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RESEARCH ARTICLE

AN *IN VITRO* ANTIOXIDANT AND ANTIBACTERIAL POTENTIALS OF ETHYL ACETATE EXTRACT OF *ENTEROMORPHA INTESTINALIS* COLLECTED FROMCOASTAL REGION OF KOVALAM, NEAR CHENNAI, TAMILNADU

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ABSTRACT

Marine algae are well-known to contain a wide diversity of bioactive compounds, many of which have profitable applications in pharmaceutical, cosmetic, nutraceutical, food and agricultural industries.Accepted antioxidants, existing in several group of algae, which are important bioactive compounds that play a vital role counter to various diseases and including anti-ageing processes and protection of cells from oxidative damage. In this high opinion, reasonably little is known about the bioactivity of Indian algae that could be a potential natural source of such antioxidants. The ethyl acetate extract of *Enteromorpha intestinalis* was evaluated for total phenolic, flavonoid contents, antibacterial and antioxidant (ABTS assay, lipid peroxidation, superoxide radical scavenging, nitric oxide radical scavenging and reducing power) activities. The results indicated that ethyl acetate extract of *E. intestinalis* was effective in inhibiting the growth of Gram positive viz; *Staphylococcus aureus*, *Bacillus subtilis*, gram negative viz; *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* and compared to antibiotic streptomycin. The ethyl acetate extract was found to be rich in total phenolic content, and high antioxidant activity as compared to Ascorbic acid. The presence of functional groups of active compounds was confirmed by Gas chromatography mass spectroscopy (GSMS) analysis of ethyl acetate extract. It was concluded that all tested ethyl acetate extract of *E. intestinalis* had antibacterial and antioxidant activities. These properties might be due to the presence of high total phenolic content or flavonoids. Hence the ethyl acetate extract of thallus of *E. intestinalis* represent a potential source of antibacterial and antioxidant compounds that may be used in food or pharmaceutical products.

INTRODUCTION

Biological systems are commonly bare to unnecessary reactive oxygen species, causing a disruption in the cells natural antioxidant safeguard systems and resultant in injury to all biomolecules, as well as nucleic acids. Indeed, oxidative DNA damage is defined as the type of impairment most probable to occur in neuronal cells. Molecules comprising unpaired electrons are known as free radicals that cause tissue breakdown by means of DNA, protein, and lipid damage (Guha *et al*., 2009). Free radicals, such as superoxide anion, lipid peroxidation and nitric oxide are known as Reactive Oxygen Species (ROS) (Rajkumar *et al*., 2010). ROS, which are caused by usual physiological processes and numerous exogenous factors, cause oxidative stress, and in this manner initiate peroxidation of membrane lipids, causing injury to a wide range of other biological molecules over and done with a process that is assumed to be involved in the etiology of numerous diseases, together with coronary artery diseases, stroke, inflammatory, diabetes, and cancer (Lefer and Grander 2000). The human body has innate defense mechanisms to counter ROS in the form of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). Consumption of dietary plants as food containing phytochemicals with antioxidant properties, such as phenolics and ascorbic acid, can help strengthen the antioxidant balance of the body (Jimenez-Estrada *et al*., 2013). H_2O_2 is an oxidizing agent that can be converted to reactive hydroxyl radicals and has been associated with DNA damage, mutations and genetic instability, which can lead to cancer development (Imlay and Linn 1988). H_2O_2 can increase cancer cell proliferation and migration (Nelson*et al*., 2003) resulting in metastasis, which is the leading cause of cancer deaths and ineffectiveness of chemotherapeutic drugs. Plant bioactives may act as cancer chemopreventive agents in normal cells by keeping H_2O_2 levels within physiological levels, thus preventing DNA damage. Natural antioxidants from algae are known to play an important role against various diseases and

