**A comparative study on the antioxidant activity of some selected seaweeds using different methods**

**ABSTRACT**

Two-thirds of the world is covered by oceans, whose upper layer is inhabited by algae, marine macroalgae, or seaweeds. Plant foods are a rich source of antioxidants. Antioxidants are molecules that fight free radicals and keep an organism free from various diseases. The present study revealed that the invitro antioxidant activity of ethanolic extracts of *Centroceras* *clavulatum* (Red), *Enteromorpha intestinalis* (Green), and *Turbinaria ornata* (Brown) collected from Mandapam. The extracts showed radical scavenging activity against DPPH, Hydroxyl, Superoxide, ABTS, and reducing power. All the concentrations of extract showed good antioxidant activity and were much higher than that of standard ascorbic acid. The various assays tested their maximum amount was approximately the same.  Seaweed extracts showed an increase with increasing concentration (between 50 to 800 μg/ml) indicating the dose dependency of these algal extracts. It indicates that it could be a potential source of natural antioxidant lead molecules. Depending on the type of extracts and the antioxidant tests, the 50% inhibitory concentration (IC50) ranged from 12.08 to 18.28 mg/ml. This suggests that *T. ornata* (brown) has the highest antioxidant activity, making it a promising candidate for future research and applications in health and nutrition.  Conversely, *E. intestinalis* (Green) displayed the least activity among the extracts tested, suggesting that its efficacy may be limited in comparison to the others. Marine algae contain natural pigments in abundant amounts, which could serve as a valuable source of natural antioxidants for potential use in the food industry, as well as in cosmetics and pharmaceuticals.

Keywords :-  DPPH, Hydroxyl, Superoxide, ABTS, and reducing power

**1.Introduction**

Macroscopic marine algae, also known as seaweeds, play a crucial role in the ocean's ecosystem. Seaweeds, or macroalgae, are a renewable resource in the marine ecosystem and have been used by humans since ancient times. Seaweeds are known to synthesise a wide range of chemical compounds, some of which are used exclusively to produce agar, carrageenan, and alginate. These substances have been used in a variety of applications, including human consumption, animal nutrition, plant fertilisation, and the extraction of various chemicals with therapeutic qualities, all with no reported side effects.

Seaweeds contain bioactive chemicals with antibacterial, antiviral, anticancer, anti-inflammatory, and antioxidant properties (Mayer *et al.,* 2011).  Organic acids, sulphated polysaccharides, and phenolic compounds are the substances that have antiviral, antibacterial, anti-cancer, and antioxidant properties (Wijffels 2008).  Common phytochemicals found in macro algae include alkaloids, catechins, flavonoids, phenols, saponin, steroids, tannins, terpenes, sugars, and amino acids. It has a good impact on human health.

The primary antioxidants found in seaweeds are phenolics, polysaccharides, vitamins, and pigments (Ismail & Ismail 2017, Michalak *et al.,*2002). Polyphenol molecules, including phenolic acid, flavonoids, and tannins, are considered potent antioxidants (Ismail *et al.,*2017). Free radicals are chemical species that can exist independently with one or more unpaired electrons in their outermost shell. They collect electrons from other substances and maintain in a neutral state. The initial attack leads the free radical to become neutral. This procedure generates another free radical, triggering a chain reaction. Covalent bonds are formed when two radicals join with unpaired electrons. Relative oxygen species (ROS) originate from molecular oxygen. It damages DNA and proteins, leading to diseases such as cancer and respiratory ailments. Compounds with antioxidant properties help prevent diseases such as cancer, cardiovascular disease, and neurological disorders.

Antioxidants may have a positive impact on human health because they protect the body from damage caused by reactive oxygen species, which attack and impair macromolecules such as DNA, proteins, and lipids, resulting in many health disorders such as diabetes, ageing, cancer, and other neurodegenerative diseases.  (Ngo DH, Wijesekara I, *et al.,*2011). Recently, marine flora and fauna have gained significant attention as natural sources and the production of antioxidants in the food and pharmaceutical industries. Marine algae are one of the most abundant sources of natural antioxidants among marine resources (Cornish ML, Garbary DJ 2010). Seaweed antioxidants act as "free radical scavengers" by preventing or repairing oxidative stress damage and have a high potential for treating a variety of ailments. (Liu and Sun 2020).

