Antioxidant Properties of the Red Seaweed *Gelidium pusillum* Extracts from the Oman Sea: an *in vitro* Study

Morteza Ebrahimi[1], Ali Taheri[1*]

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Abstract

Oxidation is a key factor in reducing the quality of aquatic feed and lead to undesirable changes in the nutritional composition of the diet. The usageof antioxidants in aquatic diets can reduce the damage caused by oxidative stress. Although synthetic antioxidants are used in livestock and aquatic feed, there are growing concerns about their toxicity and long-term effects on consumer health. Due to possessing a wide range of secondary metabolites, seaweeds have been considered as a natural source for producing natural antioxidants. Therefore, this research investigated the *in vitro* antioxidant properties of the red seaweed *Gelidium pusillum* from the Oman Sea. The seaweed was collected during low tide from the Oman Sea, dried, and powdered. Extraction was performed using methanol, chloroform, dichloromethane, and hexane solvents over 24 hours. After calculating the total phenolic content in the extracts, the antioxidant properties of the extracts were evaluated using three methods: free radical scavenging (DPPH), ferrous ion chelating activity, and reducing power. The IC50 value was calculated by plotting a graph. For statistical analysis, one-way ANOVA was used, and the Tukey post-hoc test was used for mean comparison. Experiments were performed in triplicate. Investigation of extraction yield revealed that the methanolic extract (1.5%) had the highest extraction yield compared to dichloromethane, chloroform, and hexane extracts. Examination of total phenolic compounds in *Gelidium pusillum* showed that the methanolic extract (23.14 mg GA/100g) had the highest total phenolic content, and the hexane extract (22.9 mg GA/100g) ranked second. The highest free radical scavenging activity was observed in the methanolic extract (90%), followed by the hexane extract (80%). The highest chelating activity (31.3%) was observed at a concentration of 1 mg/mL of the methanolic extract. The methanolic extract showed the highest reducing power (λ=0.56) among the extracts at different concentrations and had a significant difference with the other three extracts ($p< 0.05$). The antioxidant effect was dose-dependent and all extracts showed a significant difference in all three tests compared to the positive control ($p< 0.05$). Total phenolic content had a positive effect on the antioxidant activity of the seaweed extract, and solvents with higher total phenolic content exhibited higher antioxidant activity compared to other extracts. The high free radical scavenging activity of the methanolic extract confirmed its potential for further studies. Accordingly, investigating the *in vivo* activity

1- Faculty of Marine Sciences, Chabahar Maritime University, Iran
*Corresponding author email address: taheri@cmu.ac.ir
Doi: 10.48308/pae.2026.243889.1138

of the methanolic seaweed extract in aquatic diets is recommended.

Keywords: Chelation, Free radical, Oman Sea, *Gelidium pusillum*, Reducing power

Introduction

Algae are increasingly being considered as potential sources of bioactive compounds with pharmaceutical, biological, medical, and nutritional significance. Various macroalgal species have been traditionally used as ingredients in pharmaceutical and food products across the world. Furthermore, some of them are common sources of phycocolloids, gel-forming agents, of commercial value. In fact, there are 250 species of macroalgae that are commercially utilized worldwide, of which 150 species are consumed as human food. They are also regarded as low-calorie foods with high contents of minerals, vitamins, proteins, and carbohydrates (Kumar et al., 2011). In addition to primary metabolites, seaweeds can accumulate microelements, macroelements, and trace elements that are essential for their survival (Makkar et al., 2016; Salehi et al., 2019; Matos et al., 2021). They can also synthesize a wide array of secondary metabolites, which largely determine their bioactive potential (Øverland et al., 2019; Salehi et al., 2019).

An important group of secondary metabolites in seaweeds is phenolic compounds, which include simple phenols such as phenolic acids and polyphenols, including flavonoids and non-flavonoids such as tannins (Salehi et al., 2019). Most of these compounds exhibit antioxidant activity. Seaweed antioxidants act as free radical scavengers and prevent or repair damage induced by oxidative stress which possess high potential for treating various diseases (Liu and Sun, 2020). Seaweed-based antioxidants are primarily discussed in the context of cosmetic, pharmaceutical, and biomedical, food applications as stabilizers and preservatives, agriculture as plant growth biostimulants, as well as in livestock, poultry, and aquaculture nutrition (Cotas et al., 2020).

