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Evaluation of the antimicrobial activity of ethanol extracts from *Orthostichella rigida* (Mull. Hal.) B.H Allen & Magill (Bryophyta) on pathogenic microorganisms

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The indiscriminate use of antibiotics has led to the increase in antibiotic-resistant microorganisms, particularly the agents of high-morbidity diseases. Even though some studies report the use of bryophytes as sources of antimicrobial compounds, further research is necessary to elucidate the existence and potentialities of such bioactive compounds, especially in Brazil. This study investigated the antimicrobial properties of the ethanolic extract of *Orthostichella rigida* species of music, collected at different times of the year against bacteria and fungi that may be human pathogenic, the first of food origin and the second as opportunistic. Antimicrobial activity was verified using the broth microdilution method (MIC-MBC/MFC), assayed in triplicate. Seasonal influence was determined from the absolute values of MIC and MBC/MFC together with the triplicate average of these same methods. *O. rigida* ethanolic extract was effective in antimicrobial analysis for all microorganisms tested, being that the species less sensitive to *O. rigida* extracts was the fungus *Candida albicans* when compared to the other isolates tested. The highest sensitivity to *O. rigida* extracts were for *Listeria monocytogenes* (19.53 µg/mL), *Cryptococcus neoformans* (39.06 µg/mL), *Staphylococcus aureus* and *Salmonella enteritidis* (78.13 µg/mL). The extracts from the summer, autumn and winter seasons were more efficient to reach MIC, CBM and CFM, with values below 100 µg/mL for both, which is considered potent. Seasonal influence is statistically evident in spring, which has demonstrated minor antimicrobial activity when compared with the other seasons. Mosses are understudied regarding their compounds and *O. rigida* has demonstrated a rising potential for future research and possible use as a natural antimicrobial.

Key words: Antimicrobial activity, *Orthostichella rigida*, ethanol extracts, pathogenic microorganisms, bryophyta

INTRODUCTION

Following the World Health Organisation (WHO) guidelines, the Brazilian Ministry of Health created in 1998 a National Policy on Integrative and Complementary

Practices in Health, which defines the study of medicinal plants as a clinical research priority. In order to meet this priority, a National Program on Medicinal Plants and

Phytotherapics were created in 2008 to develop a scientific-based complementary and alternative therapy. This program established phytotherapeutic products based on the actual pharmacological value of medicinal herbs used in traditional medicine practices (DAFIE Brasil, 2006, 2009).

The most relevant topics for the development of phytomedicines are those regarding anti-cancer and antibiotic agents. Between 1980 and 2010, about 65% of registered antibiotics and 35% of registered anti-cancer agents were either from natural product origin or semisynthetic derived (Asakawa, 2012). Concerning plant-derived medicines, it is estimated that no more than 90 species are used as source of pharmacologically active compounds, and that altogether they are responsible for about 25% of the existing medicines in the global market (Calixto, 2000). Among plants, the most studied taxa are angiosperms, whilst few data are available on other plant groups such as bryophytes - which include mosses, liverworts, and hornworts (Asakawa, 1982).

The anatomical barriers of bryophytes, the simplest land plants, are less effective and therefore the "chemical barrier" established by secondary metabolites with antimicrobial activity is their most effective defence mechanism (Harborne, 1999). In the decade of 1950, the anti-bacterial effect of some species (*Anomodon rostratus* (Hedwig) Schimpe*, Orthotrichum rupestre* Schleich ex Schwägr. and *Plagiomnium cuspidatum* (Hedwig) T. J. Koponen attracted scientific attention (McCleary et al., 1960), and some years later, the first extensive study on this topic, including the analysis of 50 species, were published (McCleary and Walkington, 1966). In 1979, the antimicrobial activity of 52 species was examined in 8 bacterial strains; 56% of the tested species were active against at least one of the assayed bacteria (Banerjee and Sen, 1979). The bryophytes antibiotic activity against fungi and prokaryotic cells has also been confirmed. This is well known for *Conocephalum conicum* (L.) Dum, *Mnium undulatum* Hedw. and *Leptodictyum riparium* (Hedw.) Warnst, whose extracts have presented high antimicrobial activity against species of pathogenic bacteria (Cascaldo-Cobianchi et al., 1988). In Brazil, only two studies on the phytochemical and/or biological activity of bryophytes are known. These studies evaluated bryophytes as sources of antibiotic compounds (Pinheiro et al., 1989) and also the diversity of secondary metabolites in different populations of *Syzygiella rubricaulis* (Nees) Steph. (Costa et al., 2018).

