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Antioxidant potentialities of marine red algae *Gracillaria dura*: A search

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Abstract
Marine algae are proven sources of biologically active metabolites. The active principles produced by marine seaweeds were used in traditional and complementary medicine. Hence, phytochemical survey of the seaweeds will be a good preliminary need to reveal its secondary metabolite constituents and the resultant therapeutic values. In this study, the methanol extract of *Gracilaria dura* was subjected to phytochemical analysis to know the secondary metabolites present in the extract. The extract was purified by column chromatography. The resultant fraction was subject to GC-MS (Gas Chromatography- Mass Spectrum) analysis. A pool of terpenes were identified. Four major terpenoids were noticed in the *G.dura.* The major terpenoid component was the hexadecanoic acid, methyl ester in *G.dura* as per the concentration percentage. The purified terpenoid extract showed remarkable antioxidant activity in terms of scavenging hydroxyl radicals in a dose dependent manner. Similarly, the extract exhibited strong inhibition on the FRAP and DPPH scavenging assay. The IC_{50} values were significant and comparable with the synthetic antioxidants like ascorbate and quercetin. The obtained results suggest the possible use of red algae, *G.dura* as a good candidate in terms of functional food supplement and also in combating carcinogenesis and inflammatory disorders. Thus, *G.dura* may be considered for future application in medicine, food and cosmetic industries.

Keywords: Methanol extract, gas chromatography-mass spectrum, column chromatography, terpenoids, antioxidant

Introduction
From time immemorial, macroscopic marine algae has been closely connected with day to day human life. It has been used as a source of food, feed, fertilizer and medicine, mainly due to their economically important phycocolloids (Levering *et al*., 1969) [1]. Seaweeds are renewable living resources that are used in many parts of the world as food, feed and fertilizer. Compounds from marine red algae displays antioxidant, antimicrobial, anti-inflammatory and antidiabetic activities (Hebsibah and Dhana, 2010) [2]. Previous literatures on marine macroalgae have revealed many compounds including fatty acids, sterols, phenolic compounds, terpenes, polysaccharides, alkaloids, flavonoids and also their isolation and chemical determination. Sea weeds are usually collected for food consumption and especially known for their high nutritional value and health benefits. Among the three main divisions of macroalgae (*i.e.*, Chlorophyta, Phaeophyta, and Rhodophyta) red algae are the least exploited group. But it has been recently carried out because of their many active ingredients, particularly those that were used for medical purposes. Interestingly, various mechanisms of action have been proposed but the structural details of the active compounds was not yet properly elucidated. Recently, such phytochemical work has been carried out using purified fractions. Sea weeds contains reactive antioxidant compounds such as ascorbate, glutathione (GSH), secondary metabolites including carotenoids (alpha and beta carotenoids), aminoacids, phlorotannins (phloroglucinol), Tocopherols (alpha and beta tocopherols) (Yuan *et al*., 2005) [3].

Marine algae dominates mostly in complex habitats and were subjected to fluctuations in temperature, salinity, light, nutrients, contaminants like heavy metals etc. Thus they are prone to unfavourable environmental conditions and are capable of producing a wide range of unique primary and secondary metabolites that are not reported from other organisms surviving in terrestrial environment (Francavilla *et al*., 2013 and Rodrigues *et al*., 2015) [4, 5]. Plant terpenoids are widely used as industrially relevant chemicals, including many pharmaceutical, flavour ingredient, fragrance compound, pesticidal and disinfectants and also as feed stocks for chemical industries (Bohlmann J and Keeling, 2008) [6].

Currently, there is an increased interest in natural substances with valuable medicinal properties, such as terpenoids (Yermakov 2010) [7]. Some of the terpenes were most potent drugs against life threatening diseases. Triterpenic acids exhibit various biological and pharmacological activities, including anti-inflammatory, antimicrobial, antiviral, cytotoxic, and cardiovascular effects (Shaban *et al*., 2012) [8].

