Antioxidant capacity of phenolic acids extracted from the biomass from *Spirulina* sp.LEB-18

Capacidade antioxidante de ácidos fenólicos extraídos da biomassa de Spirulina sp.LEB-18

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ABSTRACT
This study's objective was to extract and evaluate the antioxidant activity of phenolic acids from biomass Spirulina sp.LEB-18. The analysis of phenolic compounds was made according to the Folin-Ciocalteu method, using gallic acid as the standard. The antioxidant activities were measured using different techniques such as the kidnapping of DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS• + radical capture (2.2'-azinobis (3-sulfonic acid ethylbenzothiazoline-6), the β-carotene system / linoleic acid, and the antioxidant power of reduction of iron (FRAP), its antioxidant activities determined through the different methodologies, showed more significant inhibition compared to ABTS radical.

Keywords: Inhibition, Microalgae, Phenols, Radicals.

RESUMO
O objetivo deste estudo foi extrair, avaliar a atividade antioxidante de ácidos fenólicos da biomassa Spirulina sp.LEB-18. A análise dos compostos fenólicos foi realizada pelo método de Folin-Ciocalteu, utilizando o ácido gálico como padrão. As atividades antioxidantes foram medidas usando diferentes métodos, como seqüestro de DPPH (2,2-difenil-1-picril-

hidrazil), captura de radical ABTS • + (2,2'-azinobis (ácido 3-sulfônico etilbenzotiazolina-6), o sistema β-caroteno / ácido linoléico e o poder antioxidante de redução de ferro (FRAP), suas atividades antioxidantes determinadas por diferentes metodologias, apresentaram maior inibição em relação ao radical ABTS.

Palavras-chave: Inibição, Microalgas, Fenóis, Radicais.

1 INTRODUCTION

Spirulina is a microalgae that stands out for its chemical composition, with high quality and quantity of protein, essential amino acids, minerals, polyunsaturated fatty acids, and vitamins. Moreover, it presents phenolic compounds (caffeic acid, chlorogenic, salicylic, trans-cinnamic acid), tocopherol, and pigments (carotenoids, phycocyanin, and chlorophyll) to which are assigned their potentially functional properties (Parisi *et al*., 2009; Machado *et al*., 2014), specifically reducing the risk of occurrence of physiological disorders such as diabetes, arthritis, anemia, malnutrition, obesity, premenstrual syndrome, cardiovascular diseases (Ambrosi *et al.,* 2008; Machado *et al.,* 2014) and some cancers.

Because spirulina is widely studied for its high nutritional value and the presence of bioactive compounds, this microalga is certified generally recognized as safe (GRAS) issued by the Food and Drug Administration (FDA) and can be used as food without presenting health risks (Uebel et al.,2019). The same has also been featured in the food to produce antioxidant compounds (Richa *et al*., 2011). Among the species used for this purpose stand out *platensis Spirulina, Chlorella Vulgaris, Dunaliella salina, Synechococcus sp., Nostocellipsosporum, Chlamydomonas nivalis, Porphyridiumcruentum, Phaeodactylumtricornutum* (Chacón-Lee and González-Mariño, 2010).

These bioactive compounds can be extracted from microalgae using aqueous and alcoholic solutions and present an excellent potential for inhibiting free radicals and thus acting as an antioxidant (Pyne, Bhattacharjee, & Srivastav, 2017; Souza, Prietto, Souza, & Furlong, 2015, Machado et al., 2019). Antioxidants can be defined as substances capable of inhibiting or retarding the oxidation of oxidizable substrates with the formation of free radicals.

Indeed, some antioxidants are produced by the human body and may acquire others by consuming food (Araújo, 2004). Antioxidants act by interacting with free radicals before they can react with biological molecules, thus preventing chain reactions occur avoiding the activation of highly reactive oxygen products (Ratnam *et al.,* 2006). *In vitro* methods for assessing the plant extracts' antioxidant capacity are based on the antioxidant ability to

scavenge free radicals (Assis, 2012). The excess of free radicals in the body is opposed by antioxidants produced in the body or absorbed in the diet. Among the studied methods for capturing free radicals are used much the DPPH (1,1-diphenyl-2-picrylhydrazyl) and ABTS (2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) in equivalents Trolox (TE).

