ANTIOXIDATIVE ACTIVITY OF ETHANOL EXTRACTS FROM *Spirulina platensis* AND *Nostoc linckia* MEASURED BY VARIOUS METHODS

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Abstract. The goal of this work is to determine the level of antioxidative activity of various ethanol extracts from *Spirulina platensis* and *Nostoc linckia* biomass, and also to demonstrate the possibility to select suitable methods for evaluation of these characteristics. The methods for determination of antioxidative activity were selected concerning their possible use for complex preparations: phosphomolybdenum method for evaluation of antioxidant capacity (PMRC), radical-scavenging activity by DPPH method (DPPH), antioxidant activity by the $ABTS^+$ radical cation assay (ABTS), Folin-Ciocalteu reducing capacity (FCRC). We showed the presence of antioxidative substances in ethanol extractions from 2 species of cyanobacteria, and possibility to increase their activity varying ethanol concentration. It facilitates the extraction both water- and lipid-soluble components from biomass. Regarding used methods for antioxidative activity determination, we have used only those based on reaction of electrons return (which widely used nowadays *in vitro*). Obtained in different ways results demonstrate high reduction capacity of the extracts and possibility to select suitable analytical methods for each case.

Keywords: *Spirulina platensis*, *Nostoc linckia,* antioxidative activity, ethanol extracts

INTRODUCTION

It is known that development of broad spectrum of inflammatory diseases if followed by rapid increase of free radicals level, which initiate the reactions of peroxide oxidation of lipids and bio-molecules denaturation.

First this idea was formulated by Dr. Denhim Harman, professor of Nebraska University, in 1954. Now it is a main theory explaining the reasons of origin and development for numeral and various pathological processes – so called free radicals diseases.

Necessity to cure the wide spectrum of severe diseases leads to the search of efficient pretreatment and complex therapy of free radicals diseases.

Experience of physicians in the whole world states the efficiency of use the synthetic and natural substances possessing antioxidative properties as medicinal and prophylactic remedies [7].

Regarding general trend to substitute synthetic substances with natural ones, bio-antioxidants have get more attention. Thus, most demanded now are antioxidative substances of plant origin. Their wide use is caused by high content of phenolic substances, which possess expressed oxidation and reduction properties and actively protect lipids from peroxide oxidation [11].

Specialists in human nutrition actively propose various diets including the products with antioxidative activity, which prevent different pathological conditions. Products with such properties are called "functional", and their positive influence on human health is determined by presence of "functional" compounds [3, 17].

Microalgae and cyanobacteria are also the sources of natural antioxidative substances [6, 10, 14]. They provide photosynthesis leading to the production of molecular oxygen. It generates development of the active forms of oxygen and dictates the necessity to create some protective antioxidative mechanisms. Biomass of our selected cyanobacteria strains contains complex of substances with antioxidative properties [4-6, 16].

Different methods are used to evaluate antioxidative activity of biomass or various extractions from cyanobacteria. The obtained results depend generally on suitability of the applied method for determination of antioxidative activity, and on extraction methods as well [4, 5, 8, 9].

The goal of this work is to determine the level of antioxidative activity of various ethanol extracts from Spirulina platensis and Nostoc linckia biomass, and also to demonstrate the possibility to select suitable methods for evaluation of these characteristics in each separate case.

MATERIALS AND METHODS

Preparation of sample extracts

For obtaining of all ethanol extracts we have used the lyophilized pure cultures of Spirulina platensis CNM-CB-02 and Nostoc linckia CNM-CB-03 (Laboratory of Phycobiotechnology, IMB, ASM, Chisinau, Republic of Moldova). For each sample 1g of biomass was mixed 60 min with 10 ml ethanol solutions of 10, 20, 40, 55, 65, 70% concentration in aqua dest. After filtration the samples were standardized (1 mg dry active substances in 1 ml). All extracts were kept at 0°C until further use.