The species here analysed shows that *Orthostichella rigida* (Müll. Hal.) B.H. Allen & Magill, is a moss, frequently growing as dense and hanging epiphytes. It is a member of the division Bryophyta, and is placed within the Family Neckeraceae (Goffinet et al., 2009). This species is distributed throughout the Neotropical and Palaearctic regions in Brazil, and is considered naturalised and exclusively occurs in the Atlantic Forest phytogeographic domain (Allen and Magill, 2007).

The World Health Organisation (WHO) published in 2017, a catalogue of "priority pathogens" in which were listed 12 families of antibiotic-resistant bacteria that represent the greatest threat to human health, among which are *Escherichia coli* (T. Escherich, 1885)*, Salmonella enterica* serovar Enteritidis (Le Minor and Popoff, 1987), and *Staphylococcus aureus* (Rosenbach, 1884) (WHO, 2017). These microorganisms, along with *Listeria monocytogenes* (Pirie, 1940), are transmitted through either water or food. Considering that, the WHO defines the foodborne and waterborne diseases as either infectious or toxic diseases caused by the consumption of contaminated foods or water (Figueiredo et al., 2007). According to estimates of the Agency of the United Nations, 420,000 deaths worldwide per year are due to water and foodborne diseases (WHO, 2015). Fungi that may be opportunistic pathogens responsible for fungal infections have also been studied. Among these, the most common infections are caused by *Candida* spp. *Cryptococcus neoformans* (San Felice) Vuill. and *Aspergillus* spp. (Tortora et al., 2017). It is estimated that about 11.5 million severe and superficial infections worldwide are due to fungal pathogens, leading to 1.5 million deaths per year (Giocomazzi et al., 2016).

Thus, research in plant natural products with antimicrobial action is justified since the indiscriminate use of antibiotics has led to the increase of antibiotic-resistant bacteria, particularly to the development of the agents of high-morbidity diseases – constituting, then, an important public health issue.

It is recognized that bryophytes are an important source of medicines and other pharmacological products (Cascaldo-Cobianchi et al., 1988) even though some studies have reported the use of bryophytes as sources of antimicrobial compounds (McCleary et al., 1960, McCleary and Walkington, 1966; Cascaldo-Cobianchi et al., 1988). Further research is necessary in order to elucidate the existence and potentialities of such bioactive compounds, especially in Brazil.

In this regard, this research aims to investigate the effect of antimicrobial properties of the ethanolic extract of *O. rigida* (Müll. Hal.) B.H. Allen & Magill moss species on the most common foodborne pathogenic bacteria such as *E. coli* (T. Escherich 1885), *Salmonella enterica* serovar Enteritidis (Le Minor and Popoff, 1987), *S. albicans* (Berkhout, 1923) and *C. neoformans* (San

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Felice) Vuill; which may vary by season, that is, collection period of the vegetable samples, in order to characterise the extract functionality throughout seasons.

MATERIALS AND METHODS

Plant material

The *O. rigida* samples were collected in the town of Osório, Rio Grande do Sul/Brazil, in the Environmental Protection Area of Morro de Osório (29° 52′ 54″ S, 50° 17′ 17″), southern limit of the Atlantic Forest. After the sampling, the species were identified in the Laboratory of Biology and Conservation of the State University of Rio Grande do Sul (Brazil) – North Coast Campus (LABeC). Four samplings were conducted, one per meteorologic season between October 2017 and July 2018, in the months of October, January, April and July, always in the afternoon. Approximately 100 g of wet weight of the material was collected and packed in identified packets until the extract obtention. A *voucher* specimen was deposited in the Dr. Ronaldo Wasum Herbarium of the State University of Rio Grande do Sul (Brazil) – North Coast Campus (HERW). The identification of the species was performed by Dr. Juçara Bordin of the State University of Rio Grande do Sul.

Extraction of samples

The ethanolic extract was extracted by static maceration with ethanol for seven days at room temperature. After the extraction period, the liquid was filtered, and the solvent was removed by using a rotatory evaporator in a thermostated bath at the constant temperature of 40ºC. Subsequently, the extract was placed in the lyophilizer for the complete removal of water or solvent residues. After obtaining the crude ethanolic extract, 200 mg of it was weighed and then diluted in 5 ml of the 1% Tween 80[®] diluent in order to obtain an extract with a final concentration of 40 mg/ml. (Fonseca, 2005; Handa et al., 2008; ANS, 2010).

