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IN VITRO ANTIOXIDANT AND RADICAL SCAVENGING ACTIVITY OF MARINE MICROALGA *NANNOCHLOROPSIS OCULATA*

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ABSTRACT

The study focuses on determining the antioxidant properties of the methanol extract from the microalgae *Nannochloropsis oculata*. The evaluation of the total phenolic content, total flavonoid content, total antioxidant activity, DPPH Radial scavenging assay, Deoxyribose Radial scavenging activity, Hydrogen peroxide (H_2O_2) Radical scavenging activity, ferric reducing antioxidant power assay scavenging of superoxide radical, ABTS, Lipid peroxidation assay and β-carotene linoleic assay were carried out for methanolic extract of *Nannochloropsis oculata*. The antioxidant assay was performed at the concentration ranging from 100 - 500µg/ml. The results showed that the higher antioxidant activity value was observed in methanol extract of *Nannochloropsis oculata* when compared with standard drug. Hence microalgae have received special attention as a source of natural antioxidants in comparison with macroalgae and were found to be a good source of antioxidants.

KEYWORDS: Total Phenolic Content, Total Flavonoid Content, Microalgae, *Nannochloropsis oculata.*

INTRODUCTION

Antioxidants are involved in the prevention of cellular damage as these molecules interact with free radicals and end up the reaction. It paves the way for medicinal focus on cancer, cardiovascular, aging and many diseases because of their tissue protecting effects by neutralization of ROS. Antioxidants compounds can be derived from both natural and synthetic method. There are many synthetic antioxidants like propyl gallate, butylated

hydroxytoluene are available commercially but are unsafe and toxic in nature.[1] Many researchers are interested deriving antioxidant compounds from natural sources. Therefore they are used in food supplements or food ingredients, feed additives, pharmaceutical and cosmetic industry. Algae are also used as fertilizers and medicines.

A marine alga was one of the largest producers of biomass in the marine environments.[2] Marine micro algae are focused in biomass production due to their ability to survive under environmental stresses like light intensity, dark, heat, UV exposure, nitrogen and also the metabolites production. One of the marine micro algal *Nannochloropsis oculata* exhibits notable bioactivities like antibacterial, anti-inflammatory, anti-algal, antifungal, analgesics, antioxidant activities which are effective in the prevention of many diseases and have been used in therapeutics and nutraceuticals.[1]

Nannochloropsis sp., on the other hand, is a unicellular green alga, spherical in shape, with diameter of about 2-5 μm, belonging to the *Eustigmatophyceae* class. It plays an important role in the food chain system and is also commonly used as live feed; thus, it is widely cultivated in fish hatcheries and shrimp farms.[3] Among numerous species of microalgae *Nannochloropsis oculata* has received priority attention in recent years in view of its demand for rearing operations in many hatcheries. In this study, antioxidant capacity of water, acetone, methanol, isopropyl alcohol and pyridine extracts of *Nannochloropsis oculate* were evaluated different assays were also measured to study their relation to antioxidant capacity.

MATERIALS AND METHODS

1. Sample preparation

1 gram of *Nannochloropsis oculata* was mixed with 10 ml of methanol and it is kept at room temperature for 24 hours. This mixture was filtered and stored at 4ºC for further analysis.

2. Quantification of Total Phenolics and Flavonoids[5]

Total phenolic substance was assessed by following strategy: 100 µl of methanolic extract of *Nannochloropsis oculata* was blended with 2.0 ml of 2% Na_2CO_3 and permitted to remain for 2 min at room temperature. After incubation, 100 µl of 50% Folin Ciocalteau's phenol reagent was supplemented and was mixed thoroughly. It is then allowed to stand at room temperature for 30 min. Absorbance of all the samples were measured at 720 nm using spectrophotometer. Gallic acid was used as standard to determine total phenolic activity.[6]

The total flavonoid content of tests was detected by the aluminum chloride colorimetric method.[7] 0.5 ml of methanolic extract of *Nannochloropsis oculata* were mixed with 250 µl of 5% sodium nitrite ($NaNO_2$) solution and 150 µl of 10% $AlCl_3$ solution and incubated for 5 mins. At that time, 0.5 ml of 1 mol/L sodium hydroxide (NaOH) solution was added, and was brought to 2.5 ml with double-distilled water. The mixture was allowed to stand for 15 min which was measured at 510 nm. The total flavonoid content was calculated from a calibration curve and the result was expressed as mg. Rutin was used as standard (equivalent per g dry weight).

