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Evaluation of Antioxidant and Antimicrobial Activity of Seaweed (*Sargassum* sp.) Extract: A Study on Inhibition of Glutathione-S-Transferase Activity

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Abstract: In the present study, the free radical scavenging potentials (DPPH radical and hydroxyl radical), inhibition of lipid peroxidation, and glutathione-S-transferase and antimicrobial properties of *Sargassum* sp. extract were investigated. The tested extract exhibited a dose-dependent free radical scavenging action against DPPH radical and hydroxyl radical and antimicrobial activity. In addition, inhibition of lipid peroxidation and glutathione-S-transferase activities were also observed. The overall results have established that this seaweed could be used against several diseases and, in the food processing industry, to preserve foods.

Key Words: Antimicrobial activity, free radical, glutathione-S-transferase, hydroxyl radical, lipid peroxidation

Deniz yosunu (*Sargassum* sp.) Ekstresinin Antioksidant ve Antimikrobial Aktivitesinin İncelenmesi: Glutatyon-S-transferaz Aktivitesinin İnhibisyonu

Özet: *Sargassum* sp. ekstrelerinin radikal süpürücü aktivitesi (DPPH ve hidroksil radikali), lipid peroksidasyon inhibisyonu, ve antimikrobiyal aktivitesi incelenmiştir. Buna ilave olarak lipid peroksidasyonu ve glutatyon-S-transferaz aktivitesi de araştırılmıştır. Sonuç olarak, deniz yosununun bazı hastalıklara iyi gelebileceği ve besinlerin saklanmasında kullanılabileceği ortaya çıkarılmıştır.

Anahtar Sözcükler: Antimikrobiyal aktivite, serbest radikal, glutatyon-S-transferaz hidroksil radikal, lipid peroksidasyonu

Introduction

Reactive oxygen species (ROS) (e.g., superoxide anion ($O_2^{\cdot-}$), hydroxyl radicals (OH•), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$)) are formed as a result of normal metabolic activity and exogenous sources (1). In pathological conditions, the antioxidant mechanisms are often inadequate, as excessive quantities of ROS can be generated. The ROS formed may cause cellular and subcellular damage by peroxidation of membrane lipids, by denaturing cellular proteins, and by breaking DNA strands, disrupting cellular functions (1). In vivo, cells have their own inherited antioxidative defense system, in the form of various enzymatic, as well as nonenzymatic pathways, for removing the ROS. Among enzymatic pathways $O_2^{\cdot-}$ are dismutated by superoxide dismutase (SOD) to H_2O_2, catalase (CAT) reduces H_2O_2 to water and molecular oxygen. Glutathione peroxidase (GPX) catalyzes the reduction of H_2O_2 to water and organic peroxide to alcohols at the expense of reduced glutathione (GSH), while glutathione-S-transferase (GST) conjugates xenobiotics with GSH for excretion. Among the nonenzymatic substances, β-carotene, GSH, vitamin-A, vitamin-E, and vitamin C scavenge free radicals (1).

Recently, there is a growing interest on the discovery of natural antioxidants, mainly for 2 reasons: (I) there is epidemical and clinical evidence suggesting that consumption of vegetables and fruits reduces the risk of developing chronic disease, e.g., cancer; (II) phytochemicals are generally safer than synthetic chemicals (2). Principal source of antioxidant chiefly include those of herbs, spices, and medicinal plants. There are reports that seaweeds are also rich sources of antioxidant compounds (3,4). Seaweeds provide for an excellent source of bioactive compounds such as

carotenoids, dietary fibre, protein, essential fatty acids, vitamins, and minerals (5,6).

In recent years considerable work has been done on natural products for the presence of nontoxic antioxidants that could be used in chemotherapy. Marine algae are being used as food supplement (7), source of vitamins (8), and as food additives (9). Gustafson et al. (10) reported anti-HIV activity of *Lyngbya langerheimii* and *Phormidium tenure*. Furthermore, National Facility for Marine Cyanobacteria has reported its use for treating a number of noxious effluents containing organophosphorus, pesticides, detergents, antibiotics, and other molecules (11). However not much work has been done on marine alga, particularly *Sargassum* spp. as natural source of antioxidants.