Microorganisms

The American Type Culture Collection (ATCC) strains of Gram-negative bacteria *E. coli* (ATCC 35150) and *S. enteritidis* (ATCC 13076), of Gram-positive bacteria *S. aureus* (ATCC 25923) and *L. monocytogenes* (ATCC 35152)**,** and of yeast fungi *C. albicans* (ATCC 10231) and *C. neoformans* (ATCC 32045) provided by the Laboratory of Microbiology of UNIANÁLISES of the University of Vale do Taquari (Brazil) were used to assess the antimicrobial activity of *O. rigida.*

Determination of MIC and MBC/MFC

Bacterial suspensions were standardized from a 24-h culture whilst fungi suspensions were standardized from a 48-h culture, later adjusted to a 0.5 McFarland turbidity standard (approximately 1.0 x 10^8 CFU/ml). MIC (Minimum Inhibitory Concentration), MBC (Minimum Bactericidal Concentration), MFC (Minimum Fungicidal Concentration) were determined following the broth dilution methodology of Salie et al. (1996) and Newton et al. (2000). Based on the results, the following criteria were considered: growth of the microorganisms in the culture medium, significant bacteriostatic action, absence of microorganism growth in the culture medium, and significant bactericidal action. The extracts were examined in triplicate, and the experiment for each microorganism was conducted until the absolute concentration repeated itself at least

once, for only considering the result as validated.

Statistical analyses

In order to determine the seasonal influence, both the average of the absolute values of MIC and MBC/MFC were calculated and Kolmogorov-Smirnov normality and Levene homogeneity tests were performed. The data were subsequently statistically analysed by two-way ANOVA, with the significant values then compared by Tukey's and Bonferroni's tests (α = 0.05). Analyses and graphs were made using the Statistical Analysis System – SAS 9.4[®] and the GraphPad Prism 7.0[®] statistical software programs.

RESULTS AND DISCUSSION

When compared against the isolates, all extracts presented antimicrobial activity, differing in magnitude. The MIC and MBC/MFC values of the ethanolic extracts of *O. rigida* against *E. coli, S. enteritidis, S. aureus, L. monocytogenes, C. albicans* and *C. neoformans* are represented in (Tables 1 and 2), respectively. The negative and growth controls as well as the positive and media culture sterility controls responded to the expected results: negative and growth controls returned no activity, the positive control returned antimicrobial activity and the media sterility control confirmed media culture sterility.

There is no consensus with regard to acceptable MIC values of both extracts and vegetable matter fractions (Holetz et al., 2002; Aligiannis et al., 2001; Webster et al., 2008; Lambert et al., 2011). According to the present results, we proposed to use crude extract of *O. rigida*, four different criteria suggested by Holetz et al. (2002), Aligiannis et al. (2001), Webster et al. (2008) and Lambert et al. (2011) chosen in order to compare the results (Table 3). To Holetz et al. (2002), plant extracts that display antimicrobial activity in concentrations above 500 μg/mL present weak activity, with difficult pharmacological application on either bacterial or fungal infections; to Algiannis et al. (2001) and Lambert et al. (2011), only concentrations over 1,000 μg/mL are considered weak. However, Webster et al. (2008) suggest that any concentration lower than 1,000 μg/mL is regarded to be satisfactory.

For the Gram-negative bacteria - *E. coli* and *S. enteritidis,* the lowest MIC values were obtained in summer and winter: MIC of 156.25 μg/mL against *E. coli* in both seasons and MIC of 156.25 μg/mL and 78.13 μg/mL against *S. enteritidis* in winter and summer, respectively. These values, according to the established criteria in (Table 3), fit into either a good (MIC = 78.13 μg/mL) or moderate (MIC = 156.25 μg/mL) antimicrobial activity category (Holetz et al*.,* 2002; Lambert et al*.,* 2011). Nevertheless, according to the Aligiannis et al. (2001) classification, values of MIC lower than 500 μg/mL represent potent antimicrobial activity. When analysing the obtained MBC values, equivalent results are observed. Thus, the *O. rigida* extract is bactericidal against *S. enteritidis* in concentrations of 78.13 μg/mL

Table 1. Antimicrobial effects of the ethanolic extracts of Orthostichella rigida.