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aging processes. The detected antioxidant compounds in algae from these genera and others have potential anti-aging, dietary, anti-inflammatory, antibacterial, antifungal, cytotoxic, anti-malarial, anti-proliferative, and anticancer properties (Vijayavel and Martinez, 2010; Cornish and Garbary, 2010). However, recent concern has been paramount regarding the potential detrimental side effects of synthetic additives in humans (Rajkumar *et al*., 2010). The two most commonly used synthetic antioxidants, namely butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), have been restricted because they are toxic and because DNA damage (Ito *et al*., 1982 and Sasaki *et al*., 2002). Moreover, allopathic drugs frequently have negative side effects (Clarke *et al*., 2007). Whereas, herbal medicines are comparatively much safer in this respect. Therefore, the present study was showed to quantify the antioxidant and antibacterial activities of ethyl acetate extract from *Enteromorpha intestinalis.*

MATERIALS AND METHODS

Collection of Algae

Algal material *Enteromorpha intestinalis* were collected from the littoral zone of the Kovallum region between (0.2–2.5 m depths) along the East coast of Near Chennai, Tamil Nadu. The collected algal specimen were stored in plastic bags and transported to the laboratory under iced conditions. The samples were primarily washed thoroughly with sea water to remove sand and any adhering substance and then washed thoroughly with fresh water to remove salts, and stored at 20 °C until compound extraction. The algal species were identified based on the schemes reported in the literature (Smith, 1944; Bold, 1978; Aleem, 1993).

Extraction of selected algal species

The collected fresh algae subsequently washing with distilled water for several times, the algal specimen were again washed with 5% ethanol to remove any epiphytes or salts. The samples were subjected to air drying under the shade. After drying algal specimens were ground by an electrical mixer until they became a powder. Then the powdered algal specimen were stored in a dark place, and subjected to extraction methods. Extraction of powdered algal specimens was done using ethyl acetate. Aliquots of 25 g of the powdered algal samples were soaked in 250 ml of the solvent ethyl acetate for 24 h. Later the soaked samples were homogenized in an electric blender along with the solvents at room temperature, filtered, and concentrated under reduced pressure using a rotary evaporator.

Reducing power determination

Fe (III) reduction is often used as an indicator of electron-donating activity, an important mechanism for antioxidant action (Nabavi *et al.,* 2008). The reducing power of the ethyl acetate extract from *E. intestinalis* was determined by spectrophotometric method of Yen and Chen (1995). The acetate extract (5-20µl) was mixed with 2.5 ml of 0.2 M Potassium phosphate buffer (pH-6.6) and 2.5 ml of 1% Potassium ferricyanide [$K_3Fe(CN)_6$]. The mixture was incubated at 50°C for 20 minutes, then rapidly cooled, mixed with 2.5 ml of trichloroacetic acid and centrifuged at 5000 rpm for 3 minutes. An aliquot (2.5ml) of supernatant was diluted with distilled water (2.5ml) and 0.5 ml of 0.1% Ferric chloride was added and allowed to stand for 10 minutes. The absorbance was read spectrophotometrically at 700 nm. Increased absorbance indicates increased reducing power. Vitamin C was used as positive control.

ABTS (2, 2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid) radical scavenging assay

ABTS radical scavenging activity of ethyl acetate extract from *E. intestinalis*was followed by Re *et al.* (1999). ABTS radical was newly prepared by addition 5 ml of 4.9 mM potassium persulfate solution to 5 ml of 14 mM ABTS solution and kept for 16 h in dark. This solution was diluted with distilled water to produce an absorbance of 0.70 at 734 nm and the same was used for the antioxidant activity. The final solution of standard group was made up to 1 ml with 950 µl of ABTS solution and 50 µl of Ascorbic acid. Correspondingly, in the experiment group, 1 ml reaction mixture encompassed 950 µl of ABTS solution and 50 µl of different concentration of flavonoid rich fraction. The reaction mixture was vortexed for 10 s and after 6 min, absorbance was recorded at 734 nm against distilled water by using a Deep Vision (1371) UV–Vis Spectrophotometer and compared with the control ABTS solution. Ascorbic acid was used as reference antioxidant compound.

ABTS Scavenging Effect (%) = $[(A_0 - A_1/A_0) \times 100]$

Where $A0$ is the absorbance of the control reaction and $A1$ is the absorbance of flavonoid rich fraction.

