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Evaluation of antioxidant potential and bioactive metabolites of *Nannochloropsis* sp.

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Abstract:

Marine microalgae are a photosynthetic organism which can produce diverse metabolites, biomass and O_2. The bioactive compounds produced by microalgae includes polysaccharides, polyunsaturated fatty acids, polyphenolic compounds, antioxidants, peptides, essential vitamins and minerals. The present study aimed to evaluate the antioxidant activity and bioactive metabolites of the marine microalgae *Nannochloropsis* sp. Various solvent (petroleum ether, aqueous, isopropanol, methanol and ethyl acetate) extract of *Nannochloropsis* sp. were evaluated for antioxidant activity. The ethyl acetate extract contributes maximum total antioxidant activity of 71.35 ± 0.04 mg of AAE/g of extract. The ethyl acetate extract exhibited highest on nitric oxide scavenging metal chelating and reducing power assay with % inhibition of 79.57 ± 0.215, 80.73±0.105, 0.64±0.001 at 1mg/concentration respectively. Methanolic extract showed hydroxyl radical scavenging activity of 78.72±0.21 % inhibition at a concentration of 1mg/ml. The ethyl acetate extract with highest antioxidant activity was analyzed for bioactive metabolites by GCMS. Among the 15 compounds obtained through GCMS, the major component n-Hexadecanoic acid was reported to have antioxidant activity. The results from the different types of extracts clearly indicated that the bioactive components present in the microalgae contribute significantly to the antioxidant capacity of microalgae.

Key words: Microalgae, *Nannochloropsis* sp., antioxidant, free radical scavenging, GCMS

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1. Introduction

The free radicals include reactive oxygen species (ROS) and reactive nitrogen species (RNS), are derived from both endogenous sources (mitochondria, peroxisomes, etc.) and exogenous sources (pollution, tobacco smoke, transition metals, radiation and certain drugs etc.) cause oxidative stress [1]. Free radicals are produced either directly or indirectly in the body and at high concentration, it damages all macromolecule by inducing DNA damage, alteration in protein structure, and lipid peroxidation. Imbalanced metabolism and over production of ROS generation leads to disorders such as, Parkinson's disease, Alzheimer's disease, aging and many other neural disorders [2]. Anti-oxidants are substances capable of reducing the oxidative stress by scavenging free radicals. Even at relatively low concentration, they have diverse physiological role in the body by inhibiting oxidation. Antioxidants are added in some foods to increase the shelf life and to prevent unwanted lipid oxidation [3]. Natural sources of antioxidant are flavonoids, anthocyanins, vitamins and some mineral compounds and synthetic antioxidants include butylhydroxyanisole, gallates, butylhydroxytoluene, etc. [4]. Many studies have revealed that the use of natural anti-oxidants can reduce the risk of oxidative stress induced diseases. Microalgae are microscopic unicellular or simple multicellular, photosynthetic microorganisms, which produces biomass and O_2. Diverse varieties of functional metabolites are produced by microalgae which includes oil, proteins, starch and pigments such as beta-carotene. The antioxidant compounds produced by them can be used in the prevention of age-related diseases, cardiovascular disorders, cancer and Alzheimer's disease [5]. The advantages of microalgae derived antioxidants have advantage over other source as because it is unicellular organism with high growth rate, and needs reduced space for their large cultivation. Microalgae such as *Chaetoceros* sp., a diatom, and *Nannochloropsis* sp., are reported as a potential and natural source of antioxidants [6].

2. Model and Method

2.1 Sample preparation

Dried microalgae cells of 10g were extracted in 100ml of different organic solvents specifically ethyl acetate, methanol, aqueous, isopropanol and petroleum ether for 7 days at room temperature and filtered. The crude extract was used for further studies.