The DPPH method (Brand-Williams et al., 1995) is based on the capture of DPPH (2,2-diphenyl-1-picryl-hydrazyl) antioxidants, producing a decrease in absorbance at 515 nm. This method was modified by Sánchez-Moreno *et al.* (1998) for measuring the kinetic parameters. The DPPH is a free radical that can be obtained directly by dissolving in an organic reaction medium. (Rufino *et al.*, 2007). The ABTS assay is one of the most widely used methods to measure antioxidant activity by capturing moiety generated by a chemical, electrochemical or enzymatic reaction. One can measure the activity of compounds of lipophilic and hydrophilic nature. The advantage of the ABTS test consists in its relative simplicity that allows any application in routine laboratories.

As a result, research must be conducted so that new compounds are discovered and used in the fight against free radicals, such as compounds from microalgae such as *Spirulina*, recognized worldwide because of their use in food as food functional and nutraceutical. Thus, this study aimed to extract and quantify the phenolic acids present in Spirulina sp's biomass.LEB-18, lot 2014.

2 METODOLOGY

2.1 OBTAINING THE MATERIALS

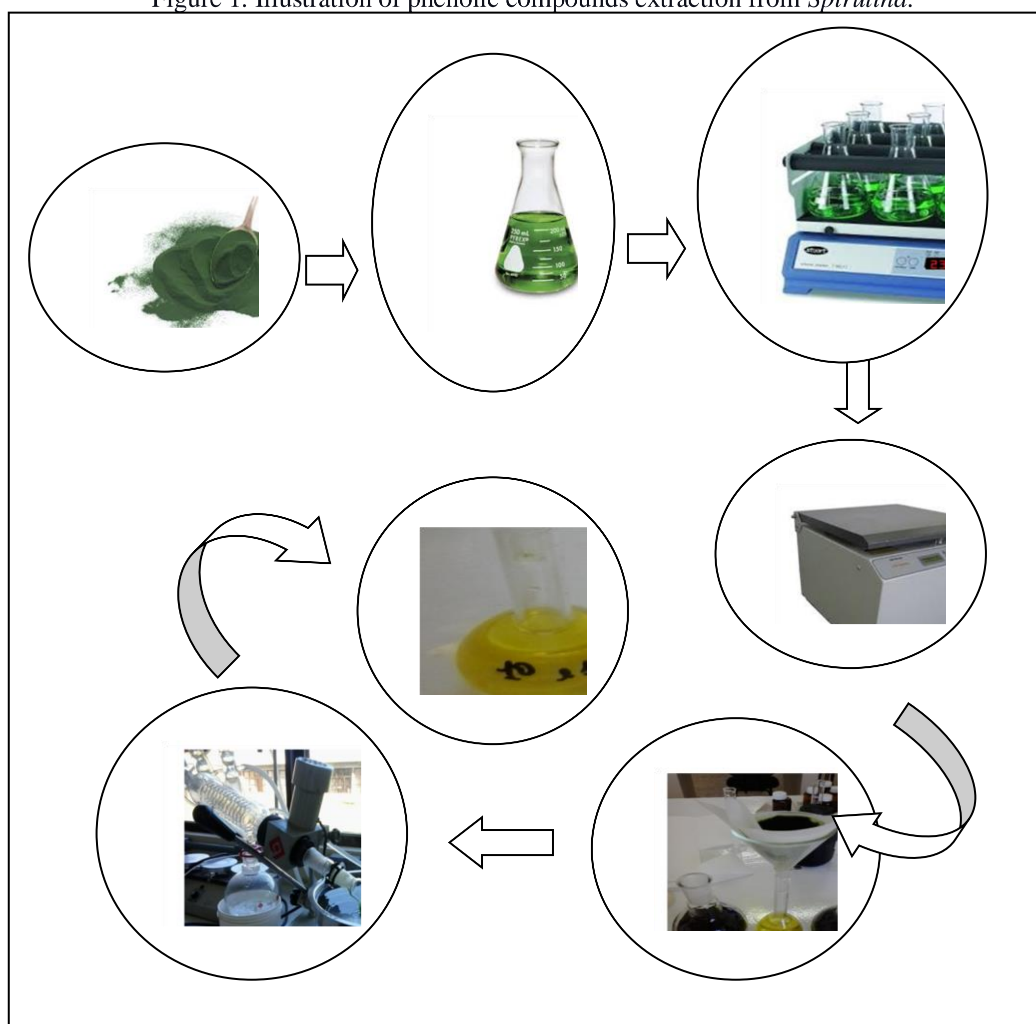
Biomass of Spirulina (LEB strain-18), batch 2014 was provided by the Biochemical Engineering Laboratory of the Federal University of Rio Grande, isolated Mangueira lagoon (33 ° 302 133 S, 53 ° 082 593 O), located in Santa Vitória do Palmar, RS, Brazil (Morais and Costa 2008) and supplemented with 20% of middle Zarrouk (Costa *et al.*, 2004). The dried biomass was provided in pellets, subsequently being chopped into a bead mill (Model Q298-2) and sieved in a sieve shaker, 200 mesh, to homogenize the particle size, reaching a particle size of 88 micrometers. Biomass in the form of powder was vacuum packed in high-density polyethylene containers (HDPE) with a capacity of 500 g and stored under refrigeration at a temperature of ± 7°C until the moment of time of analysis.