Many plant-derived antioxidants have antioxidative qualities that are largely attributed to phenolic compounds. (Canadanovic-Brunet et al., 2006, Farombi et al., 2000, Kaur & Kapoor 2001) Phenolic substances have also been shown to have a variety of biological effects, such as vasodilatory, antibacterial, anti-inflammatory, and antioxidant activities. Previous research has examined the antioxidant potential of naturally occurring phenolic components in relation to the protection of cancer, heart disease, and age-related degenerative brain illnesses.  (Stoclet *et al.,*2004, Stevenson & Hurst 2007).

Natural antioxidants used as feed supplements can improve not only animal production and health, but also their ability and resistance to many diseases. Seaweeds have antioxidant and immunomodulatory properties that can reduce oxidative damage, potentially improving the prognosis and treatment of infectious diseases for both humans and animals (Shi *et al.,* 2017; Juarez-Portilla *et al.,* 2019).   Hence, the study aimed to assess the antioxidant activity of *Centroceras clavulatum, Enteromorpha intestinalis* and *Turbinaria ornata* were obtained from Mandapam.

**2.Materials and method**

**2.1. Collection of samples**

Fresh samples of *Centroceras clavulatum , Enteromorpha intestinalis*, and *Turbinaria ornata* were gathered from the Mandapam coast Tamil Nadu, India. After being cleaned with marine water and brought to the lab in a plastic cover, the collected algal samples were washed thoroughly clean with tap water and then distilled water to get rid of any salts, epiphytes, and debris.  After draining the water, the seaweeds were spread out on blotting paper to absorb any remaining moisture. At room temperature, the samples were shade-dried. The dried substance that resulted was ground into a coarse powder. The samples that were powdered were then kept in a refrigerator.

**2.2. Preparation of Samples**

50 grams of the fine powder of each alga was extracted successively with 500 ml of organic solvent (Ethanol) in a Soxhlet apparatus for 8 hours. The extracts were filtered through Whatman No. 41 filter paper separately and all the extracts were concentrated in a rotatory vacuum evaporator (below 60oC).  The concentrated extracts were subjected to in vitro antioxidant activity.

**In vitro antioxidant activity**

**2.3. DPPH radical scavenging activity**

The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant component. This method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen donating antioxidant due to the formation of the non- radical form DPPH (Blois, 1958).

 The free radical scavenging activity of all the extracts was evaluated by 1, 1- diphenyl-2 picryl-hydrazyl (DPPH) according to the previously reported method (Blois, 1958). Briefly, 0.1 mM solution of DPPH in methanol was prepared and 1 ml of this solution was added to 3 ml of the solution of all extracts at different concentrations (50, 100, 200, 400 and 800 μg/ml). The mixtures were shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbance was measured at 517 nm using a UV-VIS spectrophotometer.  Ascorbic acid was used as the reference. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated by using the following formula.

DPPH scavenging activity (% inhibition) = (A0 - A1) / A0 X 100

Where, A0 is the absorbance of the control and A1 is the absorbance of the test samples and reference. All the tests were performed in triplicates and the results were averaged.

**2.4. Hydroxyl radical scavenging activity**

The scavenging capacity for hydroxyl radical was measured according to the modified method of Halliwell *et al.,* (1987). Stock solutions of EDTA (1 mM), FeCl3 (10 mM), Ascorbic Acid (1 mM), H2O2 (10 mM) and Deoxyribose (10 mM) were prepared in distilled deionized water. The assay was performed by adding 0.1 ml EDTA , 0.01 ml of FeCl3, 0.1 ml H2O2, 0.36 ml of deoxyribose, 1.0 ml of the extract of different concentration (50, 100, 200, 400 & 800 μg/ml) dissolved in distilled water, 0.33 ml of phosphate buffer (50 mM, pH 7.9), 0.1 ml of ascorbic acid in sequence. The mixture was then incubated at 37oC for 1 hour. 1.0 ml of the incubated mixture was mixed with 1.0 ml of 10% TCA and 1.0 ml of 0.5% TBA (in 0.025 M NaOH containing 0.025% BHA) to develop the pink chromogen measured at 532 nm. The hydroxyl radical scavenging activity of the extract is reported as % inhibition of deoxyribose. The degradation is calculated by using the following equation.

Hydroxyl radical scavenging activity = (A0 - A1) / A0 X 100

Where, A0 is the absorbance of the control and A1 is the absorbance of the test samples and reference. All the tests were performed in triplicates and the results were averaged.

