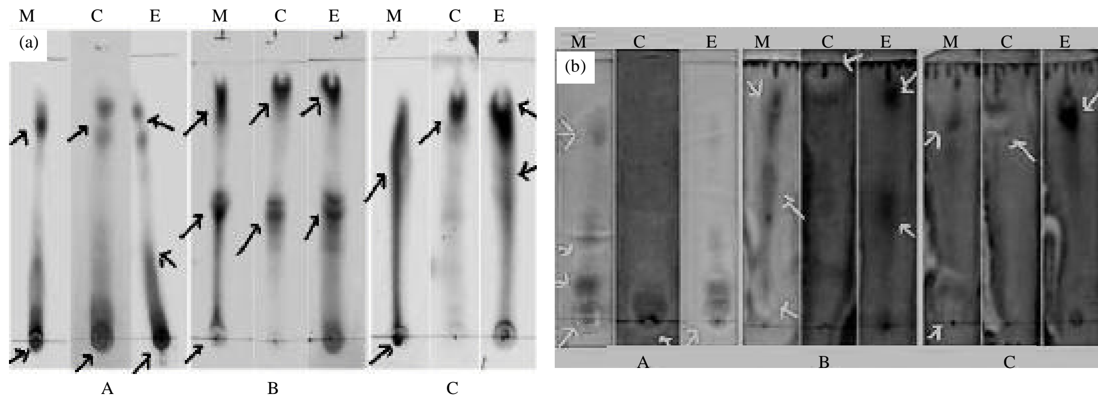

Fig. 3(a-b): TLC analysis of *E. lingulata*: methanol (M), chloroform (C) and ethyl acetate (E) extracts using different eluent systems; A: BEA, B: CEF and C: EMW (from left to right) treated with (a) iodine vapor for all organics and (b) DPPH solution for radical scavenging activity

radical was seen in the form of disappearance of purple color at some spots. Our results suggest that DPPH radical-scavenging activity was associated with the more polar components in all extracts because the R_f values of these components increased with increasing polarity of the resolving system. For all three extracts of both algae, *E. lingulata* extracts showed strong DPPH radical-scavenging activity in TLC and C and E extracts of *G. edulis* seemed to have stronger DPPH radical-scavenging activity on the TLC plates than the M extract.

Beta-carotene bleaching assay: Antioxidant activity was also examined in terms of inhibition of beta-carotene bleaching by linoleic acid (Fig. 4). The highest inhibitory activity (60%) was found

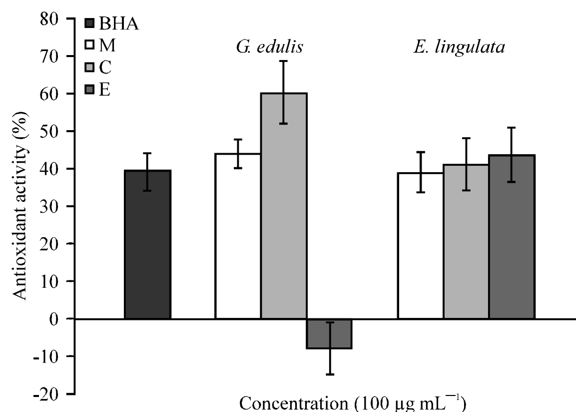

Fig. 4: Beta-carotene bleaching inhibition by *G. edulis* and *E. lingulata* extracts; M: Methanolic extract, C: Chloroform extract, E: Ethyl acetate extract

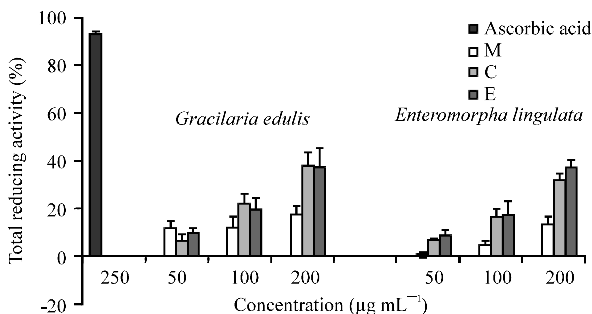

Fig. 5: Total reducing activity of the *G. edulis* and *E. lingulata* extracts; M: Methanolic extract, C: Chloroform extract, E: Ethyl acetate extract

in *G. edulis* C extract whereas E extract had negligible activity at 100 µg mL^{-1}. *G. edulis* M and *E. lingulata* M, C and E extracts had almost similar inhibitory activity of around 40%. Tukey's HSD post-hoc test showed that the differences between A549 GI and BCB group means; HCT15 GI and BCB group means and BCB and TPC group means were statistically significant ($p<0.05$).

Total reducing activity: The total antioxidant potential of marine algae was also determined by their reducing power and the results are shown in Fig. 5. The reducing power of the extracts showed concentration dependence with *G. edulis* C and E extracts having higher values of 38.16 and 37.54% at 200 µg mL^{-1}, respectively. The C and E extracts of *E. lingulata* also had similar activity (32.21 and 37.41%, respectively) whereas M extracts of both algae showed lower values: *G. edulis* M: 17.64%; *E. lingulata* M: 13.64%. Tukey's HSD post-hoc test showed that the differences between A549 GI and TRA group means were statistically significant ($p<0.05$).

