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Free radical scavenging effects of the seaweeds *Gracilaria salicornia* C. Ag. and *Gracilaria edulis* (Gmelin) Silva under *in vitro* conditions

Radha Palaniswamy*, Leninraja Dharmaraj◆, Ravi Ramasamy***, Hemalatha Palanivel***, Tilak Meenakshisundaram**** and Hemaprabha Kandasamy***

** CPMB and B, TNAU, Coimbatore-641 003, Tamil Nadu, India*
*** Horticultural College and Research Institute, Periyakulam, Theni-625 604, Tamil Nadu, India*
**** Forest College and Research Institute, Mettupalayam-641 301, Tamil Nadu, India*
***** TNAU, Coimbatore-641 003, Tamil Nadu, India*

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Abstract

Marine macroalgae are known for their high antioxidant status with probable applications in nutrition and pharmacological industries. Research was commenced to assess the ability of the red seaweeds, *Gracilaria salicornia* C. Ag., and *Gracilaria edulis* (Gmelin) Silva to scavenge free radicals. Their scavenging ability was tested by preparing extracts using solvents of varying polarity. The extracts prepared were calculated for their ability to scavenge free radicals. Antioxidant activity at different levels was observed in all the extracts, indicating that the free radical scavenging ability was due to the existence of compounds that are biologically active. Amongst all, *G. edulis* (ethanolic extract) exhibited the highest scavenging ability in all assays (DPPH: 34%; superoxide: 17.3%, nitric oxide: 86.16%; hydroxyl radical: 80%). Apparently, the scavenging abilities were similar to or greater than those of the standard antioxidant butylated hydroxytoluene (BHT). From the study's outcomes, it is apparent that *G. edulis* (ethanolic extract), is a natural source of antioxidant compounds with potential applications in pharmaceutical, nutraceutical, and functional food formulations.

1. Introduction

All living organisms require oxygen, as it is involved in oxidation processes that are fundamental to aerobic life and essential for metabolism (Aiyegoro and Okoh, 2010). A portion of the oxygen absorbed by cells is converted into destructive reactive oxygen species (ROS) and free radicals. Once generated, these free radicals can initiate a sequence of reactions, resulting in additional free radicals production (Al-Mamun *et al*., 2007). These reactive species are produced during normal metabolic processes or generated due to external features such as ultraviolet light, ionizing radiation, and chemical reactions. They have the ability to damage a varied array of vital biomolecules (Chiumiento and Bruschi, 2009). When more of reactive oxygen species is produced, it results in oxidation related stress, which leads to peroxidation of lipids, oxidation of protein and damage to DNA, and results in various diseases associated with oxidative stress (Sies *et al*., 2022).

Antioxidants have a significant role in defending biological systems from oxidative damage by scavenging free radicals, donating electrons or hydrogen atoms, and chelating pro-oxidant metal ions (Chakraborty *et al*., 2009). Antioxidants help reduce and avoid oxidative damage produced by free-radical reactions by donating electrons that neutralize free radicals (Khlebnikov *et al*., 2007; Halliwell, 2024). Although, artificial antioxidants like butylated hydroxytoluene (BHT) are widely used in nutrient and pharmacological products, concerns regarding possible toxic and carcinogenic effects have directed to the use of natural antioxidants from plant and marine sources (Blois, 1958).

Marine macroalgae are progressively known as valued sources of natural antioxidants as they get exposed to various ecological circumstances like high salinity, penetrating light and oxygen-rich surroundings. To overcome all these stress conditions, seaweeds produce a varied range of bioactive composites like phenolics, flavonoids, carotenoids and sulfated polysaccharides that possess strong antioxidant properties (Lim *et al*., 2002). Numerous studies have confirmed that red seaweed extracts prepared using solvents of differing polarity are rich in phenolic compounds and exhibit significant antioxidant activity. Red sea weeds *Gracilaria*, *Gelidium*, *Porphyra*, and *Kappaphycus* are species that are commercially important and cultivated for hydrocolloids and nutraceutical compounds extraction. In India, the red seaweeds occurring along the coastal regions of Tamil Nadu, Gujarat, and the Gulf of Mannar are significant in terms of economy and medicinal value (Kavya *et al*., 2024).