Oxidative stress represents a considerable increase in the intracellular concentration of oxidizing species, such as reactive oxygen species (ROS), which is accompanied by imbalance in antioxidant defense. This process can cause tissue damage or cell death, which occurs primarily by necrosis and apoptosis. Recent studies have shown that macro algae have become ideal candidates for sources of natural antioxidants (Karawita *et al*., 2007; Lee *et al*., 2008) [9,10]. Algae contain several enzymatic and non-enzymatic antioxidant defense systems in order to maintain the concentration of ROS for protecting the cells from damage, (Abd El-Baky *et al*., 2008) [11].

Synthetic antioxidants available in the market were widely used in food industries. The drawback of these chemical compounds are revealed by the toxicological reports in terms of the concentration used (Jayaprakasha *et al*., 2004) [12]. The search for natural antioxidants has attracted considerable attention in the last decade due to their non-toxic and cheaper value. It was also recently reported that marine algae are a source of antioxidant compounds with free radical scavenging activity (Huang and Wang, 2004) [13]. Algal biomass and algae-derived compounds have been attributed to a wide range of potential applications for human nutrition and health products. There are numerous reports on compounds derived from macro algae with broad ranges of biological activities, such as the antimicrobial, antiviral, anti-tumour, anti-inflammatory, and neurotoxic (Viswanathan *et al*., 2014) [14]. Seaweeds extracts were considered to be rich source of polyphenolic compounds. The defensive strategy of many of the red algal species suggests that they possess many anti-oxidative and anti-genotoxic constituents in their cells. For this reason, interest in marine algae as a promising potential source of pharmaceutical agents has increased during the last few years. This study aimed to analyze for the first time the terpenoids contents from the marine red algae *Gracilaria dura*, from the fractions obtained from the column by GC-MS and also to evaluate its *in vitro* antioxidant activity.

Materials and Methods

The marine algae *Gracilaria dura* was collected during June 2017, from the Mandapam coast (latitude 9˚ 17' N, longitude79˚ 22' E), Gulf of Mannar. The thalli was then cut into pieces, shade dried and powdered in a grinder to 40-mesh size powder. The ground samples were then kept in air-tight container and stored until for further analysis. Initially, 50 g each of the dried algal powder was subjected to Soxhlet extraction successively with 250 ml each of petroleum ether, chloroform, ethyl acetate, methanol and finally water. The extraction was repeated 2 to 3 times. The extracts was evaporated to total dryness by vacuum rotator and preserved in refrigerated condition for further analysis.

Phytochemical analysis was performed according to the standard protocol described by Kokate *et al*., 2003 [15]. The prepared seaweed extract were subjected to preliminary phytochemical screening for the presence of reducing sugar, flavinoids, glycosides, lignin, saponins, steroids, tannins and terpenoids. The methodologies include the estimation of total sugar by anthrone method (Jermyn, 1975) [16] and the estimation of protein by using Bradford reagent. Phenol content of the samples was estimated by Folin- ciocalteau reagent. Aluminium chloride colorimetric method was used for the determination of the total flavanoids. Terpenoid content was determined (Malik *et al*., 2017) [17]. Purification of the crude methanolic rd algal extract was done by silica gel Column chromatography and eluted using petroleum ether: ethyl acetate as solvent combinations. The eluted fractions were then subjected to TLC and followed by GC-MS. Further the antioxidant potentialities of the purified terpenoids was carried. Free radical scavenging ability was tested by DPPH radical scavenging assay as described by Zheng and Wang (2001) [18]. ABTS (2, 2-azinobis-(3-ethylbenzthiazoline- 6-sulfonate)) radical scavenging activity was done as per the protocol (Re *et al*., 1999) [19]. Subsequently FRAP (Ferric ion reducing antioxidant power) assay, H_2O_2 scavenging assay were carried as per the method of Jayaprakasha *et al*. [12]. Deoxyribose non-site specific hydroxyl radical scavenging activity was also estimated and finally the metal chelating effect of the extract was done.