Reactive oxygen species (ROS), consisting of free radicals such as the hydroxyl radical (HO•) and superoxide anion (O_2^-), as well as non-radical species such as singlet oxygen ($^1O_2$) and H_2O_2, are various forms of activated oxygen. These ROS cause irreversible oxidative damage to biomolecules, leading to a range of pathophysiological disorders (Senevirathne et al., 2006). Increased ROS disrupts redox homeostasis and leads to either enhanced ROS production or reduced ROS scavenging capacity, a condition termed oxidative stress. ROS can interfere with the expression of numerous transcription factors and signaling proteins primarily involved in stress responses and cell survival mechanisms (Trachootham et al., 2008). Oxidation in aquafeed compositions leads to damage to the aquatic consumer, negatively impacts growth and physiology, and ultimately results in economic losses (Peixoto et al., 2016). To prevent this, cells have developed various protective mechanisms to prevent ROS formation or to detoxify ROS with the aid of antioxidants. These antiox-

idants are either cellular enzymes, such as glutathione peroxidase, or antioxidant compounds that are ingested through food. Antioxidants neutralize the excess production of oxidants and convert them into less harmful or harmless species (Moylan and Reid, 2007).

The main groups of antioxidants in seaweeds include phenolic compounds, polysaccharides, and pigments. Phenolic compounds found in seaweeds consist of (a) simple phenols such as phenolic acids (de Quiros et al., 2010): hydroxycinnamic acids – caffeic, p-coumaric, ferulic, sinapic acid; and hydroxybenzoic acids – gallic, vanillic, 4-hydroxybenzoic, protocatechuic, syringic, gentisic acid (Farvin and Jacobsen, 2013); and (b) polyphenols encompassing flavonoids and non-flavonoids. Other polyphenols that can be identified in seaweeds include phenolic terpenes such as rosmanol, carnosol, carnosic acid(Zhong et al., 2020) and terpenoids including chromene, chromanol, plastoquinone(Cotas et al., 2020). Phenolic terpenoids have been determined and characterized in red and brown seaweeds (Stengel et al., 2011).

Several seaweed-based products are available on the market, such as OceanFeed® (Milltown, Ireland), which is designed for swine, equine, and cattle nutrition. A commercial seaweed product with antioxidant properties is Tasco®, produced by Acadian AgriTech™ (Dartmouth, Nova Scotia, Canada). This product is available in two forms: Tasco-Forage and Tasco-EX. The former is used as an extract applied to plant foliage and grazed by livestock, while the latter is used for direct supplementation of livestock, poultry, and aquatic animals. Both forms are responsible for enhanced antioxidant responses measured in livestock, poultry, and aquatic animals (Allen et al., 2001; Saker et al., 2001; Saker et al., 2004; Ruiz et al., 2018; Del Tuffo et al., 2019). Therefore, the commercial products derived from seaweeds indicates the high potential of these valuable aquatic organisms for the development of biotechnological products using for aquafeed.

The southern coasts of Iran, particularly the Sea of Oman in the Chabahar region, possess a remarkable biodiversity of seaweeds, which represent a genetic reservoir and a valuable source of bioactive compounds and have not yet been fully studied or scientifically exploited. To date, the antioxidant and anticancer properties of more than 30 species from this region have been investigated by the author; however, given the identification of over 157 species, a systematic study of the antioxidant activity of the remaining species is necessary. One of the red algal species found in this area is *Gelidium pusillum* from the family Gelidiaceae. This alga, purplish to dark brown in color, is observed in intertidal zones on rocky substrates. Its distribution in the coastal areas of Ramin, Chabahar, and Tang has been reported mainly during winter and spring (Gharanjik and Rohani-ghadikalaee, 2010). Considering the reported potential of related algal species and the necessity of identifying native natural antioxidant resources, the present study was designed and conducted to investigate the antioxidant activity of various organic extracts including methanolic, chloroformic, dichloromethanic, and hexanic obtained

from the red alga *Gelidium pusillum* collected from the coasts of Chabahar. In this study, the antioxidant potential of the extracts was evaluated using various assays, including DPPH free radical scavenging, ferrous ion chelating ability, reducing power, as well as measurement of total phenolic compound content, and the results were compared with standard antioxidants.