3. *In vitro* Antioxidant and Free Radical Scavenging Assays[4,5]

Total antioxidant activity

The total antioxidant capacity of methanolic extract of *Nannochloropsis oculata* was evaluated.[8] At first, 7.45 ml of sulphuric acid (0.6 mM solution), 0.9942 g of sodium sulphate (28 mM solution) and 1.2356 g of ammonium molybdate (4mM solution) was dissolved in distilled water and made up to 250 ml which was marked as TAC reagent. Then, to 300 µl of test sample 3 ml of TAC reagent was added. Reaction mixture was incubated at 95° C for 90 minutes. Absorbance was measured at 695 nm and ascorbic acid was used as standard.

DPPH radical scavenging assay

The scavenging activity of different extract of *Nannochloropsis oculata* for DPPH radical were identified by the method.[9] Concisely, 2.0 ml of test samples and 2.0 ml of 0.16 mM DPPH methanolic solution was mixed together. The mixture was vortexed for 1 min and then allowed to stand at room temperature for 30 min in the dark. The absorbance of all the sample solutions was measured at 517 nm. The scavenging effect (%) was calculated by using the formulae:

Scavenging effect (%) = (Absorbance of control –Absorbance of test solution)/Absorbance of control] × 100

Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging assay was done based on the procedure.[10] A solution of hydrogen peroxide (H_2O_2, 10 mM) was prepared in phosphate buffer (0.1 M, pH 7.4). 3.4 ml of phosphate buffer was mixed with 0.6 ml of H_2O_2 solution (0.6 ml, 43 mM) and 1ml (0.25 mg) of methanolic extract of *Nannochloropsis oculata* was added to it. The absorbance value of the mixture was recorded at 230 nm after 10 minutes and incubation at room temperature.

Blank solution contains sodium phosphate buffer without H_2O_2 was used. Ascorbic acid was used as the standard. The percentage of H_2O_2 scavenging of crude extract and standard compounds were calculated using the following equation:

H_2O_2 scavenging effect (%) = (Absorbance of control –Absorbance of test solution)/Absorbance of control) × 100

Deoxyribose Radical Scavenging Activity

Deoxyribose non-site specific hydroxyl radical scavenging activity of methanolic extract of *Nannochloropsis oculata* was estimated.[4,5] Briefly, 2.0 ml aliquots of test samples were mixed to the test tube containing reaction mixture of 2.0 ml $FeSO_4.7H_2O$ (10mM), 0.2 ml EDTA (10mM) and 0.2 ml deoxyribose (10mM). The volume was made up to 1.8 ml with phosphate buffer (0.1M, pH-7.4) and to that 0.2 ml H_2O_2 (10mM) was added. The mixture was incubated at 37°C under dark for 4 hours. After incubation, 1 ml of TCA (2.8%) and TBA (1%) were added to the mixture, and then kept under boiling water bath for 10 min. After the treatment the samples were absorbed at 532nm. If the mixture was turbid, the absorbance was measured after filtration. Ascorbic acid was used as standard. Scavenging activity (%) was calculated using the equation:

Deoxyribose radical scavenging activity (%) = Absorbance of control- Absorbance of test / Absorbance of control × 100

ABTS [2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] Radical Cation Scavenging Assay

Free radical scavenging activity was determined by ABTS radical cation decolorization assay.[13] ABTS radical cation was creäted by mixing 20mM ABTS solution with 70mM potassium peroxodisulphate and kept to stand in dark at room temperature for 24 hours before use. To, 0.6 ml of methanolic extract of *Nannochloropsis oculata* (0.25 mg), 0.45 ml of ABTS reagent was added and absorbance of these solutions was measured at 734 nm after 10 min.

ABTS radical cation scavenging assay [%] = Absorbance of control- Absorbance of test / Absorbance of control × 100

Superoxide radical scavenging activity

Scavenging of superoxide radical was calculated using the method elaborated.[14] Assay tubes contained 0.2 ml of methanolic extract of *Nannochloropsis oculata* (corresponding to 20 mg extract) with 0.2 ml EDTA (12mM), 0.1 ml Nitro blue tetrazolium, 0.05 ml riboflavin (20µg)

and 2.64 ml phosphate buffer (50 mM, 7.6 pH). The control tubes were set up with DMSO (Dimethyl sulfoxide) solution instead of the test solution. The initial optical densities of the solutions were recorded at 560 nm and the tubes were illuminated uniformly with the fluorescent lamp for 30 mins. A560 was measured again and the difference in O.D was taken as the quantum of superoxide production. The percentage of inhibition was calculated by comparing with O.D of the control tubes.

