

Production of Bioactive Lichen Compounds by Alginate-Immobilized Bionts Isolated from *Cladonia verticillaris*: An *in Vitro* Study

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Abstract

Bionts isolated from thalli of *Cladonia verticillaris*, immobilized in calcium alginate, produce two depsidones, fumarprotocetraric and protocetraric acids, and the depside atranorin, that exhibit antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae*. Organic lichen extract (acetone/ether/chloroform) shows the highest antimicrobial activity due to a possible synergism between these substances. Antioxidant capacity of soluble metabolites secreted from the immobilisates to the bath medium of incubation during the first 12 days of immobilization has been found (>80% oxidation inhibition). The concentration of soluble phenolic substances depends on the immobilization time (during 32 days), exogenous supply of acetate (1.0m mM sodium or calcium acetate) and on the type of isolated biont (phycobionts, mycobionts, whole thallus or immobilized phycobionts co-incubated with the mycobionts ones).

The role of phycobionts in phenol production has been interpreted as a possible modification of the polymalonyl pathway; for example, atranorin is actively produced and secreted during immobilization while it is not detected in thallus *in natura*. Co-incubated bionts secrete higher amounts of atranorin to the media during the first 12 days of immobilization. Immobilization of isolated bionts could be used as a biotechnological technique to obtain a potential source of biological active compounds.

On the other hand, the physiological state detected of *C. verticillaris* in the Cerrado is much better for experimentation than that of the specimens collected in Caatinga since, in this case, the thalli showed the fragility that the extreme environmental conditions of the semi-arid region of NE Brazil impose on this lichen species. To date, no priority has been defined for lichen conservation in Brazilian ecosystems. It is therefore suggested that it is important to include lichen ecophysiology studies in public conservation policies.

Keywords: *Cladoniaceae*, depsides, immobilisates, coastal tableland, caatinga, biological activities

Abbreviations: ABTS, 2,2'-Azino-di-(3-ethylbenzthiazoline sulfonic acid); ATR, Atranorin; CaOAc, calcium acetate; DMSO, dimethylsulfoxide; FUM, fumarprotocetraric acid; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; PRO, protocetraric acid; NaOAc, sodium acetate; TEAC, Trolox equivalent antioxidant capacity; Trolox, (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid).

1. Introduction

Lichens are the intriguing result of a symbiotic association between a fungus (the mycobiont) and a photosynthetic partner (the photobiont) that can be a green alga (chlorobiont) and/or a cyanobacterium (cyanobiont). The chemoergonic fungal partner gives to the phycobiont a protective structure to be able to

perform photosynthesis in an aerial rather than an aquatic environment, and the photoergonic algal partner gets energy for both symbionts through photosynthesis. The terricolous lichen *Cladonia verticillaris* is an endemic species of the east coast of Brazil and also occurs in some habitats at the interior of Pernambuco state (Tigre et al., 2012). The Caatinga is a semi-arid biome in northeastern Brazil with a significant degree of biodiversity in which *C. verticillaris* is found.

The biodiversity of the biome is highly threatened due to the progressive conversion of wild land for agriculture and livestock. Currently only 1% of its territory is well preserved (Ribeiro et al., 2009). Climate forecasts predict an increase in aridity, which could pose an additional threat to the biodiversity of the biome. The lichen *C. verticillaris* also colonizes the "Tabuleiros costeiros" (coastal plateau), considered an edaphic biome of the savannah (Cerrado), which extends over the central-west, southeast and north-northeast of Brazil. In both biomes, that of the Caatinga and that one of the savanna, the climate is tropical (Kottek and others, 2006) and the dry season is long and well defined.

C. verticillaris is chemically characterized by the production of two depsidones, protocetraric (PRO) and fumarprotocetraric (FUM) acids, and a depside, atranorin (ATR), considered as a precursor of these two depsidones (Legaz et al., 2011). These three compounds derive from methyl-3-orsellinate produced by the action of a polyketide synthase. These enzymes structurally assemble diverse products from simple acyl-CoA substrates by using a catalytic cycle involving decarboxylative Claisen condensations and variable modifications, such as reduction and dehydration of several intermediates. The occurrence of the Claisen condensation is the main step in polyketide biosynthesis (Hutchinson et al., 2000). Lichens produce sugars through photosynthesis, which relies exclusively on the photobiont, and consume carbohydrates via respiration, which takes place in both bionts, although it is suggested that total thallus respiration mainly reflects the metabolic activity of the mycobiont (Lange and Green, 2005), much more abundant in heteromerous lichens, as is the case of *C. verticillaris*, than that of the photobiont, which may constitute as little as the 10% of the total thallus biomass (Sundberg et al., 1999; Manguas et al., 2013). The main enzymes involved in the phenols production are synthesized exclusively by the fungal partner (Calcott et al., 2018). This excludes algae from the biosynthesis pathway of the orsellinate derivatives. However, some authors (Culberson and Ahmadjian, 1980; D'Áz et al., 2015) propose that the algal partner is able to regulate fungal phenol production by the action of effectors secreted to the intercellular spaces.

Compounds isolated from several lichen species have been reported to exhibit various biological activities. They are used as medicine, food, feed, perfume, spices, dyes and for various purposes worldwide (Shukla et al., 2010). The pharmacological and other biological activities of lichen substances could be divided into different categories, including antimicrobial, anticancer, antinociceptive and antioxidant (Shukla et al., 2010; Pejín et al., 2013; Kosanić et al., 2014; White et al., 2014). Recently, much attention has been paid to several lichen species as sources of natural antioxidants (White et al., 2014). The rapid emergence of antibiotic-resistant strains of bacteria currently in use and the increase in the number of immunocompromised patients represent a challenge for researchers and health professionals. In this regard, there has been a growing demand for new, highly efficient, low toxicity antimicrobial agents for host cells.

Species of the genus *Cladonia* show anti-tumoral, anti-inflammatory and antipyretic activities and have been used in folk medicine to treat respiratory diseases (throat irritation, cough, asthma and tuberculosis). De Barros et al. (2014) confirmed the expectorant and antioxidant properties of FUM produced by the lichen *C. verticillaris*. A lectin purified from this lichen species shows antibacterial activity, particularly against Gram-positive bacteria; also, it was able to inhibit the growth of the dermatophyte fungus *Trichophyton rubrum* (Ramos et al., 2014). When testing in vivo the action of the extracts with ether of *C. verticillaris*, or those obtained by a combined extraction with ether and ethyl acetate, against solid tumors (Ehrlich's carcinoma and sarcoma-180), it is concluded that both extracts were able to inhibit the development of the tumors. The extracts of the samples collected in the dry season were very active, probably by the retention of the bioactive compounds FUM and PRO acids, due to the low occurrence of rains (Santos et al., 1997).

