



Potential antifungal activity of *Cladonia* aff. *rappii* A. Evans

[Actividad antifúngica potencial de *Cladonia* aff. *rappii* A. Evans]

Claudia M. Plaza^{1*}, Celina Pérez de Salazar², Marietta Vizcaya³, C. Gabriela Rodríguez-Castillo⁴, Gerardo E. Medina-Ramírez^{1,5}, Ramón E. Plaza¹

¹Biotechnology Section; Research Institute, Facultad de Farmacia y Bioanálisis, Universidad de Los Andes, Avenida Humberto Tejera, Mérida 5101, Venezuela.

²Mycology Laboratory. Department of Microbiology and Parasitology, Facultad de Farmacia y Bioanálisis, Universidad de los Andes, Ave. Humberto Tejera, Mérida 5101, Venezuela.

³Polymer Group ULA, Chemistry Department, Facultad de Ciencias, Universidad de los Andes, Sector la Hechicera. Ave. Las Américas, Mérida 5101, Venezuela.

⁴Chemistry Department, Universidad Nacional Experimental Francisco de Miranda. Prolongación Ave. Táchira, Sector Universitario, Punto Fijo 5141, Falcón, Venezuela.

⁵Facultad de Ciencias. Escuela de Bioquímica y Farmacia. Escuela Superior Politécnica de Chimborazo (ESPOCH). Ave. Panamericana Sur, Riobamba 060155, Quito, Ecuador.

*E-mail: claudiampz@gmail.com

Abstract

Context: Lichen is a self-supporting symbiotic organism composed of a fungus and an algal partner. They have manifold biological activities like antiviral, antibiotic, antioxidant, antitumor, allergenic and inhibition of plant growth. Species of *Cladonia*, have been studied by its antifungal activity.

Aims: To evaluate the antifungal activity determination of *Cladonia* aff. *rappii* against five yeasts, four of genus *Candida* and one *Cryptococcus*, using water, ethanol and dichloromethane extracts.

Methods: The evaluation of the antifungal activity was developed by three diffusion methods such as spot-on-a-lawn, disc diffusion and well diffusion. Additionally, the values of minimal inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) were determined.

Results: Based on the experimental results obtained, the best antifungal activity was using ethanol extract at 20 mg/mL against *Candida albicans*, applying the three diffusion methods above mentioned. With ethanol extract, the lower MIC was against *Candida glabrata* and the lower MFC were with *Candida glabrata*, *C. krusei*, *C. parapsilosis* and *C. tropicalis*. The dichloromethane extract presented the lowest MIC and MFC against *C. neoformans*. Not activity was observed with aqueous extract.

Conclusions: The present study revealed antifungal and fungicidal activity in the extract of lichen *Cladonia* aff. *rappii*.

Keywords: *Cladonia rappii*; diffusion methods; lichen; minimum fungicidal concentration; minimal inhibitory concentration.

Resumen

Contexto: El líquen es un organismo simbiótico autosuficiente compuesto por un hongo y una pareja de algas. Tienen múltiples actividades biológicas como antivirales, antibióticos, antioxidantes, antitumorales, alérgicas e inhibición del crecimiento de las plantas. Especies de *Cladonia*, han sido estudiadas por su actividad antifúngica.

Objetivos: Evaluar la actividad antifúngica de *Cladonia* aff. *rappii* contra cinco levaduras, cuatro del género *Candida* y un *Cryptococcus*, utilizando extractos de agua, etanol y diclorometano.

Métodos: La evaluación de la actividad antifúngica fue desarrollada por tres métodos de difusión tales como método de gota, difusión de discos y difusión de pozos. Además, se determinaron los valores de concentración mínima inhibitoria (CMI) y la concentración mínima fungicida (CMF).

Resultados: Sobre la base de los resultados experimentales obtenidos, la mejor actividad antifúngica fue usando el extracto etanólico a 20 mg/mL contra *Candida albicans*, aplicando los tres métodos de difusión arriba mencionados. Con el extracto de etanol, la CMI más baja fue contra *Candida glabrata* y las CMF más baja fueron contra *Candida glabrata*, *C. krusei*, *C. parapsilosis* y *C. tropicalis*. El extracto de diclorometano presentó la menor CMI y CMF contra *C. neoformans*. No se observó actividad con el extracto acuoso.

Conclusiones: El presente estudio reveló actividad antifúngica y fungicida en el extracto de líquen *Cladonia* aff. *rappii*.

Palabras Clave: *Cladonia rappii*; concentración mínima fungicida; concentración mínima inhibitoria; líquen; métodos de difusión.

ARTICLE INFO

Received | Recibido: April 19, 2017.

Received in revised form | Recibido en forma corregida: July 10, 2017.

Accepted | Aceptado: July 15, 2017.