Recent advances in marine biotechnology highlighted the status of red seaweeds as sources of bioactive compounds that find applications in prevention of diseases, drug discovery, and health-promoting functional foods. Their properties such as their rapid growth, cultivation in an eco-friendly way and renewable nature have made red seaweeds as promising in nutraceutical and pharmaceutical applications (Eladl *et al*., 2024). Genus *Gracilaria* are extensively spread along tropical and subtropical coastlines and serve as an imperative source of agar and efficient food constituents.

Corresponding author: Dr. D. Leninraja
Assistant Professor (SS & AC), HC&RI, Periyakulam, Theni-625 604, Tamil Nadu, India
E-mail: leninraja@tnau.ac.in
Tel.: +91-9486623111

Previous studies have shown that the seaweeds *G. salicornia* and *G. edulis* are rich in biologically active secondary metabolites. Phytochemical analyses of different solvent extracts have described the occurrence of phenolics, flavonoids, tannins, alkaloids, saponins, steroids, terpenoids, glycosides and sulfated polysaccharides in these seaweeds. The antioxidant capacity of the seaweeds are mostly due to the existence of phenolics and flavonoids as they strongly scavenge and reduce free radicals. Of the many extracts, the extracts of *Gracilaria* species prepared using ethanol and methanol possess higher content of polyphenols and is responsible for the antioxidant, antimicrobial, anti-inflammatory and cytotoxic potential. The existence of these phytoconstituents stresses the pharmacological implication of *G. salicornia* and *G. edulis* as a good source for application in therapeutics and functional food (Ghannadi *et al*., 2016).

Neutralizing free radical action of the red seaweeds is to scavenge reactive oxygen species and thereby prevent cellular injury triggered due to oxidative stress. Sulfated polysaccharides derived from *Gracilaria* species have additionally been shown to possess antiviral, immunomodulatory and anticoagulant effects. Furthermore, ethanolic and methanolic extracts of *G. edulis* and *G. salicornia* have demonstrated notable inhibitory activity against pathogenic microorganisms and free radical-mediated oxidative damage. These observations emphasize the pharmacological importance of these marine macroalgae as promising natural sources of therapeutic agents for pharmaceutical, nutraceutical, and functional food applications (Holdt and Kraan, 2011). Although, antioxidant properties of certain *Gracilaria* species have been reported, comparative evaluation of different solvent extracts of *G. salicornia* and *G. edulis* against multiple free radical systems is limited. Hence, an effort was made to value and compare the free radical scavenging effects of these two seaweeds using antioxidant potential screening assays involving DPPH, nitric oxide, superoxide, and hydroxyl radicals.

2. Materials and Methods

2.1 Collection of marine algae

Fresh seaweeds, *G. salicornia* and *G. edulis*, were acquired from the Manamelkudi coastal region of Pudukkottai District, Tamil Nadu. Collected seaweeds were identified and authenticated by the Botanical Survey of India located in Coimbatore, India and the corresponding voucher specimens are BSI/SRC/5/23/2016/Tech/1137 and BSI/SRC/5/23/2017/Tech/3552.