Lichen growth is slower than that of most vascular plants because they have a shorter period of activity. Therefore, the replacement in nature of the biomass consumed by the different industries is extremely slow and difficult. To minimize this problem, biotechnological methods are being developed, consisting mainly of tissue culture and cell immobilization (Behera et al., 2009; Vivas et al., 2006). The technique of cell immobilization have great advantages, such as the very small biomass required for immobilization, its inexpensive maintenance, longevity of cells in immobilized systems, and the very fast rate of metabolite production (Pereira et al., 2002; Martins et al., 2017). This methodology shows the advantage to be economical and effective for the production of substances of pharmacological interest from lichens without causing environmental damage or collection of large amounts of material for obtaining significant amounts of substances.

Metabolic activity of both partners, photobiont and mycobiont, must be mutually tuned in a very complicated manner in order to achieve and maintain structure, function and growth of the thallus (Honneger, 1993). In experimental lichenology, it is essential to understand the roles of different lichen components in this process.

Based on previous studies, this research attempt to confirm the influence of the algal cells on the production of FUM, PRO and ATR in *C. verticillaris*. At the same time, new biotechnological approaches, using immobilisates of isolated bionts, have been established in order to produce soluble and organic bioactive compounds with antioxidant and antimicrobial properties.

2. Materials and Methods

2.1 Biological Material and Area Selection

Cladonia verticillaris samples were collected from soils of Cerrado, in Mamanguape-PB (Paraíba, NE of Brazil, Latitude 06°44'27" S, Longitude 35°08'24" W, altitude 180 m) and in Caatinga: Buíque-Catimbau-Parna-PE (Pernambuco, NE of Brazil, Latitude 08°30'00" S, Longitude 37°15'00" W, altitude 960 m). All the samples were collected from a unique environment to avoid changes in the concentration of bioactive compounds derived from the different soils or exposure degrees. The lichen material was separated in the laboratory from its substrate and stored in paper bags at room temperature in the dark. Determination of the lichen species was accomplished using chemical and morphological thallus characters. Dried specimens were deposited in the Herbarium UFPE, Dept. of Botany, Universidade Federal de Pernambuco (Brazil), register N° UFP 82995 (Mamanguape) and UFP 82997 (Caatinga). Different spectrophotometric assays were used to monitorize the physiological status of lichen. The loss of plasma membrane integrity was evaluated by use of Evans blue stain as described Baker and Mock (1994) and red vitality reagent was used for determining the number of living cell. Chloroplast pigments, extracted with dimethylsulphoxide, were quantified according to Pompelli et al. (2013).

2.2 Isolation of Lichen Bionts

Bionts were isolated from thalli of *C. verticillaris* thalli according to Fontaniella et al. (2000a) with modifications. Isolated phycobionts and mycobionts were observed under light microscopy (Fig. 1). Both myco and phycobionts were separated by centrifugation in a density gradient of sucrose-potassium iodide and phosphate buffer-sucrose, as described. Sufficiently rehydrated thalli (1.0 g) were ground to a fine powder in a mortar. Powder was resuspended in 15 mL of 10 mM phosphate buffer, pH 7.2 (Fig. 1A). Homogenates were filtered through four-layered cheese-cloth and centrifuged at 1500 rpm for 1 min. The pellet obtained was resuspended in 5 mL of phosphate buffer, the mixture was strongly stirred and newly centrifuged. This step was four times carried out. All the supernatants (Fig 1B) were mixed and centrifugation at 5000 rpm for 10 min, the pellet was resuspended in 4 mL 0.25 M sucrose, recovered with a micropipette and then this suspension was gently overlaid on the top of 5.0 mL of 80 % (w/v) potassium iodide, which was centrifuged at 1500 rpm for 45 s. The layer containing algal cells and hyphal fragments was recovered with a micropipette and placed on 5.0 mL KI solution. Then, 2.0 mL of 10 mM phosphate buffer were added and centrifuged at 3000 rpm for 90 s. The interphase containing algal cells was recovered with a micropipette, deposited on 5 mL KI and, then, 3.0 mL of phosphate buffer were added and centrifuged then at 3200 rpm for 3 min. This last step was repeated until yielding a pure preparation of algal cells. The fungal fraction obtained as a pellet during the last centrifugation was also recovered and 80 % KI was added to a final volume of 4.0 mL. The mixture was strongly stirred and 4.0 mL 10 mM potassium phosphate buffer, pH 7.2, was added. The mixture was then centrifuged at 3200 rpm for 3 min until a gradient is formed. Algal contaminations were removed with a micropipette and added to the algal preparation (Fig. 1C). The protocol was repeated until obtain a pure preparation of fungal cells (Fig. 1D).

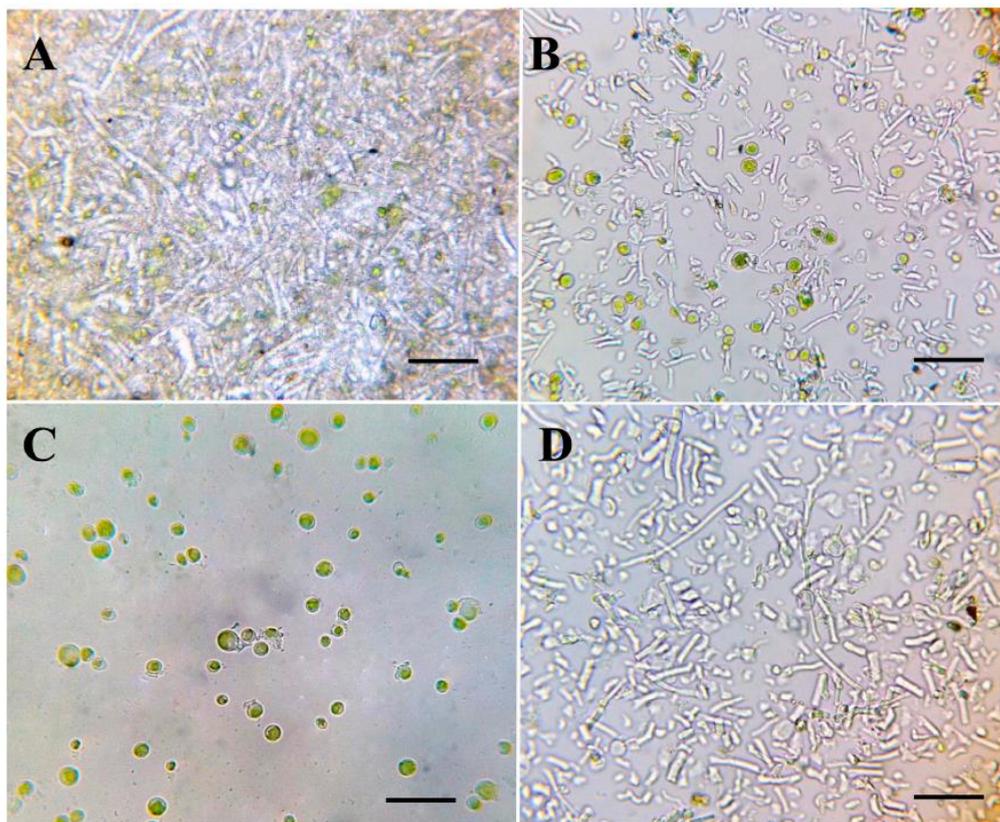


Figure 1. Light micrographs of macerated. (A), algal and hyphal fragments (B), phycobiont (C) and mycobiont (D) of *Cladonia verticillaris*. Bar = 20 μm