Material and methods

Algae collection

In this study, sampling of the seaweed species *G. pusillum* was conducted during autumn and early winter of 2024 (1403 Persian calendar) at maximum low tide. Samples were collected from the coastal station of Pelag-e-Tiss (coordinates 25°17′71″ N and 60°37′17″ E) and Daryaye Bozorg station (coordinates 25°16′37″ N and 60°39′59″ E). To determine the appropriate sampling time (maximum low tide), weekly data from the EasyTide online database were used. Following transfer to the laboratory, the samples were first washed with seawater and then placed in labeled plastic bags containing some seawater. Subsequently, they were rinsed again with freshwater to remove impurities such as mineral particles, epiphytes, and other associated organisms. Species identification was performed using valid taxonomic keys, specialized image resources (Gharanjik and Rohani-ghadikalaee, 2010), as well as searches in international scientific databases such as Algaebase (www.algaebase.org). The samples were then air-dried for several days in a shaded area away from direct sunlight until a constant weight was achieved. Subsequently, they were ground into a powder using an electric grinder and stored at -20°C until further experiments were conducted (Mosaddegh et al., 2014).

Extraction and preparation

To isolate the active compounds, 25 g of dried algal powder was mixed with 80–100 mL of four different organic solvents (methanol, chloroform, dichloromethane, and hexane). The containers holding these mixtures were placed in an incubator shaker for 24 hours at room temperature with shaking at 100 rpm. The mixtures were then filtered using Whatman No. 1 filter paper. To ensure complete extraction, this process (addition of fresh solvent, shaking, and filtration) was repeated twice more on the solid residue. After three successive extraction steps, the filtered solutions obtained from each solvent were collected in separate, labeled glass Petri dishes according to the algal species and the solvent used. Excess solvent was removed by placing the Petri dishes under a chemical fume hood and allowing evaporation at room temperature. The final concentrated extracts were stored at -20°C until subsequent experiments. The extraction yield for each extract was calculated using Formula 1. This method was performed according to the protocol described in the study by Lim et al. (2002).

Extraction yield (%) = (Dried extract weight / Initial dried algal weight) × 100 (Formula 1)

Evaluation of antioxidant activity of extracts

DPPH free radical scavenging activity

The antioxidant activity of the obtained extracts was assessed using the DPPH free radical scavenging assay. Initially, different

concentrations of each extract (starting from a stock solution of 1000 µg/mL) were prepared in 50% methanol and homogenized using a vortex mixer. Concurrently, a working solution of DPPH radical was freshly prepared daily by dissolving 2 mg of the solid compound in 50 mL of 95% methanol. According to the standard method (Blois, 1958), 1.5 mL of the DPPH solution was added to an equal volume of each extract concentration, and the mixture was vortexed for one minute. The samples were then kept in the dark at room temperature for 30 minutes. After this period, the optical absorbance of each mixture was measured at 517 nm using a spectrophotometer. A decrease in the absorbance of the DPPH solution indicates the antioxidant capacity of the extract and its ability to neutralize free radicals (Ganesan et al., 2011).

The percentage of free radical inhibition for each extract was calculated using Formula 2.

$$\% \text{ Free radical inhibition} = [(A_{Control} - A_{Sample}) / A_{Control}] \times 100 \quad \text{(Formula 2)}$$

In the above equation, A_{Sample} represents the absorbance of the mixture containing different concentrations of the extract and the DPPH solution, and $A_{Contrxol}$ is the absorbance of the control sample (containing DPPH solution and 50% methanol, without the extract). To correct for the possible effect of the intrinsic color of the extracts on the assay results, a blank sample (containing the same amount of extract and 95% methanol, but without DPPH) was prepared for each extract concentration, and its absorbance was measured. To investigate the relationship between extract concentration and antioxidant activity, the experiment was performed at various concentration levels. Additionally, an ascorbic acid solution at a concentration of 0.02 mg/mL was used as a positive control and a strong antioxidant to compare the performance of the samples.

Ferrous ion chelating activity

To evaluate the ferrous ion chelating capacity of the extracts, the standard method described by Dinis et al. (1994) was used. First, extract solutions of different concentrations were prepared from the stock solution (1000 µg/mL) in distilled water. To 3.7 mL of each extract concentration, 0.1 mL of ferrous chloride ($FeCl_2$) solution was added, and the mixture was kept for 3 minutes. Subsequently, 0.2 mL of ferrozine reagent was added, and after thorough mixing, the samples were incubated for 10 minutes at room temperature. Finally, the optical absorbance of each sample was measured at 562 nm using a spectrophotometer. The percentage inhibition of iron-ferrozine complex formation, which indicates the chelating activity of the extract, was calculated according to Formula 3.

$$\% \text{ Inhibition} = [(A_0 - A_1) / A_0] \times 100 \quad \text{(Formula 3)}$$

In this equation, A_0 is the absorbance of the control group, and A_1 is the absorbance of the sample containing the extract or positive control. An EDTA solution at a concentration of 0.1 mg/mL was used as a positive control.