2.2 Determination of Total antioxidant activity

The total antioxidant activity was evaluated by phosphomolybdenum method described by Prieto *et al.* 1999 [7]. 1.0 ml of the microalgal extract was mixed with 1.0 ml of the standard reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated at 95°C for 90 minutes. Cooled it to room temperature, measured the absorbance at 695 nm against a reagent blank. The total antioxidant capacity was expressed as milligram of Ascorbic Acid Equivalence (AAE) per gram of extract.

2.3 Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity of the extracts was determined according to the method reported by Klein *et al.*[8] .The reaction mixture contained 1.0 ml of different concentration of extracts (200µg -1000µg), 1.0 ml of iron-EDTA solution (0.13% ferrous ammonium sulphate 0.26% EDTA), 0.5 ml of 0.018% EDTA, 1.0 ml of DMSO (0.85 % in 0.1 M phosphate buffer pH7.4) and 0.5 ml of 0.22% ascorbic acid. The tubes were capped tightly and heated in a water bath at 80-90ºC for 15 minutes, the reaction was terminated by adding 1.0 ml of ice-cold TCA (17.5 %). To the above reaction mixture 3.0 ml of Nash reagent (75.0 g of ammonium acetate, 3.0 ml of glacial acetic acid and 2.0 ml of acetyl acetone were mixed and distilled water was added to a total volume of 1 L) was added and incubated at room temperature for 15 minutes for color development. The intensity of the yellow color formed was measured at 412 nm against a reagent blank. Ascorbic acid was taken as standard. The hydroxyl radical scavenging activity was calculated by the following formula. % of inhibition = Absorbance of control – Absorbance of test/Absorbance of control × 100.

2.4 Nitric oxide (NO) radical scavenging assay

Nitric oxide generated from sodium nitroprusside interacts with oxygen to produce nitrite ions which was measured by the Griess reaction. This assay was done by the procedure described by Green et al. [10]. The reaction mixture contained 3.0 ml of 10 mM sodium nitroprusside in phosphate buffered saline (pH 7.4) and various

concentration of (200µg -1000µg) extracts. The resulting solution was then incubated at 25ºC for 1 hour. To the incubated sample 5.0 ml of Griess reagent (1% sulphanilamide, 0.1% NED in 2% H3PO4) was added and the absorbance was measured at 546 nm against a reagent blank. Percentage inhibition of the nitrite ions generated was observed. Ascorbic acid was taken as standard. The free radical scavenging activity was determined by evaluating % inhibition as above.

2.5 Metal chelating activity

The ferrous ion chelating potential of the extracts was evaluated by the method followed by Dinis *et al* [9]. The reaction mixture contained 1.0 ml of various concentrations of the extracts (200µg -1000µg) and 0.05 ml of 2 mM FeCl3. The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine. The reaction mixture was shaken vigorously and left at room temperature for 10 min and the absorbance of the reaction mixture was measured at 562 nm against a reagent blank. A lower absorbance of the reaction mixture indicated a higher ferrous ion chelating ability. Ascorbic acid was taken as standard. The chelating ability was calculated by the following formula.

% of inhibition = Absorbance of control – Absorbance of test/Absorbance of control × 100.

2.6 Reducing power assay

The reducing power ability of the extracts was evaluated by the method described by Oyaizu [11]. The reaction mixture contained 1.0 ml of various concentrations of extracts (200µg - 1000µg), 2.5 ml of 1% potassium ferricyanide and 2.5 ml of 0.2 M sodium phosphate buffer. The mixture was incubated at 50°C for 30 min and the reaction was terminated by the addition of 2.5 ml of 10% TCA, followed by centrifugation at 3000 rpm for 10 minutes. 2.5 ml of the upper layer was mixed with 2.5 ml of deionized water and 0.5 ml of 0.1% ferric chloride. The absorbance was measured at 700 nm against blank. The reducing power ability of the sample is determined by increase in absorbance of the sample.