2.3 Immobilization of Isolated Bionts

Mechanically disgregated thalli (100 μg), or isolated phyco- or mycobionts (100 μg) were dispersed in autoclaved 5.0 mL 4% (w/v) sodium alginate and the different cell suspensions were added drop-to-drop, by means of a syringe, on 25 mL of a sterile 20 mM calcium chloride solution. After 24 h, the calcium alginate spheres (immobilisates) were ready for their use. The beads of calcium alginate were supplemented with 20 mL 1.0 mM sodium acetate (NaOAc) and maintained, gently stirred, at 25 ± 1 $^{\circ}\text{C}$ for 32 days in the light of a photon flux rate of $125 \mu\text{mol m}^{-2} \text{s}^{-1}$. A second series of immobilized cells were prepared and maintained in the same conditions but 1.0 mM calcium acetate (CaOAc) was used as the bath solution. Aliquots of 5.0 mL from these bath solutions were collected at different times (on alternate days) to extract lichen metabolites and replaced with the same volume of fresh medium. Four types immobilisates were designed as isolated phycobionts, isolated mycobionts, lichen thallus and immobilized phycobionts co-incubated with the mycobionts ones. For this type of bioreactor, 80 μg of mycobiont and 20 μg of phycobiont were used according to their proportion found in thalli. Bioreactors washes of immobilization systems were employed to evaluate phenolic, proteins, carbohydrates content and in vitro antioxidant and antimicrobial activity.

2.4 Phenolics Extraction and Analysis from Immobilized Samples

Phenolics were extracted by mixing aliquots of the bath solution with organic solvents. Samples were placed in a separation funnel and treated with two solvent systems: 5.0 mL of diethyl ether/ethyl acetate (60:40, v/v) for the first extraction and 5.0 mL of chloroform/acetonitrile (65:35, v/v) for a second extraction. Organic phases were mixed when indicated. Phenolic content was analyzed by using high performance liquid chromatography (HPLC) method.

2.5 Phenolic Quantification by HPLC

The organic extracts from each immobilized system were dried in air-flow. The residue was redissolved in 250 μL methanol and analysed by HPLC according to Legaz and Vicente (1983) using a Hitachi Chromatograph (655 A-11, Tokyo, Japan) coupled to a CG437-B UV detector. For the separation, a C-18 reverse phase column MicroPack MCH-18 de 300 mm \times 4 mm, Berlin, Germany (Merck® KGaA, Darmstadt, Germany) was used.

Organic extracts from cell immobilization and standard FUM, PRO and ATR were developed in an isocratic mode using as mobile phase methanol/deionized water/acetic acid, 80:19.5:0.5 (v/v). Detection was carried out by a UV set at 254 nm. Other analytical parameters were: volume of injection 10 μL , attenuation 0.16, pressure 90 atm, flow rate 1.0 mL min^{-1} at room temperature ($28 \text{ }^\circ\text{C} \pm 3 \text{ }^\circ\text{C}$). The results were analyzed by determining the retention time of substance in the column, and peak area, taking the standards, isolated and purified as described thereupon, as reference.

2.6 Isolation and Purification of FUM, PRO and ATR

FUM and PRO acids were purified from *C. verticillaris*. Thalli (30g) were successively extracted by soaking with diethyl ether, chloroform and acetone at room temperature for 3h in movement (three times). The last extract was kept cold during 24 h, after which it was filtered through a Whatman filter paper and concentrated using a rotary evaporator at $40 \text{ }^\circ\text{C}$. Finally, FUM and PRO were isolated using the vacuum flask. Cold acetone was added to the extract and centrifuged at 5000 rpm for 5 min. Then, the pellet was washed five times in G4 funnel with cold acetone.

ATR was kindly provided by the Dra. Neli Kika Honda of Department of Chemistry of Mato Grosso \acute{s} Federal University, Brazil.

2.7 Protein and Carbohydrate Quantification from Immobilisates

Bioreactors washes of immobilized systems were employed to evaluate proteins and carbohydrates content. A mixture of solvents, as above described, was required to discard phenolics acids and aqueous phase was used to evaluate proteins and carbohydrates according to Warburg and Christian (1941) and Dubois et al. (1956), respectively.

2.8 Antioxidant and Antimicrobial Assay

Aqueous phases collected at different times from immobilisates were frozen at $-20 \text{ }^\circ\text{C}$, lyophilised at $-80 \text{ }^\circ\text{C}$ and 10 mbars vacuum and stored at $-20 \text{ }^\circ\text{C}$ until determining antioxidant and antimicrobial capacity. Lichen acids (FUM, PRO and ATR) detected in bath solution and organic extract of lichen also were employed to determination of biological activities. The lyophilized samples and phenolic standards were redissolved in distilled water and methanol, respectively, to antioxidant assay or in 0.9% DMSO to antimicrobial assay.

The method of free radicals elimination by ABTS was used for antioxidant assay, according to Uch \acute{a} et al. (2015). The ABTS⁺ solution (3 mL) was added to the aqueous sample of immobilisates or lichen phenolic standards (30 μL of 1 $\text{mg}\cdot\text{mL}^{-1}$ solution) and reacted at different time intervals (6, 15, 30, 45, 60 and 120 min) before reading at 734 nm, room temperature. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a reference standard. The values of oxidative inhibition percentage were calculated and plotted as a function of the reference antioxidant concentration (Trolox) and expressed as Trolox equivalent antioxidant capacity (TEAC, μM). All determinations were carried out in triplicate.