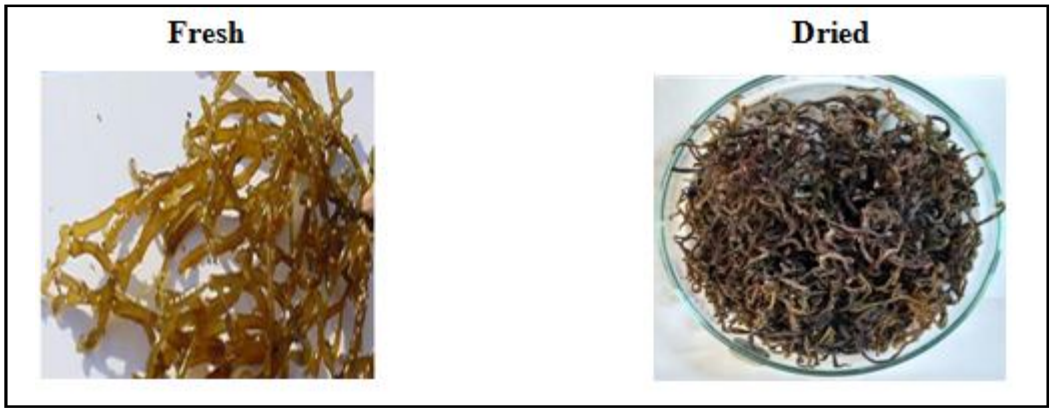

Plate 1: ***Gracilaria Salicornia.***

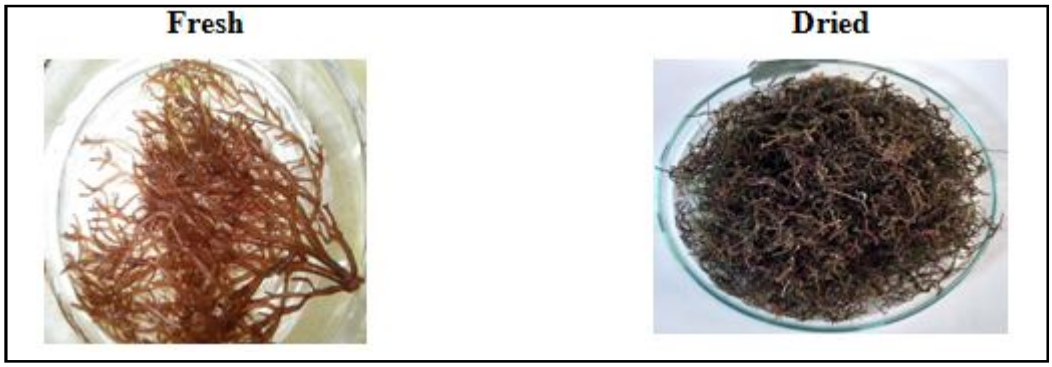

Plate 2: ***Gracilaria edulis.***

2.2 Preparation of seaweed extracts

The seaweeds collected were carefully cleaned to get rid of adhering sand and epiphytes, shade-dried and powdered. About five grams of the plant material were extracted separately using polar and non-polar solvents (chloroform, ethyl acetate, methanol, ethanol and distilled water). The filtered extracts were concentrated and the solvent was completely evaporated. Then dissolved 20 mg of the concentrated extract in 5 µl of dimethyl sulphoxide for antioxidant efficacy. The phytochemical composition or secondary metabolite profile differs among species. Preliminary phytochemical screening of secondary metabolites of various solvent extracts was performed using standard qualitative methods. Findings demonstrated the occurrence of saponins, flavonoids, alkaloids, tannins, and phenolics. These phytoconstituents are widely recognized for their medicinal significance and diverse physiological activities.

2.3 Antioxidant efficacy

2.3.1 Diphenyl picryl hydrazyl (DPPH) radical scavenging assay

The ability of the seaweed extracts to scavenge DPPH (stable free radical) was assessed following Mensor *et al*. (2001), due to its reduction to diphenyl picryl hydrazine. Leaf extract (20 µl) or BHT was added to DPPH (0.5 ml) in methanol and methanol (0.48 ml). Incubated for 30 min at room temperature. With methanol as the blank, DPPH dissolved in methanol (excluding the sample) served as the control. The drop in intensity of purple colour was quantified at 517 nm after incubation. The standard antioxidant used was BHT.