Inhibition of lipid peroxidation activity

Lipid peroxidation induced by Fe^{2+}ascarbate system in egg yolk was assessed as thiobarbituric acid reacting substances (TBARS) by the method of Ohkawa *et al.*1979. The experimental mixture contained 0.1 ml of egg yolk (25% w/v) in Tris-HCl buffer (20 mM, pH 7.0); KCl (30 mM); $FeSO_4$ $(NH4)_2SO_4.7H_2O$ (0.06 mM); and different concentrations of ethyl acetate extract from *E. intestinalis*in a final volume of 0.5 ml. The experimental mixture was incubated at 37^{o}Cfor 1 hour. After the incubation period, 0.4 ml was collected and treated with 0.2 ml sodium dodecyl sulphate (SDS) (1.1%); 1.5 ml thiobarbituric acid (TBA) (0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The final volume was made up to 4.0 ml with distilled water and then kept in a water bath at 95 to 100 °C for 1 hour. After cooling, 1.0 ml of distilled water and 5.0 ml of n-butanol and pyridine mixture (15:1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The absorbance of butanol-pyridine layer was recorded at 532 nm in Deep Vision (1371) UV–Vis Spectrophotometer) to quantify TBARS. Inhibition of lipid peroxidation was determined by comparing the optical density (OD) of test sample with control. Ascorbic acid was used as standard. Inhibition of lipid peroxidation (%) by the flavonoid rich fraction was calculated according to 1-(E/C) X 100, where C is the absorbance value of the fully oxidized control and E is absorbance of the test sample (Abs_{532}+TBA–Abs_{532}+TBA).

Superoxide radical scavenging assay

This assay was based on the capacity of the flavonoid rich fraction to inhibit the photochemical reduction of Nitroblue tetrazolium (NBT) (Beauchamp and Fridovich, 1971) in the

presence of the riboflavin-light-NBT system, as described earlier (Tripathi and Pandey, 1999; Tripathi and Sharma, 1999). Each 3 ml reaction solution contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 µM riboflavin, 100 µM Ethylene diamine tetra acetic acid (EDTA), NBT (75 µM) and different concentration of ethyl acetate extract from *E. intestinalis*. It was kept visible in fluorescent light and absorbance was taken after 6 min at 560 nm by using a Deep Vision (1371) UV–Vis Spectrophotometer. Identical tubes with reaction mixture were kept in the dark served as blanks. The percentage inhibition of superoxide radical activity was measured by comparing the absorbance of the control with test sample solution.

% Super oxide radical scavenging capacity= $[(A_0-A_1)/A_0] \times 100$

Where A_0 was the absorbance of control and A_1 was the absorbance of flavonoid rich fraction.

Nitric oxide radical scavenging activity

Nitric oxide scavenging ability of acetate extract were measured according to the method described by Olabinri *et al* 2010. 0.1 ml of sodium nitroprusside (10 mM) in phosphate buffer (0.2 M, pH 7.8) was mixed with different concentration of ethyl acetate extract from *E. intestinalis*and incubated at room temperature for 150 min. After treated period, 0.2 ml of Griess reagent (1% Sulfanilamide, 2% Phosphoric acid and 0.1% N-(1-Naphthyl) ethylene diamine dihydrochloride) was added. The absorbance of the experimental sample was read at 546 nm against blank. All readings were taken in triplicate and ascorbic acid was used as standard. The percentage of inhibition was calculated by following equation.

% Nitric oxide radical scavenging capacity= $[(A_0-A_1)/A_0] \times 100$

Where A_0 was the absorbance of control and A_1 was the absorbance of flavonoid rich fraction.

Antibacterial Properties

Bacterial strains

The bacteria used for the assessing antibacterial activities were Gram positive viz; *Staphylococcus aureus* MTCC 29213, *Bacillus subtilis* MTCC 1771, gram negative viz; *Pseudomonas aeruginosa* MTCC 2488, and *Klebsiella pneumoniae* MTCC 25922. The bacterial strains were obtained from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology Sector 39-A, Chandigarh-160036, India. All bacterial strains were sub cultured on nutrient agar medium, incubated at 37 °C for 24 h and stored at 4 °C in refrigerator to maintain stock culture.

