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Original article

Chemical characterization and evaluation of the biological activity of Spirulina extracts

Caracterización química y evaluación de la actividad biológica de extractos de Spirulina

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ABSTRACT: The increase in the yield and productivity of crops, without affecting the environment, is one of the challenges that farmers experience today. The use of Spirulina (*Arthrospira platensis)* extracts as a plant biostimulant is one of the most viable options to use for these purposes. The objective of this work was to obtain, characterize and evaluate the biological activity of some alcoholic extracts of Spirulina. For this, extracts were prepared from Spirulina powder using ethanol as solvent in two concentrations (70 and 90 %), two mass:solvent ratios (1:20 and 1:10) and two maceration times (10 and 21 days) and the content of proteins, phenols and flavonoids of each was determined by spectrophotometric determinations. Biological activity was evaluated using a rice seed germination assay from seed imbibition for 24 h and the germination process was carried out in Petri dishes with distilled water for seven days. The results obtained from the chemical characterization will allow to adapt the extraction parameters depending on the components that are to be favored and the physiological effect that is to be achieved, since the concentration of proteins, phenols and flavonoids in the extracts significantly influenced the percentage end of germination of rice seeds.

Key words: *Arthrospira platensis*, composition, germination, rice.

RESUMEN : El incremento en el rendimiento y la productividad de los cultivos, sin afectar el medio ambiente, es uno de los desafíos que experimentan los agricultores en la época actual. El empleo de extractos de Spirulina (*Arthrospira platensis*) como bioestimulante vegetal, es una de las opciones más viables a utilizar con estos fines. El objetivo del presente trabajo fue la obtención, caracterización y evaluación de la actividad biológica de algunos extractos alcohólicos de Spirulina. Para ello, se prepararon los extractos a partir de Spirulina en polvo utilizando etanol como solvente en dos concentraciones (70 y 90 %), dos relaciones masa-solvente(1:20 y 1:10) y dos tiempos de maceración (10 y 21 días) y se determinó el contenido de proteínas, fenoles y flavonoides de cada uno mediante determinaciones espectrofotométricas. La actividad biológica fue evaluada utilizando un ensayo de germinación de semillas de arroz a partir de la imbibición de semillas durante 24 h y el proceso de germinación se llevó a cabo en placas Petri con agua destilada durante siete días. Los resultados obtenidos de la caracterización química permitirán adecuar los parámetros de extracción en función de los componentes que se quieran favorecer y el efecto fisiológico que se quiera lograr, puesto que, la concentración de proteínas, fenoles y flavonoides en los extractos influyó significativamente en el porcentaje final de germinación de las semillas de arroz.

Palabras clave: *Arthrospira platensis*, composición, germinación, arroz.

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INTRODUCTION

In recent years, natural products based on algae and cyanobacteria are being used as agrochemical substitutes and have acquired great importance due to the benefits they have on crops and the reduced impact they have on the environment. It has been proven that their application increases certain metabolic and physiological expressions in plants. These products, such as algae and cyanobacteria extracts, are generally obtained by the use of a solvent and an adequate extraction process, and are mainly marketed as biofertilizers due to their high content of macro and micronutrients or as biostimulants because they contain, among other compounds, plant growth promoting hormones (1-4).

Spirulina (*Arthrospira platensis*), one of the most widely used for these purposes, is a microscopic blue-green cyanobacterium, where the blue color comes from the phycocyanin present and the green from the chlorophyll, and it was considered a microalgae until very recently. It derives its name from the spiral nature of its filaments and has become an object of scientific study due to its bioavailability of nutrients, since 85-95 % are assimilable (5).

It has approximately 60-70 % of its dry mass in proteins with high bioavailability, it also contains chlorophylls, as well as phenolic compounds and flavonoids that can act as natural antioxidants (6-8). It is the terrestrial and aquatic organism with the highest protein content and the best aminogram and digestibility (7); therefore, it is widely used as a source of amino acids for humans, animals and plants. It also contains essential polyunsaturated fatty acids and vitamins, as well as xanthines, phycobiliproteins (9,10), carbohydrates, nitrogen, phosphorus, potassium, calcium, iron, manganese, zinc (10). It also has a high content of vitamins B12, B1, B2, B6 and E, biotin, pantothenic acid, folic acid, inositol and niacin (10), halogenated compounds, polyketides, agar agar, alginic acid and carrageenan (11), great richness in α- and ß-carotenes (7,12), phycocyanin, considerable amounts of α-linolenic acid (polyunsaturated fatty acid with different beneficial effects) (13), a high concentration of phytohormones, trace elements, antioxidants and polysaccharides, therefore, it is an excellent biological supplement (14).

