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RESEARCH ARTICLE

IN VITRO ANTIOXIDANT ACTIVITY OF METHANOLIC EXTRACT OF *CHLORELLA VULGARIS.*

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Abstract

The present study focuses on the quantification of total phenolics and flavonoids using Gallic acid and Rutin as standard respectively. The antioxidant activity of methanolic extract of *Chlorella vulgaris* was also determined. The antioxidant and free radical scavenging potential was due to the high content of phenolics [0.022 ± 0.015 µg/ml] and flavonoids [0.054 ± 0.031 µg/ml]. The microalga extracts with good antioxidant capacities can be explored for new natural antioxidant sources.

Introduction:-

Chlorella vulgaris is a single-celled eukaryotic green micro algae, known to be first form of a plant with a well-defined nucleus emerged over 2 billion years ago. *Chlorella vulgaris* contains the highest amount of chlorophyll of any known plant. *Chlorella vulgaris* is well determined nutrient-dense superfood containing 60% protein, 18 amino acids, 20 vitamins and minerals like iron, calcium, potassium, magnesium and phosphorous. One of its unique properties is a phytonutrient called Chlorella Growth Factor (Nick, 2003).

In recent times, studies suggest that there is an inverse relationship between dietary intake of antioxidant rich foods and the incidence of human disease. The search to replace these synthetic antioxidants with natural antioxidants has become an essential deed in immune pharmacy discovery since these components are suspected carcinogen (Goiris*et al.*, 2012). Antioxidants are presumed to have several positive health effects that include prevention of cardiovascular disorders, ageing related diseases such as Alzheimer and certain types of cancer (Shibata *et al.*, 2006).

However, some recent studies showed that several classes of flavonoids, such as isoflavones, flavanones, flavonols, and dihydrochalcones found in microalgae demonstrates that they are able to produce more complex phenolic compounds. Therefore, characterization and identification of phenolic compounds in microalgae are required, especially as they may contain novel phenolic compounds. Based on this suggestion it is founded that both phenolic and carotenoids contributed significantly to the antioxidant capacity of micro algae (Natrah, 2007). Therefore, the present work was carried out to assess the antioxidant and free radical scavenging potential of methanolic extract of *Chlorella vulgaris.*

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Materials and Methods:-

1. Sample preparation

1 gram dried powder of *Chlorella vulgaris* was mixed with 10 ml of methanol solvent and it is kept at room temperature for 24 hours. This mixture was filtered using Whatmann No.1 filter paper and stored at 4ºC for further analysis.

2. Quantification of Total Phenolics and Flavonoids

Total phenolic contents were assessed by following strategy: 100 µl of methanol extract of *Chlorella vulgaris* 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% FolinCiocalteau'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. (Taga, *et al*., 1984).

The total flavonoid content of tests was detected by the aluminum chloride colorimetric method (Chang, *et al*., 2002). 0.5 ml of methanol extract of *Chlorella vulgaris* 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 (Suganya, *et al.*, 2017)

Total antioxidant activity

The total antioxidant capacity of methanol extract of *Chlorella vulgaris*was evaluated by Prieto P *et al*., 1999. 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. The concentration in the range of 100 to 500 µl were taken for both standard and test samples.

DPPH radical scavenging assay

The scavenging activity of methanol extract of *Chlorella vulgaris* for DPPH radical were identified by the method of (Yen and Chen, 1995). 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 varied concentration in the range of 100 to 500 µl were taken for both standard and methanol extract of *Chlorella vulgaris*. 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 of Ruch*et al*., 1989. 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 methanol extract of *Chlorella vulgaris* 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

Nitric oxide scavenging activity

Nitric oxide scavenging activity was performed based on the following procedure: 3ml of 10 mM of sodium nitroprusside was prepared in phosphate buffer saline (pH 7.4, 0.2 M) which was mixed with 1 ml of methanol extract of *Chlorella vulgaris* and incubated at 25°C for 180 mins. By mixing equal amounts of 1% sulphanilamide in 2.5% phosphoric acid and 0.1% naphthylethylenediaminedihydrochloride in 2.5% phosphoric acid the Griess reagent was prepared immediately before use. The sample was mixed with an equal volume of freshly prepared Griess reagent. The absorbance was measured at 546 nm. Ascorbic acid was used as the positive control. The

percentage inhibition of the extract and standard was calculated and recorded (Fadzai Boora*et al.,* 2014). The percentage nitrite radical scavenging activity of the ethanol extracts and Gallic acid were calculated using the following formula:

Nitric oxide activity (%) = Absorbance of control- Absorbance of test / Absorbance of control × 100.