Antibacterial assay

Antibacterial activity was assessed using disc diffusion method followed by Sathyabama *et al*., 2011. Petriplates were prepared with 20 ml of sterile nutrient agar (HIMEDIA). The tested cultures were swabbed on top of the solidified media and allowed to dry for 10 min. The test was conducted in four different concentrations of the ethyl acetate extract from *E. intestinalis*(5, 10, 15 & 20 µl/ml) and treated discs (Whatman No.1 filter paper was used to prepare discs) were air dried. The treated discs were placed on the surface of the medium and incubated at room temperature for 24 h. The relative inhibition of organisms to the flavonoid rich fraction was indicated by a clear zone of inhibition around the discs. It was then detected, measured and documented in millimeters with three replicates.

GC–MS analysis

The flavonoid fraction was analyzed by GC–MS (QP-2010, Shimadzu Co., Kyoto, Japan) equipped with 30- mX 0.25 mm DB-5MS column (Agilent Technologies, J& W Scientific Products, Folsom, CA). The carrier gas was helium. The temperature program was set as follows: 100 °C hold for 5 min, raised at 4 °C/min to 280 °C, and hold for 5 min. The injector and detector temperatures were set at 250 and 280 °C, respectively. The ion source and interface temperatures were set at 200 and 250 °C, correspondingly. The mass range was scanned from 50 to 900 amu. The control of the GC–MS system and the data peak processing were controlled by Shimadzu's GC–MS solution software, version 2.4. Compound identification was confirmed based on the relative retention time and mass fragmentation pattern spectra with those of standards and the NIST147. LIB. Database of the GC–MS system (Shimadzu).

Statistical Analysis

The impact of the ethyl acetate extract of *E. intestinalis*on its antioxidant activity was measured by the ABTS assay, lipid peroxidation, superoxide scavenging, metal chelating and nitric oxide radical were determined using one-way analysis of variance (ANOVA). Likewise, Duncan's post hoc test was applied, so as to determine the statistically significant different values. All statistical handling was performed using SPSS software, v. 14.0 (SPSS, Chicago, Ill., U. S. A.).

RESULT AND DISCUSSION

Phytochemical screening of ethyl acetate extract of *E. intestinalis*

The phytochemical screening of the*E. intestinalis* studied presently showed the presence of alkaloids, flavonoids, phenol, Terpenoids, and absence of saponin and glycosides (Table -1).In present study agreement with that reported by Premalatha *et al*., (2011) phytochemical screening of *Chaetomorpha antennina* revealed the presence of antioxidant compounds such as phenolics, tannins, and glycosides.

Table 1. Phytochemical screening of ethyl acetate extract of *E. intestinalis*

S/No.	Constituents	Ethyl acetate extract of *E. intestinalis*
1.	Alkaloids-Dragendroffs reagent	+
2.	Flavonoids- Ammonia test	+
3.	Tannin- $Fecl_3$ test	+
4.	Saponins- Frothing test	-
5.	Terpenoids - Nollers test	+
6.	Glycosides- Keller-Killiani Test	-
7.	Polyphenols	+

The Partial Characterization of ethyl acetate extract of *E. intestinalis*

TLC profile of *E. intestinalis*were adsorbed on precoated siliga gel TLC plates (60 F254, Merk, USA). Efficient solvent system used Toluene: dioxan: acetic acid in the ratio 95:25:4 were used for the mobile phase of flavonoid and followed by colour development of the separated compounds of ethyl acetate extract was viewed under UV 240 nm and 360 nm (Table-2 and Fig-1).TLC chromatographic fingerprints could be useful for the quality assessment and also act as a biochemical marker for these medicinally important seaweeds in the pharmaceutical industry (Babu *et al*., 2015).