**2.5. Superoxide radical scavenging activity**

The superoxide anion scavenging activity was measured as described by Srinivasan *et al.,* (2007). The superoxide anion radicals were generated in 3.0 ml of Tris - HCl buffer (16 mM, pH 8.0) containing 0.5 ml of NBT (0.3 mM), 0.5 ml NADH (0.936 mM) solution, 1.0 ml extract of different concentrations (50, 100, 200, 400 & 800 μg/ml) and 0.5 ml Tris - HCl buffer (16 mM, pH 8.0). The reaction was started by adding 0.5 ml PMS solution (0.12 mM) to the mixture, incubated at 25oC for 5 min and the absorbance was measured at 560 nm against a blank sample, ascorbic acid. The percentage inhibition was calculated by using the following equation.

Superoxide radical scavenging activity = (A0 - A1) / A0 X 100

Where, A0 is the absorbance of the control and A1 is the absorbance of the test samples and reference. All the tests were performed in triplicates and the results were averaged.

**2.6. Antioxidant Activity by Radical Cation (ABTS+)**

ABTS assay was based on the slightly modified method of Huang *et al*., (2011). ABTS radical cation (ABTS+) was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12 - 16 hrs before use. The ABTS+ Solution were diluted with ethanol to an absorbance of 0.70 + 0.02 at 734 nm. After addition of 100 μg/ml of sample or Trolox standard to 3.9 ml of diluted ABTS+ solution, absorbance was measured at 734 nm by Genesys 10S UV-VIS (Thermo scientific) exactly after 6 minutes. Results were expressed as Trolox equivalent antioxidant capacity (TEAC).

ABTS+ radical cation activity = (A0 - A1) / A0 X 100

Where, A0 is the absorbance of the control and A1 is the absorbance of the test samples and reference. All the tests were performed in triplicates and the results were averaged.

**2.7. Reducing Power Assay**

The reducing power of the different solvent of sea weed extract was determined by the method of Kumar and Hemalatha (2011). 1.0 ml of solution containing 50, 100, 200, 400 & 800 μg/ml of extract was mixed with sodium phosphate buffer (5.0 ml, 0.2 M, pH 6.6) and potassium ferricyanide (5.0 ml, 1.0%). The mixture was incubated at 50oC for 20 minutes. Then 5ml of 10% trichloroacetic acid was added and centrifuged at 980 rpm (10 minutes at 5oC) in a refrigerated centrifuge. The upper layer of the solution (5.0 ml) was diluted with 5.0 ml of distilled water and ferric chloride and absorbance read at 700 nm. The experiment was performed thrice and results were averaged.

**3.RESULT AND DISCUSSION**

The pharmaceutical, medical, cosmetic, nutraceutical, food, and agricultural sectors could all benefit economically from the numerous bioactive compounds present in marine algae.  Natural antioxidants, which are present in many types of algae, are important bioactive substances that protect cells from oxidative damage, hence reducing the risk of disease and ageing. Many studies have been conducted on the antioxidant properties of marine algae. They possess various antioxidant functions, which include scavenging free radicals like DPPH, Hydroxyl, Superoxide, ABTS, lipid peroxidation, and ferric reducing antioxidant capacity.

This study examined the antioxidant activity of *Centroceras clavulatum , Enteromorpha* *intestinalis*, and *Turbinaria ornata*, revealing that the extracts exhibited the activity in a concentration-dependent manner. The *T.ornata* extract (75.31%) had the highest DPPH activity, while the *C.clavulatum* extract (61.86%) had the lowest activity at 800 mg/ml. (Fig.1).  The *C. clavulatum , E. intestinalis*, and *T. ornata*, concentrate;’ions required for 50% inhibition (IC50) were 15.87, 16.42, and 17.09 mg/ml. While ascorbic acid required 16.21 mg/ml. (Table 1)

Fig.1 DPPH radical scavenging Activity of seaweeds

The DPPH radical scavenging assay is a rapid method for assessing the antioxidant capacity of a compound. When reduced in the presence of hydrogen-donating antioxidants, DPPH (1, 1-diphenyl-2-picryl-hydrazyl) is a persistent free radical that causes ethanol to change from a purple colour to colourless. (Garcia *et al.,*2012) Consequently, the typical absorption of DPPH decreases as the concentration of the antioxidant compound increases, suggesting that the compound can scavenge DPPH (Dore *et.al.,*2013, Swathi *et al.,*2024) 800 μg /ml was found to exhibit the highest percentage of activity. The ascorbic acid standard was used to compare the algal extract's results. The present findings are consistent with those of a prior study. (Khan *et* *al.,* 2021, Si-Min *et al.,* 2022)