Ferric reducing antioxidant Power (FRAP):-
Reducing power of methanol extract of *Chlorella vulgaris* was carried out by the method prescribed by Oyaizu M, 1986. To 1.0 ml of methanol extract of *Chlorella vulgaris*, 2.5 ml of Phosphate buffer (0.2 M, pH 6.6) and 2.5 ml Potassium ferricyanide (1%) was mixed. Reaction mixture was incubated at 50°C for 20 min. After incubation, 2.5 ml of Trichloroacetic acid (10%) was added and centrifuged (650 g) for 10 min. From the upper layer, 2.5 ml solution was taken and mixed with 2.5 ml distilled water and 0.5 ml $FeCl_3$ (0.1%). Absorbance of all the sample solutions was measured at 700 nm. Increased absorbance is indicated increased reducing power.

Deoxyribose Radical Scavenging Activity:-
Deoxyribose non-site specific hydroxyl radical scavenging activity of methanol extract of *Chlorella vulgaris* was estimated (Indu and Seenivasan, 2013). 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 cationdecolorization assay (Re R *et al*., 1999). 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 methanol extract of *Chlorella vulgaris*(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 by (Winterbourn*et al*., 1975). Assay tubes contained 0.2 ml of methanol extract of *Chlorella vulgaris*(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 (Nishimiki*et al.,* 1972), with slight modifications. To, 1ml of NitroblueTetrazolium (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 Phenazinemethosulphate (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 (200 to 1000 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 (Badmus*et al.*, 2013). Egg homogenate (0.5 ml, 10% in distilled water, v/v) was mixed with 0.1 ml of methanol extract of *Chlorella vulgaris* 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 based on (Zargar*et al.*, 2011). 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 methanol extract of *Chlorella vulgaris* 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

Result:-

Quantification of Total Phenolics and Flavonoids

Figure 1:-Total phenolic content of Gallic acid standard

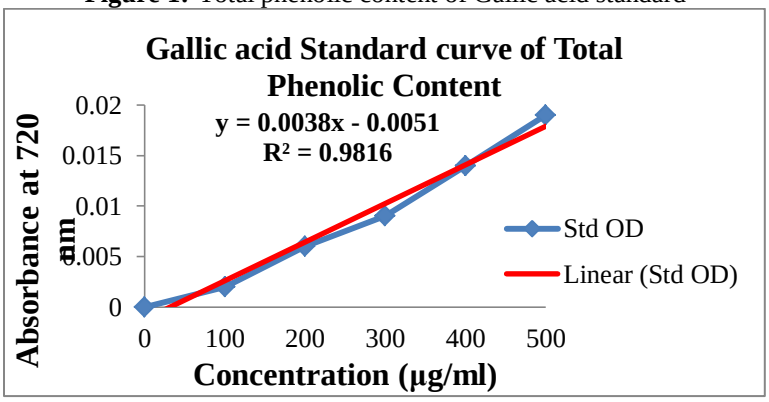

Figure II:-Total flavonoids content of Rutin standard

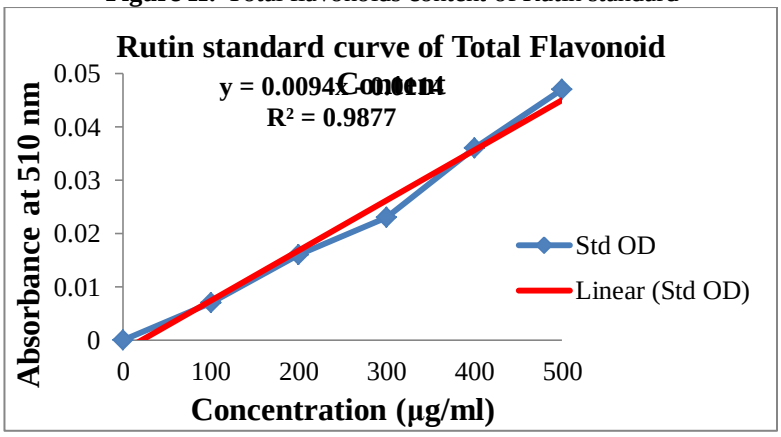

Table (1):-Total Phenolic Content and Total Flavonoid Content of methanol extract of *Chlorella vulgaris*