The antimicrobial activity of *C. verticillaris* lyophilized samples from immobilisates, the phenolic acids, FUM, PRO and ATR, and organic extracts were tested against *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae*. Strains were provided by Department of Antibiotics of Pernambuco Federal University, Brazil, and maintained in Nutrient Agar and stored at $4 \text{ }^\circ\text{C}$. Minimum inhibitory concentration (MIC) was determined by the microdilution method following the protocol established by the CLSI (2011) for bacteria. A two fold serial dilution of the lichen samples (at concentrations ranging from 0.02 to 5.0 mg mL^{-1}) was prepared in Mueller Hinton Broth and 10 μL of bacterial suspension was added. Inoculates were prepared in the same medium at a density adjusted to a 0.5 McFarland turbidity standard [108 colony forming units (CFU) mL^{-1}] and diluted 1:10 for the broth microdilution procedure. Microtiter plates were incubated at $37 \text{ }^\circ\text{C}$ and the MICs were recorded after 24 h of incubation. MIC corresponded to the minimum sample concentration that inhibited visible bacterial growth. Resazurin solution (0.01% w/v) was used as an indicator by color change visualization: any color changes from purple to pink were recorded as bacterial growth. Afterwards, cultures were seeded onto Mueller Hinton Agar and incubated for 24 h at $37 \text{ }^\circ\text{C}$ to determine the minimum bactericidal concentration (MBC) which corresponded to the minimum concentration of samples that caused the bacteria elimination. The assay was performed in quadruplicate.

2.9 Statistical Analysis

The results were submitted to analysis of the Student's t-test, differences were considered to be significant at ($p < 0.05$).

3. Results and Discussion

3.1 Selection of the Geographical Area

The northeast of Brazil is divided into four phytogeographic zones from which Caatinga and Cerrado lato senso were chosen to carry out this work. These biomes present a great biodiversity and innumerable endemisms, such as the lichen species *C. verticillaris*, which is known to modify the chemical composition of the soil (Vasconcellos et al. 2015; de Armas et al., 2016). Some ecophysiological responses of *C. verticillaris* in the Caatinga and Cerrado biomes were evaluated, such as plasma membrane integrity (Fig. 2A), cell vitality (Fig. 2B), chloroplast pigment content (Fig. 2C) and phaeophytin content (Fig. 2D). The integrity of the plasma membrane (monitored with Evans Blue dye) and the mortality rate of algal cells (monitored with red vitality reagent) in thallus *in natura* indicated that *C. verticillaris* collected in Caatinga exhibited a higher mortality rate ($31 \pm 1.3\%$) in addition to a damage of its structure (optical density at 600 nm was 0.043 ± 0.02) compared to the lower values detected for specimens collected from Cerrado biome (Fig. 2A and B).

Analyzing the content of photosynthetic pigment in the thallus at zero time, before immobilization, the results indicated that *C. verticillaris* from Caatinga contained an amount of total chlorophylls higher than those from Cerrado (Fig. 2C), with a rate of chlorophyll a to chlorophyll b of 1:1 for the first and 2:1 for the second location. This appears to be a normal behaviour for lichens living in water-limiting habitats, such as arctic- and alpine environments or mediterranean areas (Pintado et al., 1997), where the short periods of water availability severely affects survival of photosynthetic organisms. Similarly, Schipperges et al. (1995) observed a higher chlorophyll content in lichens from southern, more xeric zones, than in others coming from higher-latitude, more mesic areas. Degradation of chlorophyll (b in Fig. 2C) to pheophytin (b in Fig. 2D) was observed only in the Caatinga biome, although similar values of pheophytin (a in Fig. 2D) were observed in both biomes (Fig. 2D).

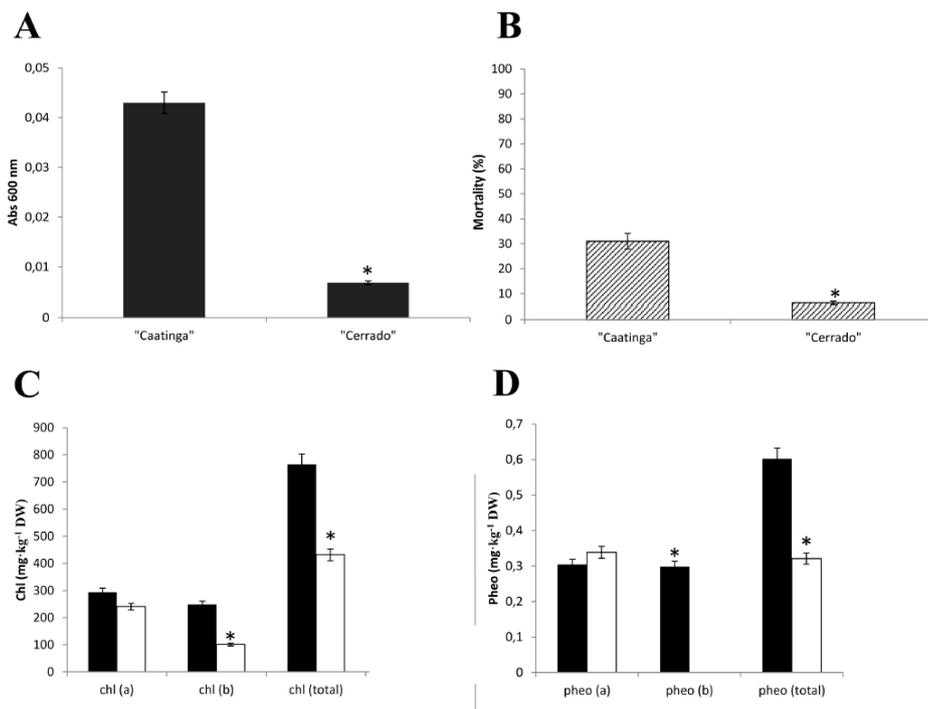


Figure 2. Ecophysiological response of *Cladonia verticillaris* to different Brazilian biomes. A) Plasma membrane integrity (OD at 600 nm), B) vitality (% mortality), C) chloroplast pigments (mg kg^{-1} DW) of thalli from “Caatinga (black) and Cerrado (white), and D) phaeophytin (mg kg^{-1} DW) of thalli from “Caatinga (black) and Cerrado (white). Values are the mean of three replicates, vertical bars give standard error. * indicates significant differences ($p < 0.05$)

The better physiological status of *C. verticillaris* in Cerrado biome is in agree to the fragility of Caatinga, the semi-arid region of Brazilian NE. For this reason, experiments were carried out using lichen thalli collected in Cerrado. To date, no definitions of priority of lichen conservation have been developed for the Brazilian

ecosystems. This would suggest the importance of including lichen ecophysiology studies in public conservation policies. The study and description of the physiological behaviour of one single species from geographically isolated places may be very useful as a means to increase our understanding of the climatic conditions, which are more favourable for the success of this species. Living organisms cope with changing environmental conditions in different ways. Differing selective pressures may contribute to the evolution of local adaptation (Nicotra, 2007), so that different populations of the same species growing in habitats with contrasting conditions often have different phenotypes. They usually show morphological, anatomical and/or physiological differences, which are a response to varying abiotic factors such as climatic conditions, nutrient limitation or water availability (Bradshaw, 1965; Cordell et al., 1998; Nicotra, 2007), or to interactions with other species (Davis et al., 1998). So things become more complicated for lichens. As dual organisms, the metabolic activity of both lichen partners must be tuned to each other in a very complicated way to achieve and maintain the structure, function and growth of the thallus. Therefore, lichens can be more competitive than their isolated counterparts and capable of colonizing new habitats.