Total phenolic content: As phenols from natural sources are considered to be good antioxidants, the antioxidant potential observed in the two marine algae in this study was postulated to be linked

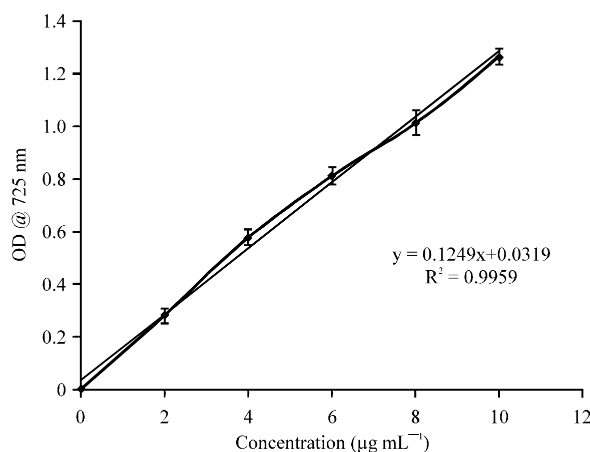

Fig. 6: Gallic acid standard curve for total phenolic content

Table 1: Total phenolic content

	Gallic acid equivalents (µg mL^{-1}) (GAE)		
Marine algae	M	C	E
Gracilaria edulis	0.142±0.025	0.155±0.017	0.216±0.032
Enteromorpha lingulata	0.262±0.031	0.332±0.085	0.540±0.069

M: Methanolic extract, C: Chloroform extract, E: Ethyl acetate extract, Quercetin was used as positive control with GAE 8.25±0.15 at 10 µg mL^{-1}. All values are represented as Mean±SEM (n = 3)

to the phenolic constituents. The phenolic content of the algal extracts was measured in terms of Gallic Acid Equivalents (GAE) from the regression equation $y = 0.1249x+0.0319$ of the gallic acid standard curve (Fig. 6). The E extract of *E. lingulata* had the highest phenolic content of 0.54 µg mL^{-1} GAE, whereas *G. edulis* M extract had the least phenolic content of 0.14 µg mL^{-1} GAE for 1 mg mL^{-1} of the extracts (Table 1). Tukey's HSD post-hoc test showed that the differences between TPC and BCB group means were statistically significant ($p<0.05$). Also, a high positive correlation (Pearson correlation coefficient) was observed between the means for GI values in HCT15 and TPC value, at $p<0.05$, suggesting a link between the TPC and GI observed in HCT15 cells for all these extracts (in particular, E of *E. lingulata*).

Growth inhibition assay: The antiproliferative activity of *G. edulis* and *E. lingulata* M, C and E extracts showed different trends in HCT 15 and A549 cell lines (Fig. 7). The *E. lingulata* E extract showed maximal inhibition in the HCT15 cell line of about 19.37% followed by C extract (15.66%); the M extract showed no growth inhibition. *G. edulis* (E, C and M) extracts showed low growth inhibition of about 7.12, 4.22 and 0.79%, respectively. Neither alga had any growth inhibitory effects on the A549 cell line (Fig. 7). Tukey's HSD post-hoc test showed that the differences between DPPH radical scavenging and A549 GI group means; A549 GI and TRA group means; A549 GI and BCB group means; A549 GI and HCT15 GI group means and HCT15 GI and BCB group means were statistically significant ($p<0.05$).

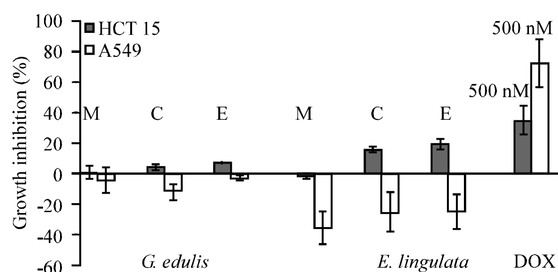

Fig. 7: Growth inhibition by *G. edulis* and *E. lingulata*-M, C, E extracts in HCT15 and A549 cell lines at 100 μg mL^{-1}

DISCUSSION

Reactive oxygen species (free radicals) are highly reactive chemical molecules that are formed as by-products of normal metabolism and whose levels increase dramatically due to various environmental stresses or damage. They finally cause great damage to the cells by interacting with vital cellular components like DNA or the cell membrane. Antioxidants play an important role in preventing cellular damage-a major feature of cancer, ageing and various other diseases-by neutralizing these free radicals (Chanda *et al*., 2011; Lompo *et al*., 2007). Several molecules and antioxidants from natural sources, such as mushrooms and higher plants have been reported and used for therapeutic benefit (Dimitrios, 2006; Samchai *et al*., 2009; Anokwuru *et al*., 2011).