S.No	Test name	mg equivalent Standard drug/ g dw
I	Total Phenolic Content	2.13 mg equivalent Gallic acid/ g dw
II	Total Flavonoid content	2.49 mg equivalent Gallic acid/ g dw

Total Phenolic Content and Total Flavonoid Content of methanol extract of *Chlorella vulgaris* was estimated along with standard. Gallic acid and Rutin standard curve was prepared based on the OD values with different concentration in the range of 100 – 500 µg/ml represented in **Fig I & Fig II** . When compared with standard Gallic acid and Rutin, the test samples produces 2.13 mg equivalent of Gallic acid/ g dw for Total Phenolic Content and 2.49 mg equivalent of Rutin/ g dw for Total Flavonoid Content **(Table 1)** based on the OD obtained.

3. *In vitro* Antioxidant and Free Radical Scavenging activity of methanol extract of *Chlorella vulgaris*

Total antioxidant activity

Table (2):-Total antioxidant activity of standard and methanol extract

S.No	Concentration µg/ml	Standard OD Mean ± SD	Methanol extract OD Mean ± SD
1	**100**	0.002 ± 0.006	0.006 ± 0.004
2	**200**	0.006 ± 0.013	0.011 ± 0.016
3	**300**	0.008 ± 0.009	0.013 ± 0.123
4	**400**	0.012 ± 0.005	0.017 ± 0.012
5	**500**	0.017 ± 0.011	0.021 ± 0.009

Table 2 denotes the total antioxidant activity of Ascorbic acid standard and methanol extract of *chlorella vulgaris*. The total antioxidant activity of *chlorella vulgaris* at the concentration of 500 µg/ml (0.021 ± 0.009) indicates higher activity whereas, at the concentration of 100 µg/ml (0.06 ± 0.004) indicates lower activity and the concentration of 300 µg/ml and 400 µg/ml (0.013 ± 0.123 to 0.017 ± 0.012) indicates moderate activity when related with standard ascorbic acid (0.008 ± 0.009 to 0.012 ± 0.005).

Dpph Radical Scavenging Assay

Table (3):-DPPH radical scavenging assay of standard and methanol extract

S.No	Concentration µg/ml	Standard OD Mean ± SD	Standard % Mean ± SD	Methanol extract OD Mean ± SD	Methanol extract % Mean ± SD	Control OD
1	**100**	0.956 ± 0.782	13.72 ± 0.153	0.921 ± 0.098	16.87 ± 0.172	1.108
2	**200**	0.821 ± 0.624	25.90 ± 0.108	0.765 ± 0.087	30.95 ± 0.183	
3	**300**	0.685 ± 0.054	38.17 ± 0.387	0.615 ± 0.067	44.49 ± 0.123	
4	**400**	0.612 ± 0.542	44.77 ± 0.472	0.557 ± 0.046	49.72 ± 0.201	
5	**500**	0.391 ± 0.298	64.71 ± 0.186	0.325 ± 0.034	70.66 ± 0.187	
IC 50 Values		-	403.815	-	359.058	

In present study, the DPPH activity of ascorbic acid and methanol extract were determined and the results (OD values and percentage) are presented in **Table 3.** All these samples possessed the ability to scavenging DPPH at various degrees. The methanol extract showed the maximum DPPH radical scavenging activity (16.87% to 70.66%) with the IC 50 values 359.058 µg/ml. The scavenging effect of standard ascorbic acid was founded to be from 13.72 to 64.71 % with IC 50 values 403.815 µg/ml.