2.7 GC-MS Analysis of microalgae extract

The GC-MS analysis of ethyl acetate extract of *Nannochloropsis* sp. (EAN) was carried out using GC-MS QP2010 Ultra (Shimadzu) with Rxi-5Sil MS fused-silica capillary column of 0.25 mm diameter, 30 m length, and 0.25 mm film thickness. Helium gas (99.99%) was used as the carrier gas at the constant flow-rate of 1 ml/minute. An electron ionization system with ionization energy of 70 eV was used for analysis. The temperatures of the injector and mass transfer line were set at 250°C and 240°C, respectively. The oven temperature was programmed from 80°C to 200°C at 3°C /minute, and finally increased to 260 °C at 10°C /minute. Aliquots of 1 ml of the diluted samples were injected in split mode with a split ratio of 1:10 and with a mass scan range of 45 - 900 AMU. The total running time of the GC-MS analysis was 51 minutes.

2.8 Statistical analysis

The data were analyzed and expressed as means ± SD. The variance was analyzed using one-way ANOVA (SPSS.ver.22).

3 Result and discussion

3.1 Antioxidant activity

Reactive oxygen species (ROS) includes free radicals such as hydroxyl radical, nitric oxide ferric ion etc. and over production of these radical can lead to diseases. External supply of antioxidants may overcome the effect of free radicals on the body, and in turn can prevent the occurrence of many diseases. Some algae are considered as rich sources of natural antioxidants [12].The various solvent extract of *Nannochloropsis* sp.: ethyl acetate (EN), methanol(MN) , isopropanol(IN), aqueous(AN) and petroleum ether (PN) were determined for the total antioxidant activity. All experiments are done in triplicate.

3.2 Determination of total antioxidant activity

The ethyl acetate extract exhibited maximum total antioxidant activity of 71.35 ± 0.04 mg of AAE/g of extract. The total antioxidant activity was found to be highest in EN followed by MN, IN, AN, PN. Antioxidant property was determined through phosphomolybdenum method, which is based on the subsequent formation of Mo (IV) to Mo(V) with a maximum absorption at 695nm and the absorbance is directly proportional to the antioxidant activity [13]. Antioxidant activity involves free radical scavenging which relay upon the tendency of electron or hydrogen donations [14]. Anuradha *et al* reported that the total antioxidant activity of methanolic extract of *Nannochloropsis oculate* was 12.41 AAE/g of extract [15]. In the present study, EN was found to have better antioxidant activity when compared to previous studies. Figure. 1 shows the total antioxidant activity of various extract of *Nannochloropsis* sp.