Cancer is the most commonly occurring "disease" worldwide of which, lung and colon cancers are widely prevalent (Garcia *et al*., 2007). Most of the anticancer drugs in clinical use possess deleterious side effects. Hence, the discovery of novel anticancer drug leads with better toxicological and pharmacokinetic profiles is of great importance. Phytochemicals, such as polyphenols, are considered to be good chemo-preventive agents for colorectal cancer (Sale *et al*., 2005). Several anticancer drugs from plant sources, such as taxol, vinblastine, vincristine and etoposide phosphate, have been in use for the treatment of cancer (Da Rocha *et al*., 2001). Antioxidant and antiproliferative studies have been performed on various marine algae (seaweeds) all over the world (Vinayak *et al*., 2011; Zubia *et al*., 2009; Yuan and Walsh, 2006; Ismail and Hong, 2002). In India too, reports have demonstrated antioxidant activity of marine algae found along the Indian coastline but only few reports describe antiproliferative activity (Delma *et al*., 2008; Sachindra *et al*., 2010; Ganesan *et al*., 2008). Hence, the current study has been conducted to determine the antioxidant and antiproliferative activities of M, C and E extracts of two marine algae from the Chennai coast in Tamil Nadu, India.

Gracilaria edulis and *Enteromorpha lingulata* are edible marine algae. There are no reports, to date, on the *in vitro* antiproliferative activity of *G. edulis* although three fatal poisoning cases have been reported during 2002-2003 in Phillipines, due to ingestion of *G. edulis* and *Acanthophora spicifera* (Yotsu-Yamashita *et al*., 2004). Our TLC results suggest that the relatively polar compounds that have been extracted by all three solvents (methanol, chloroform, ethyl acetate) from both algae are likely to be strong contributors to the observed DPPH radical scavenging activity. While it is difficult to correlate the qualitative results of TLC with the quantitative ones of the DPPH radical-scavenging assay, *E. lingulata* extracts showed strong DPPH radical-scavenging activity in both TLC and the assay. M extract of *G. edulis* showed greater DPPH radical-scavenging activity at all concentrations in the assay in contrast to the

results of TLC analysis. In contrast, Devi *et al.* (2008) had reported a value of 25% for the DPPH radical-scavenging activity of the methanolic extract of *G. edulis* at 100 μg mL^{-1}. However, they used different concentrations of DPPH and a different incubation period in their assay. No reports were found on the antioxidant activity of *E. lingulata*.

In contrast to the higher activity seen for *E. lingulata* in other assays, the BCB assay showed that *G. edulis* C extract had the highest inhibitory activity of about 60% at 100 μg mL^{-1}, with the others being almost comparable to the positive control, BHA (100 μg mL^{-1}, 39%), except for E extract of *G. edulis* which showed slight pro-oxidant activity.

Total reducing activity of the E extract of *E. lingulata* was the highest of all, followed by C and E extracts of *G. edulis* and the C extract of *E. lingulata*, with the M extracts of both algae showing the least total reducing activity. Total phenolic content was higher for *E. lingulata* (E>C>M for both algae).

Reports suggest that the widely prevalent cancer of the colon can metastasize into other organs, such as the lungs and *vice versa* (Dishop and Kuruvilla, 2008). Hence, the antiproliferative effects of the algae were tested on HCT15 (colon carcinoma) and A549 (lung adenocarcinoma) cells. The results show no growth inhibitory effects of any of the extracts on A549 cells; in fact, the *E. lingulata* extracts (M>C>E) showed varying growth-promoting effects. However, the C and E extracts (E>C) of *E. lingulata* showed very modest growth inhibition (15.7 and 19.4%) in HCT15 cells. The M, C, E extracts of *G. edulis* and the M extract of *E. lingulata* showed very negligible or no inhibitory effects on the growth of HCT15 cells. A higher Pearson correlation coefficient (>0.8) between TPC and HCT15 GI means suggests a role for phenolic compounds in the antiproliferative activity of the E extract of *E. lingulata*.

CONCLUSION

In summary, the above results suggest that the compounds extracted by methanol, chloroform and ethyl acetate into M, C and E extracts of both algae show different levels of antioxidant activities. Regardless of the differences observed for the various assays, it appears that the relatively more polar components extracted from *Enteromorpha lingulata* by ethyl acetate and chloroform showed higher antioxidant (DPPH radical-scavenging, TRA) activities than the others. In contrast, the methanolic (M) extracts of both algae, especially of *G. edulis*, showed the lowest antioxidant activities (DPPH, TRA). The similar trend of total phenolic content (E, C>M and *E. lingulata*>*G. edulis*) warrants further investigation into the nature of the compounds in the E and C extracts of *E. lingulata* that have shown the observed antioxidant and antiproliferative activities (in HCT15 cells).

ACKNOWLEDGMENT

The authors would like to thank the Department of Science and Technology (DST), India, for financial support. We are also grateful to Dr. Baluswami, Madras Christian College, Chennai, for his valuable help in identifying the algae and to Ms. S. Sowmya, VIT University, Vellore, for her valuable inputs into the statistical analysis.

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