Available Online | Publicado en Línea: July 25, 2017.

Declaration of interests | Declaración de Intereses: The authors declare no conflict of interest.

Funding | Financiación: The authors confirm that the project has no funding or grants.

Academic Editor | Editor Académico: Gabino Garrido.



INTRODUCTION

Medicinal plants continue to be major resources for therapeutic compounds and are receiving greater attention (Babiah et al., 2014). Many modern medicines were inspired by constituents found in traditional medicinal plants, and some modern drugs are still isolated from plants materials. The synthetic drugs have emerged to pose damage harmful for environment and human health. Therefore, the plant's products when compared to their synthetic counterparts minimize the adverse side effects (Hoda and Vijayaraghavan, 2015).

At the present the infectious diseases by pathogenic and opportunistic microorganisms remain a major threat to public health, also the continuous and uncontrolled use of antibiotics in general, have allowed the emergence of multidrug resistant pathogens, permitting that these are progressing towards final line of antibiotic defence. This has led to the search of new molecules and targets that shown structural intricacy and chemical diversity required to interact with antibacterial protein targets and provide vast opportunities for new drug development (Verma et al., 2011; Hoda and Vijayaraghavan, 2015).

Just as plants are used as alternative substances to control diseases, lichens have been used for medicinal purpose since time immemorial and are known to produce unique secondary metabolites exhibiting considerable biological activities such as antimicrobial, antimycobacterial, antifungal, antiviral, antioxidant, analgesic, cytotoxic, fungicidal, antiherbivore, herbicidal and antibiotic properties (Ranković et al., 2009; Sinha and Biswas, 2011).

Likewise, the genus *Cladonia* has been used in the traditional medicine to treat fevers, diarrhea, infections, pains, wounds and others (Açıkğöz et al., 2013). Reason why it has been studied its biological properties, like antifungal activity.

For this purpose, there are a variety of methods that are used to determine the sensitivity of microorganisms to antibiotics and since not all of them is based on same principles. Results obtained are highly affected not only by the selected method, but also by the microorganisms used to carry out the test, and by the degree of solubility of each tested compound (Valgas et al., 2007).

The aim of this study was to evaluate the *in vitro* antifungal property of *Cladonia* aff. *rappii*. For this purpose, the effect of lichen extract (water, ethanol and dichloromethane) was tested at two concentrations, evaluated by three different diffusion methods (spot-on-a-lawn, disk diffusion and well diffusion method). Finally, minimum inhibitory concentration and minimal fungicidal were evaluated against six yeast species.

MATERIAL AND METHODS

Collection and identification of lichen

The lichen sample of *Cladonia* aff. *rappii* A. Evans (Cladoniaceae), was collected in February 2014 at 2395 m altitude in Mérida state (8° 26' 36" N, 71° 11' 18" O) Venezuela. The initial determination of the lichen sample was made using several identification keys, e.g., Ahti, (2000) and Nash et al. (2002; 2004). The selected sample was subsequently subjected to molecular phylogenetic analysis, which revealed that the material commonly identified as *Cladonia rappii* represents an undescribed specie (no published data). Voucher sample was deposited in the MERF herbarium of the Facultad de Farmacia y Bioanálisis of the Universidad de Los Andes and in the Field Museum (F), with the number C2.

Chemicals

Sabouraud dextrose agar (BBL™), Müller Hinton agar and broth (Difco™), were purchased from Becton, Dickinson and Company (BD) USA, dimethyl sulfoxide (DMSO) was obtained of Sigma Chemicals, USA. Fluconazole (Laboratorio Colmed International®). All other chemicals used, including the solvents, were of analytical grade.

Extraction from lichen sample

The lichen material was air-dried at room temperature for one week. Then, it was grinded into a uniform powder. The extracts (in water, ethanol and dichloromethane) were prepared by soaking 10 g of material separately with 250 mL of each solvent at room temperature. Aqueous extracts left overnight were obtained to avoid decomposition of the extract, and extracts of ethanol and dichloro-

methane for seven days. All extracts were filtered using filter paper (Whatman No. 1). The aqueous extract was concentrated under reduced pressure and lyophilized. The ethanol and dichloromethane extracts were concentrated by evaporation of the solvent at room temperature with air flow.

Fungal strains and media

Six fungal yeasts were used as test organisms in the study: *Candida albicans* ATCC 90028, *C. glabrata* ATCC 90030, *C. krusei* ATCC 6258, *C. tropicalis* ATCC 50628, *C. parapsilosis* ATCC 22019 and *Cryptococcus neoformans* as clinical isolate. These were obtained from mycological collection maintained by the Mycological Laboratory Dr. Corrado Capretti of the Department of Microbiology of the Universidad de Los Andes, Venezuela. The yeasts cultures were kept on Sabouraud dextrose agar and were transferred to Müller-Hinton agar. All cultures were stored at 4°C and subcultured every 48 h for *Candida* yeasts and 72 h for *Cryptococcus*.