SOD activity

Measurement of superoxide anion scavenging activity was performed based on the method.[4,6] To, 1ml of Nitroblue Tetrazolium (NBT) solution containing 156µM NBT dissolved in 1.0 ml of phosphate buffer (100mM, pH 7.4) and 1ml of NADH solution containing 468 µM of NADH which is dissolved in 1ml of phosphate buffer (100 mM, pH 7.4) with 0.1 ml of test samples were added and the reaction was started by adding 100 µl of Phenazine methosulphate (PMS) solution containing 60 µM of PMS 100 µl of phosphate buffer (100 mM, pH 7.4). The reaction mixture was incubated at 25°C for 5 min and the absorbance at 560nm was measured against the control samples. BHT was used as the reference compounds (100 to 500 µg/ ml). The percentage of inhibition was calculated as mentioned below.

% of SOD = (Absorbance of control- Absorbance of test) / Absorbance of control × 100

Estimation of lipid peroxidation using egg yolks

Inhibitions of lipid peroxidation in the egg of hen were determined using a modified method thiobarbituric acid- reactive species (TBARS) assay.[15] Egg homogenate (0.5 ml, 10% in distilled water, v/v) was mixed with 0.1 ml of methanolic extract of *Nannochloropsis oculata* in a test tube and the volume was made up to 1 ml, by adding distilled water. Lastly, 0.05 ml $FeSO_4$ (0.07 M) was added to the above mixture to induce lipid peroxidation and incubated for 30 min. Then, 1.5 ml of 20% acetic acid and 1.5 ml of 0.8% TBA (w/v) in 1.1% sodium dodecyl sulfate (SDS) and 0.05 ml 20% TCA was added, vortexed and then heated in a boiling water bath for 60 min. After cooling, 5.0 ml of butanol was added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm.

% Inhibition= Absorbance of control- Absorbance of test / Absorbance of control × 100

β carotene linoleic acid assay

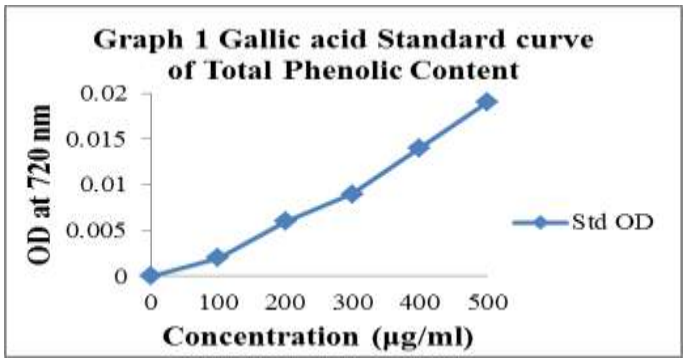
β- Carotene linoleic acid assay was carried out.[16] Briefly, in 10 ml of chloroform, 2 mg β-carotene, 200 mg linoleic acid and 20 mg Tween 40 were dissolved which was taken in flask. Chloroform was evaporated using vacuum evaporator apparatus. At that time, 50 ml of distilled water saturated with oxygen by shaking for 30 mins. This mixture is used as stock solution. 200 µl of methanolic extract of *Nannochloropsis oculata* were mixed with 2.5 ml of stock solution in the test tube. After that, the samples were placed in an oven at 50°C for 3 hours. The absorbance was read at 470 nm.