The effects that the Spirulina application has caused in different plant species have been reported by several authors. Thus, in *Amaranthus gangeticus*, it has been found that imbibition of seeds and foliar application of Spirulina extracts increased protein (14) and iron levels in plants (15). Similarly, it was reported that imbibition of *Phaseolus aureus* and *Solanum lycopersicum* L. seeds in extracts of this cyanobacterium increased Zn levels in plants (16). In the species *Solanum melongena* L., the application of a commercial fertilizer based on Spirulina increased plant yield without affecting foliar levels of N, P, K and Na or quality indicators (17). In addition, it was found that Spirulina platensis extract has positive effects on wheat and barley seed germination, as well as on root and stem lengths (18).

In Cuba, Spirulina has been cultivated for more than three decades; however, its use for agricultural purposes has been very limited and there is no information available about the use of extracts for these purposes. For this reason, the objective of the present work was to obtain, characterize and evaluate the biological activity of some alcoholic extracts of Spirulina.

MATERIALS AND METHODS

The experiment was carried out at the Department of Plant Physiology and Biochemistry of the National Institute of Agricultural Sciences (INCA). Extracts were prepared from Spirulina powder from the Génix Company of LABIOFAM S.A., using ethanol (absolute ethanol M= 46.07) as solvent.

Two ethanol concentrations (70 and 90 %), two mass-solvent ratios (1:20 and 1:10) and two maceration times (10 and 21 days) were studied for the elaboration of the extracts. The extracts obtained were as follows:

1. Mass-solvent ratio 1:20 using EtOH 90 % for 21 days.
2. Mass-solvent ratio 1:20 using EtOH 90 % for 10 days.
3. Mass-solvent ratio 1:20 using EtOH 70 % for 21 days.
4. Mass-solvent ratio 1:20 using EtOH 70 % for 10 days.
5. Mass-solvent ratio 1:10 using EtOH 90 % for 21 days.
6. Mass-solvent ratio 1:10 using EtOH 90 % for 10 days.
7. Mass-solvent ratio 1:10 using EtOH 70 % for 21 days.
8. Mass-solvent ratio 1:10 using EtOH 70 % for 10 days.

Chemical characterization of the extracts

The chemical characterization of the extracts was carried out by biochemical analysis methods with spectrophotometric determinations of proteins, flavonoids and phenols from the representation of the standard curve corresponding to each technique with the measurement of four repetitions of absorbances of each standard. Three calibration curves were obtained, where absorbance values of each standard were plotted as a function of concentration (19).

The quantification of proteins of each extract was performed using the Micro-Lowry method (20), the phenol content according to the Folin-Ciocalteau method (21) and flavonoids were determined using a spectrophotometric method (22).

Evaluation of the biological activity of the extracts

To determine whether the composition of proteins, phenols and flavonoids influenced the biological activity of the extracts, biological evaluation was performed on the extracts with the lowest (A) and highest (B) content of these compounds. For this purpose, an experiment was carried out in which rice seeds cv. INCA LP-7, for 24 h, with different concentrations (5; 1; 0.5; 0.05; 0.005 mg L^{-1}) of A and B extracts.

For germination, seeds were placed in Petri dishes (20 seeds per dish and four dishes per treatment) containing distilled water. The treatments were as follows:

1. Imbibition with distilled water (Control).

2. Imbibition with 5 mg L^{-1} of extract A.
3. Imbibition with 1 mg L^{-1} of the extract A.
4. Imbibition with 0.5 mg L^{-1} of extract A.
5. Imbibition with 0.05 mg L^{-1} of extract A.
6. Imbibition with 0.005 mg L^{-1} of the extract A.
7. Imbibition with 5 mg L^{-1} of extract B.
8. Imbibition with 1 mg L^{-1} of extract B.
9. Imbibition with 0.5 mg L^{-1} of extract B.
10. Imbibition with 0.05 mg L^{-1} extract B.
11. Imbibition with 0.005 mg L^{-1} of extract B.

The plates were placed in the germination chamber at 28 °C for seven days, evaluating the number of germinated seeds per plate at 24, 48, 72 and 144 hours, determining the final germination percentage and germination speed, and after ten days, the dry mass of the radicles (25 radicles per treatment, five samples of five radicles each).

For data processing, means, standard deviations and confidence intervals at α=0.05 were calculated using Excel software.

RESULTS AND DISCUSSION

Chemical characterization of the extracts

Table 1 shows the content of proteins, phenols and flavonoids present in the extracts analyzed.