Evaluation of antioxidant capacity by phosphomolybdenum method (PMRC method)

The total antioxidant capacity of ethanol-water extracts were evaluated according to the method described by Prieto, Pineda, and Aguilar (1999) [12]. An aliquot of 0.1 ml of sample solution was combined with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). In control 0.1 ml of ethanol solution was used instead of sample. The tubes were incubated at 95ºC for 90 min. After cooling to the room temperature, the absorbance was measured at 695 nm against blank in UV-VIS spectrophotometer (PG Instruments Limited, Alma Park, Wibtoft, England). Antioxidant capacity was expressed as the equivalents

of ascorbic acid (mg AA/g dry extract). The calibration curve of ascorbic acid from 1 to 6 µg/ml is linear, n=8, r2=0.999.

Each trial was performed three times.

Radical-scavenging activity by DPPH method (DPPH method)

Antioxidant activity of extracts was determined according to Brand-Williams, Cuvelier, and Berset (1995) [1]. DPPH (1,1 diphenyl-2-picryl hydrazyl radical) solution of 0.6 M was prepared in ethanol. Aliquots of extracts were added to 1 ml DPPH solution. After 60 min of incubation in the dark at room temperature, the absorbance was read against a blank at 517 nm. The reduction of the absorbance (inhibition %) for DPPH reagent was calculated using the following equation:

Inhibition % = $\mathbf{(Abs_{t=0} - Abs_{t=30\ min})/Abs_{t=0} * 100;}$

where $\mathbf{Abs_{t=0min}}$ was the absorbance of DPPH reagent at 0 min and $\mathbf{Abs_{t=30\ min}}$ was the absorbance of DPPH reagent after 30 min.

Antioxidant activity by the ABTS+ radical cation assay (ABTS method)

The total antioxidant activity of extracts was measured by the ABTS+ (2,2 azinobis 3-ethylbenzothiazoline-6-sulfonic acid) radical cation decolorization assay of Re et al. 1999 [13]. ABTS+ was generated by oxidation of ABTS with potassium persulphate. ABTS was dissolved in deionized water to a 7mM concentration, and potassium persulphate added to a concentration of 2.45 mM. The reaction mixture was left at room temperature overnight (12-16 h) in the dark before use. Prior to assay, the ABTS+ stock solution was diluted with ethanol to an absorbance of 0.700 ± 0.020 at 734 nm. Then 1 ml of diluted ABTS+ solution was mixed with 10 µl of the test sample (1.0 mg/ml), and the absorbance was measured at 734 nm after 6 min. The percentage inhibition of absorbance was calculated using the formula mentioned in the DPPH method. All trials and measurements were performed three times. TEAC (trolox equivalent antioxidant activity) value was expressed as mM Trolox/g dry extract, using the calibration curve of Trolox. Linearity range of the calibration cure was 20 to 1000 µM (r2 = 0.9976).

Folin-Ciocalteu reducing capacity (FCRC method)

FCRC was determined using the Folin-Ciocalteu's reagent (Singleton & Rossi, 1965) [18]. Samples (0.3 ml, triplicate) were introduced into test tubes followed by 1.5 ml of Folin-Ciocalteu's reagent (diluted 10 times with water) and 1.2 ml of sodium carbonate (75 g/l). The tubes were vortexed and incubated for 5 min at 50 ºC, and then cooled. The absorbance was measured at 760 nm. The results were expressed in gram of gallic acid per gram of extract (g GA/g dry extract). Linearity range of the calibration cure was 0.01 to 0.1 mg/ml (n=6, r2=0.999).

Reagents: All chemicals and reagents were analytical grade purchased from Sigma, Merck or Fluka.

In this study was used the statistical calculations that includes the calculation of their average and their deviation. In this way were compared the maximal values of the activity of each other value for the each used method. We considered that are significant the cases when the value is $p<0.05$. In the article are proposed for discussion only the differences that meet the same condition.