2.2 EXTRACTION AND TOTAL PHENOLIC COMPOUNDS OF MEASUREMENT

In the preparation of extracts, the methodology used was according to Souza *et al.* (2009), with modifications, Figure 1. The extraction was performed in an orbital shaking

incubator at 230rpm, 35°C for 2h using 2 g of biomass and 25ml methanol solvent, followed by centrifugation at 5000 x*g* for 15 min. After the supernatant was collected and held by evaporation of the solvents with subsequent dissolution in distilled water. The extracts were clarified with 20 ml of barium hydroxide 0.1 M and 20 ml of 5% zinc sulfate, filtered and the volume adjusted with distilled water to 100 ml in a volumetric flask. Quantification of total phenol content of the extracts was performed by mass spectrometry using the Folin-Ciocalteu method. The procedure consisted of adding vials in aliquots of 500µl of the phenolic extract, 500µl of distilled water, and 4.5 ml of an alkali solution (sodium carbonate 4%, 2% copper sulfate, and tartrate of sodium and potassium 2% in the ratio of 100: 1: 1). The tubes were incubated at 40°C for 15 min. Then was added 500µl of 2N Folin-Ciocalteu reagent diluted 1: 2, left to stand for 10 min, to measure the absorbance at 750nm. The total phenolics content was determined by interpolating samples' absorbance to an analytical curve constructed with a standard solution of gallic acid at 20 to 80µg / ml.

Figure 1. Illustration of phenolic compounds extraction from *Spirulina*.



Source: Authors.

2.3 ANTIOXIDANT ACTIVITY

2.3.1 Scavenging of free DPPH radical (2,2-diphenyl-1-picrylhydrazyl)

The antioxidant activity of phenolic extracts from microalgae was measured using the procedure described by Herrero *et al*. (2005) with modifications by monitoring the consumption of the free radical DPPH through the sample by determining the decrease in absorbance unit (AU), the solutions containing the phenolic extracts. The measurements were performed in a UV-Vis spectrophotometer (VARIAN/CARY-100) at wavelength 515 nm. The tubes containing 3.0 mL of the methanol solution of DPPH (5.2x10-5 moles/L) were added 0.5 mL methanol and 0.5 mL of phenolic extracts. The reactive mixture to stand at room temperature without light and change color to yellow violet was measured after 30 min. of reaction. The DPPH solution was prepared and stored in amber bottles covered with aluminum foil, kept in the dark at 4°C until determinations. The kidnap free radical capacity was expressed as a percentage of the radical oxidation inhibition and calculated according to the following equation:

% Inhibition = ((ADPPH - AExtr) / ADPPH) * 100 (1)

Where ADPPH is the absorbance of DPPH and AExtr, a solution is the absorbance sample solution. AExtr.was calculated based on the difference between the sample solution's absorbance under test with its background.