2.3.2 Superoxide quenching activity

Superoxide anion generation was calculated by the technique developed by Winterbourn *et al*. (1975). The efficacy of the seaweed extracts to suppress NBT reduction to formazan induced by superoxide ions was measured at 560 nm. A total reaction volume of 3.0 ml, containing seaweed extract (0.02 ml), EDTA (0.2 ml), NBT (0.1 ml), riboflavin (0.05 ml), and phosphate buffer (2.63 ml) was added. All the reagents in the mixture, substituting the extract with DMSO served as the control. The tubes were vortexed, and the absorbance was recorded at 560 nm. Again, the reaction tubes were measured at the same absorbance after exposure to fluorescent light for 30 min. The superoxide anion scavenging activity was exhibited by the variation in absorbance following illumination and calculated on comparison with the optical density of the control. BHT served as the standard antioxidant compound.

2.3.3 Nitric oxide neutralizing activity

The process involved studying the *in vitro* inhibition of nitric oxide radical generation (Green *et al*., 1982). The reaction mixture consisted of sodium nitroprusside (2.0 ml) along with phosphate-buffered saline (0.5 ml), and seaweed extract (0.5 ml). Incubated the mixture for 30 minutes at 25°C. Next, added 0.5 ml of Griess reagent, and further maintained under incubation for 30 minutes. The mixture in the absence of the extract was considered the control. Measured the optical absorbance at 546 nm. The standard antioxidant compound used was BHT.

2.3.4 Hydroxyl radical quenching assay

The capacity of seaweed extracts to scavenge hydroxyl species was assessed as per the procedure of Elizabeth and Rao (1990). A total reaction volume of 1.0 ml was prepared with 0.1 ml of deoxyribose, $FeCl_3$ (0.1 ml), EDTA (0.1 ml), hydrogen peroxide (0.1 ml), ascorbic acid (0.1 ml), and KH_2PO_4-KOH buffer (0.48 ml), with seaweed extract (20 µl) and incubated (37°C) for 1 h. Added thiobarbituric acid (1.0 ml) after incubation, and heated for 20 min at 95°C for color development. Upon cooling, the thiobarbituric acid reactive substances (TBARS) produced was quantified at 532 nm. The TBARS production (%) of the extract-treated groups was calculated by taking the positive control (H_2O_2) as 100%. The standard antioxidant compound used was BHT.

2.4 Statistical interpretation

All analyses were performed in three independent replicates, presented as Mean ± SD, and subjected to ANOVA followed by means of Duncan's Multiple Range Test (DMRT).

3. Results

3.1 DPPH radical scavenging activity

G. salicornia and *G. edulis* extracts revealed significant DPPH scavenging potential, which is an indication of the presence of antioxidant compounds that contribute hydrogen atoms or electrons and neutralize free radicals. Of all the solvents analysed, the highest percentage inhibition ($p<0.05$) was in the ethanolic extract, followed by methanolic extract. When compared to the methanolic extract, the ethanolic extract of *G. edulis* revealed significantly greater scavenging activity than *G. salicornia* ($p<0.05$). Phenolic and flavonoid compounds are known to be extracted competently by ethanol, and they are very much responsible for the antioxidant activity in seaweeds.

Table 1: DPPH free radical quenching activity of *G. salicornia* and *G. edulis* extracts

Extract	*G. salicornia* (% inhibition)	*G. edulis* (% inhibition)
Chloroform	5.34 ± 0.04^a	8.20 ± 0.07^b
Ethyl acetate	4.98 ± 0.03^a	7.78 ± 0.02^b
Methanol	6.00 ± 0.10^b	18.00 ± 0.20^c
Ethanol	8.90 ± 0.10^c	34.00 ± 0.10^d
Aqueous	7.27 ± 0.06^b	11.70 ± 0.02^b
BHT	61.00 ± 1.00	–

Values represent mean ± SD of triplicate observations (n = 3). Within each column, values with different superscript letters are statistically significant at $p<0.05$.

3.2 Superoxide and nitric oxide scavenging activity

The extracts (ethanol and methanol) of both seaweeds showed significantly higher ability to scavenge superoxide radicals ($p<0.05$) when compared to non-polar solvent extracts. *G. edulis* steadily showed greater inhibition than *G. salicornia*.