RESULTS

Described above methods for determination of antioxidative activity were selected concerning their possible use for complex preparations [9].

The phosphomolybdenum method is based on the reduction of molybdenum (VI) to molybdenum (V) by the antioxidant compounds and the formation of a green molybdenum (V) complex. In this way the ability of obtained extracts to participate in the oxidative-reduction reactions is determined. PMRC is one of the routine methods for the determination of antioxidative activity. Finally, its result depends exclusively on the amount of the substances, possessing such activity. Antioxidative compounds from Nostoc belong mostly to the proteins and polysaccharides. Their extraction is more active by lower ethanol concentrations. Spirulina biomass contains also a considerable amount of carotenoid pigments and tocoferol, which were better extracted by higher ethanol concentrations. Our results confirm above mentioned.

Antioxidative activity of water-ethanol extracts from Spirulina platensis biomass increases proportional to the concentration of ethanol used for the extraction (Fig. 1). Antioxidative activity of 10% ethanol extract is 38 mg AA/g of active substance, 70% extract increases it twice. Increase of activity is not linear; most active increase we observed in range of 55-70% of ethanol concentration, for lower range (10-55%) the increase is slower.

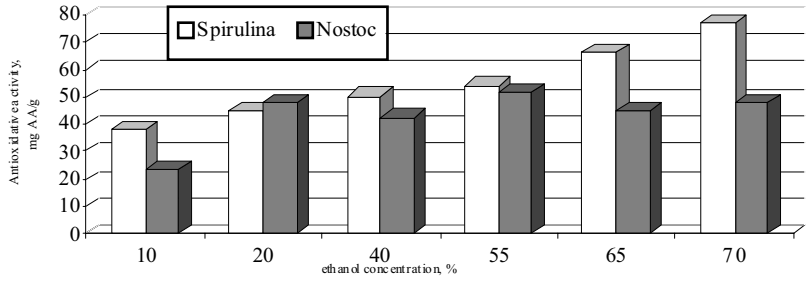

Figure 1. Antioxidative activity of ethanol extracts from *Spirulina and Nostoc*, determined with phosphomolybdenum reagent, in mg of ascorbic acid per gram of active substance (AA - ascorbic acid).

Ethanol extracts from *Nostoc linckia* biomass have lower antioxidative activity comparing to *Spirulina platensis* extracts. We have not observed any strict correlation between activity and ethanol concentration in the extract. The highest activity (55 mg AA/g of active substance) was observed for the extract obtained with 55% of ethanol.

Radical scavenging activity using radical DPPH is based on reduction ability of antioxidant which serves as a donor of hydrogen in such case. Activity of water-ethanol extracts obtained from *Spirulina plateansis* biomass with 10-40% ethanol causes 30% of DPPH inhibition. Increase of ethanol concentration to 55 and 70% increases also DPPH (46 and 52% correspondently). Activity of preparations obtained with 10-40% ethanol from *Nostoc linckia* biomass is higher comparing to those ones from *Spirulina platensis* and causes 40% of inhibition. Maximal activity was observed for 70% extracts.

Regarding above mentioned it is possible to expect that ethanol soluble components have main antioxidative properties and ability to return hydrogen in complex extracts obtained from *Spirulina* and *Nostoc* biomass.

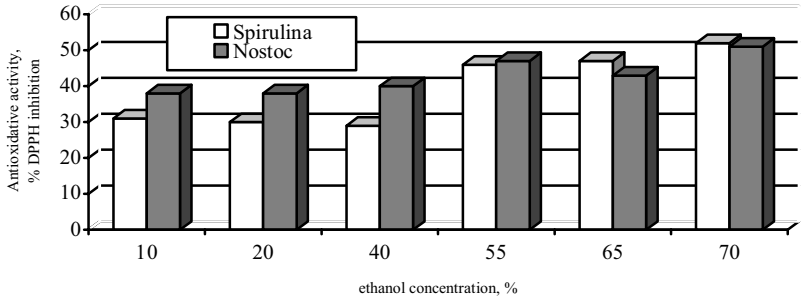

Figure 2. Antioxidant activity of ethanol extracts from *Spirulina* and *Nostoc* expressed in % of DPPH inhibition (DPPH -1,1 diphenyl-2-picryl hydrazyl radical).