2.3.2 Capture of radical abts• + (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid))

The capture capacity of the cationic radical ABTS • + the samples was performed according to Rufino *et al.* (2007). First, ABTS was dissolved in deionized water at a concentration of 7 mM (w/v), and potassium persulfate was added to the concentration of 2.45mM (w/v). The radical ABTS• + was generated by the oxidation of ABTS with potassium persulfate. The solution was left for 16 h in the dark at room temperature. Then, the solution was diluted with ethanol to achieve an absorbance of 0.700 ± 0.020 at 734 nm. It was placed concentration of extract from *Spirulina* (9.98) in the test-tube and added 3.0 mL of dilute ABTS • +. Absorbance measurements were performed at 734nm after 6 min of reaction. Values were expressed as the Trolox equivalent antioxidant activity (TEAC) as µM Trolox.g-1 microalgae using a Trolox calibration curve (from 100 to 2000 uM/ml).

2.3.3 Determination of the antioxidant activity using the system β-Carotene / Linoleic acid

This method was originally described by Marco (1968) and subsequently modified by Miller (1971). To prepare the reactive mixture was added 27μL of linoleic acid, 0.1 ml of Tween 80, and 1 ml of β-carotene solution at 0.1 mg/mL in chloroform. Subsequently, the mixture was subjected to complete evaporation of the chloroform. To this mixture, free chloroform was added 50 mL of deionized water under vigorous stirring. The reactive mixture introduced itself clearly, with absorbance between 0.6 and 0.7nm to 470nm. The procedure consisted of adding a 5 ml aliquot of the reactive mixture into test tubes containing 0.5 ml of the extract, and the absorbance at 470nm was measured immediately. After the first reading, the tubes were incubated in a water bath at 50°C and measured absorbance in 15 min - 150 min. Until completion. The white was held along with extracts reading, and the preparation of reactive white mixture was only added linoleic acid and Tween 80. The percentage of antioxidant activity was determined by Equation 2:

% AA = white Abs 0 '- Abs white 150' / Abs sample 0 '- Abs sample 150' x 100(2)

2.3.4 Power iron reduction antioxidant (FRAP)

The method FRAP (Ferric Reducing Antioxidant Power) is also used to measure antioxidant capacity. In this method, the complex tripiridiltriazina ferric (Fe III-TZP) complex is reduced to the ferrous (Fe II-TZP) in the presence of an antioxidant and under acidic conditions. The complex formed by this reaction has an intense blue color with maximum absorption at 593 nm (Rufino *et al.*, 2006).

2.4 STATISTICAL ANALYSIS

All determinations were performed in triplicate, and the results were statistically analyzed by ANOVA and Tukey test at the 5% level of significance, with the aid of STATISTIC® program, version 7.0.

3 RESULTS AND DISCUSSION

Microalgae are a potential source for various valuable chemicals for commercial applications ranging from nutraceuticals to fuels (Scaglioni and Badiale-Furlong, 2017). Phenolic compounds are dominant contributors to antioxidant activity and also possess many biological activities (Machu *et al.*, 2015).

3.1 QUANTIFICATION OF PHENOLIC COMPOUNDS

The total content of phenols in *Spirulina* LEB-18 was 2.28mg gálico. g acid microalgae -1. Some work has been mentioning the presence of phenolic compounds, other sources of alimentary. With this, the composition of phenolic compounds for various tissues evaluated in studies found in the literature has a great advantage. The levels can be influenced, like all other components, the variety, species, the planting procedure, storage conditions, and the industrial process to which the fabric is subjected (Kim *et al*., 2003; Souza *et al*., 2010).