Therefore the present study was undertaken to evaluate the free radical scavenging potential and antimicrobial activities, such as inhibition of mitochondrial and microsomal peroxidation, scavenging of OH•, inhibition of GST activity, free radical scavenging by DPPH assay and antimicrobial activity taking *Sargassum* sp. extract, a common seaweed found in Arabian sea coast.

Materials and Methods

Preparation of methanol extract of seaweed

The leafy parts of *Sargassum* sp. were collected during summer in March 2006 at low tide from intertidal zone of Dona Paula, a rocky coast of Goa, India. The samples were dried at room temperature, powdered, and sieved. The powder was dissolved with methanol (1:10 w/v) and kept at room temperature for overnight. The residue was filtered through a 4-layered cheesecloth and concentrated in Heidolph Laborata 4000 rotary evaporator. The extract was then dried at room temperature. The dried sample was dissolved in Millipore filtered double distilled water and stored in -20 °C until further use.

Preparation of mitochondrial and microsomal fractions

Sheep livers, brought from slaughterhouse of Panaji, Goa, were washed with ice-cold phosphate buffer (50 mM, pH 7.4) and homogenized (20% w/v) in the same buffer with motor driven homogenizer. Homogenates were filtered through a 2-layered cheesecloth. The filtrate was centrifuged at 700 × g for 10 min followed by centrifugation for 10 min at 10,000 × g in order to get mitochondrial fractions (MTF). The supernatants were considered as microsomal fractions (MSF). The separation was performed according to Lin and Fishman (12) with slight modifications. Their protein contents were estimated by Lowry et al. (13).

Lipid peroxidation inhibition evaluated by thiobarbituric acid test

LPO of MTF and MSF were induced using a 200 µM ferrous sulphate solution. The end product of lipid peroxidation was quantified by the method of Ohkawa et al. (14). In acidic medium, MDA reacts with thiobarbituric acid (TBA) upon boiling and resultant MDA-TBA adduct as other TBA reactive substances (TBARS) absorb at 532 nm. Formation of MDA-TBA adduct was monitored by a UV-Vis Spectrophotometer in both presence and absence of extracts. Percent inhibition in lipid peroxidation was calculated by the following expression:

$$\text{Percentage of inhibition} = [(A_o - A_1) / A_o] \times 100$$

where A_o is absorbance of control and A_1 is absorbance of sample - turbidity factor.

Scavenging of hydroxyl radical

The degradation of deoxyribose generated by Fenton reaction was measured spectrophotometrically in the presence and absence of test compound as in the method of Kaur and Saini (15). The final reaction mixture in each test tube consisted of 0.3 ml each of deoxyribose (30 mM), ferric chloride (1 mM), EDTA (1 mM), ascorbic acid (1 mM), H_2O_2 (20 mM) in phosphate buffer pH 7.4, and 0.3 ml of test compound (200-800 µg/ml) in distilled water. The test tubes were then incubated for 30 min, at 37 °C. After incubation, trichloroacetic acid (0.5 ml, 5%) and thiobarbituric acid (0.5 ml, 1%) were added and the reaction mixture was kept in a boiling water bath for 30 min. It was then cooled and the absorbance was measured at 532 nm. Results were expressed as percentage of scavenging of OH• by following expressions:

$$\text{Percentage of Scavenging} = [(A_o - A_1) / A_o] \times 100$$

where A_o is absorbance of control and A_1 is absorbance of sample- turbidity factor.

DPPH assay

The free radical scavenging activity of seaweed extract was measured by the 2,2-Diphenyl-1-picrylhydrazyl

(DPPH) method proposed by Hatano et al. (16). The extract (200-800 µg/ml) was added to 0.5 ml solution of DPPH (0.25 mM in 95% ethanol). The mixture was shaken and allowed to stand at room temperature for 30 min. It was mixed with 2 ml of doubled distilled water and absorbance was measured at 517 nm. Percentage inhibition was calculated by following expressions:

$$\text{Percentage of Scavenging} = [(A_o - A_t) / A_o] \times 100$$

where A_o is absorbance of control and A_t is absorbance of sample-turbidity factor.

Determination of sheep liver GST activity toward CDNB

GST activities were determined spectrophotometrically by monitoring the thioester formation at 340 nm using 1-chro-2, 4-dinitrobenzene (CDNB) as the substrate (17). Sheep liver cytosolic fractions were prepared and used as the enzyme source to measure GST activity towards CDNB (12). The enzyme activity was calculated by taking the extinction coefficient of CDNB 9.6 mM^{-1} cm^{-1}. The percentage of GST inhibition was calculated by following expressions:

$$\text{Percentage of inhibition} = [(A_o - A_t) / A_o] \times 100$$

where A_o is activity of control and A_t is activity of sample.