The hydroxyl radical, the most reactive free radical, can be produced by superoxide anions and hydrogen peroxides in the presence of metal ions like copper and iron. Hydroxyl radicals can harm various biomolecules, such as proteins, DNA, polyunsaturated fatty acids, and nucleic acids.  (Aruoma 1998).  The Fenton reaction was used to examine the scavenging effect of OH, and the 50% inhibition (IC50) was 12.08,15.48,15.11 mg/ml while the ascorbic acid exhibited 16.11 mg/ml*. E. intestinalis* was recorded as the highest percentage (69.23%) followed by *T.ornata* (63.16%) and *C.clavulatum* (59.31%), respectively, which was shown in the table 1 and Fig.2.

Fig.2.Hydroxyl radical scavenging activity of seaweeds

H2O2 is a non-radical compound with biological significance due to its ability to penetrate biological membranes. H2O2 is not very reactive, but it can sometimes be toxic to the cell because it can give rise to hydroxyl radicals in the cells (singlet oxygen and HO radicals) (Ma *et al.,*2011).  Thus, removing H2O2 is critical to protecting the biological system in general, and food components in particular. Extracts of brown seaweeds *T.ornata* have been shown to have over 90% H2O2 scavenging activity (Heo *et al.,* 2005) indicating their high content of natural antioxidants capable of scavenging H2O2 radicals. Many other seaweed species have been reported in the literature as having potential H2O2 scavenging activity (Gupta & Abu-Ghannam 2011). The hydroxyl radical is a highly reactive species that targets almost every biological compound. It is made from hydrogen peroxide by the Fenton reaction, which is catalysed by metal ions (Cu2+ or Fe2+) (Heo *et al*.,2006.,). The scavenging activity of algal extracts was assessed using the percentage of inhibition of hydroxyl radicals produced in the Fenton reaction mixture; the results are shown in table 1.

**IC 50 Values of seaweeds**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| ASSAYS | *C.clavulatum* | *E.intestinalis* | *T.ornata* | STANDARD (Ascorbic acid) |
| DPPH | 15.87 | 16.42 | 17.09 | 16.21 |
| Hydroxyl | 12.08 | 15.48 | 15.11 | 16.11 |
| ABTS | 14.16 | 18.28 | 12.34 | 16.88 |
| Super oxide | 15.45 | 16.31 | 12.98 | 13.57 |

Table.1 IC 50 value of seaweed extracts

In the present study, *C.clavulatum, Eintestinalis, & T.ornata* showed the maximum percentage of inhibition was noticed. In *E.intestinalis* at 80.16% at 800 μg/ml concentration and is slightly lower than that of the standard ascorbic acid, which was 79.33% (Fig3). The minimum was noticed in *T.ornata* 60.16%. The IC50 value of ABTS of the above experimental extracts was 14.16,18.28 and 12.34, whereas the standard was 16.86. (Table 1) Where the IC50 value was low, the antioxidant activity was higher.

Fig.3. ABTS radical scavenging Activity of seaweeds

The ABTS+ test serves as a valuable method for identifying hydrogen-donating and chain-breaking antioxidants. The reduction of the radical cation was utilized to decolorize ABTS+ as a percentage inhibition of absorbance at 734 nm (Fellegrini *et al.,*1999) ABTS+ was produced by incubating the ABTS chromophore during the reaction. This extract antioxidant properties may be due to the neutralization of radicals (ABTS), either by transferring hydrogen atoms or electrons (Matkowski & Tasarz 2008). The current study's findings show that the extract from brown seaweed demonstrated greater ABTS radical activity. The ABTS scavenging activity was evaluated using differential extraction methods. This possesses chain-breaking antioxidant properties (Subashini & Prasanth 2014) As shown in Fig. 3, the percentage efficiency of ABTS scavenged by seaweed extract was found to increase with concentration.  According to El-Sheekh *et al*.,2020 and Ismail 2017 brown seaweeds possess greater antioxidant activity than red seaweeds. This explains why the brown algae *T. ornata* and *P. indica* exhibited the highest levels of antioxidant activity.