Hydrogen peroxide scavenging activity

Table (4):-Hydrogen peroxide scavenging activity of standard and methanol extract

S.No	Concentration µg/ml	Standard OD Mean ± SD	Standard % Mean ± SD	Methanol extract OD Mean ± SD	Methanol extract % Mean ± SD	Control OD
1	**100**	1.615 ± 0.003	23.68 ± 0.002	1.557 ± 0.009	26.41 ± 0.008	2.116
2	**200**	1.259 ± 0.006	40.50 ± 0.010	1.213 ± 0.008	42.67 ± 0.005	
3	**300**	0.954 ± 0.012	54.91 ± 0.005	0.907 ± 0.004	57.13 ± 0.004	
4	**400**	0.612 ± 0.021	71.08 ± 0.008	0.546 ± 0.006	74.19 ± 0.122	
5	**500**	0.333 ± 0.021	84.26 ± 0.012	0.269 ± 0.012	87.28 ± 0.007	
IC 50 Values		-	267.800	-	250.829	

The hydrogen peroxide scavenging activity of test samples along with standard the concentration in the range of 100 – 500µg/ml was shown in **Table 4**. The minimum activity was founded for methanol extract at the concentration 100 µg/ml (26.41%). Similarly, the standard ascorbic acid possesses 23.68%. At concentration, 500 methanol extract (87.28%) showed higher activity than standard (84.26%). The IC 50 values of both standard and test samples were calculated which was found to be 267.800 µg/ml and 250.829 µg/ml based on their percentage of inhibition.

Nitric oxide scavenging activity:-

The OD values, Percentage of inhibition and IC 50 values were noticed in **Table 5.** The control values were also recorded (1.934) which is used to calculate the percentage of samples. The nitric oxide scavenging activity was done for methanol extract of *Chlorella vulgaris* along with standard ascorbic acid. The highest inhibition was shown at the concentration 500 µg/ml with inhibition of 74.30 %. The lowest inhibition was found at the concentration 100 µg/ml (12.66%). At all concentration test samples possesses lower percentage of inhibition when compared with standard ascorbic acid which produced 14.53% to 78.75% of inhibition.

Table (5):-Nitric oxide scavenging activity of standard and methanol extract

S.No	Concentration µg/ml	Standard OD Mean ± SD	Standard % Mean ± SD	Methanol extract OD Mean ± SD	Methanol extract % Mean ± SD	Control OD
1	**100**	1.653 ± 0.010	14.53 ± 0.006	1.689 ± 0.006	12.66 ± 0.003	1.934
2	**200**	1.291 ± 0.003	33.25 ± 0.002	1.301 ± 0.011	32.73 ± 0.005	
3	**300**	0.889 ± 0.101	54.03 ± 0.120	1.009 ± 0.006	48.19 ± 0.002	
4	**400**	0.632 ± 0.005	67.32 ± 0.011	0.765 ± 0.002	60.44 ± 0.132	
5	**500**	0.411 ± 0.021	78.75 ± 0.008	0.497 ± 0.120	74.30 ± 0.011	
IC 50 Values		-	302.609	-	328.717	

Ferric reducing antioxidant Power (FRAP)

Table (6):-Ferric reducing antioxidant power of standard and methanol extract

S.No	Concentration µg/ml	Standard OD Mean ± SD	Methanol extract OD Mean ± SD
1	**100**	0.012 ± 0.322	0.006 ± 0.101
2	**200**	0.026 ± 0.120	0.019 ± 0.235
3	**300**	0.039 ± 0.005	0.028 ± 0.121
4	**400**	0.051 ± 0.111	0.041 ± 0.210
5	**500**	0.062 ± 0.158	0.055 ± 0.146

Standard in the range of 0.012 ± 0.322 to 0.062 ± 0.158 was recorded at concentration 100 – 500 µg/ml which shows higher activity than methanol extract whose OD ranges from 0.006 ± 0.101 to 0.055 ± 0.146. The values were denoted in **Table 6.**

- **Deoxyribose Radical Scavenging Activity**

From the **Table** 7, it is concluded that the standard (23.00% to 82.45%) showed less activity when matched with the test samples (27.15% to83.79 %) which possessed higher Deoxyribose Radical Scavenging Activity. The IC 50 value of methanol extract was found to be 247.066 µg/ml and the IC 50 values of standard was found to be 279.100 µg/ml.