Antimicrobial properties of the methanol extract

Microbial strain

Three human pathogenic microorganisms, such as *Staphylococcus aureus* (MTCC-96), *Escherichia coli* (MTCC-443), and *Bacillus subtilis* (CCR-12), were used in the present study for evaluation of the antimicrobial activity. The strains *Staphylococcus aureus* and *Bacillus subtilis* are Gram positive while *E. coli* is Gram negative, out of which 2 strains (MTCC-96 and MTCC-443) were obtained from Institute of Microbial Technology, Chandigarh and another strain (CCR-12) was a laboratory isolate. All the strains were maintained in nutrient agar slants in the Department of Botany, North Orissa University.

Disc diffusion method

Nutrient agar plates were inoculated with 0.1 ml of 24-h-old cultures from *S. aureus, B. subtilis,* and *E. coli,* each in 3 replicates and spread properly throughout the solid media in a petri dish (100 mm diameter) with the help of a spreader. Three sets of sterile filter paper discs of 5 mm diameter (2 sets were prepared and dipped in 10 µl of methanol extract of 2 different concentrations, namely 2000 µg/100 µl and 4000 µg/100 µl, and another set was dipped in 10 µl of Ampicillin at 1000 µg/100 µl) were placed apart from each other on each agar plate aseptically to test the antimicrobial activity. The plate was incubated at 37 °C for 24 h. In the case of positive antimicrobial activity, the zone of inhibition was measured and expressed in millimeters. Three nutrient agar plates were inoculated with each type of bacterial culture and the average zone diameter was expressed in millimeters (18).

Statistical analysis

Each data point was obtained by making at least 3 independent measurements. The results were expressed as mean ± SD and levels of significance were assessed using ANOVA test and the coefficient of variance and the critical differentiation value were obtained (Table 1).

Results and Discussion

Aqueous extract of *Sargassum* sp. (800 µg/ml) inhibited lipid peroxidation (LPX) significantly in MTF and MSF respectively (Figure 1A). The peroxidation of membrane lipids by ROS or by other factors affects the

Table 1. ANOVA test.

Doses/Tests	Inhibition of LPX		DPPH Assay	Hydroxyl Radical Scavenging	GST Activity
	MTF	MSF			
200 µg/ml	0*	8.0*	43.76667*	18.45333*	63.7667*
400 µg/ml	22.2*	13.0*	52.62667	33.39667*	74.433
800 µg/ml	29.0*	47.5*	68.41667*	46.14333*	79.433*
Mean	17.067	22.166	54.93667	32.664443	72.54423
$C.V._{(E)}$	6.409379		10.8157	11.11616	7.172193
$C.D._{(P=0.05)}$	2.3281869		13.4870032	8.2418382	11.8100794

N:B: $C.V._{(E)}$ = Coefficient of variation due to error, C.D. = Critical differentiation at probability 0.05, * = significant data

structure and function of a cell. Induction of LPX by iron in vivo and in vitro has been well studied (19). It can react with the other reactants via hydrogen abstraction, hydrogen addition, or electron transfer (20). In the present study, the extract has high DPPH and OH• scavenging capacity, which increased with increasing concentrations (Figure 1B and 1C). The percentage of GST inhibition also increased with increasing concentration of *Sargassum* sp. (Figure 1D). Interestingly, when TBARS inhibition plotted against OH•, a positive trend was observed (Figure 2), which implies that methanol extract of *Sargassum* sp. might be playing an important role in protecting cells against ROS.

The experiments for the inhibition of LPX, DPPH assay, hydroxyl radical scavenging assay, and the inhibition of glutathione-S-transferase were carried out at different concentrations of algal samples, namely 200 µg/ml, 400 µg/ml, and 800 µg/ml. ANOVA was carried out in completely randomized design (CRD) in triplicates (Table 1). It indicates that there is significant difference for LPX and hydroxyl radical scavenging assay among all the concentrations used in the experiments. DPPH assay did not show any significant difference at 200 µg/ml and 400 µg/ml concentrations; however, it was significant for 200 µg/ml and 800 µg/ml. Similarly, the concentrations of 200 µg/ml and 400 µg/ml were not significant for GST inhibition assay. But it was significant for 200 µg/ml and 800 µg/ml. There are sufficient variations among different experiments for all the concentrations of the samples taken for the experiment.