Superoxide anion radicals arise from cellular oxidation processes in various organisms, including humans. Even though it is an oxidant of relatively low strength, it decomposes via dismutation and other reactions to form stronger oxidative agents like hydrogen peroxide and hydroxyl radicals. It is also responsible for the generation of free radicals formed in vivo. SOA radicals and their derivatives damage cells, harming DNA and cell membranes. Thus, scavenging SOA radicals (Sarikurkc *et al.,* 2010, Yangthong *et al.,* 2009) is more important. Superoxide is a molecule with high reactivity that interacts with different substances generated through metabolic processes.

The percentage of superoxide anion radicals examined at different concentrations of *C.clavulatum, E.intestinalis & T.ornata* was revealed in Fig. 4. The extract of the above seaweeds shows an effect of 75.16, 72.19, & 64.31, respectively (at 800 μg/ml). This activity is dose-dependent; when the concentration increases, the percentage of inhibition also increases. The IC50 value was 15.45, 16.31, and 12.18, while standard ascorbic acid was 13.57.(Table1).

Fig.4. Superoxide radical scavenging activity of seaweeds

The findings of this investigation coincide with those of Pandithurai and Murugesan (2014), who examined the antioxidant activities of *Spatoglossum asperum* (brown alga) and found it exhibited more significant antioxidant activities than L-ascorbic acid and Butylhydroxy Toluene (BHT). It is a widely recognized fact that among the various types of algae, brown algae typically exhibit the highest antioxidant activity, with red and green algae following behind (Vladkova *et al.,* 2022).  The significant superoxide anion scavenging activity of the chosen algal extracts could be beneficial for both medical and functional food applications.

The reduction of power, which serves as a measure of antioxidant activity, can be assessed through a reaction that transforms Fe 3+ in potassium ferricyanide into Fe 2+. The ferric reducing assay measures the capability of an antioxidant to diminish a reactive oxygen species in comparison to that species oxidative strength. Compounds exhibiting reducing power demonstrate that they serve as electron donors capable of reducing oxidized intermediates involved in lipid peroxidation, thereby functioning as primary and secondary antioxidants (Chanda & Dave 2009).

The reducing power of all the three extracts increased in a dose-dependent manner, as illustrated in Fig 5. In this study reducing power was increased with increasing concentration. Maximum reducing power value was observed in 800 μg/ml concentration of *T.ornata* (0.436) and followed by *C.clavulatum* (0.421) & *E.intestinalis* has minimum value (0.406)

Fig 5. Reducing power Activity of seaweeds.

The current findings accept with those of Matsukawa *et al*., 1997, Farghl *et al*., 2021 who observed that brown algae had higher antioxidant activity than either red or green groups. Most of the species under study are therefore thought to have high antioxidant content. According to (El-Sheekh *et al.,* 2020 and Ismail 2017) brown seaweeds have higher antioxidant activity than red seaweeds, which explains why brown algae *T. ornata* and *P. indica* had the highest antioxidant activity.

**CONCLUSION**

In addition to being a good way to treat or prevent chronic diseases, seaweeds are a valuable source of bioactive compounds that could be used to prepare new functional ingredients for food. Since consumers have recently shown a great deal of interest in natural bioactive compounds as functional ingredients in foods, it can be argued that seaweeds are a substitute source of synthetic ingredients that can improve consumer health by appearing in novel functional foods and medications. Additionally, the diverse biological activities linked to bioactive compounds derived from marine algae may increase their health benefits in the food and pharmaceutical industries.

In the current study, *C.clavulatum, E.intestinalis & T.ornata* extracts were found to have strong antioxidant activity. Seaweed extracts antioxidant mechanisms may be due to their ability to scavenge free radicals. Furthermore, it seems that phenolic compounds are the source of the antioxidant activity found in seaweed extracts. The results obtained indicate that seaweeds can be utilized for various beneficial chemo-preventive effects. This study may provide a rational basis for using marine algal extracts in the potential treatment of oxidative stress-related diseases. It further supports the idea that these antioxidant-rich extracts or fractions can serve as dietary supplements to promote good health.

**DISCLAIMER (ARTIFICIAL INTELLIENCE)**

Authors hereby declare that no generative AI technologies such as large language, models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

**COMPETING INTERESTS**

Authors have declared that no competing interests exist.

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