Statistical Methods

The data was mean ± standard deviation (SD) after six replicates. The statistical significance was determined by one-way analysis of variance with the level of significance at $P < 0.05$.

Result and Discussion

Phytochemicals such as flavonoids, terpenoids and phenolic acids were growing interest because of their potent antioxidant potentiality and are distributed throughout the plant world (Cornish and Garbary, 2010)[20]. These compounds together with other phenolic structures of origin have been reported as scavengers of reactive oxygen species and are promising therapeutic drugs for free radical mediated pathologies including diabetic, cardiovascular diseases (Velavan, 2011)[21]. Phytochemical screenings of the various extracts of *G.dura* revealed the presence of reducing sugar, flavonoids, glycosides, tannins and terpenoids. However, steroids, lignin, saponins were poorly present in all the solvent extracts. Methanolic extract showed strong colour reaction for most of the major phytochemicals. Carbohydrate content forms the major component in *G. dura* (dry weight basis) i.e., 110 mg/g DW. The protein content was 221 mg /g DW. These results were almost similar to previous reports by other researchers i.e., generally higher carbohydrate and protein content like that of green and red sea weeds (10% to 47% DW) (Polat and Ozogul, 2009) [22]. Preliminary phytochemical screening in the seaweed showed the presence of phenols in substantial amount i.e., 1.305 ± 0.03 mg gallic acid equivalent / g dry seaweed. Significant level of secondary metabolites was also found in sea weed extracts (Table: 1). The proximate chemical composition and nutrient profile of the sea weed species was comparable to that of other sea weeds traditionally used in human and animal nutrition.

Table 1: Phytochemical analysis of the sea weed *G.dura*

Phytochemicals	Concentration (mg /g tissue)
Carbohydrate	110
Protein	221
Phenols	1.305
Flavanoids	1.162
Terpenoids	3.46

The methanolic extract was purified by silica gel column chromatography. The fraction eluted from the column was further quantified for the presence of terpenoids (i.e 3.46 mg/g). Subsequently, fraction was subjected for GC -MS analysis. Each fraction was eluted using petroleum ether: ethyl acetate as solvent combinations. The fraction eluted using 95:5 solvent combination in *G. dura* showed significant amount of terpenoids which was confirmed using GC-MS spectra technique. Parallelly, the fractions eluted by column chromatography were subjected to thin layer chromatography for confirming the presence of terpenoids. Retention time and the relative abundance of each compound were recognized. Figure 1 shows the terpenes composition of *G.dura* as detected by using GC-MS spectra technique. The GC-MS analysis of the purified fraction revealed the presence of 4 major peaks of terpenoids such as n- hexadecanoic acid, methyl ester, n-hexadecanoic acid, octadecanoic acid, phytol. n-hexadecanoic acid followed by octadecanoic acid (RT = 47.64 min, 80.78 %) was most abundant. Table 1 summarized the terpene components, retention time (Rt), molecular weight (M.W.), molecular formula (M.f) and concentration percentage in *G.dura.*