Sample (μg/mL)	Gram-negative bacteria		Gram-positive bacteria		Fungi	
	MIC		MIC		MIC	
	E. coli	*S. enteritidis*	*S. aureus*	*L. monocytogenes*	*C. albicans*	*C. neoformans*
Spring	-	-	78.13	156.25	-	625.00
Summer	156.25	78.13	19.53	19.53	156.25	39.06
Autumn	312.50	312.50	19.53	19.53	312.50	78.13
Winter	156.25	156.25	39.06	19.53	156.25	39.06

-, Values > 1,000 μg/mL.

Table 2. Minimum bactericidal and fungicidal concentration (MBC/MFC) of the ethanolic extracts of *Orthostichella rigida.*

Samples (μg/mL)	Gram-negative bacteria		Gram-positive bacteria		Fungi	
	MBC		MBC		MFC	
	E. coli	*S. enteritidis*	*S. aureus*	*L. monocytogenes*	*C. albicans*	*C. neoformans*
Spring	-	-	156.25	312.50	-	625.00
Summer	156.25	78.13	78.13	39.06	156.25	39.06
Autumn	312.50	312.50	78.13	19.53	625.00	78.13
Winter	156.25	156.25	78.13	78.13	312.50	39.06

-, Values > 1,000 μg/mL.

Table 3. Criteria for crude extract evaluation on antimicrobial activity.

MIC (μg/mL)	Holetz et al. (2002)	Aligiannis et al. (2001)	Webster et al. (2008)	Lambert et al. (2011)
≤10	-	-	-	Excellent
< 100	Good	Potent	-	Good
100 - 500	Moderate	Potent	-	Moderate
500 - 1000	Weak	Moderate	-	Weak
≤1000	-	-	Satisfactory	-
≥1000	Inactive	Weak	-	Inactive

-, Absent classification. Source: Adapted from Holetz et al. (2002), Aligiannis et al. (2001), Webster et al. (2008) and Lambert et al. (2011).

and against *E. coli* in concentrations of 156.25 μg/mL.

The antibacterial activity of mosses against Gram-negative bacteria was also cited in a study that demonstrated that these bryophytes present a better inhibitory capacity against Gram-negative bacteria than against Gram-positive bacteria (Basile et al*.,* 1999). The *O. rigida* extract was particularly active against antibiotic-resistant species (*S. aureus, S. enteritidis* and *E. coli).* The fact that the Gram-negative bacteria here assessed are sensible to the *O. rigida* extract is important in a clinical perspective, since antibacterial substances are primarily active against Gram-positive bacteria (Amato et al*.,* 2007).

Gram-positive bacteria presented lower values when compared to the other tested microorganisms. As shown in Table 1, apart from the spring sample against *L.*

monocytogenes, which returned a MIC value of 156.25 μg/mL, all other results fit the criteria established in Table 3 as displaying either good or potent antimicrobial activity, once the values were lower than 100 μg/mL.

Still regarding Gram-positive bacteria, all extracts obtained in different periods of the year returned low MIC concentrations; however, it is possible to observe that the lowest concentrations were obtained in summer and autumn against *S. aureus*, and in summer, autumn, and winter against *L. monocytogenes.* Analysing the MBC results, it was observed that with the exception of the sample of *L. monocytogenes* collected in October both MBC and MIC were similar and the MCB values of both bacteria were higher than their MIC values. Therefore, when tested on *S. aureus*, the summer and autumn samples of the extract returned to the lowest MIC values

Table 4. Average of concentration (µg/mL) obtained from MIC and MBC/MFC tests.

Season	Microorganism					
	Gram-negative		Gram-positive		Fungi	
	E. coli	*S. enteritidis*	*S. aureus*	*L. monocytogenes*	*C. albicans*	*C. neoformans*
Spring	208.30±360.80B	0.00±0.00B*	104.17±45.11B	130.21±45.11B	6250.00±2500.00Aa	520.83±180.42B
Summer	156.30±0.00*	78.13±0.00*	16.27±5.64	21.97±12.29	130.21±45.11b	39.06±0.00*
Autumn	312.50±0.00*	312.50±0.00*	26.04±11.28	19.53±0.00*	253.91±117.19b	78.13±0.00*
Winter	156.30±0.00*	156.30±0.00*	32.55±11.28	19.53±0.00*	208.33±90.21b	52.08±22.55