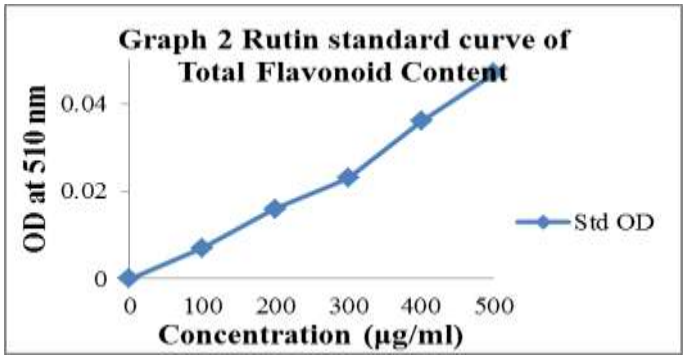
The percent of antioxidant activity was calculated from the following equation:

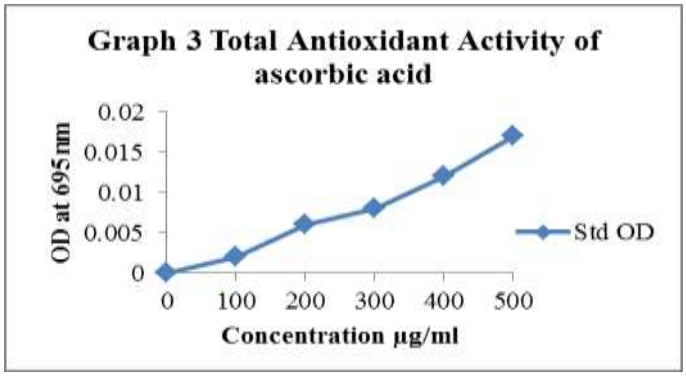
% Inhibition= Absorbance of control- Absorbance of test / Absorbance of control × 100

RESULTS

Quantification of Total Phenolics and Flavonoids





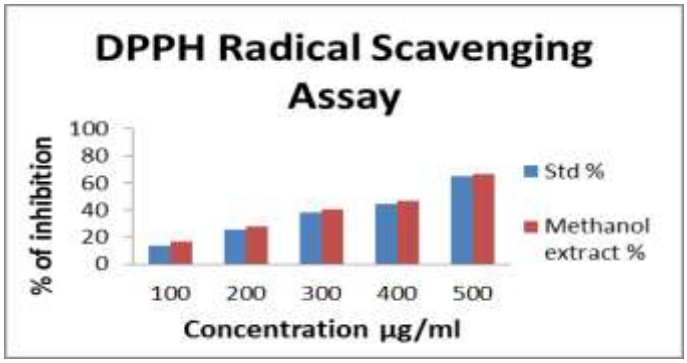


The different extract of *Nannochloropsis oculata* was estimated to test the Total Phenolic, Total Flavonoids and Total Antioxidant content along with standard Gallic acid, Rutin and Ascorbic acid. Based on the comparison with standard Gallic acid the better Phenolic content was founded in methanolic extract (4.12 mg equivalent standard drug/g dw) of *Nannochloropsis oculata* (**Table 1 and Graph 1**). The total flavonoid content was founded to be high in methanol extract i.e. 5.78 mg equivalent standard drug/g dw. (**Table 1 and Graph 2**). The total antioxidant activity of methanol extract of *Nannochloropsis oculata* was determined and the result was present in **Table 1** which is compared to the standard ascorbic acid represented in **Graph 3**. The highest total antioxidant was found in methanol extract which produces above 12.41 mg equivalent standard drug/g dw.

Table 1: Total Phenolic Content, Total Flavonoid Content and antioxidant activity of different extract of *Nannochloropsis oculata.*

Test name	**mg equivalent Standard drug/ g dw**
	Methanol extract of *Nannochloropsis Oculata*
Total Phenolic Content	4.12
Total Flavonoid content	5.78
Total antioxidant activity	12.41

DPPH radical scavenging assay

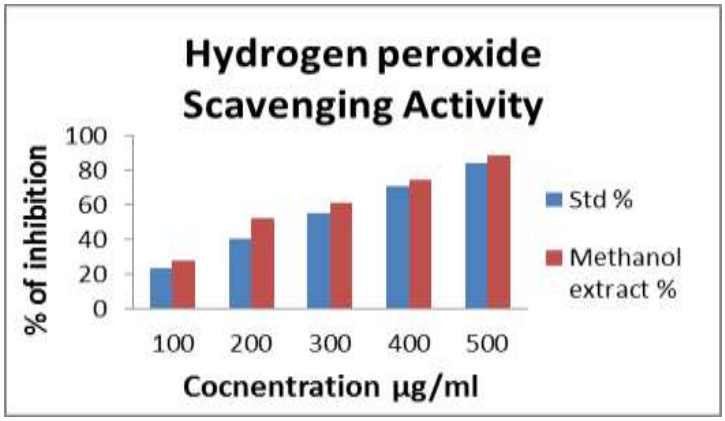


Graph 4: DPPH scavenging activity of standard and methanol extract of *Nannochloropsis Oculata.*