Table 2. Partial characterization of ethyl acetate extract of *E. intestinalis* by TLC

Component No.	UV Light 360nmRf value	UV light 240 nmRf value	Normal Light Rf value
1.	0.23	0.23	-
2.	0.36	0.36	-
3.	0.56	-	-
4.	0.76	-	-
5.	0.85	-	-

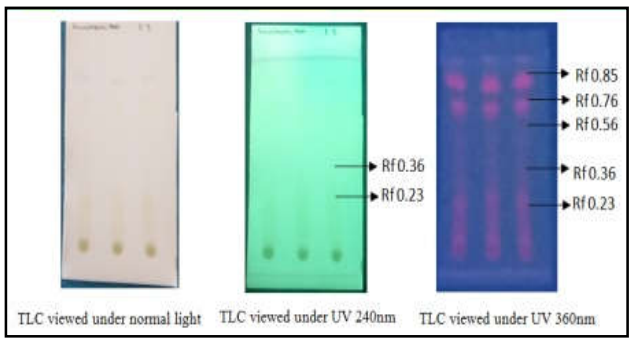

Fig. 1. Phyco-compounds profile of *Enteromorpha intestinalis* ethyl acetate extract by TLC

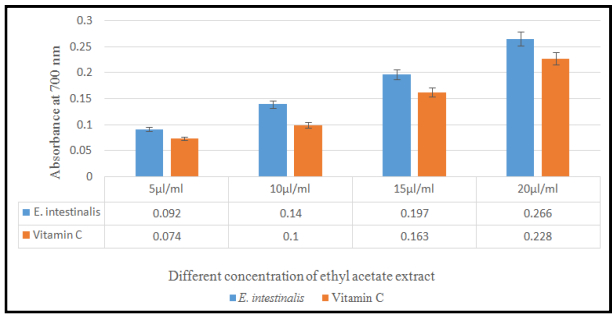

Fig. 2. Reducing power determination of ethyl acetate extract of *E. intestinalis*

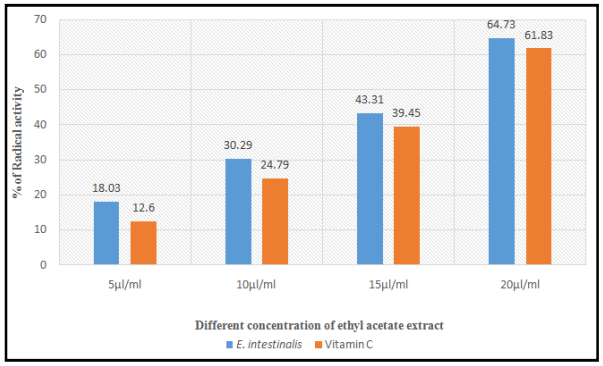

Fig. 3. ABTS radical activity of ethyl acetate extract E. intestinalis

Reducing power determination of ethyl acetate extract of E. intestinalis

The reducing power assay, the presence of reductants as antioxidants in tested samples would result in reducing Fe^{3+} ferricyanide complex to the ferrous form. The Fe^{2+} can therefore be examined by measuring the formation of Perl's prussian blue at 700 nm. Fig-2 shows the reducing powers of *E. intestinalis*ethyl acetate extract, and standard as Vitamin-C. The reducing power increased with the concentration of the *E. intestinalis*extract to a certain extent, then leveled off with further increase in the concentration than Vitamin-C.Pandima Devi*et al*., (2008) have been reported that algal compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants.

ABTS radical activity of ethyl acetate extract from E. intestinalis

Ethyl acetate extract of *E. intestinalis*exhibited a powerful scavenging activity for ABTS radical cations in a concentration dependent manner. Fig-3 showing a direct role in catching free radicals. Maximum inhibition was observed with the*E. intestinalis*extract ranges from 18.03 to 88.93% at 5-20 µl/ml than positive control vitamin-C. In the presence of antioxidant reductant, the coloured radical is converted back to colourless ABTS. In the present study compared with previously reported that Indu and Seenivasan (2013) the brown seaweed *S.wightii* showed the highest activity in ABTS radical scavenging assay compared to green and red seaweed. ABTS test, such as the ability of a test sample to respond with ABTS radical somewhat than to inhibit the oxidative progression and slow reaction of many phenolics require well-matched evaluation of antioxidant activity (Roginsky and Lissi, 2005).