Table (7):-Deoxyribose Radical Scavenging Activity of standard and methanol extract

S.No	Concentration µg/ml	Standard OD Mean ± SD	Standard % Mean ± SD	Methanol extract OD Mean ± SD	Methanol extract % Mean ±SD	Control OD
1	**100**	1.891 ± 0.021	23.00 ± 0.012	1.789 ± 0.006	27.15 ± 0.021	2.456
2	**200**	1.559 ± 0.021	36.52 ± 0.009	1.334 ± 0.122	45.68 ± 0.004	
3	**300**	1.111 ± 0.008	54.76 ± 0.002	1.001 ± 0.004	59.24 ± 0.008	
4	**400**	0.759 ± 0.004	69.10 ± 0.011	0.718 ± 0.008	70.76 ± 0.003	
5	**500**	0.431 ± 0.012	82.45 ± 0.003	0.398 ± 0.012	83.79 ± 0.004	
IC 50 Values		-	279.100	-	247.066	

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

Table 8:-ABTS [2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] Radical Cation Scavenging Assay of standard and methanol extract

S.No	Concentration µg/ml	Standard OD Mean ± SD	Standard % Mean ± SD	Methanol extract OD Mean ± SD	Methanol extract % Mean ± SD	Control OD
1	**100**	0.512 ± 0.009	29.96 ± 0.018	0.489 ± 0.013	33.10 ± 0.004	0.731
2	**200**	0.432 ± 0.121	40.90 ± 0.005	0.412 ± 0.021	43.63 ± 0.011	
3	**300**	0.310 ± 0.010	57.59 ± 0.010	0.289 ± 0.007	60.46 ± 0.007	
4	**400**	0.237 ± 0.006	67.58 ± 0.004	0.215 ± 0.002	70.58 ± 0.012	
5	**500**	0.156 ± 0.014	78.66 ± 0.122	0.137 ± 0.017	81.25 ± 0.005	
IC 50 Values		-	260.203	-	236.682	

Table 8 indicates the percentage of inhibition against concentration in the range of 100 – 500 µg/ml for both Gallic acid standard and test samples. The methanol extract of *chlorella vulgaris* showed maximum of 81.25 % of inhibition at 500 µg/ml concentration with IC 50 values 236.682 µg/ml and they are slightly higher than that of the standard Gallic acid (78.66%) with IC 50 values of 260.203 µg/ml.

Superoxide radical scavenging activity:-

Table 9 explains the superoxide radical scavenging activity of standard and test samples which shows 29.14 % at 100 µg/ml and 88.70% at 500 µg/ml with IC 50 value 230.799 µg/ml for standard and shows 30.72 % at 100 µg/ml and 89.90 % at 500 µg/ml which is slightly higher than the standard drug with IC 50 values of 214.507 µg/ml.

Table (9):- Superoxide radical scavenging activity of standard and methanol extract

S.No	Concentration µg/ml	Standard OD Mean ± SD	Standard % Mean ± SD	Methanol extract OD Mean ± SD	Methanol extract % Mean ± SD	Control OD
1	**100**	0.715 ± 0.012	29.14 ± 0.007	0.699 ± 0.005	30.72 ± 0.005	1.009
2	**200**	0.579 ± 0.004	42.62 ± 0.011	0.538 ± 0.002	46.67 ± 0.132	
3	**300**	0.342 ± 0.011	66.11 ± 0.003	0.321 ± 0.013	68.18 ± 0.006	
4	**400**	0.239 ± 0.004	76.31 ± 0.012	0.213 ± 0.006	78.88 ± 0.002	
5	**500**	0.114 ± 0.006	88.70 ± 0.011	0.101 ± 0.001	89.90 ± 0.114	
IC 50 Values		-	230.790	-	214.507	

Superoxide Dismutase scavenging activity (SOD)

Table (10):-Superoxide Dismutase scavenging activity (SOD) of standard and methanol extract

S.No	Concentration µg/ml	Standard OD Mean ± SD	Standard % Mean ± SD	Methanol extract OD Mean ± SD	Methanol extract % Mean ± SD	Control OD
1	**100**	1.005 ± 0.012	17.01 ± 0.003	0.699 ± 0.005	30.72 ± 0.005	1.211
2	**200**	0.814 ± 0.003	32.78 ± 0.001	0.538 ± 0.002	46.67 ± 0.012	
3	**300**	0.607 ± 0.004	49.88 ± 0.121	0.321 ± 0.013	68.18 ± 0.006	
4	**400**	0.398 ± 0.012	67.13 ± 0.101	0.213 ± 0.006	78.88 ± 0.002	
5	**500**	0.213 ± 0.011	82.41 ± 0.004	0.101 ± 0.001	89.90 ± 0.114	
IC 50 Values		-	300.957	-	214.507	

Table 10 demonstrates the OD values, percentage and IC 50 values 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 BHT (17.01%) and highest values at 500 µg/ml (89.90%). When compared with standard, the test sample shows low percentage of inhibition at 500 µg/ml (82.41%) with IC 50 values of 214.507 µg/ml.