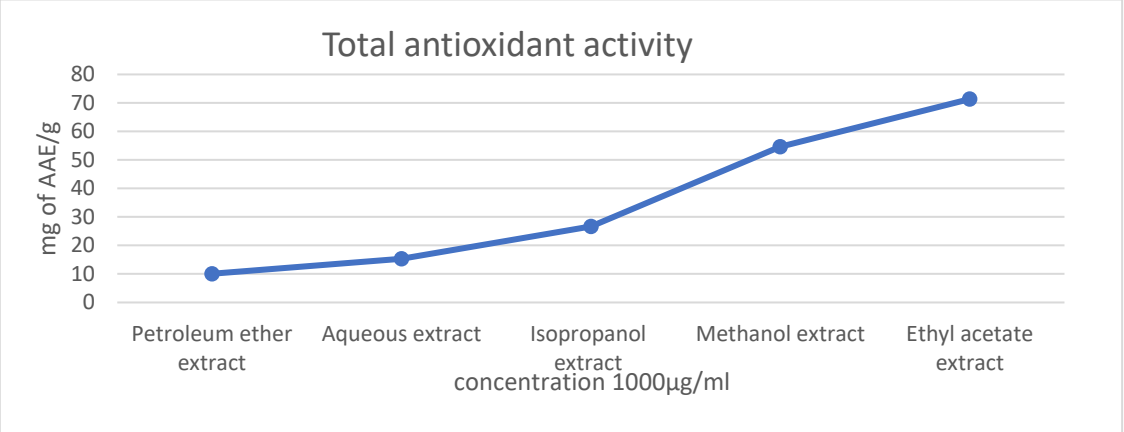

Fig. 1. Total Antioxidant activity

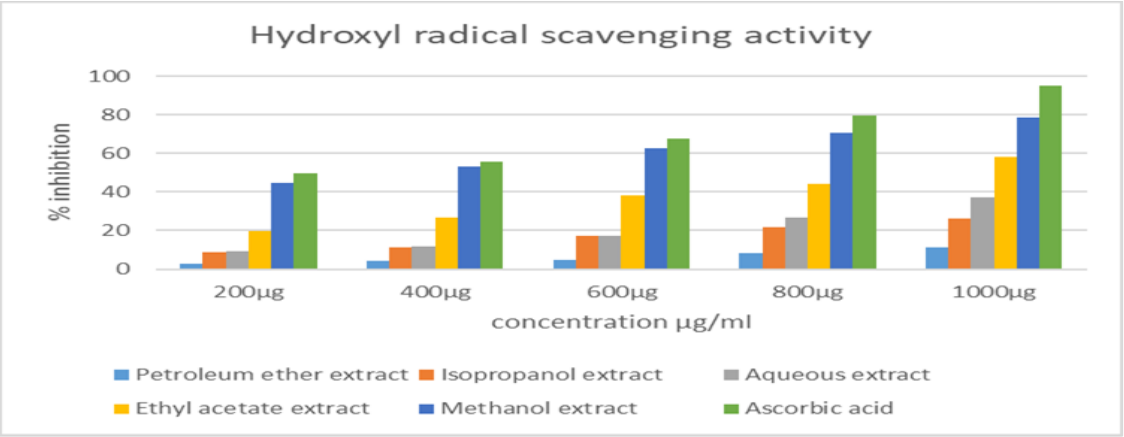

Fig.2. Hydroxyl radical scavenging activity

3.3 Hydroxyl radical scavenging assay

One of the most reactive oxygen centered species is hydroxyl radical which causes severe damage to nearby biomolecule. The activity of the extract was found in the order: MN> EN> AN> IN and PN. The level of inhibition is between 2.63 ± 0.05 to 78.72 ± 0.21 % at varying concentration between various extracts. All the extracts scavenged hydroxyl radicals in a concentration dependent manner. Variance in hydroxyl radical scavenging assay between various concentrations was found to be significant ($P<0.01$). Hydroxyl radical cause severe damage to the cells compared to other ROS [16]. It induces severe damage to biomolecules in the body, leading to pathological condition such as ageing, cancer and several other diseases [17]. The EN was found to contain better activity when compared to aqueous extracellular polysaccharides of *Graesiella* sp. (66% inhibition) reported by Lamia etal [18]. Figure 2 shows the inhibition of hydroxyl radical by various solvent extract.

3.4 Nitric oxide radical scavenging assay

NO radical scavenging activity of various extracts is presented in Figure 3. The NO production was effectively reduced by most of the extracts of *Nannochloropsis* sp. EN (79.57± 0.21) and MN (78.72± 0.21) showed almost same level of inhibition of NO radical when compared to other extracts. All the extract showed dose dependent activity. The inhibitory effect among various extract in the order was found to be EN >MN >IN > AN > IN. Variance among the various level of extract was found to be significant. ($P<0.01$). NO is an intercellular messenger molecule with diverse role such as modulating blood flow, thrombosis, and neural activity. Excess generation of NO due to deregulation can neurotoxic cascade[19].When NO reacts with superoxide radical, it is converted to toxic and highly reactive peroxynitrite anion ($ONOO^-$) [20]. Anantharaman *etal* reported that the methanolic extract of microalgae *I. galbana* showed 37.33% NO scavenging activity [21] and was found to be lower than the activity of EN.