The DPPH scavenging activity was done for methanol extract of *Nannochloropsis oculata* along with standard ascorbic acid (**Graph 4).** The control OD value was recorded as 1.108 which is used to calculate the percentage of samples. For the standard, highest inhibition was shown at the concentration 500 µg/ml with inhibition of 64.71% and the lowest inhibition was found at the concentration 100 µg/ml (13.72%). At all concentration test samples possesses higher percentage of inhibition i.e 16.88% to 66.88% when compared with

standard drug. The IC 50 value of both standard and methanol extract was founded to be 403.815 and 387.030 µg/ml.

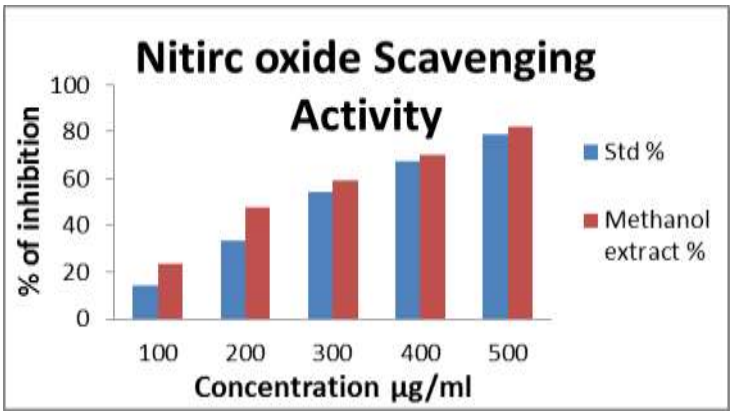
Hydrogen peroxide scavenging activity



Graph 5: Hydrogen peroxide scavenging activity of standard and methanol extract of *Nannochloropsis Oculata.*

From the **Graph 5**, it is concluded that the standard (23.68% to 84.26%) showed less activity when compared with the methanol extract (27.74% to 88.56%) which possessed higher Hydrogen peroxide Scavenging Activity. The IC 50 value of methanol extract was found to be 254.084µg/ml and the IC 50 values of standard was found to be 267.800µg/ml.

Nitric oxide scavenging activity



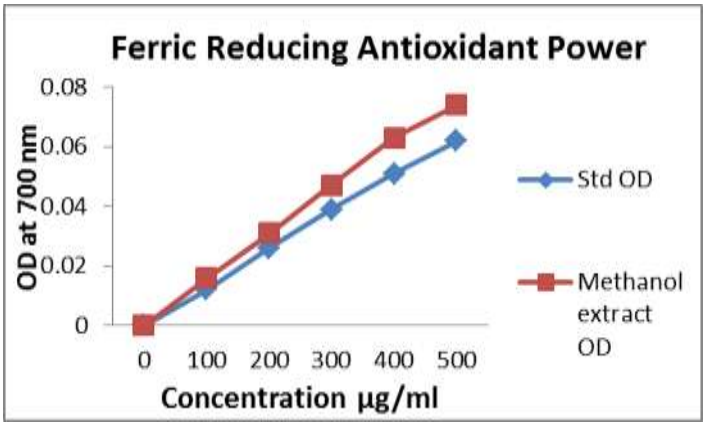
Graph 6: Nitric oxide scavenging activity of standard and methanol extract of *Nannochloropsis Oculata.*

In present study, the Nitric oxide activity of standard drug and methanol extract of *Nannochloropsis oculata* were determined and the results are presented in **Graph 6.** All these samples possessed the ability to scavenging Nitric oxide at various concentrations (100-

500µg/ml). The methanol extract showed the maximum scavenging activity (23.54% to 82.11%) with the IC 50 values 253.375 µg/ml. The scavenging effect of standard was founded to be from 14.53 to 78.75% with IC 50 values 302.609 µg/ml.