Test substances

The lichen extracts were dissolved in dimethyl sulfoxide (DMSO), to obtain concentrations of 20 mg/mL and 100 mg/mL (Yilmaz et al., 2004; Aslan et al., 2006; Ranković et al., 2011); except the aqueous extract. The antimycotic fluconazole (25 µg/disc) was dissolved in sterile distilled water and used as positive control and DMSO as negative control.

In vitro antifungal assays

Determination of antifungal activity

The antifungal activity of extracts obtained from *Cladonia* aff. *rappii* were evaluated against six test yeasts using three diffusion methods: the spot method on the grass, the disk diffusion and the well diffusion method. In the diffusion methods, a 1 mL of fresh yeast culture was used and inoculated into 15 mL of Müller Hinton agar. All experiments were performed in triplicate for the calculation of standard deviations. The sensitivity of the microorganisms to the extracts of the examined lichen was tested by determining the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC).

Suspension preparation

The yeast suspensions were prepared by the direct colonies method (Andrews, 2005). The colonies were extracted directly from the fresh plate culture and rinsed with sterile distilled water, used to determine the turbidity spectrophotometrically at 530 nm (Milton Roy, Spectronic 20D+, Pont-Saint-Pierre, France) and then diluted further to approximately 10⁶ CFU/mL according to the procedure recommended by NCCLS (2002), adjusting to the turbidity of the 0.5 McFarland standard.

Spot-on-a-lawn method

The protocol used was estimated using the method described by Vera et al. (2007). An amount of 3 µL of each extract at 20 mg/mL and 100 mg/mL was placed on this lawn and after the plates were incubated for 48-72 h at 37°C. After the incubation period, the inhibition zones were measured. The fluconazole and DMSO controls were used in the same manner.

Disc diffusion method

The methodology was carried out according to Kirby-Bauer as described in Vizcaya et al. (2014) with slight modifications. Sterile filter paper disks (Whatman No 1) of 6 mm diameter were impregnated with 15 µL and 3 µL of each extract at 20 mg/mL and 100 mg/mL, respectively. In addition, individual disks soaked with 3 µL and 15 µL of fluconazole as a positive control and DMSO as a negative control. They were plated on previously inoculated plates and incubated at 37°C for 48-72 h. The inhibition zone was measured.

Well diffusion method

This method was used as described by Kageröz et al. (2009). The agar was perforated using a sterile cork perforator, wells of 3 mm and 6 mm diameter were made in the inoculated medium and then filled with 15 µL and 3 µL at 20 mg/mL and 100 mg/mL, respectively. The same protocol was used with the fluconazole and DMSO as positive and negative controls. The plates were allowed to stand for 30 minutes and then, were incubated at 37°C for 48-72 h. The inhibition zone was recorded.

Determination of minimum inhibitory concentration

The MIC of the extracts was tested using the microdilution method described by Mitrović et al. (2011). The MIC was determined in samples showing activity with any of the diffusion techniques. This was done in 96-well bottom plates "V", which were prepared by dispensing 100 µL in Müller Hinton broth into each well. A 100 µL of a stock solution (200 mg/mL) of each extract was added into the first column of the plate. Then, twofold serial dilutions were performed between the first and tenth columns. Finally, 10 µL of the diluted yeast suspension was added to each well to give a final concentration of 5×10^5 CFU/mL, making a final volume of 210 µL in each well. The concentration range obtained was 0.186 to 95.23 mg/mL. Each test included growth control and sterility control. The fluconazole as positive control was evaluated between 0.125 to 64 µg/mL and DMSO was performed to study the effect on the growth of microorganism. The inoculated plates were incubated at 37°C for 48-72 h. After incubation period, the plate was observed using a mirror. The lowest concentration of the extract that did not produce visible growth (no turbidity) was considered as MIC (Verma et al., 2011). All tests were performed in duplicate.

Determination minimum fungicidal concentration

The MFC was determined by plating 10 µL of samples from each well where no visible growth was recorded, on the Sabouraud Dextrosa agar medium. Plates were incubated at 37°C for 48-72 h. At the end of the incubation period the lowest concentration without growth was defined as MFC (Mitrović et al., 2011). The MFC was the minimum concentration of compound or drug that can inhibit 100% microbial growth (Goodman and Gilman et al., 1991; Espinel-Ingroff et al., 2002).

Statistical analysis

The data were expressed as the means \pm standard deviation (SD). All statistical analyzes were performed using SPSS package (SPSS for Windows ver. 15, Chicago, IL, USA). Mean differences were established by Student's *t*-test. Data were analyzed by unidirectional analysis of variance (ANOVA). In all

cases *p* values <0.05 were considered statistically significant.