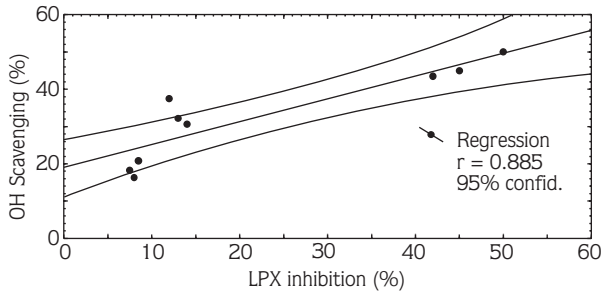

Figure 2. Correlation curves between LPX vs. $OH^{\cdot}$ (% scavenging capacity of seaweed extract).

DPPH is a relatively stable free radical. DPPH radicals react with suitable reducing agents, the electrons become paired off, and the solution loses color stoichiometrically depending on the number of electrons taken up (21).

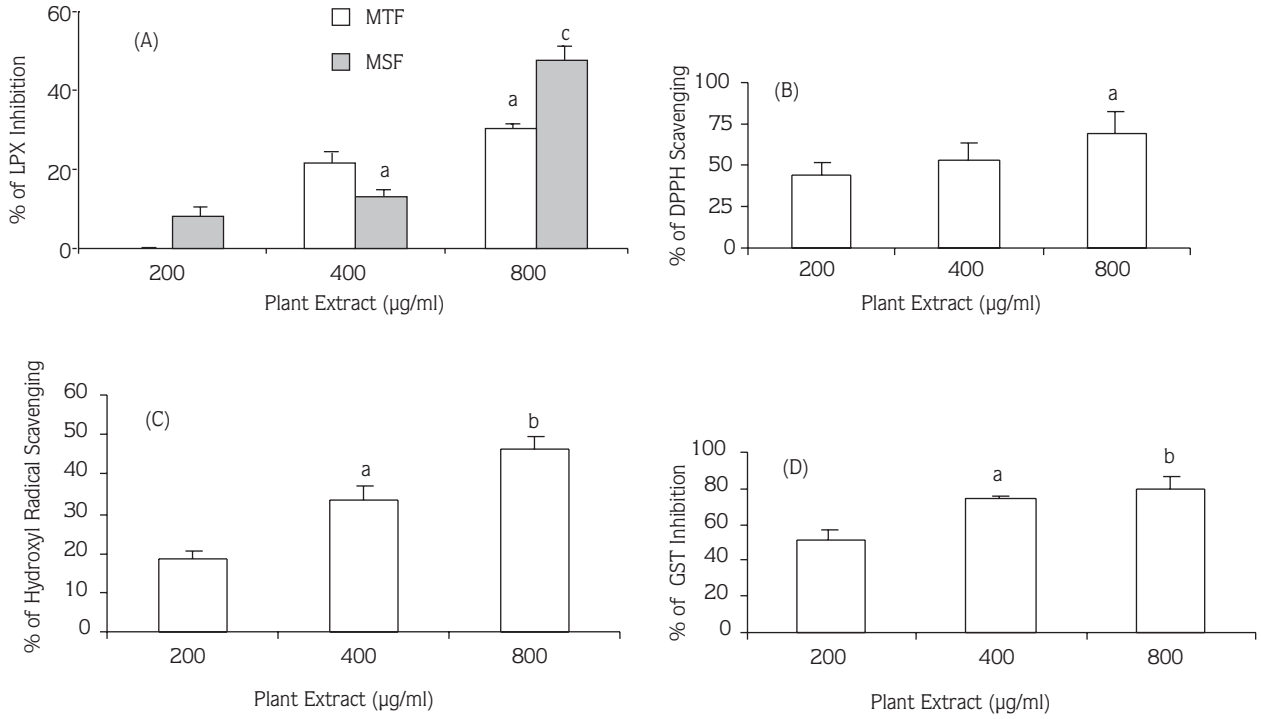

Figure 1. Effect of the methanolic extract on (A) Inhibition of lipid peroxidation (B) Scavenging of DPPH, (C) Hydroxyl radical scavenging, and (D) Inhibition of glutathione-S-transferase. Values are mean of triplicate determination ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$.