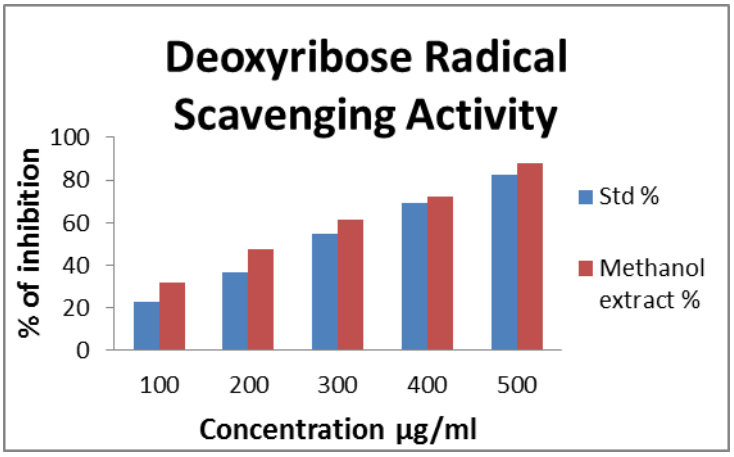
Ferric reducing antioxidant Power (FRAP)

The OD value of the standard in the range of 0.012 to 0.062 was recorded at concentration 100 – 500 µg/ml which shows lower activity than methanol extract which possess OD ranges from 0.016 to 0.074. The values were represented diagrammatically in **Graph 7.**



Graph 7: Ferric reducing antioxidant Power (FRAP) of standard and methanol extract of *Nannochloropsis Oculata.*

Deoxyribose Radical Scavenging Activity



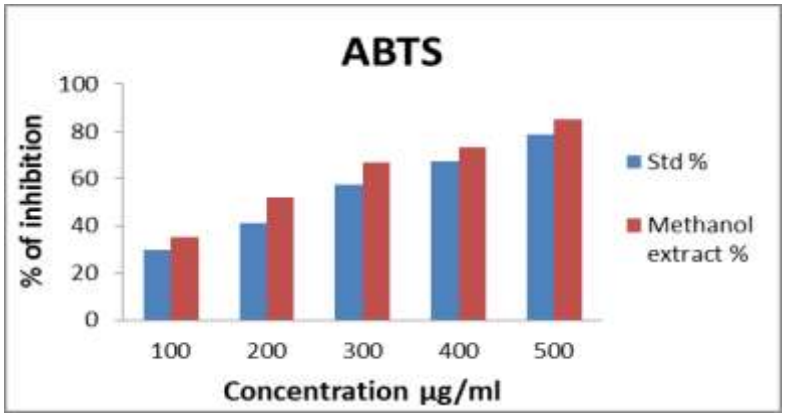
Graph 8: Deoxyribose Radical Scavenging Activity of standard and methanol extract of *Nannochloropsis Oculata.*

Table 8 indicates the percentage of inhibition against concentration in the range of 100 – 500 µg/ml for both standard and test samples. The methanol extract of *Nannochloropsis oculata* showed maximum of 87.62% of inhibition at 500 µg/ml concentration with IC 50 values

225.137 µg/ml and they are higher than that of the standard (82.45%) with IC 50 values of 279.100 µg/ml.

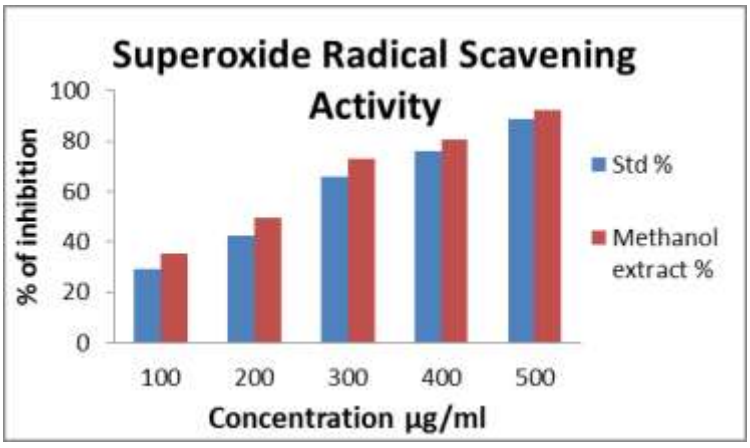
ABTS [2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] Radical Cation Scavenging Assay

Graph 9 demonstrates the ABTS activity of test samples and standard with different concentration (100- 500 µg/ml). From the result, it is determined that lowest percentage of inhibition was gained at 100 µg/ml for standard (29.96%) and highest values at 500 µg/ml (78.66%). When compared with standard, the test sample shows high percentage of inhibition at 500 µg/ml (85.23%) with IC 50 values 196.168µg/ml. Standard drug possess 260.203 µg/ml of IC 50 value.