Determination of IC_{50}

The effective concentration of the extract required to inhibit 50% of ferrous ions (IC_{50}) was calculated using linear regression analysis between the extract concentration and

the percentage of inhibition (chelating activity).

Reducing power assay

To assess the reducing power of the extracts, the protocol described by Oyaizu (1986) was employed. Extract solutions at various concentrations were prepared from the stock solution (1000 µg/mL) using distilled water. A mixture was prepared containing 1 mL of the extract, 1 mL of 0.2 M phosphate buffer (pH 6.6), and 1 mL of 1% potassium ferricyanide solution. The mixture was then incubated for 20 minutes at 50°C. Subsequently, 1 mL of 10% trichloroacetic acid (TCA) was added to the mixture. Then, 2 mL of the supernatant was separated and mixed with 2 mL of distilled water. Finally, 0.4 mL of 0.1% ferric chloride ($FeCl_3$) solution was added, and the mixture was kept at room temperature for 10 minutes. The optical absorbance of the samples was measured at 700 nm using a spectrophotometer. An ascorbic acid solution at a concentration of 0.02 mg/mL was used as a positive control. In this method, an increase in absorbance at the specified wavelength indicates a higher reducing power of the sample.

Total phenolic content (TPC)

The total phenolic content of the extracts was measured using the Folin-Ciocalteu spectrophotometric method according to the protocol of Taga et al. (1984). In this method, 200 µL of the extract sample was mixed with 4 mL of 2% sodium carbonate solution and kept at room temperature for 2 minutes. Then, 200 µL of 50% Folin-Ciocalteu reagent was added to the mixture, and after stirring, it was incubated for 30 minutes at room temperature (26–28°C) in the dark. After this period, the absorbance of the samples was read at 720 nm using a spectrophotometer. A standard gallic acid solution at concentrations of 0.002, 0.01, and 0.05 mg/mL was used to prepare a calibration curve. The final results were reported as milligrams of gallic acid equivalent (GAE) per gram of dried algal powder. The linear equation derived from the standard curve was as follows:

$Y = 0.0141x \ (R^2 = 0.98)$

In this equation, Y represents the absorbance, and x represents the gallic acid concentration in µg/mL.

Statistical analysis

After confirming normality using the Shapiro-Wilk test, one-way analysis of variance (One-way ANOVA) was used for statistical evaluation. Tukey's test was employed for multiple mean comparisons using GraphPad Prism 9 software.

Results and Discussion

Extraction yield

The extraction yields are presented in figure 1. The methanolic extract exhibited the highest extraction yield. The hexanic extract showed the lowest extraction yield ($p < 0.05$). The dichloromethanic and chloroformic extracts did not show a significant difference from each other, but they differed significantly from the hexanic and methanolic extracts ($p < 0.05$).

The extraction yield in this study was consistent with reported values for other types of seaweeds, ranging from 2.9% to 12.7% (Ganesan et al., 2008). In a study by Sharifian et al. (2019) on the extraction of phenolic compounds and antioxidant properties

of the seaweeds *Padina australis* (2.62%) and *Nizimuddinia zanardinii* (6.71%), the highest extraction yield was reported for the methanolic solvent. In another study on the antioxidant properties of the red alga *Laurencia snyderiae*, the methanolic extract (6.12%) also showed the highest yield compared to chloroform and ethyl acetate (Karimzadeh and Zahmatkesh, 2021).

DPPH free radical scavenging activity

The results of the free radical scavenging activity are presented in Table 1. The highest free radical scavenging activity was observed in the methanolic extract, followed by the hexanic, dichloromethanic, and chloroformic extracts ($p < 0.05$). The lowest activity was measured at a concentration of 0.1 mg/mL for the chloroformic extract. Free radical scavenging activity was dose-dependent for each extract, and the activity decreased significantly with decreasing extract concentration ($p < 0.05$). All extracts differed significantly from the positive control (97.77 ± 0.5%) and exhibited lower