*Average values identical to absolute values. Distinct capital letters in the same row indicate significant difference between the microorganisms in each season returned by the Turkey test (p<0.05). Distinct lowercase letters in the same column indicate significant difference among seasons, also returned by the Turkey test (*p*<0.05).

and it was verified that they displayed bacteriostatic activity in concentrations of 19.53 µg/mL. Tested against *L. monocytogenes,* the bactericidal activity was also observed at this same concentration during winter. Nevertheless, against *L. monocytogenes,* the lowest MBC value registered for the autumn sample was 19.53 µg/mL, whilst against *S. aureus* the lowest value was 78.13 µg/mL registered for the summer, autumn, and winter samples.

The antibacterial activity against Gram-positive bacteria such as *S. aureus* must be valued, once these bacteria present high rates of resistance against all beta-lactam antibiotics. Dealing with methicillin and vancomycin-resistant *S. aureus* isolates has been challenging, and its susceptibility spectrum has become critical, contributing to high mortality rates (Cowen, 2008). Furthermore, the antimicrobial action against *L. monocytogenes* and *S. aureus* might be related to the sensibility of Gram-positive bacteria. Gram-positive bacteria have a single layered, with no outer cell membrane, in contrast to Gram-negative bacteria, whose cell wall is surrounded by an outer membrane containing lipopolysaccharides and proteins that function as a physical barrier with low permeability to antimicrobial agents (Forsythe, 2013). The results obtained here corroborate a previous study that analysed six species of the genus *Bryum* against pathogenic fungi and bacteria (Krishman et al*.,* 2012). Similar to what observed in our results, this study observed a more efficient antibiotic activity of the species against Gram-positive bacteria and a minor sensibility of Gram-negative bacteria to the antimicrobial agents (Krishman et al*.,* 2012).

When tested on the fungi, the lowest concentrations observed were 156.50 and 39.06 µg/mL for *C. albicans* and *C. neoformans,* respectively, whether obtained during the summer or winter (Table 1). According to the established criteria (Table 3), these values fit to the moderate/potent category (156.50 µg/mL) and to the good/potent category (39.06 µg/mL). The MFC results against *C. neoformans* were similar to the MIC results; while against *C. albicans*, however, except for the summer sample, the MFC values were higher than the

MIC values. Therefore, the summer extract of *O. rigida* is bactericidal against *C. albicans* in concentrations of 156.50 µg/mL, whereas against *C. neoformans*, the bactericidal action occurs in concentrations of 39.06 µg/mL.

Compared to bacteria, *C. albicans* is less sensitive to the *O. rigida* extract, possibly due to the difference between fungi and bacteria regarding cell wall and membrane composition (Cowen, 2008). The existence of a major antibacterial activity in comparison to antifungal activity indicates a specificity of action against the bacterial metabolism in the *O. rigida* extract (Sanglard and Odds, 2002). It must be also considered that *C. albicans* has been showing a problematic antifungal susceptibility profile. This resistance involves several molecular mechanisms, among them, the capability to change the lipid composition of its cell membrane (Sanglard and Odds, 2002). Also, other fungi here evaluated are sensitive to the *O. rigida* extract, which demonstrates an antifungal potential of less documented mosses, since studies on antifungal activity are more common on liverwort species (Sabovljevié et al*.,* 2001).

It is important to emphasize that it is impossible to directly compare the results here obtained with the results of the aforementioned work, since the species, methodology and microbiological strains evaluated were not the same. Elementally, the *O. rigida* extract has demonstrated a promising potential for novel studies and for application as a natural antimicrobial agent.