3.3 Hydroxyl radical quenching activity

The capacity of the ethanolic extracts of both seaweeds to deoxyri-bose degradation exposed their significance more effectively ($p<0.05$) in defending against hydrogen peroxide-induced oxidative damage. The antioxidant activity of the ethanolic extract of *G. edulis* against hydroxyl radical scavenging activity was comparable to that of the standard BHT.

Table 2: Antioxidant effects of *G. salicornia* and *G. edulis* extracts against superoxide and nitric oxide radicals

Extract	Superoxide (% inhibition)		Nitric oxide (% inhibition)	
	G. salicornia	*G. edulis*	*G. salicornia*	*G. edulis*
Chloroform	5.15 ± 0.15^{a}	6.90 ± 0.02^{b}	25.10 ± 0.04^{a}	29.00 ± 0.16^{b}
Ethyl acetate	4.80 ± 0.01^{a}	5.62 ± 0.05^{a}	25.60 ± 0.60^{a}	27.10 ± 0.02^{a}
Methanol	10.40 ± 0.30^{b}	13.30 ± 0.06^{c}	36.17 ± 0.15^{b}	68.00 ± 0.20^{c}
Ethanol	14.70 ± 0.66^{c}	17.30 ± 0.56^{d}	55.70 ± 0.50^{c}	86.16 ± 0.21^{d}
Aqueous	5.73 ± 0.25^{a}	7.30 ± 0.36^{b}	25.60 ± 0.60^{a}	33.50 ± 0.56^{b}
BHT	29.00 ± 0.07	–	39.00 ± 1.05	–

Values represent mean ± SD of triplicate observations (n = 3). Within each column, values with different superscript letters are statistically significant at $p<0.05$.

Table 3: Influence of *G. salicornia* and *G. edulis* extracts against oxidative damage to deoxyribose

Extract	*G. salicornia* (% scavenging)	*G. edulis* (% scavenging)
Chloroform	41^{a}	52^{b}
Ethyl acetate	40^{a}	48^{b}
Methanol	54^{b}	75^{c}
Ethanol	60^{c}	80^{d}
Aqueous	44^{a}	56^{b}
BHT	81	–

Values with different superscript letters differ significantly at $p<0.05$.

4. Discussion

Oxidative damage caused due to free radicals are minimized by the antioxidants that act as reducing agents by means of electron donation, thereby neutralizing them (Herrling *et al*., 2008). The antioxidants react with free radicals and neutralize them before they cause any cellular damage (Halliwell, 2012). The secondary metabolites, which are produced by the plants, have antioxidant properties. Hence, an attempt was made to assess the ability of *G. salicornia* and *G. edulis* to scavenge free radicals under laboratory conditions. DPPH, a coloured crystalline compound with steady free radicals, is widely used as a standard radical in antioxidant assays. The DPPH radical, when in solution, shows a dark violet colour, when neutralized loses its violet colour and forms DPPH-H (Goldschmidt and Renn, 1922).

G. salicornia and *G. edulis* extracts revealed variable DPPH radical scavenging activity. Comparable remarks have been described for other red seaweeds, wherein ethanolic extracts proved advanced DPPH scavenging activity. Research carried out by El-Rafie *et al*. (2024) on edible marine macroalgae from the Egyptian Red Sea coast showed that red seaweeds such as *Digenea simplex*, *Laurencia papillosa*, and *Galaxaura oblongata* showed higher DPPH radical scavenging activity. *Actinotrichia fragilis*, a red sea weed exhibited a significant inhibition of DPPH scavenging activity *in vitro* (Krishnan *et al*., 2019). *Manilkara zapota* extracted with methanol indicated its effective free radical scavenging potential (Kaneria *et al*., 2009). Polyphenol rich tea extracts displayed effective DPPH radical neutralizing activity (Tejero *et al*., 2014).