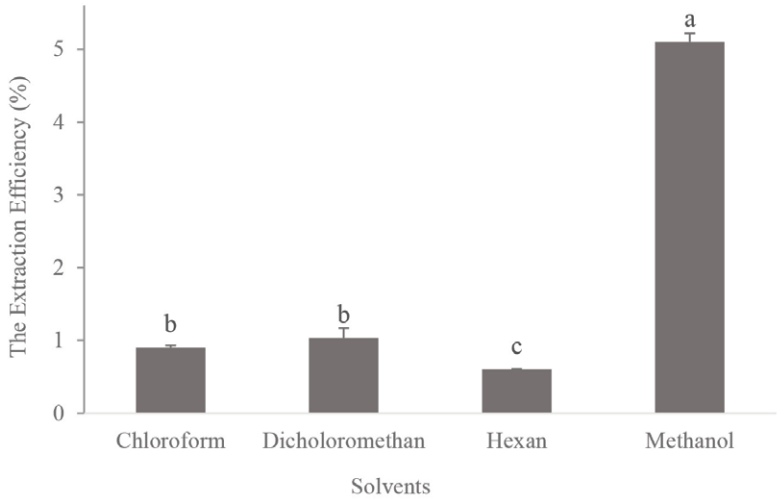

Fig. 1. The extraction efficiency of *Gelidium pusillum* (%) with 4 differenct organic solvents (Chloroform, Dichloromethan, Hexan, and Methanol).The results are Mean value from three replicates ± standard error. The letters a, b, c represent statistical significant differences betwheen means, as determines whit Tukey's post hoc test ($p< 0.05$).

Table 1. Effect of different solvents and different concentrations of organic extracts of algae *Gelidium pusillum* at DPPH free radical scavenging assay (%)

	Concentration of organic extracts (mg/mL)			
Solvent	0.1	0.3	0.5	1
Methanol	10.22 ± 0.9^{dA}	22.33 ± 1.55^{cA}	59.1 ± 3.22^{bA}	90 ± 1.76^{aA}
Hexan	6.6 ± 0.77^{dAB}	15.88 ± 4.30^{cB}	50.1 ± 2.67^{bB}	80.1 ± 1.49^{aB}
Dicholoromethan	4.2 ± 0.19^{dB}	12.18 ± 1.10^{cB}	32.91 ± 1.19^{bC}	55.16 ± 1.44^{aC}
Chloroform	1.4 ± 0.27^{cB}	6.1 ± 1.44^{cC}	12.2 ± 0.33^{bB}	22.1 ± 3.40^{aD}

The results are as Mean±SD from 3 repetition. The letters a, b, c in each row and A, B, C in each columns represent statistical differences ($p<0.05$).

free radical scavenging activity. Free radical scavenging activity was significantly dose-dependent for each extract.

The DPPH free radical, due to its unpaired electron, exhibits a characteristic purple color with maximum absorbance at 517 nm. In the presence of an antioxidant compound capable of acting as a hydrogen donor, this unpaired electron is neutralized, and the radical is converted to its reduced, stable form. The single electrons on the nitrogen atoms of DPPH are reduced to hydrazine (DPPH-H) by abstracting hydrogen atoms from antioxidants (Febrina et al., 2025). Previous research on the DPPH free radical scavenging activity of methanolic extracts of red algae revealed that Gracilaria corticata (44.32%), Gracilaria dura (33.03%), Gracilaria debilis (53.34%), and Gracilaria salicornia (53.43%) exhibited good free radical scavenging activity (Kumar et al., 2011; Karimzadeh and Zahmatkesh, 2021). Similar to the present study, in the research by Samir et al. (2019), *Bifurcaria bifurcata* with a methanolic extract, which was the richest in phenolic compounds and showed the highest inhibition percentage (81%), was the best, followed by *Cystoseira tamariscifolia* (50%) and *Fucus spiralis* (37.74%). Furthermore, in the study by Abdul Hamid et al. (2024), DPPH scavenging values ranged from 15.25% to 64.83%, where the methanolic extract of brown algae recorded the highest inhibition percentage, followed by red algae. Most extracts exhibited higher inhibitory activity when extracted with polar solvents. In this study, the concentration-dependent free radical scavenging activity was confirmed for the different algal species studied, but they showed weaker scavenging performance compared to the positive control.