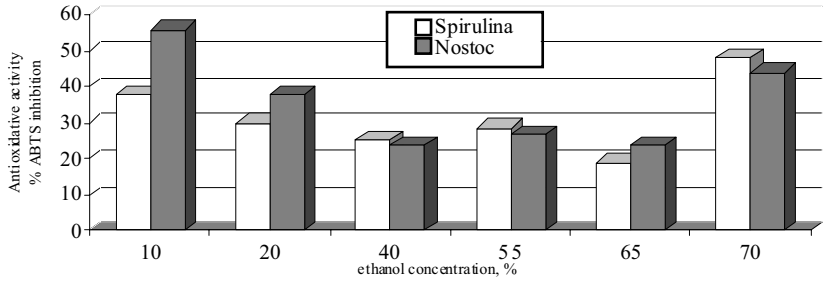

Figure 3. Antioxidant activity of ethanol extracts from *Spirulina* and *Nostoc* expressed in % ABTS inhibition ($ABTS^+$ - 2,2 azinobis 3-ethylbenzothiazoline-6-sulfonic acid).

Activity of radical cation ($ABTS^+$) scavening reaction (Fig. 3) of alcochol extracts from *Spirulina platensisi* and *Nostoc linckia* biomass does not depend on ethanol concentration used for extraction. For both the highest activity was observed using preparations obtained with 10% ethanol (38 and 56% inhibition $ABTS^+$ for *Spirulina* and *Nostoc* correspondently) and 70% ethanol (48 and 44 % inhibition $ABTS^+$ for *Spirulina platensis* and *Nostoc linckia* correspondently). Observed variations of activity have similar pattern, independent on the raw material used. Thus, the lowest activity was observed in preparations with 40 and 65% ethanol, obtained from both species.

Trolox equivalent antioxidant capacity assay (TEAC) for the extractions of *Nostoc linckia* and *Spirulina platensis* was considerable high (Tab.1). For *Spirulina platensis* extracts it was 1.3-1.8 mM Trolox for the 20-65%-ethanol preparations, and 2.2-2.8 mM Trolox for 10 and 70% extracts correspondently. Similar pattern was observed for *Nostoc linckia* extractions: activity 1.5-1.7 mM Trolox for the 20-65%-ethanol preparations, and 3.2-2.6 mM Trolox for 10 and 70% extracts correspondently.

Table 1. Trolox equivalent antioxidant capacity (TEAC) mM/g of ethanol extracts from *Spirulina platensis* and *Nostoc linckia.*

SPECIES	***ETHANOL EXTRACTS, ETHANOL CONCENTRATION, %***					
	10	20	40	55	65	70
Spirulina platensis	2.185±0.02	1.524±0.06	1.336±0.10	1.800±0.04	1.288±0.06	2.819±0.08
Nostoc linckia	3.220±0.05	1.524±0.08	1.556±0.02	1.692±0.10	1.552±0.07	2.584±0.08

Reagent Folin-Ciocalteu is widely used for the determination of phenol compounds based on oxidation-redox reaction and it is used for testing of plant biomass, including microalgae. However it is worth to mention that this reagent is not specific for phenols and can be reduced also by non-phenolic substances as ascorbic acid, aromatic amines, several metals, etc. Therefore reagent Folin-Ciocalteu was proposed for the determination of reduction capacity of our samples [19]. Some authors mention the dependance of first range between ability to reduce Folin-Ciocalteu reagent and antioxidative activity,

determined by reduction of DPPH and ABTS (reactions of hydrogen and electrons tranfer) [15]. Definitely, this dependence exists, but it is characteristic only for the plant biomass, where phenol compounds dominate qualitatively and quantitatively [2]. Used method gives also a good approximation of protein-bound phenol content [20]. Regarding that better results were obtained in the extracts with higher content of protein compounds. Considering *Spirulina* as a super-producer of protein and more efficient extraction of oligopeptide components with ethanol of lower concentrations, we expect higher activity in *Spirulina* extracts with low ethanol content (up to 55%).