Superoxide radicals ($O_2^{\bullet -}$) are produced in living systems during cellular respiration and, when compared showed lower toxicity; although, in the presence of iron, they can be changed into highly sensitive hydroxyl radicals (•OH) (Lushchak, 2014). Furthermore, superoxide anions generated from incomplete oxygen metabolism cause harm to biomolecules either directly or indirectly by producing reactive species like H_2O_2, •OH, peroxynitrite, and singlet oxygen (Kirkinezosa and Morae, 2001). Hence, the elimination of superoxide anion radicals is essential to safeguard cells against their harmful actions. Different extracts from *Schima wallichii* suppressed the generation of superoxide radicals in a dose-responsive manner **(**Lalhminghlui and Jagetia, 2018). Superoxide free radical is a primary reactive oxygen molecule that can generate more harmful radicals. Comparable superoxide scavenging activity has been reported in Indian red seaweeds extracted with polar solvents. The *Iris germanica extracts (*aqueous and ethanolic) showed potent superoxide radical (SO•–) scavenging activity (Nadaroglu *et al*., 2007). The marine algae *Grateloupia filficina* exhibited a higher superoxide radical scavenging activity as reported by Athukorala *et al*. (2007).

Nitric oxide, an important cell signaling mediator has a major role in numerous biological and disease-related processes. It acts as a potent blood vessel dilator and has a very short half-life just a few seconds in the bloodstream (Hou *et al*., 1999). It also has a significant part in inflammatory processes when produced in excess. When compared, the ethanolic extract of *G. edulis* showed remarkably high nitric oxide scavenging activity, which was significantly elevated ($p<0.05$) relative to the standard BHT. This observation is consistent with earlier studies reporting effective nitric oxide scavenging by seaweed-derived phenolics and sulfated polysaccharides (Kumar *et al*., 2008). The scavenging of nitric oxide by *Actinotrichia fragilis* was reported by Rajakumaran *et al*. (2019). The nitric oxide scavenging ability of *Porphyra tenera* extract was demonstrated by Lee *et al*. (2021). Sree *et al*. (2007) showed that the crude extract obtained from the bark (ethanol) of *Terminalia arjuna* was rich in phenolic compounds and

showed strong reducing power, along with pronounced free radical scavenging activity, including effective inhibition of nitric oxide radical. Hydroethanolic extract of *Alocasia indica* showed strong nitric oxide quenching activity (Mulla *et al*., 2010). Strong nitric oxide scavenging activity suggests a potential anti-inflammatory role of *G. edulis* extracts.

Hydroxyl radicals, extremely reactive intermediates, are able to induce severe harm to biomolecules like lipid molecules, proteins and DNA. Similar protective effects have been reported in red seaweeds rich in polyphenols, which act through metal ion chelation and inhibition of Fenton-type reactions. A novel red pigment isolated from *Osmanthus fragrans* seeds showed strong, concentration-dependent hydroxyl radical inhibition, demonstrating higher activity at low concentrations than ascorbic acid and quercetin (Pan *et al*., 2009). *Stachytarpheta angustifolia* ethanolic leaf extract effectively inhibited hydroxyl radical activity (Samak *et al*., 2009). Heo *et al.* (2006) reported potentially high hydroxyl radical scavenging activity in the extracts of red seaweeds, *Gracilaria verrucosa*, *Gracilaria textorii*, *Grateloupia filicina* and *Polysiphonia japonica*.

Overall, the results demonstrate that *G. edulis* possesses significantly higher antioxidant potential ($p<0.05$) than *G. salicornia*, particularly in ethanolic and methanolic extracts. The enhanced activity can be caused by the high content of polyphenolic and additional polar antioxidant compounds, as supported by previous studies on *Gracilaria* species.

5. Conclusion

The present investigation demonstrates that both *G. salicornia* and *G. edulis* exhibit significant antioxidant activity. The presence of phytochemicals viz. phenolics, flavonoids and other bioactive secondary metabolites, may be responsible for the observed pharmacological properties. The findings thus provide scientific support for the therapeutic and nutraceutical potential of the seaweeds and find light in their applications in phytomedicine and functional food formulations.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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