3.2 Production of Lichen Compounds during Bionts-immobilization

C. verticillaris bionts were isolated by density gradient centrifugation. Algal and fungal preparations were examined by light microscopy. Results showed homogeneity of the samples (Fig. 1). The physical separation of lichen symbionts was necessary to analyze the role of lichenized algae in the production of phenolic acids.

Fungal, algal partners and whole thalli were immobilized in calcium alginate and mixed with aqueous solutions of a phenolic precursor, 1.0 mM NaOAc or 1.0 mM CaOAc, during 32 days. Bioreactors washes after this time of immobilization were employed to evaluate lichen phenolic acids by HPLC.

Thallus before immobilization, at zero time, contains two metabolites separated by HPLC: PRO ($t_R = 3.3$) and FUM ($t_R = 3.5$), whereas the washes of immobilisates also contain ATR ($t_R = 18.6$). This is not the only difference between thallus recently collected and that after immobilization. Time-course of phenolic production showed differences between thallus *in natura* and the different types of immobilisates. Time-course of phenolic recovering from the different washes is shown in Figs. 3, 4 and 5. FUM, PRO and ATR concentration was different depending on time of immobilization, the chemical nature of the precursor (NaOAc or CaOAc) and the type of biont-isolated (mycobiont, phycobiont, thallus, or co-incubated bionts immobilisates).

During the first 8 days of phycobionts immobilization and incubation on NaOAc, secretion of PRO decreased whereas that of FUM increased in an inverse way (Fig. 3A). Similar behavior was detected after 15 days of thallus immobilization and incubation on CaOAc. In this last assay, the maximum concentration of FUM recovery from the bath medium was reached (Fig. 4C). This suggested that both depsidones could be transformed one in the other one in a reaction catalyzed by an esterase/oxido-reductase complex (Fontaniella et al., 2000b). The maximum concentration (mg g^{-1} dry weight) of PRO recovery from the bath medium was reached after 5 days of mycobiont immobilization supplied with NaOAc, to be decreased after 8 days. In this case, the amount of FUM showed a similar pattern of accumulation (Fig. 3B). However, when immobilized isolated mycobionts were incubated on CaOAc, recovered amounts of both FUM and PRO were lower and similar during all the immobilization time (Fig. 4B). Similar values and time-course accumulation of FUM and PRO were obtained using whole thallus immobilisates and those which contained co-incubated bionts on NaOAc (Fig. 3C and D) or phycobionts and co-incubated bionts immobilisates on CaOAc (Fig. 4A and D).

Depside and depsidone are exclusively produced by lichenized fungi (Culbertson et al. 1985) or by isolated mycobionts (Culbertson & Armaleo 1992). However, Hamada & Ueno (1987) supposed that the algal partner could collaborate to depside-depsidone biosynthesis producing chemical modifications of the basic structure. Diaz et al. (2016) confirmed that the photobiont could also have some influence on the secondary metabolism of the mycobiont. New studies have revealed that the algal partner could use fungal enzymes secreted during isolation from the intact thallus to produce lichen substances (unpublished data). Diffusible enzymes from the mycobiont could be entrapped together the isolated phycobionts in the alginate beads. Under these conditions, stabilized fungal enzymes after entrapment (Guzik et al., 2014) could have a continuous source of acetyl-CoA and malonyl-CoA from the phycobionts and/or type of carbon supply used (NaOAc, CaOAc). In this way, the production of FUM and PRO would be explained by the action of phycobionts immobilisates. Enzymes entrapped in calcium alginate are more stable than those free enzymes during the storage in solution (Busto et al., 1987; Danial et al., 2015). The fungal enzyme that produced FUM from PRO depends on the algal photoassimilates and on the supply of succinyl-CoA from the fungal respiration (Fontaniella et al., 2000b). For these reasons, the production and secretion of depsidones drastically diminished from mycobionts immobilized on NaOAc in the absence of the algal partner (Fig. 3B). These results would be in agreement with those obtained

by Díaz et al. (2016). However, immobilisates of isolated phycobionts maintained the pool of acetyl-CoA and, so, FUM and PRO were secreted to the medium mainly from immobilized isolated phycobionts and thallus.

Metabolism of *C. verticillaris* was modified to produce other compounds when bionts were isolated and immobilized, since the occurrence of ATR in the incubation media was observed whereas it was not found in thallus before immobilization. Co-incubated bionts secreted to the medium higher amounts of ATR after 8 and 12 days of immobilization when the immobilisates were supplied with NaOAc (Fig. 5A) and CaOAc (Fig. 5B), respectively. ATR was then neosynthesized and stored during immobilization process. This could explain the lower amount of FUM and PRO detected in the media of immobilized co-incubated bionts. In this case, the conversion of ATR into depsidones might be inhibited.