In comparison with this study, Sari (2011), that S. platensis obtained results in relatively low total phenols of 0.468 mg / g extract. Comparing the total phenol content in Spirulina LEB-18 with Souza *et al.* (2009) with other plant tissue species, where they found the total phenol content ranging from 0.077 mg/g (bs) in extract (bs) in aqueous onion extract, as well as in the rice bran study obtained a mean CF content of 0.75 mg/g of rice bran (Souza *et al*., 2012).

According to the literature on the amount of phenol found in many plant tissues as 0.99 mg/g (tyrosine equivalents), phenolic compound content obtained in the potato from 0.061 to 0.084 mg/g (ferulic acid) in orange peel, also found 1.0 mg/g and 0.31 mg/g of phenols in apple and banana respectively (Zhou *et al*., 2004). In addition, other studies state that the phenolic acids of Spirulina, besides acting as antioxidants, act as natural fungicides (Pagnussatt *et al*., 2014).

Therefore, it can be justified that *Spirulina* is a promising source of phenols by presented more content than food consumed by the population.

According to Safafar *et al.* (2015), the phenolic content based on the Folin presented 7.72 (mg/gGAE) to the concentration of microalgal biomass *Desmodesmus* (De.S). Larrosa *et al.* (2016), in their research regarding *Spirulina* dried using the vacuum dryer, met for total phenolics 326mg / 100g-1 for *Spirulina in nature.*

However, several authors have published results on the content of phenolic compounds in microalgae (Li *et al*., 2007; Colla *et al*., 2007; Souza *et al*., 2010), but there is little information about the content of phenols in particular microalgae.

3.2 ANTIOXIDANT ACTIVITY OF THE PHENOLIC COMPOUNDS OF SPIRULINA LEB-18

Antioxidants are compounds that inhibit the oxidation of other molecules and compounds and, they have an important impact on the prevention of different diseases like cardiovascular disease (Samarakoon and Jeon, 2012).

Table 1 shows the content of phenolic compounds in *Spirulina* sp LEB-18 and its antioxidant activity against different methods.

Table 1.Assessment antioxidant DPPH (%) IC50 (mg / mL) ABTS (%) β-Carotene (%) and FRAP (Ferric Reducing Antioxidant Power)

Analysis	**Phenolic extract of Spirulina LEB-18 (Lot 2014)**
DPPH (%)	***20.43***
IC 50 (mg/mL)	***8.19***
ABTS(µM Trolox.g -1 microalgae)	***74.85***
β-Carotene(%)	***61.64***
Ferrous sulfate concentration (µM/g sample)	***91.37***

Source: Authors.

In this study, we found 20.43% of ASR (Table3) with the use of methanol extracts of *Spirulina* LEB-18 at a concentration of 2.28 mg/g gallic acid microalgae.

The greater the absorbance, the higher the antioxidant activity of the sample. DPPH is a stable radical which has been widely used to test the ability to capture free radicals in different samples (Cao *et al*., 2012; Panzella *et al.,* 2012; Bukman *et al*.,2013). Sudha *et al*. (2011) studied the antioxidant activity of *Spirulina platensis* sample at a concentration of 15mg/ml, which showed 51.94% of DPPH scavenging activity.

Assis (2012) found 28.65% ASR with the use of methanol extracts in the concentration of *Chlorella* microalga 8.42mg/mL to pyranoidosa.

Ahmed *et al*. (2010) stated that antioxidants produced in the body are insufficient to fight free radicals produced by the body, it will suffer degenerative actions through the disorder known as oxidative stress, and the trend is that these radicals increase, causing great damage and harm to the body, even cell death.

The free radicals can attack the polyunsaturated fatty acids of the membranes, which are very vulnerable. These reactive molecules trigger oxidation reactions in the fatty acids of the lipoprotein membrane called lipid peroxidation, which affect the structural and functional integrity of the cell membrane, changing its fluidity and permeability. Moreover,

the oxidation of membrane lipids products can cause changes in certain cell functions (Kumar *et al*., 2012).