Chelating activity of Organic algal extracts

The results of the chelating activity percentage of the organic extracts of *Gelidium pusillum* at different concentrations are presented in Table 2. Based on the results, all extracts at all concentrations differed significantly from the EDTA positive control and showed lower chelating activity ($p< 0.05$). Chelating

Table 2. The percentage of chelating activity of algae *Gelidium pusillum* organic extracts at different concentrations compared with the positive control of EDTA

	Concentration of organic extracts (mg/mL)			
Solvent	0.1	0.3	0.5	1
Methanol	4.14 ± 1.12^{dB}	11.5 ± 2^{cB}	18 ± 4^{bB}	31.3 ± 2^{aB}
Hexan	1.4 ± 0.35^{bB}	2.7 ± 0.98^{bC}	9 ± 2^{aC}	13 ± 6.8^{aC}
Dicholoromethan	1.8 ± 1.0^{bB}	3.3 ± 0.7^{bC}	6.3 ± 2.3^{abC}	10 ± 1.1^{aC}
Chloroform	0.98 ± 0.8^{dB}	2.5 ± 0.66^{cC}	7 ± 1^{bC}	12 ± 0.22^{aC}
EDTA	83 ± 3.1^{A}	83 ± 3.1^{A}	83 ± 3.1^{A}	83 ± 3.1^{A}

The results are as Mean±SD from 3 repetition. The letters a, b, c in each row and A, B, C in each columns represent statistical differences ($p< 0.05$).

activity did not exceed 34% even under optimal conditions. The highest value (31.3%) was observed at a concentration of 1 mg/mL for the methanolic extract. The chelating activity of the methanolic extract at concentrations of 1, 0.5, and 0.1 mg/mL showed significant differences compared to hexane, dichloromethane, and chloroform ($p< 0.05$), but no significant difference was observed among these three extracts ($p > 0.05$). The chelating activity of each extract was also dose-dependent.

The extracts of *G. pusillum* exhibited moderate to weak chelating properties, with the highest ferrous ion chelating activity belonging to the methanolic extract (31.3%). Chelating activity was directly correlated with total phenolic content in the extract. The ability of seaweeds to adsorb and chelate ferrous ions may be attributed to the presence of endogenous chelating agents, mainly phenolic compounds; because some phenolic compounds possess functional groups with appropriate orientations that can chelate metal ions (Wang, 2009). Similarly, in the study by de Alencar et al. (2016), it was found that the methanolic (54.7%) and hexanic (52.27%) extracts of the red alga *Pterocladiella capillacea* and the hexanic (33%) and methanolic (27.7%) extracts of the red alga *Osmundaria obtusiloba* exhibited the best ferrous ion chelating activity. In the study by Krishnan et al. (2019), the metal chelating activity of the alga *Actinotrichia fragilis* was 77.09 µg/mL.

According to studies by Lindsay (1996), chemical compounds containing functional groups such as hydroxyl (–OH), carboxyl (–COOH), thiol (–SH), ether (–O–), amino ($–NR_2$), phosphonate (PO_3H_2), and sulfide (–S–) have the ability to adsorb metal ions under favorable environmental conditions and can act as effective secondary antioxidants. Therefore, the presence of different functional groups in different solvents may account for the differential chelating performance of the extracts. Nevertheless, the algal extracts in the present study exhibited moderate to weak chelating activity and showed less type II antioxidant properties compared to free radical scavenging activity.

Reducing power of organic algal extracts

Table 3. Organic extracts reduction power of alga *Gelidium pusillum* at different concentrations (λ) compared with the control of ascorbic acid

	Concentration of organic extracts (mg/mL)			
Solvent	0.1	0.3	0.5	1
Methanol	0.1 ± 0.03^{dB}	0.21 ± 0.03^{cB}	0.4 ± 0.05^{bB}	0.56 ± 0.12^{aB}
Hexan	0.01 ± 0.0^{cB}	0.05 ± 0.02^{bcC}	0.12 ± 0.03^{abC}	0.17 ± 0.02^{aC}
Dicholoromethan	0.04 ± 0.01^{cB}	0.09 ± 0.01^{bcC}	0.16 ± 0.03^{abC}	0.22 ± 0.13^{aC}
Chloroform	0.0 ± 0.0^{cB}	0.05 ± 0.01^{bC}	0.09 ± 0.01^{aC}	0.12 ± 0.02^{aC}
ascorbic acid	0.9 ± 0.006^{A}	0.9 ± 0.006^{A}	0.9 ± 0.006^{A}	0.9 ± 0.006^{A}

The results are as Mean±SD from 3 repetition. The letters a, b, c in each row and A, B, C in each columns represent statistical differences ($p< 0.05$).