Nitric oxide scavenging assay of ethyl acetate extract of E. intestinalis

Nitric oxide is a potent pleiotropic inhibitor of physiological process such as smooth muscle relaxation, neural signalling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical that plays many roles as an effectors molecule in diverse biological systems including neuronal messenger, vasodilation and antimicrobial and antitumor activities (Hagerman *et al*., 1998).

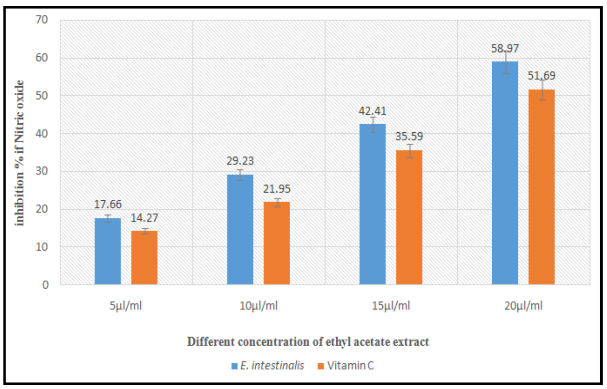

Fig. 4. Nitric oxide scavenging assay of ethyl acetate extract of *E. intestinalis*

In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (Nabavi *et al*., 2008). In present study ethyl acetate

extractof *E. intestinalis*showed Nitric oxide scavenging activity. Virtuous result was observed at *E. intestinalis*extract with scavenging ranges 58.97±0.91%at 25µg/ml than vitamin-C ranges 51.69% which served as positive control (Fig. 4). The present study demonstrated ethyl acetate extract acts as Nitric oxide scavenging due to extracts contain poly phenol compounds; free radicals are scavenged and therefore can no longer react with nitric oxide, resulting in less damage.

Superoxide anion scavenging activity of ethyl acetate extract of Enteromorpha intestinalis

Superoxide radicals by photochemical decrease of nitro blue tetrazolium (NBT) in the occurrence of a riboflavin-light-NBT system, which is one of the standard methods. The total fractions ethyl acetate extractof *Enteromorpha intestinalis*exhibited potent scavenging activity for superoxide radicals in a concentration dependent manner (Fig. 5). The *Enteromorpha intestinalis*extract had highest Superoxide radicals scavenging percentage 61.38 at 20µg/ml and Vitamin-C was least potent with 57.97 value at 20µg/ml. Removal of superoxide in a concentration dependent manner by any solvent fractions may be attributed to the direct reaction of its phytomolecules with inhibition of the enzymes. The present study compared with previous report Shanab *et al*. (2011), that the acetone extracts of *U. lactuca* and *E. intestinalis*confirmed a comparatively powerful antioxidant activity, possibly due to the higher content of antioxidant active compounds in extracts. These results may direct the potential contribution of bioactive metabolites which reveal antioxidant activity such as pigments chlorophyll, carotenoids, essential oils, and low molecular weight polysaccharides (Murthy *et al*., 2005).

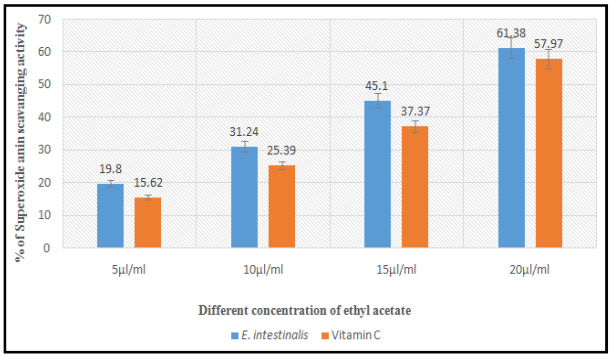

Fig. 5. Superoxide anion scavenging activity of ethyl acetate extract of *Enteromorpha intestinalis*