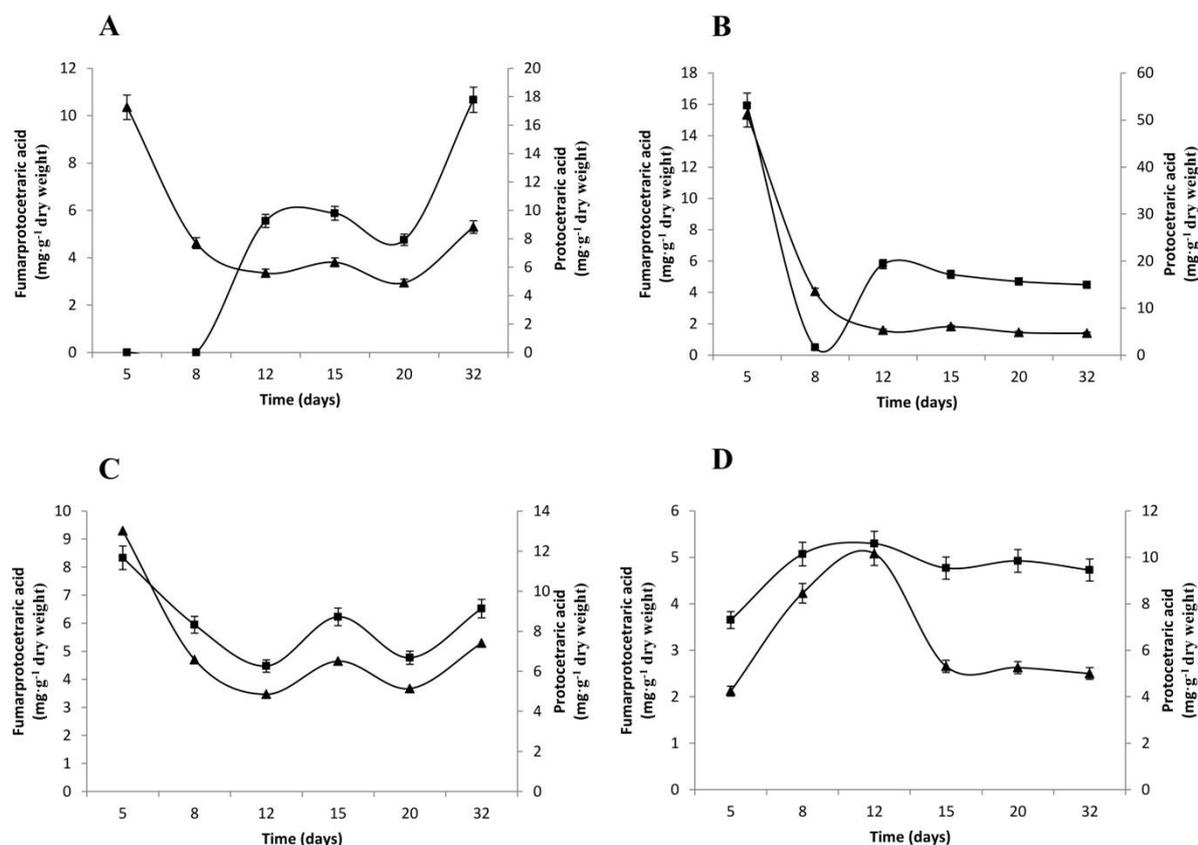


Figure 3. Time-course of FUM (■) and PRO (▲) acids production by alginate-immobilized phycobionts (A), mycobionts (B), lichen (C) and mycobionts co-incubated with algal immobilisates (D). Cells were supplemented with 1.0 mM NaOAc. Values are the mean of three replicates, vertical bars give standard error where larger than the symbols

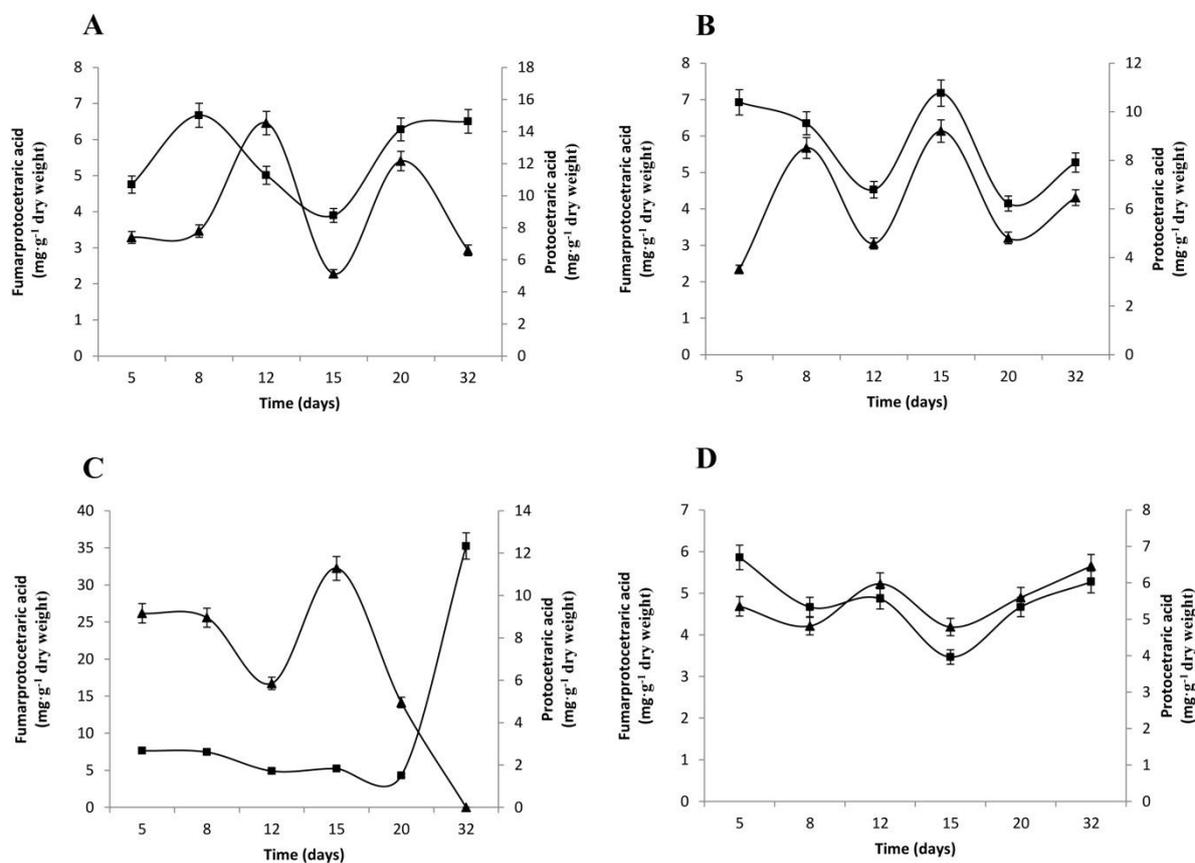


Figure 4. Time-course of FUM (■) and PRO (▲) acids production by alginate-immobilized phycobionts (A), mycobionts (B), lichen (C) and mycobionts co-incubated with algal immobilisates (D). Cells were supplemented with 1.0 mM CaOAc. Values are the mean of three replicates, vertical bars give standard error where larger than the symbols