Graph 9: ABTS [2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] Radical Cation Scavenging Assay of standard and methanol extract of *Nannochloropsis Oculata.*

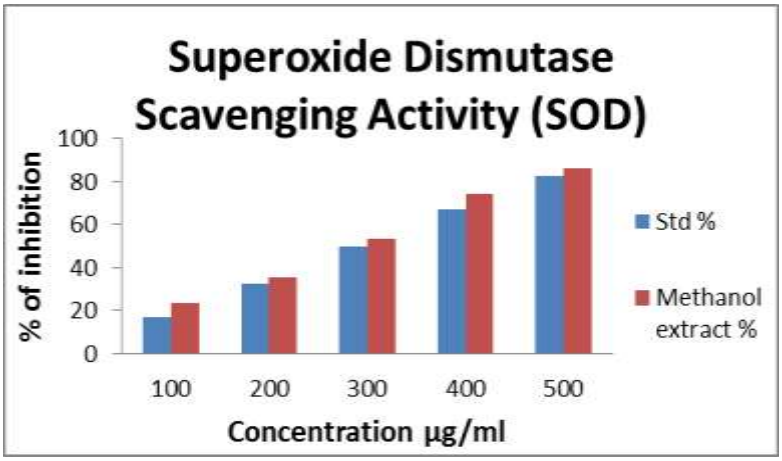
Superoxide radical scavenging activity



Graph 10: Superoxide radical scavenging activity of standard and methanol extract of *Nannochloropsis Oculata.*

Percentage of inhibition by the test sample was founded to be 92.27% which is slightly higher than standard drug 88.70% at concentration 500µg/ml. Similarly, the IC 50 value of standard was 230.790µg/ml which is higher than the IC 50 value of methanol extract (188.792µg/ml) **(Graph 10)**.

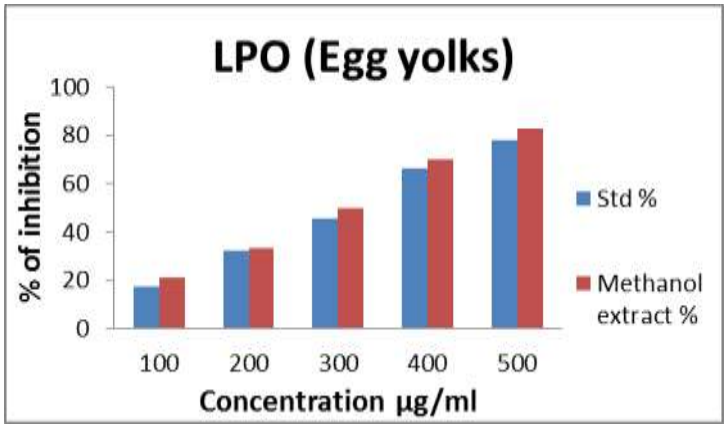
Superoxide Dismutase scavenging activity (SOD)



Graph 11: Superoxide Dismutase scavenging activity (SOD) of standard and methanol extract of *Nannochloropsis Oculata.*

Percentage of inhibition for standard showed 17.01% at 100 µg/ml and 82.41% at 500 µg/ml concentration which is compared with the methanol extract of *Chlorella vulgaris* which possess 23.70% at 100 µg/ml and 86.46% at 500 µg/ml concentration respectively. Therefore, the percentage of inhibition by test sample is higher than the standard drug **(Graph 11).** The IC 50 value of standard was 300.975 µg/ml and the test sample was 271.608 µg/ml.