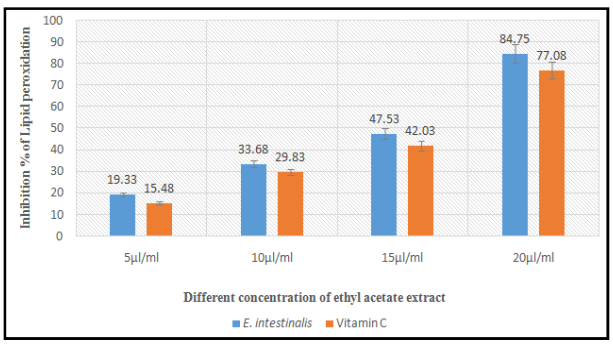

Fig. 6. Inhibition of lipid peroxidation by ethyl acetate extract of *E. intestinalis*

Inhibition of lipid peroxidation by ethyl acetate extract of E. intestinalis

Ethyl acetate extract of *E. intestinalis*also inhibited the lipid peroxidation induced by ferrous sulfate in egg yolk homogenates. Maximum inhibition was observed with total *E. intestinalis*extract with inhibition percentage 19.33 to 84.75 at 20µg/ml than Vitamin-C (Fig. 6). This inhibition of lipid peroxidation possibly either due to chelation of Fe or by corner of the free radicals. Iron also is playing a major role for the formation of lipid peroxidation in the body. In this experiment inhibited the lipid peroxidation related to their abilities of phenolic and flavonoid compounds presence in ethyl acetate extract of *E. intestinalis*. Droge (2002) stated that certain flavonoids and phenols have protective effect on radical species due to its antioxidant properties. The attained results of the present study was in agreement with those of Sanaa and Shana (2007), dichloromethane extracts of *Sargassum dentifolium* had the greatest free radical scavenging activity (86%) and that of *Laurencia papillosa* showed the greatest anti-lipid peroxidation efficiency (87%) comparing with those of silymarin (92 & 96%, respectively), which was used as reference control.

Antibacterial activity of ethyl acetate extract of *E. intestinalis* against pathogenic bacteria

The antibacterial activity of the investigated ethyl acetate extract of *E. intestinalis* against Gram positive and Gram negative bacteria used by agar disk diffusion and micro-dilution methods were shown in Table-3.

Table 3. Antibacterial activity of the *Enteromorpha intestinalis*ethyl acetate extract by disc diffusion method

Pathogenic organism	*Enteromorpha intestinalis* ethyl acetate extract concentration			
	5µl/ml	10µl/ml	15µl/ml	20µl/ml
Staphylococcus aureus	8.3±0.45	12.3±0.49	15.7±0.35	19.3±0.50
Bacillus subtilis	9.3±0.41	13.4±0.36	16.6±0.30	19.6±0.66
Klebsiella pneumoniae	9.2±0.20	12.7±0.70	14.7±0.40	17.3±0.20
Pseudomonas aeruginosa	10.5±0.45	13.73±0.30	16.4±0.36	20.6±0.51

All the values are mean ± SD; SD: standard deviation.

The algae ethyl acetate extract differ in their activities against the bacteria tested. Results achieved in the present study relieved that Ethyl acetate extract of *Enteromorpha intestinalis*was found to be active maximum against *P. aeruginosa, B.subtilis, S. aureus* than *K. pneumoniae.* Highest antibacterial activity was observed with ethyl acetate extract of *Enteromorpha intestinalis*against *P. aeruginosa,* (20 mm) respectively while lowest activity was observed against *K. pneumoniae*with the inhibition zone of 17 mm at the concentration of extracts of 20 µg/mL. The antibacterial results of current study are in accordance with literature, where acetone extracts of the tested *U. lactuca* and *E. intestinalis*, which suggests that these species contain components toxic to microorganisms and, therefore, in control for their antimicrobial activity (Marijana *et al*., 2015).

Conclusion

Green algae are rich and easily renewable source of biologically active metabolites, including prototannin, polyphenol, minimized protein and carotenoids. These unique metabolites have been interestingcare as pharmaceuticals, food and cosmetic ingredients. Based on the results, tested macroalgae *E. intestinalis* appear to be good natural antioxidant and antimicrobial agents. Identification of the

active compounds of *E. intestinalis* algal will lead to their evaluation of considerable commercial potential in medicine, food production and cosmetic industry.

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