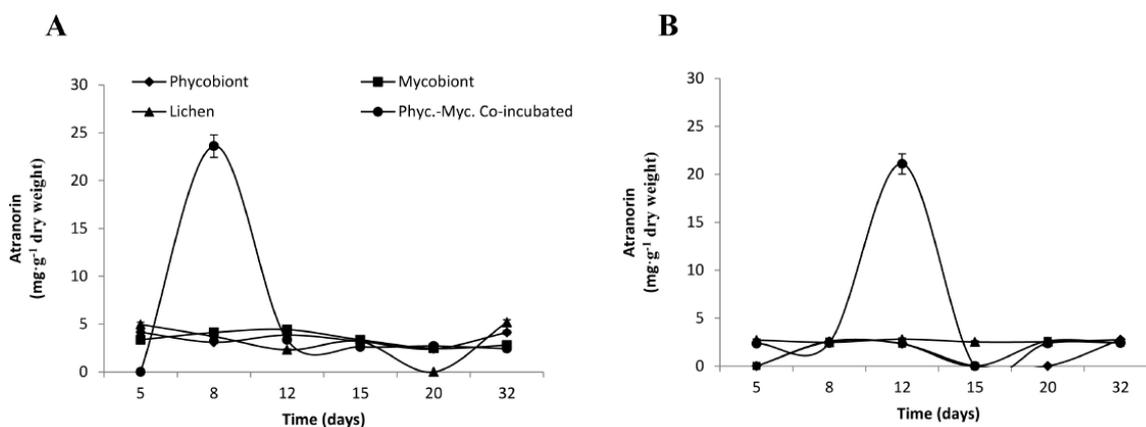


Figure 5. Time-course of ATR production by alginate-immobilized phycobionts (♦), mycobionts (■), lichen (▲) and mycobionts co-incubated with algal immobilisates (●). A) represents cells supplemented with 1.0 mM NaOAc and B) cells supplemented with 1.0 mM CaOAc. Values are the mean of three replicates; vertical bars give standard error where larger than the symbols

Isolated and immobilized bionts synthesize different secondary metabolites, the accumulation of which is different from that found in symbiosis before immobilization, as occurs with bionts and/or thalli cultures

(Yoshimura et al., 1994; Stocker-Wörgötter, 2002; Fazio et al., 2007). Polymalonyl pathway seems to be modified to synthesize those compounds required for symbiosis or isolated bionts, as long as acetate is available. These facts could be explained by a possible heterogeneity with respect to the ability to use acetate. According to these results, the isolation and maintenance of bionts on CaOAc or NaOAc will influence phenolic metabolism. Different results observed for other *Cladonia* species justified these results (unpublished data).

3.3 *In Vitro* Antioxidant and Antimicrobial Properties of Organic and Soluble Metabolites Produced by Immobilized Isolated Bionts

In vitro biological activities of lichen phenolics were evaluated using bath media of immobilization. Elimination of free radicals by ABTS in the antioxidant assay was analyzed. The antioxidant activity, measured as the equivalent antioxidant capacity of Trolox (TEAC), is based on the scavenging of ABTS by Trolox. Figure 6 shows the samples that have antioxidant activity. Data for values below 50% oxidation inhibition are not shown. After 12 days of immobilization, no samples showed antioxidant capacity. The concentration of the soluble extracts used in the test was always 1.0 mg·mL⁻¹.

The soluble extracts obtained from immobilized phycobionts and mycobionts incubated in NaOAc showed a potent antioxidant capacity with an inhibition of the oxidation capacity >80% (after a reaction time of 15 minutes) during the first 12 days of immobilization. Thallus and co-incubated bionts secreted to the bath medium soluble metabolites with similar potent antioxidant capacity during 5 and 8 days of incubation, like mycobionts and co-incubated bionts on CaOAc. However, when phycobionts and whole thallus were incubated on CaOAc, soluble metabolites with potent antioxidant capacity were secreted only during the first 5 days.

The maximum value of proteins and carbohydrates content is shown in Fig. 7. During the first 12 days of immobilization, only accumulation of proteins was detected in the media. The results showed that immobilisates of bionts isolated of *C. verticillaris* have antioxidant activity (Fig. 6) and this seems to be related to the soluble and diffusible proteins. These results mostly agree with the those from the literature, where we can find a number of reports for the antioxidant activity of lichen compounds (White et al., 2014). Proteins can inhibit lipid oxidation by biologically designed mechanisms (e.g. antioxidant enzymes and iron-binding proteins) or by nonspecific mechanisms. Amino acid residues that can scavenge free radicals and chelate pro-oxidative metals (Elias et al., 2009). Regarding the scavenging of water-soluble radicals, de la Coba et al. (2009) concluded that the antioxidant activity of microsporine-like amino acids isolated from three red macroalgae and a marine lichen was dose-dependent and increased with the alkalinity of the medium. According to Brewer et al (2011), the effectiveness of a large number of antioxidant agents is proportional to the number of hydroxyl groups present in their aromatic rings. On the other hand, some authors suggested that the antioxidant activity of different lichens may also depend on other, non-phenolic molecules (Odabasoglu et al., 2004). Weissman et al (2005) detected superoxide dismutase and catalases synthesized by the phycobionts and/or mycobionts. In addition, the authors also detected glutathione reductase and glucose-6-phosphate dehydrogenase activities in response to rehydration.

Antioxidant activity of depside (ATR) and depsidones (FUM and PRO) detected on bath medium of bioreactors also was assayed. Most of the reports relate the antioxidant activity of extracts with high content of phenolic compounds (Behera et al., 2009). However, FUM and PRO did not show to have antioxidant capacity *in vitro* in this study. ATR at the same concentration (1.0 mg mL⁻¹) had antioxidant capacity (47.77 ± 3.2%) lower than soluble extracts analyzed showed in Fig. 6.

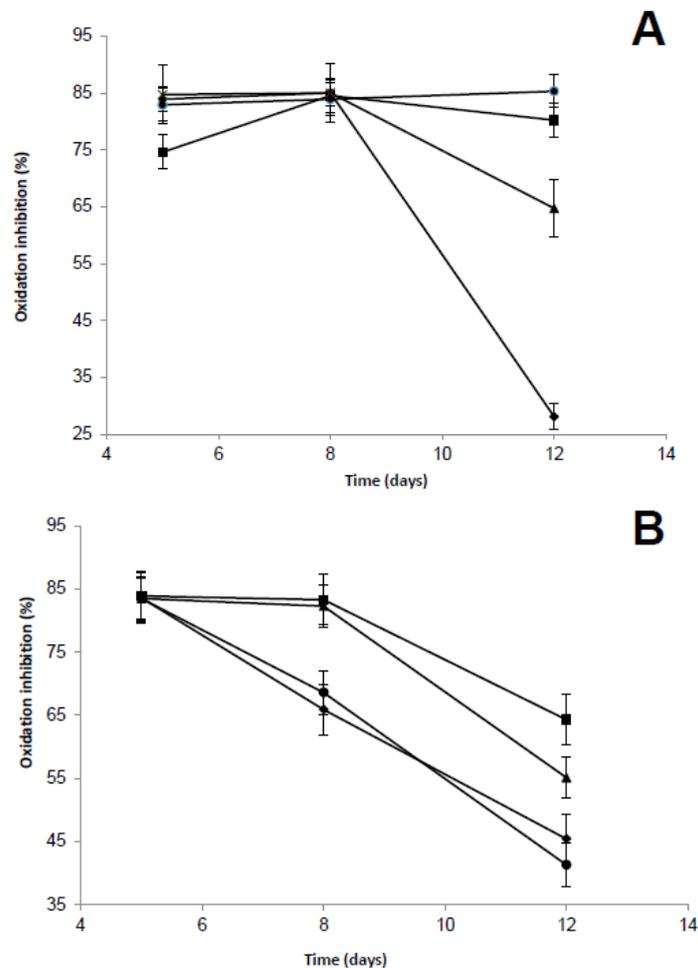


Figure 6. Time-course the oxidation inhibition produced by soluble extracts obtained by alginate-immobilized phycobionts (◆), mycobionts (■), lichen (▲) and mycobionts co-incubated with algal immobilisates (●). A) represents cells supplemented with 1.0 mM NaOAc and B) cells supplemented with 1.0 mM CaOAc. Values are the mean of three replicates; vertical bars give standard error where larger than the symbols