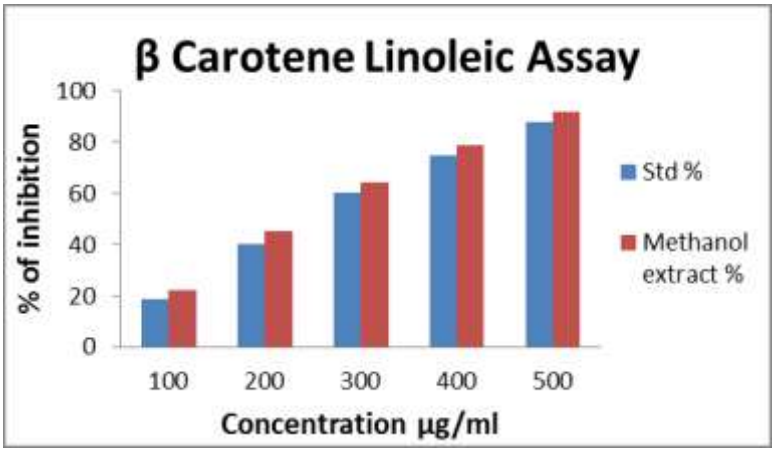
Estimation of lipid peroxidation using egg yolks



Graph 12: Estimation of lipid peroxidation using egg yolks of standard and methanol extract of *Nannochloropsis Oculata.*

Graph 12 explains the LPO scavenging activity by egg yolk of standard and test samples which shows 17.70% at 100 µg/ml and 78.01% at 500 µg/ml with IC 50 value 313.030 µg/ml for standard and test samples shows 21.34% at 100 µg/ml and 82.54% at 500 µg/ml which is slightly higher than the standard drug and possess IC 50 values of 291.410 µg/ml.

β carotene linoleic acid assay



Graph 13: β carotene linoleic acid assay of standard and methanol extract of *Nannochloropsis Oculata.*

The β carotene linoleic activity of test samples along with standard the concentration in the range of 100 – 500µg/ml was shown in **Graph 13**. The minimum activity was founded for methanol extract and the standard at the concentration 100 µg/ml was founded to be 22.11% and 18.48%. At concentration 500 µg/ml methanol extract (91.88%) showed higher activity than standard (87.74%). The IC 50 values of both standard and test samples were calculated which was found to be 263.549 µg/ml and 239.613 µg/ml based on their percentage of inhibition.

DISCUSSION

The use of antioxidants to prolong the shelf life of foodstuffs is ubiquitous. Today, mostly synthetic antioxidants, such as butylated hydroxytoluene (BHT) or butylated hydroxyanisole (BHA), are used. As these components are suspected carcinogens, there has been a search in recent years to replace these synthetic antioxidants with natural antioxidants.[19] Hence, in the present study the antioxidant activity of *Nannochloropsis oculata* was determined by various methods.

The antioxidant activity of *Chaetoceros* sp., a diatom and *Nannochloropsis* sp., a unicellular green microalga, both has been reported.[17] In their study, methanol extract of

Nannochloropsis sp., possess 30.60% which confirm the good antioxidant potential than other extract.

The present study was also correlated with the study of[18] which has been carried out to evaluate potential applications of aqueous extracts of two microalgae *Isochrysis galbana* and *Nannochloropsis oculata*. Antioxidant activity was evaluated from the DPPH scavenging activity. Microalgae *Isochrysis galbana* and *Nannochloropsis oculata* showed a concentration dependent DPPH radical scavenging activity. At concentration of 10 mg/mL, both microalgae *Isochrysis galbana* and *Nannochloropsis oculata* exhibit an antioxidant activity of 41.45% and 59.07%, respectively. *Nannochloropsis Oculata* produces 2.04±0.35 mg $GAEg^{-1}$ D.W of phenolic content and 40.68 ± 1.61 µmol trolox $eq.g^{-1}$ D.W of Ferric reducing antioxidant power was reported[19] which is compared with our present study.

On the other hand, studies microalgae are more common as the example reported that the DPPH scavenger activity on microalgal extracts for *Tetraselmis chuii*, *Nannochloropsis oculata*, *Chlorella minutissima* and *Rhodomonas salina*. The results obtained for the DPPH scavenger activity were higher for the higher concentrations (5 and 10 mg/ml) than those described by for methanol and hexane microalgae extracts, where the higher concentration was achieved by the hexane extract of *Nannochloropsis oculata* (70.3%) which was reported.[20]

CONCLUSION

Natural antioxidants plays potential role in protecting the cells against free radical induced damage. The diet rich in antioxidants from natural source can prevent and protect the cells in combating several diseases. *Nannochloropsis oculata*, among the Microalgae were underexplored for its nutritive value and radical scavenging potential. The present study proved the efficacy of *Nannochloropsis oculata* as antioxidants. Thus, microalgae can be used as natural antioxidant supplements.

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