Studying the extracts from cyanobacteria we have observed quite different pattern.

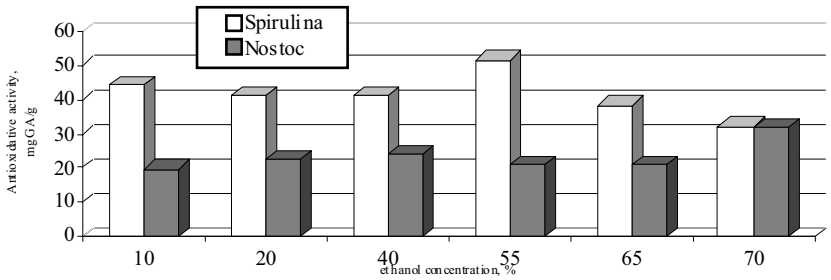

Figure 4. Folin-Ciocalteu reducing capacity of *Spirulina* and *Nostoc* ethanol extracts from expressed in mg GA/g (GA – Gallic acid).

Folin-Ciocalteu reducing capacity of ethanol extractions from *Spirulina* biomass is double comparing to *Nostoc* ones. For 10-40% *Spirulina* extracts FCRC is 41-44 mgGA/g of active substance. Maximal meaning of reduction capacity 51.5 mgGA/g of active substance was observed for 55% extract. Further increasing of ethanol concentration caused the decrease of reduction capacity (38.5 and 32 mgGA/g of active substance for 65 and 70% ethanol extracts).

There was no significant difference between the means of FCRC in extracts obtained from *Nostoc* biomass using ethanol in concentrations 10 to 65%. It contains approximately 20-24 mgGA/g. Activity of 70% preparation from *Nostoc* is higher and contains 32.0 mgGA/g.

DISCUSSION

We have compared values of antioxidative activity, obtained using 4 above mentioned methods. For analysis, evaluation and comparing of the obtained results and values, maximal value of activity for each used method was taken as 1. The rest of values were estimated comparing to the maximal one. The results of this analysis are shown in the Fig. 5 & 6.

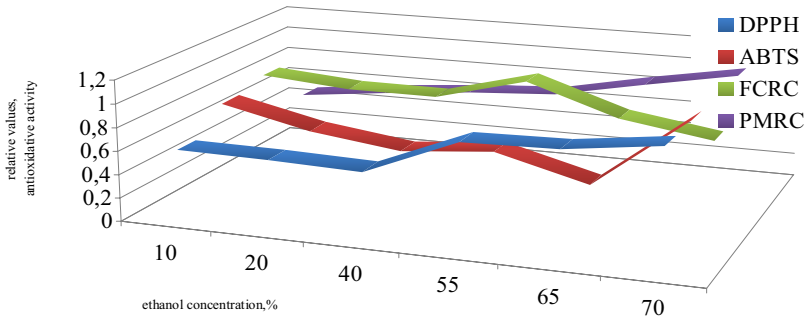

Figure 5. Comparison of antioxidative activity of ethanol extractions from *Spirulina platensis* biomass, determined by: DPPH, ABTS FCRC, PMRC methods.