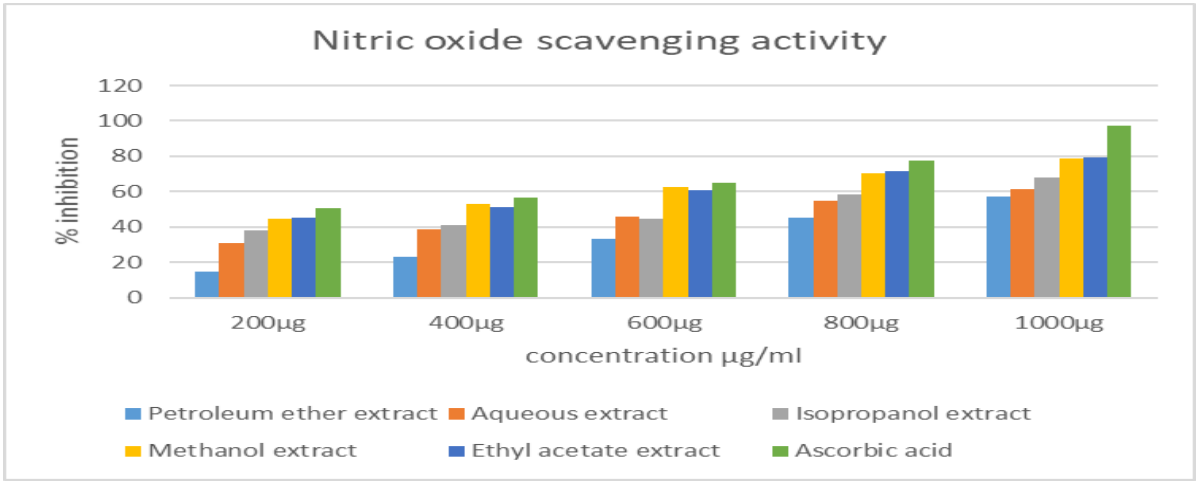

Fig.3. NO scavenging activity

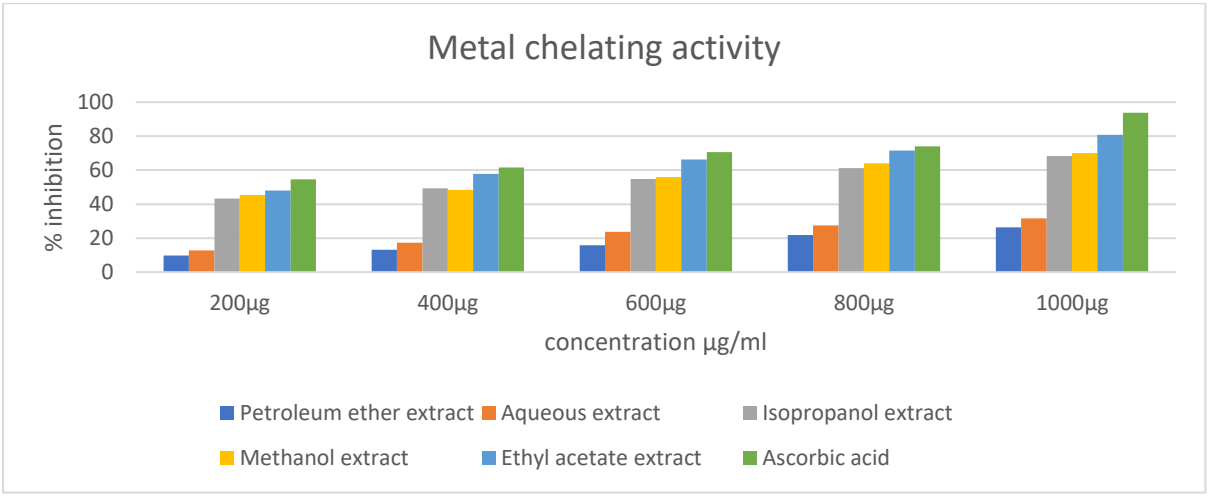

Fig.4. Metal Chelating activity

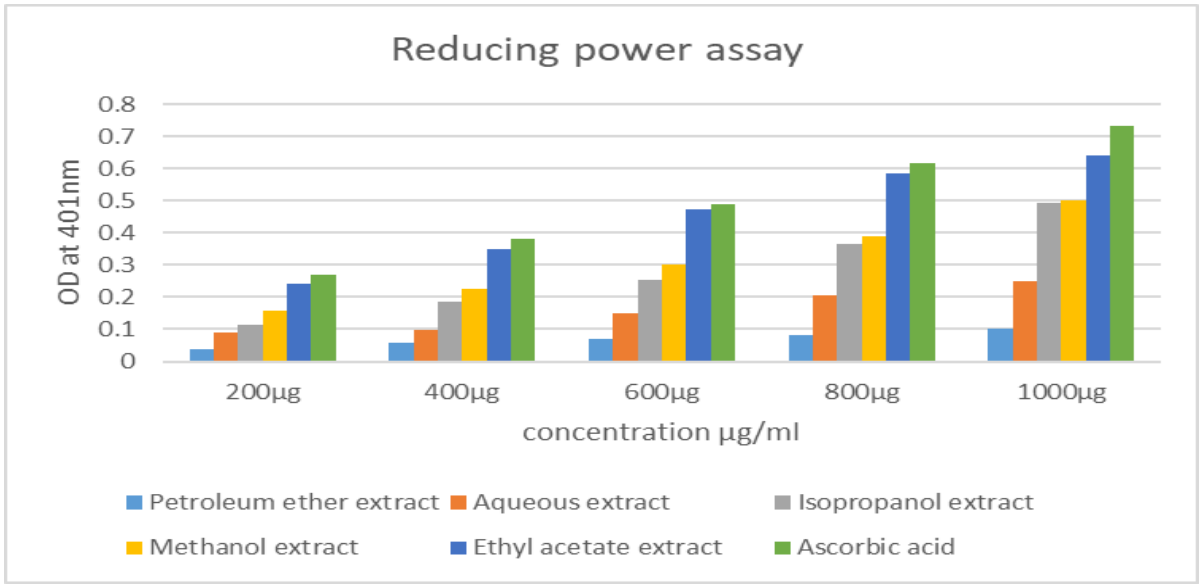

Fig.5. Reducing power assay

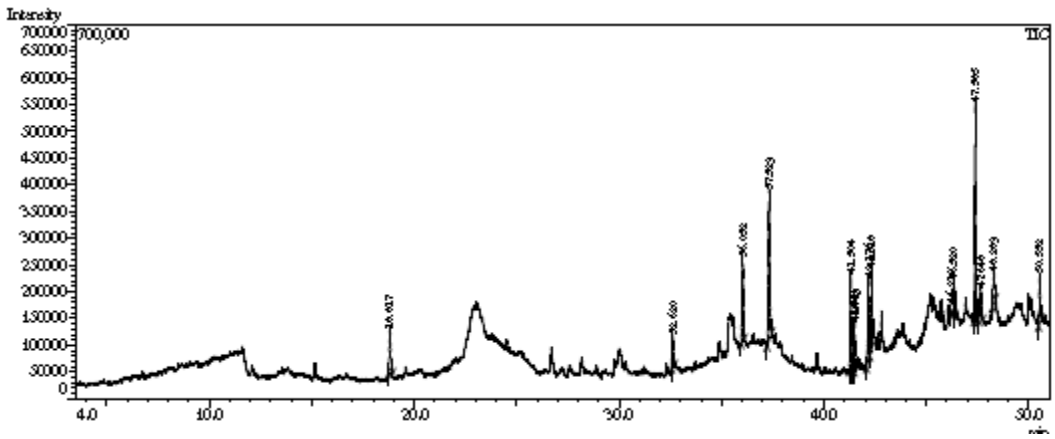

Fig. 6. GCMS analysis of EN

Table.1. GCMS analysis of EN

Peak	R.time	Area	Area %	Compound name
1	18.817	423664	5.51	Phenol, 4-ethenyl-2,6-dimethoxy-
2	32.620	218543	2.84	i-Propyl 12-methyltetradecanoate
3	36.032	677462	8.82	Hexadecanoic acid, methyl ester
4	37.329	1312708	17.08	n-Hexadecanoic acid
5	41.304	554943	7.22	9,12-Octadecadienoic acid (Z,Z)-, methyl ester
6	41.4292	264704	3.45	7,10,13-Hexadecatrienoic acid, methyl ester
7	41.489	257344	3.35	9-Octadecenoic acid, methyl ester
8	42.176	615861	8.02	9,12-Octadecadienoic acid (Z,Z)-
9	42.318	736610	9.59	Oleic Acid
10	46.210	211882	2.76	3-Hydroxy-6-isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydro-2-naphthalenyl acetate
11	46.320	298726	3.89	Lup-20(29)-en-3-ol, acetate, (3.beta.)-
12	47.385	1080422	14.06	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester
13	47.648	384107	5.00	1,2-Benzenedicarboxylic acid
14	48.289	306895	3.99	Gamma.-Sitosterol
15	50.532	339633	4.42	Octadecanoic acid, 2,3-dihydroxypropyl ester

3.5 Reducing power assay