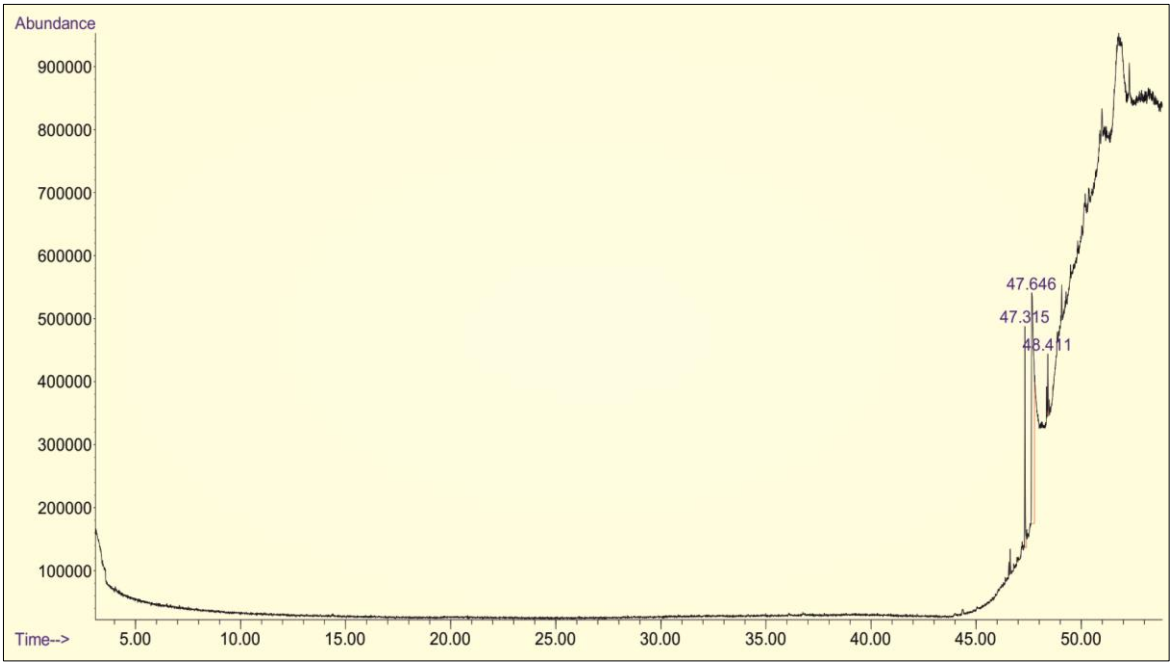

Fig 1: GC-MS spectra showing the terpene composition in *G.dura*

Table 1: Terpene components identified from *G.dura* by GC-MS.

Compound Name	Molecular weight	Molecular formula	Area%	RT
Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	15.58	47.315
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	80.78	47.646
Octadecanoic acid	$C_{18}H_{36}O_2$	284	80.78	48.411
Phytol	$C_{20}H_{40}O$	296	3.65	50.177

Claude (2012) [23] mentioned that the diterpenes are exceptionally open chained molecules found in phytol which forms a part of chlorophyll. Eman *et al*., (2015) [24] also reported the presence of terpenoids from the filamentous green algae *Spirogyra*. Marine algae such as *Laurencia* was first noted for the presence of terpenoids. Ghazala and Shameel (2005) [25] identified diterpene phytol from brown algae. Gupta and Abu Ghannam (2011) [26] confirmed several types of diterpenoids and sesquiterpenoids as the main secondary metabolites of the species from Dictyotales. Terpenoids have multiple functions like inhibition of tumour proliferation via apoptosis triggered activity, and cation channel regulation. n-hexadecanoic acid, palmitic acid were reported to possess antioxidant, hypocholesterolemic, nematicide, pesticide, lubricant activities and hemolytic 5-alpha reductase inhibitor potentials. Hexadecanoic acid was reported earlier as a component of the alcohol extract from the leaves from *Kigelia pinnata* and *Melissa officinalis* for their medicinal potentialities (Sharafzadeh *et al*., 2011). [27]

Purified terpenoids was subjected to evaluate the antioxidant potentiality using various assays. DPPH radical assay is a suitable method for determining the radical scavenging activities of the plant extracts. The activity was based on the colour changes from purple to yellow. The purified terpenoid extract shows a dose dependent inhibition of DPPH free radicals. The bleaching of purple-coloured DPPH was observed with increasing concentration of terpenoids. Ferric-reducing power is another indicator of the antioxidant potential of a plant extract. The ability to reduce ferric ions indicates that the antioxidant compounds were electron donors and further reduce the lipid peroxidation processes, thus acting as primary and secondary antioxidants. The reducing power was found to be higher as the concentration of extract increased. ABTS scavenging results were comparative less i.e., 38.6% at 200 µg/ml extract (Table: 2).