As the Fig. 5 shows, maximal values of of DPPH radical-scavenging activity, ABTS radical reducing capacity, and phosphomolybdenum reducing capacity were observed in 70% extract from *Spirulina platensis*. Only for Folin-Ciocalteu reducing capacity maximal value was observed in 55% extract. Also the relative values of DPPH radical-scavenging activity and reducing of phosphomolybdenum reagent capacity in this extract were 0.88 and 0.70 respectively. In the same time relative value of ABTS radical reducing capacity was 0.59. For the extracts, obtained using lower ethanol concentrations (10-40%) the highest values of antioxidative activity were obtained using the method of Folin-Ciocalteu reducing capacity (approximately 0.80). The other methods for the same concentrations gave lower values. Relative values of antioxidative activity in 65% ethanol extract, obtained with DPPH and PMRC methods, were higher than 0.80, but the value for ABTS method was the lowest for the same extract (0.40). Summarizing the results we can state, that 55 and 70% extracts possess the highest activity. Outgoing from the specificity of these methods, we can assume, that antioxidative activity of 55% extract is a result of reduction potential of its

active compounds. However activity of 70% ethanol extract is caused by the components – donors of hydrogen and electrons, which confirm its complex antioxidative activity extract's nature.

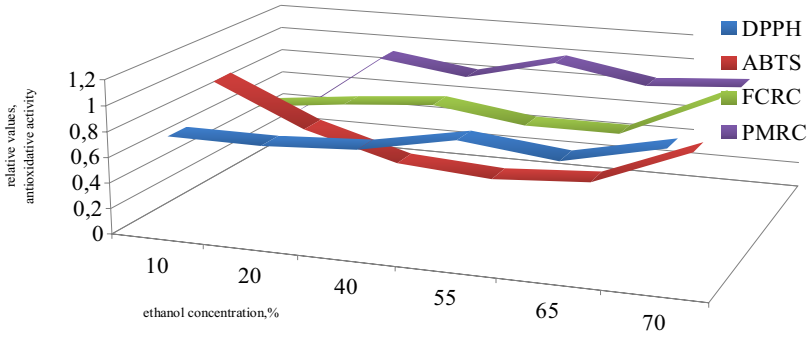

Figure 6. Comparison of antioxidative activity of ethanol extractions from *Nostoc linckia* biomass, determined by: DPPH, ABTS FCRC, PMRC methods.

Relative values of antioxidative activity of *Nostoc linckia* extracts, obtained by the same methods, are presented in Fig. 6. For 2 methods: DPPH radical-scavenging activity and Folin-Ciocalteu reducing capacity the maximal values were observed in 70% ethanol extract. Relatively high activity in ABTS and PMRC methods (0.78 and 0.92 correspondently) might be, which is an evidence of high complex antioxidative activity.

As in the case with *Spirulina*, 55% ethanol extract of *Nostoc* biomass had high relative antioxidative activity – 1.0 in case of PMRC and 0.92 in case of DPPH method.

Analogically to the extractions from *Spirulina* biomass 10, 20 and 40% ethanol extractions from *Nostoc* showed low relative activity. An exception was only 10% ethanol extract, which showed maximal antioxidative activity, in ABTS method. Activity of 40 and 65% ethanol extractions from *Nostoc* did not differ from each other. Thus, as in case with *Spirulina*, the most expressed antioxidative activity was observed in 55 and 70% ethanol extracts from *Nostoc*, which is determined also by specific properties of the components.

Thus, the method Folin-Ciocalteu reducing capacity is more informative for the determination of antioxidative activity, then for the determination of phenol compounds. ABTS method, which is widely used for the determination of antioxidative activity for complex preparations, seems to be not very useful in case of cyanobacteria, but we have observed high values of TEAC (Tab. 1), comparable with the values obtained for polyphenolic plant extracts [2, 6, 19].

Thus, our main aim was to show the presence of antioxidative substances in ethanol extractions from 2 species of cyanobacteria , and possibility to increase their activity varying ethanol concentration. It facilitates the extraction both water- and lipid-soluble components from the biomass. Regarding used methods for antioxidative activity determination, we have used only those based on reaction of electrons return (which widely used nowadays *in vitro*). Obtained in different ways results demonstrate high reduction capacity of the extracts and possibility to select suitable analytical methods for each case.

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