Based on the reducing power results presented in Table 3, the methanolic extract, except at the concentration of 0.01 mg/mL, exhibited the highest reducing power among the extracts at different concentrations and showed a significant difference from the other three extracts ($p < 0.05$). However, the hexanic, dichloromethanic, and chloroformic extracts did not show significant differences from each other. A dose-dependent effect was observed for all four extracts to varying degrees, with the most significant difference between concentrations observed for the methanolic extract. All extracts differed significantly from the positive control and showed lower reducing activity than ascorbic acid ($p < 0.05$).

The methanolic extract (0.56) exhibited the highest reducing power, while the other three extracts showed no significant differences among themselves. Reducing power, like the other two assays, was dose-dependent. In the study by de Alencar et al. (2016), the methanolic (0.136) and hexanic (0.167) extracts of the red alga *Pterocladiella capillacea* and the hexanic (0.101) and methanolic (0.180) extracts of the red alga *Osmundaria obtusiloba* exhibited reducing power that was weaker than the positive control. Reducing power has been reported for the species *Hypnea musciformis* (absorbance 1.46), *Hypnea valentiae* (0.48), and *Jania rubens* (0.45) (Chakraborty et al., 2015). In the species *Turbinaria ornata*, reducing power increases with increasing extract concentration, with values measured in the range of 0.2 ± 0.04 to 0.72 ± 0.07 (Vijayabaskar and Shiyamala, 2012). Other studies have shown that polyphenols extracted from *Gracilaria edulis* and *Hypnea valentiae* have reducing power of 80.56% and 75.09%, respectively (Mahendran et al., 2021).

Compounds with reducing power are electron donors and can reduce oxidized intermediates in lipid peroxidation processes. Therefore, these compounds are capable of acting as both primary and secondary antioxidants. Reducing agents present in a solution facilitate the reduction of the Fe^{3+}/ferricyanide complex to the ferrous (Fe^{2+}) form, which can be measured by absorbance at 700 nm (Ganesan et al., 2011). Red algae contain antioxidant compounds such as phenolic compounds (phenolic acids, bromophenols, flavonoids, phlorotannins), pigments (beta-carotene, bromophenol, phycobiliproteins, chlorophyll), sulfated galactans (carrageenan, agar), vitamins (B1, B3, C, and E), terpenoids, tannins, and peptides (Kumar et al., 2021; Wells et al., 2017). According to studies, the antioxidant activity of red algae is not limited to phenolic compounds, and other bioactive compounds also contribute to their antioxidant properties (Yabuta et al., 2010; Ngo et al., 2011).

Total phenolic content (TPC)

The total phenolic content is presented in figure 2. The phenolic content of the methanolic extract was significantly higher than that of the other three extracts ($p < 0.05$). Furthermore, the hexanic extract showed higher phenolic content than the dichloromethanic and chloroformic extracts ($p < 0.05$), but there was no significant difference in phenolic content between the dichloromethanic and chloroformic extracts ($p > 0.05$). The lowest total phenolic content was observed in the chloroformic extract.

In a study by Ismail et al., the seaweeds *Turbinaria decurrens*, *Ulva lactuca*, *Padina pavonica*, *Pterocladia capillacea*, *Sargassum muticum*, and *Sargassum acinarium* were investigated (Ismail et al., 2020). Total phenolic content was measured in methanolic, acetone, and aqueous extracts, and the results showed that the methanolic extract contained a higher amount of total phenols compared to the acetone and aqueous extracts. The polarity of the solvent and the solubility of the target compounds play a crucial role in determining the yield of polyphenols (Wakeel et al., 2019). In the study by Abdul Hamid et al. (2024), the TPC of methanolic algal extracts showed the highest values, ranging from 30.54 to 50.67 mg phloroglucinol equivalent (PGE)/g sample. The methanolic extract of *Caulerpa lentillifera* (35.77 mg PGE/g sample) exhibited a relatively high value. In the study by Bouzenad et al. (2024), the highest total phenolic content was also reported in the polar ethyl acetate extract of the seaweeds *Sargassum muticum*, *Cladophora laetevirens*, *Corallina officinalis*, *Dictyota dichotoma*, and *Ulva lactuca* (TPC ranging from 158.89 to 235.67 µg gallic acid/mg).