Regarding the influence of seasons, overall, summer and winter extracts presented the lowest absolute values of MIC and MBC/MFC tested against all assayed microorganisms. Nonetheless, when performing the statistical analysis in which the average of the triplicates was considered, the influence of seasons is only evident for spring, the only season with a significant higher MIC against *C. albicans* (Figure 1). Hence, statistically, the most appropriate period for collecting the vegetable matter of *O. rigida* is summer, autumn, and winter, since there is no significant difference between the MIC values of these seasons. A significant difference (*p*<0.05) was also observed among MIC values tested on
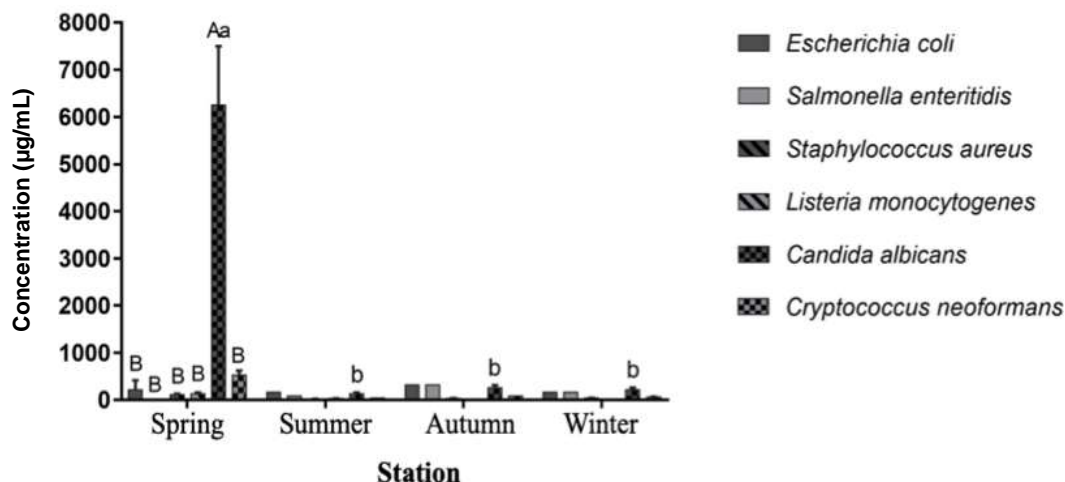
Figure 1. Average concentration (µg/mL) obtained by MIC and MBC/MFC tests. Capital letters indicate significant difference among the microorganisms within seasons. Lowercase letters indicate significant difference among the seasons.

microorganisms in summer, with *C. albicans* presenting significantly higher MIC values than any of the other microorganisms (Figure 1). This result is consistent with the work conducted by Ilhan et al. (2006), who compared the extracts of the *Palustriella commutata* (Brid.) Ochyra moss collected in two different periods of the year in the northern hemisphere – October 2004 and May 2005 – and demonstrated a major antimicrobial activity against tested strains in October, whilst the May samples lacked activity. Our results support the findings of Ilhan et al. (2006) as the least effective antimicrobial activity also occurred in spring for both studies.

The pattern observed here was predicted, seeing that many authors consider seasons as an influence factor on metabolism and on metabolite production in plants, as well as solar radiation, UV rays, and drought/wet periods (Becho et al., 2009). Besides these physical factors, the biological characteristics of *O. rigida* also help us understand the results presented here. The reproductive behaviour of bryophytes is a relevant topic that has been analysed in mosses species and on which a pattern was established: gametangia development in autumn and winter, fecundation in spring and summer, sporophyte growth in summer, followed by spore dispersion also in summer (Lloret and Pérez, 1984). This pattern is seen in mosses species of environments with distinct seasonal changes, such as Rio Grande do Sul, a subtropical location that has a subtropical/temperate climate with four distinct seasons. The results found here suggest that *O. rigida* repeats this reproductive pattern, considering that the best MIC results were observed in winter and summer seasons in which the plant is undergoing a critical reproductive period and therefore produces specific substances for gametangia development as well as for sporophyte growth and spore liberation.

According to the results obtained in this study, the ethanolic extract of *O. rigida* was effective in antimicrobial analysis for all microorganisms tested. The species less sensitive to *O. rigida* extracts was the *C. albicans* fungus when compared to the other isolates tested. The highest sensitivity to *O. rigida* extracts were for *L. monocytogenes* (19.53 µg/mL), *C. neoformans* (39.06 µg/mL), *S. aureus* and *S. enteritidis* (78.13 µg/mL). The extracts from the summer, autumn and winter seasons were more efficient to reach MIC, CBM and CFM, with values below 100 µg/mL for both, which is considered potent.

These environmental and physiological factors, the methodology employed, and the solvent used for the extract preparation justify the results obtained in the antimicrobial assays that were here demonstrated, as suggested by other authors (Haida et al., 2007). The interaction between the substances in each extract is another pertinent topic: it is known that countless biological activities are attributed to phytochemical compounds present in plants; in crude extracts, all the chemical compounds are present, being expected interaction among them either through synergism or antagonism, influencing the antimicrobial activity of each extract (Funari and Ferro, 2006).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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