When compared to other extracts EN exhibited highest reducing power ability at 0.64 ± 0.001 OD at a concentration of 1mg/ml. Reducing power ability of various extracts was found in the order same as that of metal chelating activity. At 1mg concentration, IN and MN exhibited almost similar activity of 0.49 ± 0.001 and 0.5 ± 0.001 OD respectively. The extracts showed good reducing power ability in a dose dependent manner. Compounds having reducing power can donate electrons and can inhibit lipid peroxidation by reducing the oxidized intermediate [21]. The reductones present in the extract exhibit the antioxidant activity. Reducing power of the extracts increases with the increase in concentration. Figure 5 shows the reducing power of various extract. Variance in reducing power assay between various levels of extract is ($P<0.01$). Previous study reported that ethyl acetate extract of *N. oculate* showed an OD values of 0.73 at 1600µg [24] and the result found in the present study reveals that EN is having significant reducing power when compared with previous studies.

3.6 GC-MS analysis

The highest antioxidant activity was noticed in ethyl acetate extract and was analyzed through GCMS. The GC-MS analysis showed a total of 15 metabolites. Table 1 shows the list of compounds analyzed through GCMS and the figure 6 shows the graph obtained by GCMS analysis. The major compounds and its area percentage are n-Hexadecanoic acid (17.08%), Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester (14.06%) and oleic acid (9.59%). Most of the compounds obtained through GCMS analysis were reported to have biological activities. n-Hexadecanoic acid are reported to have antioxidant, hypocholesterolemic nematicide, pesticide, anti-androgenic flavor, hemolytic, 5-Alpha reductase inhibitor [25] and the result suggests that this compound contributes the antioxidant property.

4. Conclusion

The various solvent extracts of microalgae, *Nannochloropsis* sp. was found to contain antioxidant property. Of the various extracts analyzed, the ethyl acetate extract was found to contain highest activity on nitric oxide scavenging, metal chelating activity and reducing power. The highest hydroxyl radical scavenging was noticed in methanolic extract. The potent extract, EN analyzed through GCMS shows that most of the compounds were fatty acid origin and were reported to have bioactivities. The compound n-Hexadecanoic acid found in the microalgae analyzed through GCMS was reported to have antioxidant activity. The results suggest that the microalgae, *Nannochloropsis* sp. possess antioxidant potential, which could be considered for future applications in medicine against free radical induced diseases.

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