According to Wu *et al*. (2005), they had found that the *Spirulina* extract had higher antioxidant activity than other commercial algae, such as *Chlorella*, attributing this effect to the higher content of phenolic compounds that species. Custódio *et al*. (2012) found 35.3% ASR with the use of methanol extracts (10 mg/ml) for microalga chlorella *minutissima.*

According to Table 1, the greatest inhibition of ABTS radical by the phenol extract was from *Spirulina* LEB-18. According to Yeh and Yen (2006), they concluded that among all benzoic acids present in vegetables, gallic acid is the one with greater effectiveness in inactivating the radical ABTS • +. Gallic acid was the major phenol found in extracts evaluated by Assis (2012).

But Cepoi *et al.* (2009) found no proportionally greater the ABTS• + radical scavenging activity, the extent to which increased the concentration of the solvent in ethanol extracts of *Spirulina platensis*.

According to Chu *et al*. (2010), it was found a strong ability of the aqueous extract from *Spirulina* significantly reduce cell death induced by free radical DPPH • and ABTS • +, but also an activity of the crude extract much higher than the bioactive compound alone, suggesting that a mixture compounds more active than a single pure compound.

The compounds with antioxidant activity can act in different phases of the oxidative process (Damodaran et al., 2010). According to Tessmer Scaglioni *et al*. (2018), in their research with Spirulina sp. and Nannochloropsis sp, consider that the phenolic extracts of microalgae are efficient in inhibiting the propagation phase, using the ABTS and DPPH methods.

Evaluation of antioxidant activity can be performed by spectrophotometric-based discoloration or oxidation of β-carotene induced oxidative degradation products of a fatty acid such as linoleic acid, used in this analysis. In this study, there was a high antioxidant activity of the compounds of *Spirulina* LEB-18, being suitable for inhibiting the oxidation of β-carotene (Table 1), according to Rodriguez-Garcia and Guil-Guerrero (2008), also had high values, using a similar antioxidant method, the ethanol extract of *Chlorella Vulgaris*, compared to two other microalgae (*P. cruentum, P. tricornutum*), and attributed the result to the chemical composition of *Chlorella*, as well as its high content of polyunsaturated fatty acids.

Machado *et al.* (2017), in their study on the evaluation of the antioxidant potential of phenolic extracts of microalgae *Spirulina* sp. LEB-18 and *Chlorella pyrenoidosa* found that

the antioxidant activity using the β-carotene / linoleic acid system, wherein the inhibition concentration was higher phenolic extracts from *Spirulina* (58%) compared to *Chlorella* (47%), meaning higher capacity inhibit the peroxidation of linoleic acid. Pulido *et al.* (2000) describe the method FRAP (Ferric Reducing Antioxidant Power) - Antioxidant Power Iron Reduction developed as an alternative to determining the reduction of iron in biological fluids and aqueous solutions of pure compounds.

However, it was found through Table 1, the values obtained are very low and inefficient microalgae for the reduction of iron (Fe3 + to Fe2 +), or phenolic extracts derived from *Spirulina*, did not show antioxidant activity to reduce Fe3+ in reducing Fe 2+ as to achieve an acceptable activity it had to increase the phenolic extract concentration of microalgae.

These data corroborate various studies such as Madhyastha *et al*. (2009), who found that the C-phycocyanin extracted from *Spirulina* fusiform showed less antioxidant activity to the method for reduction of iron (FRAP). As the O'Sullivana (2011), with its research where the phenolic compounds from seaweed L. Hyperborea demonstrated low antioxidant activity by FRAP. Therefore, according to the results of phenolic acids of the extracted biomass of *Spirulina* LEB-18, comparing the antioxidant activity determined by different methodologies has been to the method of ABTS.

4 CONCLUSION

The phenolic extract of *Spirulina* LEB-18 shows good antioxidant activity, inhibiting up to 74.85% for ABTS• +, using as a methodology (2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid)). To capture this radical. These results of inhibiting the extracts of phenolic compounds are an alternative that can prevent the degradation of food. As a suggestion for future work, a study of the flavored phenolic extract with different food formulations is suggested, evaluating its effects *in vivo.*

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