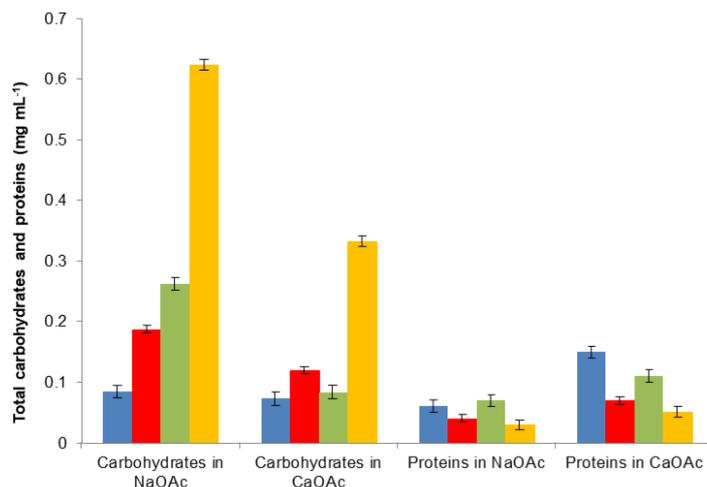


Figure 7. Maximum recovery of carbohydrates and proteins from the incubation media of alginate-immobilized phycobionts (blue), mycobionts (red), whole thallus (green) and mycobionts co-incubated with algal immobilisates (yellow). Values are the mean of three replicates; vertical bars give standard error

The antimicrobial activities of the lichen extracts (acetone/ether/chloroform), isolated substances (FUM, PRO and ATR) and water-soluble metabolites produced by immobilized isolated bionts against microorganisms such as *S. aureus*, *E. coli* and *K. pneumoniae* are shown in Table 1. The organic extract of *C. verticillaris* developed bacteriostatic (they inhibited the growth of microorganisms tested at concentrations from 0.039 to 0.625 mg mL⁻¹) and bactericidal activities against *S. aureus* at 2.5 mg mL⁻¹. FUM, PRO and ATR also inhibited growth of *E. coli* and *K. pneumoniae* at higher concentrations (0.625 to 5 mg mL⁻¹). The intensity of the MIC or MBC also depends on the species of microorganisms tested. Often, the activity of the extracts may be the result of synergist or antagonist effects of several compounds. In this work, lichen extracts show relatively strong antimicrobial activity. Lichen extracts are mixtures of different compounds, and their antimicrobial activity is not only the result of the different activities of individual components but may be the result of their interactions, which can have different effects on the overall activity of extracts. Ribeiro et al. (2006) also reported action of ether extract from *C. substellata* against human and plant pathogenic bacteria and supposed a synergic action between their components. Martins et al. (2010) also showed a synergic action of organic extract of *C. aggregata* against *S. aureus*. Similar results about the synergism between lichen substances have been observed for ether extract of *Heterodermia leucomela*, that contained high content of ATR and zeorin as an accessory substance, against *B. subtilis* (Falcão et al., 2002).

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the organic extract *Cladonia verticillaris* and FUM, PRO, and ATR standards, and soluble extract of immobilisates. Negative control was DMSO 0.9%

Bioactive fraction from <i>C. verticillaris</i>	Mean of MIC and MBC in <i>Staphylococcus aureus</i>		Mean of MIC and MBC in <i>Escherichia coli</i>		Mean of MIC and MBC in <i>Klebsiella pneumoniae</i>	
	MIC (mg mL ⁻¹)	MBC (mg mL ⁻¹)	MIC (mg mL ⁻¹)	MBC (mg mL ⁻¹)	MIC (mg mL ⁻¹)	MBC (mg mL ⁻¹)
Organic extract	0.625	2.5	0.039	-	0.3	-
FUM	-	-	2.5	2.5	2.5	-
PRO	-	-	5.0	-	0.625	-
ATR	-	-	-	-	2.5	-
Soluble extracts from immobilisates	-	-	-	-	-	-

Soluble compounds secreted to the medium of immobilization showed neither bacteriostatic nor bactericidal activity of the microorganisms tested. In the negative control, DMSO had no inhibitory effect on the tested organisms. The bactericidal activity was also found for FUM, which inhibited *E. coli* bacteria in 2.5 mg mL⁻¹ concentration. The best activity against bacteria was observed for organic extract (MIC ≤ 0.039 mg mL⁻¹; MBC ≤ 2.5 mg mL⁻¹). Yilmaz et al. (2003) observed antimicrobial activity of the lichen *Cladonia foliacea* extract containing usnic acid, ATR and FUM.

Numerous lichens and lichen compounds were screened for antimicrobial activity in search of the new antimicrobial agents (Goel et al., 2011; Ranković et al., 2012; Lucarini et al., 2012). The action mechanism of lichen phenolics against microorganisms could be based on their capacity to dissociate H⁺ which can lead to an acidification of plasma membrane surface of the microorganism, resulting in H⁺-ATPase rupture, required for ATP synthesis (Maeda et al., 1999), and causing the intracellular coagulation of cytoplasm constituents.

4. Conclusions

The better physiological status of *C. verticillaris* in Cerrado biome than Caatinga showed the fragility of the semi-arid region of Brazilian NE. Production of depsidones (FUM and PRO) and depside (ATR) depends on types of bionts-isolated immobilisates and exogenous nutritional supply. The results reveal the role of *C. verticillaris* phycobionts in the production of phenolic compounds. Polymalonyl pathway seems to be modified according to synthesize those compounds required as long as acetate is available. Antioxidant capacity of soluble metabolites secreted to the bath medium was also demonstrated. Our results indicate that organic extracts of *C. verticillaris* and their isolated compounds (FUM, PRO and ATR) could be used as antimicrobial agents. Bionts-isolated immobilization could be a biotechnological technique to obtain promissory active compounds.

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