Parallelly, the antioxidant activity in terms of deoxyribose assay was carried. The terpenoid extracts of *G.dura* inhibit the formation of OH radicals and was comparatively lesser than the synthetic antioxidants such as ascorbate and quercetin. Many marine macroalgae had the ability to scavenge hydrogen peroxide. H_2O_2 can cross membranes and may slowly oxidize many vital bio molecules. Hydrogen peroxide

is generally not very reactive, but sometimes it can be toxic to cells because it leads to the formation of the hydroxyl radicals in the cells. Here also the terpenoid extract showed concentration dependent activity. Finally, metal chealating activity of the terpenoid extract was also analysed. A dose dependent increase in percentage of inhibition was observed which was lesser than that of the standard drugs ascorbate and quercetin (Table II).Thus, the AOX potentiality was significant against FRAP, DPPH and metal chelating potential.

Table 2: Reducing power assays of methanolic extract of *G. dura*

Concentration (µg/ml)	FRAP % inhibition	DPPH % inhibition	ABTS % inhibition	Hydroxy Radical	H2O2 Scavenging	Metal chealating effect
50	19.72	32.91	18.11	23.22	15.44	25.62
100	43.01	54.60	23.54	47.51	50.02	35.44
150	74.64	65.71	36.57	52.43	51.64	69.20
200	76.21	66.2	38.66	53.0	52.72	70.13

In a comprehensive literature review by Yoshiki *et al.* (2009) [28] identified a number of compounds from marine algae contributed to their antioxidant activity. Antioxidant effect was reported with sulfoglyco lipid fraction isolated from *Porphyridum cruentum* (Berge *et al*., 2002) [29]. Extracts from several macro algae harvested from Spain, Korea, China and Japan have demonstrated antioxidant activity under *in vitro* conditions. Among the marine organisms, seaweeds are considered to be attractive sources, due to their enormous biodiversity and safety and were used in traditional Asian foods. The extracts of macroalga *Taonia atomaria* exhibited high radical scavenging activity due to stypodiol and stypoldione components. Shimazu *et al.* (2007) [30] revealed the relationship between the traditional Japanese dietary patterns and cardiovascular diseases. They concluded that a diet high in antioxidant foods, including sea weeds, decreased the risk of cardiovascular diseases mortality. Hwang *et al.* (2006) [31] demonstrated that brown algal polyphenols from *Ecklonia* sp. decreased UVB-induced skin tumor development in mice regardless of whether the polyphenols were administered topically, or ingested as a dietary component, suggesting that the viability of these seaweed based antioxidants is unaffected by digestive processes. Sea weeds are indeed suitable natural agents for producing and delivering multi-functional secondary metabolites and also a wide variety of associated non-toxic antioxidants (Smit 2004, Bocanegra *et al*. 2009) [32,33]. Phenolic compounds in particular were considered to be one of the most important classes of natural antioxidants. Polyphenolic compounds, isolated from sea weeds exhibit a broad spectrum of beneficial biological potentialities [34]. Terpenoidal fractions of phenolic compounds were found in essential oil possess many biological effects including antioxidant, antiapoptosis, antiaging and anticarcinogenic properties and therefore used as dietary nutraceuticals and chemo protective agents.

Conclusion

The phytochemical analysis of the methanolic extract of *G.dura* revealed metabolites with higher medicinal activities such as flavonoids, alkaloids and terpenoids. GC-MS analysis results of the purified fraction showed 4 different terpenoid compounds of varied nature. In the present study, the work summarizes that the purified terpenoid fraction from *G. dura* showed remarkable antioxidant activity in terms of FRAP, DPPH and metal chelating effect. Thus, the marine red algae *G.dura* can be a significant source of important compounds which can be used in formulating drugs by the pharmaceutical industries. Future studies are warranted in animal model to confirm the obtained *in vitro* data.

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