Hence this assay provided information on reactivity of test samples with a stable free radical. The DPPH scavenging capacity of seaweed extract increased with increasing concentration (Figure 1B). The decrease in the absorbance of the DPPH radical caused by test samples was due to the scavenging of radical by electron donation.

The primary role of GST is to facilitate conjugation of endogenous glutathione with electrophiles, thus resulting in more polar compounds to be excreted or further metabolized (1). In the present study, inhibition of GST activity was observed on exposure of sheep liver microsomal fraction (MSF) to seaweed extract. The inhibitory effects of plant phenols such as tannic acid, ellagic acid, ferrulic acid, caffeic acid, silybin, quercetin, curcumin, and chlorogenic acid against GST activity have been reported (22,23). Haslam (24) reported that phenolic compounds such as tannic acid have the potential to bind proteins including GST enzymes through hydrogen bond formation causing steric hindrance and hence enzyme inactivation. However, the reason for inhibition of GST activity by *Sargassum* sp. extract is not known, and further study is required to find the inhibitory mechanism. The search for novel antioxidants with GST inhibitory capacity has become an important issue because of their role in tumor cells (25,26).

Disc diffusion methods are extensively used to investigate the antibacterial activity of natural substances and plant extracts. In the present study, the crude extract of *Sargassum* sp. evaluated for antimicrobial activity against 3 pathogenic bacteria, namely *S. aureus, B. subtilis,* and *E. coli,* showed zones of inhibition ranging from 8 to 18 mm (Table 2). Among the 3 strains tested, the extract of *Sargassum* sp. was more effective against *B. subtilis* and *E. coli,* showing 18 mm and 16 mm zone of inhibition respectively at 4000 µg/100 µl concentration in comparison to *S. aureus* showing 10 mm zone of inhibition at the same concentration. These results indicate that the extracts contained different antibacterial substances and reflect the variety of secondary metabolites (27). Methanol extracts of *Cylindrospermopsis raciborskii*, *Tychonema bourrellyi*, *Anabaena lemmermannii*, *Microcystis aeruginosa,* and *Aphanizomenon flos-aquae* showed similar significant results against *B. subtilis,* whereas moderate response was found against *E. coli* (28). These assays show that agar diffusion methods using different test bacteria are valuable tools for describing the antibacterial effects of aqueous and methanol extracts of cyanobacteria (28). In the case of solutions with low activity a large concentration or volume is needed. Important factors affecting the size of inhibition zones are the chemical and physical properties of agar and the size and ionic charge of the antibiotic molecules (29). In the present study, out of the 3 microbial species used to screen the possible antimicrobial activities of methanol extract of *Sargassum* sp. *S. aureus* is one of the most common gram-positive bacteria causing food poisoning. Its source is not the food itself but the humans who contaminate foods after they have been processed (30).

The result shows that the methanol extract possesses a strong antimicrobial activity against both gram-positive, as well as gram-negative, bacteria (*E. coli*) when compared with Ampicillin as standard. However, the exact mechanism and the compound responsible for the antimicrobial activities are currently unclear. Therefore, it is suggested that further works should be performed on the isolation and characterization of the compound.

Conclusion

The methanol extract of *Sargassum* sp. showed strong antioxidant activity. In addition, this extract possessed noticeable antimicrobial activity against gram-positive and gram-negative bacteria when compared with

Table 2. Antibacterial activity of seaweed (*Sargassum* sp.) extract.

Sl. No.	Pathogenic Strains Used	Inhibition Zone in mm		
		Ampicillin (10 µg/100 µl)	Plant Extract (2000 µg/100 µl)	Plant Extract (4000 µg/100 µl)
1	*Bacillus subtilis* (CCR-12).	23	10	18
2	*Escherichia coli* (MTCC-443).	10	09	16
3	*Staphylococcus aureus* (MTCC-96).	10	08	10

standard Ampicillin. It is evident from the present study that the methanol extract of *Sargassum* sp. could be utilized as a good natural source of antioxidants and a possible food supplement or as an antimicrobial agent in pharmaceutical industry. However, the active components responsible for the antioxidant and antimicrobial activities need to be evaluated. Therefore, it is suggested that further works may be performed on the isolation and identification of the antioxidant and antimicrobial components in *Sargassum* sp. for its industrial and pharmaceutical application.

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