Estimation of lipid peroxidation using egg yolks

Percentage of inhibition by standard BHT was founded to be 78.01% which is slightly higher than the test sample 76.07%. Similarly, the IC 50 value of standard was 313.030 µg/ml which is lower than the IC 50 value of methanol extract (326.024 µg/ml)**(Table 11)**.

Table (11):-Estimation of LPO (Egg yolks) for standard and methanol extract

S.No	Concentration µg/ml	Standard OD Mean ± SD	Standard % Mean ± SD	Methanol extract OD Mean ± SD	Methanol extract % Mean ± SD	Control OD
1	**100**	1.018 ± 0.003	17.70 ± 0.010	I.032 ± 0.010	16.57 ± 0.012	1.237
2	**200**	0.837 ± 0.012	32.34 ± 0.004	0.856 ± 0.006	30.80 ± 0.006	
3	**300**	0.671 ± 0.013	45.76 ± 0.012	0.714 ± 0.002	42.27 ± 0.007	
4	**400**	0.419 ± 0.003	66.13 ± 0.006	0.440 ± 0.011	64.43 ± 0.002	
5	**500**	0.272 ± 0.001	78.01 ± 0.002	0.296 ± 0.004	76.07 ± 0.011	
IC 50 Values		-	313.030	-	326.024	

βcarotene linoleic acid assay

Table (12):-β carotene linoleic acid assay of standard and methanol extract

S.No	Concentration µg/ml	Standard OD Mean ± SD	Standard % Mean ± SD	Methanol extract OD Mean ±SD	Methanol extract % Mean ±SD	Control OD
	100	0.490 ± 0.006	15.37 ± 0.002	0.472 ± 0.010	18.48 ± 0.100	0.579
2	**200**	0.368 ± 0.105	36.44 ± 0.021	0.345 ± 0.003	40.41 ± 0.003	
3	**300**	0.289 ± 0.003	50.08 ± 0.101	0.231 ± 0.012	60.10 ± 0.007	
4	**400**	0.178 ± 0.001	69.25 ± 0.011	0.146 ± 0.002	74.78 ± 0.012	
5	**500**	0.112 ± 0.121	80.65 ± 0.004	0.071 ± 0.011	87.74 ± 0.002	
IC 50 Values		-	297.809	-	263.549	

Percentage of inhibition for standard BHT showed 15.37 % at 100 µg/ml and 80.65% at 500 µg/ml concentration which is compared with the methanol extract of *Chlorella vulgaris* which possess18.48% at 100 µg/ml and 87.74% at 500 µg/ml concentration respectively. Hence, percentage of inhibition by test sample is higher than the standard drug **(Table 12)**.

Discussion:-

In present investigation, methanol extract of *Chlorella vulgaris* shows higher antioxidant activity in all the tests except Nitric oxide scavenging, Ferric reducing, LPO and β-Carotene when compared with the different standard drug. This was related with other findings whose results were similar to our present analysis.

In (Dantas*et al.*, 2015). studies, the solvents with higher efficiency of extraction of compounds with antioxidant activity of the species *Chlorella vulgaris* were done. The solvents DMSO (dimethyl sulfoxide) and water showed the DPPH percentage of inhibition of 64.6% and 68.5%, respectively, higher than the standards Catechin (49.6%) and Gallic acid (28.7%), showing that they are potential inhibitors of cellular oxidation by free radicals.

Antioxidant activity of the methanol extracts of *Chlorella vulgaris* was determined in terms of IC50 value based on the percentage of free radical scavenging activity. In both the extracts, higher scavenging activity was observed at the concentration of 1000 µg/mL. IC50 values for the *C. vulgaris* determined by H_2O_2 and Thiocyanate assay were 26.31µg/mL and 28.18µg/mL respectively. The presence of flavonoids and phenols in the methanol extract might been responsible for free radical scavenging activity individually or by synergistic action (Annamali and Nallamuthu, 2014).