As shown in Table 1, the highest protein concentrations were obtained when 70 % EtOH was used (extracts 3, 4, 7, 8), regardless of the mass-solvent ratio used, so that a lower ethanol concentration favors the presence of proteins in the extracts. Note that with a mass-solvent ratio of 1:20 and a 10-day maceration (extract 4), which is the most economical extract, a good protein content was obtained, suggesting that this extract could be used for crop biofortification.

With the phenol content, something similar occurred, while the highest concentrations of flavonoids were obtained in the extracts obtained with 90 % EtOH (extracts 1, 2, 5, 6), independently of the mass-solvent ratio and maceration time.

It follows that extraction with EtOH 70 % favored the content of soluble proteins and total phenols in the extracts, which is logical since the higher amount of water favors polarity. According some authors (23), the extraction of antioxidant compounds from Spirulina biomass was more effective with a 70 % ethanol solution. Likewise, antioxidant activity was favored in Spirulina extracts obtained with ethanol as solvent over other solvents such as H_20, methanol and petroleum ether (24). On the other hand, the extraction of total phenolic compounds was favored in extracts prepared with 50 % ethanol over those prepared with 50 % dimethyl sulfoxide (25).

Maceration with 90 % EtOH solution increased the content of total flavonoids, which could be useful to favor crops subjected to environmental stress, since the role of these compounds in increasing antioxidant activity is known (26). Other authors have obtained high concentrations of flavonoids, alkaloids and saponins from Spirulina biomass with absolute ethanol extraction (27).

It is interesting to note that the concentrations of the compounds evaluated did not differ significantly when comparing the two maceration times used. The time factor becomes more significant when using methods such as ultrasonication or microwave-assisted extraction, not so much maceration (28).

The presence of proteins, phenols, flavonoids, etc. in Spirulina extracts favors seed germination, the formation of healthy and balanced plants, higher crop yields, longer post-harvest life, greater leaf vigor, less incidence of diseases and greater resistance to stress due to climatic factors or drought (29-31). It should be taken into account that in the extracts, as mentioned above, there are many other compounds that were not determined in this work and that can influence their biological activity.

Nevertheless, with this limited chemical characterization, it was decided to select extracts 1 and 3 as the extracts with the lowest and highest protein and phenol content, respectively, to evaluate their biological activity as a stimulator of rice seed germination.

Biological activity of the extracts

The speed and final percentage of seed germination for each of the treatments are shown in Table 2. As can be seen, there was an influence on the final germination percentage with two concentrations (5 and 0.5 mg L^{-1}) of

Table 1. Concentration of total soluble proteins, phenols and flavonoids of the different alcoholic extracts of Spirulina

Extracts°	Proteins (µg µL^{-1})	Phenols (µg µL^{-1})	Flavonoids (µg µL^{-1})
1	5.5663 ± 1.1764	0.6513 ± 0.0420	**3.1232 ± 0.1822***
2	6.7619 ± 0.8062	0.6255 ± 0.0339	**2.9988 ± 0.1405***
3	**26.4102 ± 1.7561***	**1.3309 ± 0.0184***	1.8640 ± 0.1030
4	**13.0988 ± 2.2376***	0.7109 ± 0.0181	0.6049 ± 0.0849
5	10.2957 ± 1.9360	0.8627 ± 0.0389	**4.4198 ± 0.1523***
6	7.7716 ± 0.5229	0.7421 ± 0.0426	**3.3150 ± 0.0842***
7	**17.8415 ± 0.3408***	**1.1201 ± 0.0181***	1.8506 ± 0.0719
8	**21.0298 ± 2.4684***	**1.0707 ± 0.0244***	1.4563 ± 0.0473

1. Mass ratio: solvent 1:20 using EtOH 90 % for 21 days. 2. Mass ratio: solvent 1:20 using EtOH 90 % for 10 days. 3. Mass ratio: solvent 1:20 using EtOH 70 % for 21 days. 4. Mass ratio: solvent 1:20 using EtOH 70 % for 10 days. 5. Mass ratio: solvent 1:10 using EtOH 90% for 21 days. 6. Mass ratio: solvent 1:10 using EtOH 90 % for 10 days. 7. Mass ratio: solvent 1:10 using EtOH 70 % for 21 days. 8. Mass ratio: solvent 1:10 using EtOH 70 % for 10 days. *Means differing significantly from the control treatment according to confidence interval at α=0.05

extract 3, although there were no significant differences in the germination speed, so that the higher concentration of soluble proteins and total phenols presented by this extract significantly increased the final germination percentage of rice seeds cv. INCA LP-7.