Higher phenolic content in algae indicates higher antioxidant activity, which is attributed to their ability to act as reducing agents. Many studies have reported a significant correlation between antioxidant activity and phenolic compound content (Honey et al., 2024; Li et al., 2007). However, in contrast to these findings, some other studies, such as that by Lim et al. (2002) on the alga *S. siliquastrum*, did not observe a direct relationship between the antioxidant effect of the extract and its total phenol content. It should be considered that in addition to phenolic compounds, other compounds such as

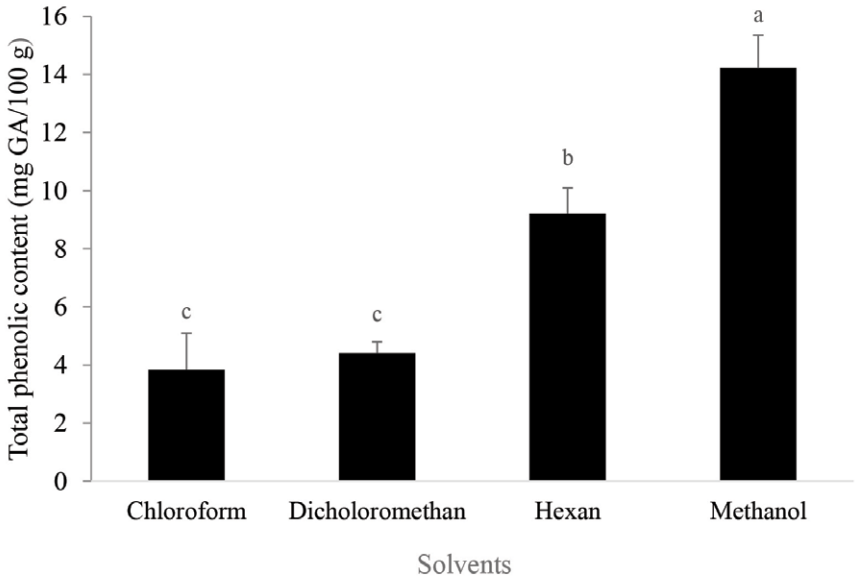

Fig. 2. Total phenolic content of *Gelidium pusillum* algae organic extracts with 4 differenct organic solvents (Chloroform, Dichloromethan, Hexan, and Methanol) represented as mg GA/100g. The results are Mean value from three replicates ± standard error. The letters a, b, c represent statistical significant differences betwheen means, as determines whit Tukey's post hoc test ($p < 0.05$).

carotenoids, unsaturated fatty acids, as well as low-molecular-weight compounds like polysaccharides, peptides, and some chromophytes, may also play a role in free radical scavenging activity.

IC_{50} values of organic algal extracts

Based on the results presented in Table 4, the methanolic extract, with an IC_{50} value of 0.54, exhibited the best DPPH free radical scavenging performance, followed by the hexanic, dichloromethanic, and chloroformic extracts, which required higher concentrations of active compounds to achieve the same effect. Regarding metal ion chelating activity, methanol again showed the best performance with 1.63 mg/g, followed by hexanic > chloroformic > dichloromethanic extracts.

Conclusion

The highest free radical scavenging activity of *Gelidium pusillum* algal extract was observed in the methanolic extract (90%), followed by the hexanic extract (80%). The highest chelating activity (31.3%) was observed in the methanolic extract. The methanolic extract exhibited the highest reducing power (λ = 0.56) among the extracts at different concentrations. Antioxidant activity was dose-dependent for each extract, and the activity decreased significantly with decreasing extract concentration. All extracts showed significant differences from the positive control in all three assays. The red alga *G. pusillum* demonstrated acceptable in vitro antioxidant properties, which were similar to or better than those reported for other red algae. Higher antioxidant activity might be achieved from this alga through purification or extraction with other solvents such as ethyl acetate and acetone, or through solvent-solvent fractionation.

Author contributions

M.E. was responsible for conceptualizing the study, planning the experiments, conducting the experimental procedures, interpreting the results, and writing the initial draft of the manuscript; A.T. supervised the research, assisted in the experimental design, offered critical feedback, and gave final approval for the manuscript. Both authors reviewed and endorsed the final version of the manuscript.

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Table 4. The IC_{50} values of the organic extracts of alga *Gelidium pusillum in vitro* antioxidant assays

Test	Chloroform	Dicholoromethan	Hexan	Methanol
DPPH Free Radical Scavenging (mg/mL)	2.19	0.88	0.61	0.54
Metal Chelating (%)	3.98	5.28	3.69	1.63

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