Based on our present result, the highest phenolic content, hydrogen peroxide Radical Scavenging Activity was also obtained by Anantharaman*et al.*, 2013. The highest phenolic content was observed in methanolic extract of *C. marina* (0.78 ± 0.032 mg/g gallic acid equivalent) and methanol extract of *C. marina* (23.08%) and *D. salina*(17.66%) and acetone extract of *C. marina* (20.54%) was found to be the most potent scavenger. Hydrogen peroxide Radical Scavenging Activity (%) was found to be maximum in methanol (61.33%) and hexane extract (55.3%) of *C. marina,* whereas minimum in acetone extract (15.54%) of *N. clavata*. The reducing power was found to be higher in methanolic extract of *C. marina* (0.73 ± 0.026 mg/g). The highest nitric oxide scavenging activity was also observed in methanol and hexane extract of *C. marina* (25.76% and 21.73%) respectively.

According to Manivannan*et al.*, 2012 methanol extract of *Chlorella marina* exhibited higher activity which as followed by diethyl ether and hexane extract. This may be due to the differencesin the polarity of the solvents used.The highest antioxidant activity of *C. marina* methanol extract is (1.03 ± 0.02 mg/g).

Antioxidant potential of microalgae in relation to their phenolic and carotenoid content was carried out by Goiris*et al.*, 2012 and founded that industrially cultivated samples of *Tetraselmissuecica, Botryococcusbraunii, Neochlorisoleoabundans, Isochrysis sp., Chlorella vulgaris* and *Phaeodactylumtricornutum* possessed the highest antioxidant capacities and thus could be a potential new source of natural antioxidants. The results from various studies with different types of extracts clearly indicated that next to the well-studied carotenoids, phenolic compounds also contribute significantly to the antioxidant capacity of microalgae.

Similarly, Sivakumar andRajagopal, 2011 reported that the highest antioxidant activity was observed in methanol extract from eight green algal species. Uma *et al.*, 2011 observed that the methanolic extracts displayed greater potential in all antioxidant assays when compared to ethanolic and acetone extract of green microalgae *Desmococcusolivaceous*and *Chlorococcumhumicola* and it also clearly explain that the methanolic extract found to be having higher phenolic content in *D. olivaceous*and flavonoid content was high in acetone extract of *C. humicola*. The hexane extract of *N. clavata*showed the minimum DPPH radical scavenging activity at 9.1% respectively. Similarly, both methanolic and acetone extracts of *D. olivaceous*and *C. humicola*showed a significant dose dependent reduction of DPPH radicals. The methanolic extracts of *D. olivaceous*exhibited 39% scavenging activity and the acetone extracts of *C.humicola*exhibited 15% scavenging activity.

The present study was related with the finding of Lee *et al*., 2010, who found that the ethyl acetate of *H. porphyrae*(30.1%) and the 80% of methanol extract of *O. unicellularis*(49.3%) exhibited significantly higher nitric oxide radical scavenging effects than those of the commercial antioxidants. The implications are important as radical scavengers may protect cell tissues from free radicals, thereby preventing diseases such as cancer.

Wang *et al.*, 2010 studied the species *Chlorella vulgaris* and they observed that in the DPPH assay using ultrasonic extraction with ethanol found the lowest percentage of inhibition (0.74%). This fact might be related to the variation in strains of microalgae and with the appropriate constituents. The result found using aqueous extract proves satisfactory because in addition to reducing processing costs, resulting in a product without the potentially toxic residues found in other solvents.

Similarly, Kuda*et al*., 2005 reported that the highest amount of reducing power was observed in the highly polar water extract of *S. lomentaria*and the minimum reducing power was observed in ethanol extract and crude fucoidan, these were dose dependent. Supportively, Herrero*et al*., 2005 explained that these polar compounds can be extracted to a higher extent, in this way increasing the yield of extract.

Conclusion:-

The antioxidant activities of various microalga species, their phenolic and carotenoid contents were evaluated extensively during the past two decades. In the present study, methanolic extract of *Chlorella vulgaris* showed potent antioxidant activity. Microalga biomass can therefore be considered as potential source of natural antioxidants, such as the carotenoids and the phenolic compounds.

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