The results of radicle dry mass showed that there were no significant differences between treatments; however, the immersion of the seeds in 5 mg L^{-1} and 0.05 mg L^{-1} of extract 3 increased the dry mass of the radicles by 8.2 and 10.3 %, respectively. Note that the treatment with 5 mg L^{-1} of this extract was one of those that significantly increased the final germination percentage; confirming the influence that a higher concentration of soluble proteins and total phenols exerted on the germination and initial growth of rice seedlings.

Microalgae and their extracts are natural stimulants that accelerate seed germination and increase seedling vigor when used at relatively low doses (32). Several studies describe their beneficial effects on germination percentage, rate and mean germination time, as well as plumule and radicle length. These results are attributed to the activation of key enzymatic pathways for germination physiology. For example, α-amylase is an enzyme synthesized in the aleurone layer and its gene expression is regulated by gibberellins. This enzyme is responsible for the mobilization of reserve substances, such as starch, from the endosperm to support embryo growth and differentiation (33). To demonstrate this hypothesis, the significant effects that an extract of Spirulina platensis exerted on peanut (*Arachis hypogaea* L.) crop on indicators such as seed germination percentage, radicle length, and protein and carbohydrate contents were recently reported (32). In another study, enhanced vigor, quality and germination of black bean (*Vigna mungo* L.) seeds were reported to be correlated with increased gibberellic acid and α-amylase enzyme activity, when soaked in 1.5 *% Spirulina platensis* extract for 3 hours (34). On the other hand, it was found that the use of a methanolic extract (0.25 %) favored almost all growth indicators, nutrient content, yield components and phytohormone level of *Lupinus luteus* (35). In another investigation, it was found that when tomato seeds were treated with 0.2 g 100 mL of Spirulina extract, a germination rate of 86.7 % was obtained (36), and concentrations of 25 and 50 % of this extract had a positive effect on seed germination and the development of lettuce seedlings (37).

The results obtained in this research, although still preliminary, showed the importance of ethanol concentration

Table 2. Effect of different concentrations of two alcoholic extracts of Spirulina on seed germination of rice cv. INCA LP-7(Means ± confidence intervals)

Concentrations mg L^{-1}	Alcoholic extracts of Spirulina	Final germination	Germination rate (seeds germinated day^{-1})
0	-	72.50 ± 2.83	11.95±0.39
5	Extract 1	72.50 ± 6.33	10.95±1.59
1		75.00 ± 8.95	10.62±0.92
0.5		82.50 ± 10.20	12.91±1.29
0.05		78.33 ± 5.66	12.05±0.83
0.005		75.00 ± 4.00	11.08±0.78
5	Extract 3	**82.50 ± 6.33***	12.40±1.42
1		71.25 ± 4.69	9.29±0.80
0.5		**83.33 ± 71.48***	11.44±0.92
0.05		68.75 ± 14.07	10.04±2.52
0.005		67.50 ± 8.49	9.54±0.82

Extract 1: Mass ratio: solvent 1:20 using EtOH 90 % for 21 days. Extract 3: Mass-solvent ratio 1:20 using EtOH 70 % for 21 days *Means differing significantly from control treatment according to confidence interval at α=0.05

Table 3. Effect of different concentrations of two alcoholic extracts of Spirulina on the dry mass of radicles of rice seedlings cv. INCA LP-7 (Means ±confidence intervals)

Concentracions mg L^{-1}	Spirulina alcoholic extracts	Dry mass of radicles (mg $plant^{-1}$)
0	-	4.37 ± 0.54
5	Extract 1	4.00 ± 0.23
1		4.50 ± 0.69
0.5		4.02 ± 0.57
0.05		4.15 ± 0.34
0.005		4.44 ± 0.69
5	Extract 3	4.73 ± 0.35
1		3.92 ± 0.60
0.5		3.81 ± 0.30
0.05		4.82 ± 0.49
0.005		4.04 ± 0.27

Extract 1: Mass ratio: solvent 1:20 using EtOH 90 % for 21 days. Extract 3: Mass-solvent ratio 1:20 using EtOH 70 % for 21 days

and maceration time in the composition and activity as agricultural biostimulant of Spirulina extracts; therefore, it is necessary to continue deepening in this sense, with a view to obtaining biologically active extracts that can be satisfactorily used in agriculture.

CONCLUSIONS

- The chemical characterization of the alcoholic extracts of Spirulina allowed the selection of two extracts for the evaluation of their biological activity.
- A higher concentration of soluble proteins and phenols in the extracts favored germination and dry mass of rice seedling radicles.

RECOMMENDATION

- To continue to characterize the extracts and evaluate the biological activity of the extracts in other crops.

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