RESULTS

Diffusion methods: Spot-on-a-lawn method, disc diffusion and well diffusion

The results of screening for antifungal activity using ethanol and dichloromethane extracts were observed at 20 mg/mL, except with aqueous extract, which showed no activity. The ethanol extract inhibited a greater number of yeast than the dichloromethane extract. Additionally, it was observed that the results with the ethanol extract was repeated in the three diffusion methods applied (Table 1) whereas in the dichloromethane extract it was observed only with the spot-on-a-law method (Table 2).

Although the ethanol extract at 20 mg/mL inhibited all tested yeasts, there were significant differences ($p < 0.05$) between the used methods. *Candida glabrata* was inhibited only by the spot-on-a-low method and *Cryptococcus neoformans* only with the well diffusion method (Table 1). Significant differences ($p \leq 0.05$) were further observed in dichloromethane extract results (Table 2).

After observing the three methods, the ethanol extract showed the greatest areas of inhibition zones against *Candida albicans* and the lowest with *C. parapsilosis*. Variability was observed with *C. krusei* (Table 1). The dichloromethane extract was only active against three yeasts: *Candida albicans*, *C. krusei* and *Cryptococcus neoformans* (Table 2).

The standard fluconazole showed inhibition against all yeasts pathogens tested by the three methods used. Also, they showed uniformity in the inhibition zones (Table 3).

Minimum inhibitory concentration and minimum fungicidal concentration

The lowest MICs values were obtained against *Candida glabrata* with the ethanol extract at 2.2 ± 0.7 mg/mL and against *Cryptococcus neoformans* 2.9 ± 0.0 mg/mL with dichloromethane extract and viceverse. On the other hand, MFC values for ethanol extract were almost all at 11.9 ± 0.0 mg/mL except against *C. albicans* and *C. neoformans* at $17.9 \pm$

2.2 mg/mL. The lowest MFC were obtained with dichloromethane extract against *C. glabrata* at 8.9 ± 2.2 mg/mL and *C. neoformans* at 7.4 ± 3.3 mg/mL (Table 4).

The MIC and MFC that resulted the same value for both extracts were observed against *Candida krusei*, *C. parapsilosis* and *C. tropicalis* (Table 4).

Table 1. Antifungal activity of ethanol extract of *Cladonia* aff. *rappii* (20 mg/mL) against tested yeasts using three diffusion methods.

Yeasts	Spot-on-a-lawn	Disc diffusion	Well diffusion
<i>C. albicans</i>	23.0 ± 2.0	24.0 ± 1.0	24.6 ± 0.5
<i>C. glabrata</i>	12.3 ± 1.5*	-*	-*
<i>C. krusei</i>	12.3 ± 0.5*	24.6 ± 0.5*	19.0 ± 1.0*
<i>C. parapsilosis</i>	11.6 ± 1.1*	16.3 ± 1.5*	17.6 ± 0.5*
<i>C. tropicalis</i>	22.6 ± 1.1	18.6 ± 0.5	20.0 ± 1.0
<i>C. neoformans</i>	-*	-*	14.0 ± 1.0*

Values are mean inhibition zones ± SD (in mm) of three replicates; - no inhibition observed. All the results at 100 mg/mL were negatives. The analysis of ANOVA reflected *p < 0.05 represents the statistical difference between the three diffusion methods.

Table 2. Antifungal activity of the dichloromethane extract of *Cladonia* aff. *rappii* (20 mg/mL) against tested yeasts by the spot-on-a-law method.

Yeasts	Inhibition zone
<i>C. albicans</i>	7.0 ± 1.0
<i>C. glabrata</i>	-
<i>C. krusei</i>	6.3 ± 1.1
<i>C. parapsilosis</i>	-
<i>C. tropicalis</i>	-
<i>C. neoformans</i>	7.3 ± 1.1

Values are mean inhibition zones ± SD (in mm) of three replicates; - no inhibition observed. All the results at 100 mg/mL were negatives. Student's t-test analysis reflected p < 0.05 represents the statistical difference between all the yeasts used.

Table 3. Inhibition zones of the fluconazole (25 µg/disc).

Yeasts	Spot-on-a-lawn	Disc diffusion	Well diffusion
<i>C. albicans</i>	55.0 ± 0.5	55.0 ± 0.5	50.3 ± 0.5
<i>C. glabrata</i>	30.0 ± 1.5	32.0 ± 0.5	32.0 ± 1.5
<i>C. krusei</i>	17.0 ± 1.5	18.2 ± 1.0	18.0 ± 0.5
<i>C. parapsilosis</i>	35.0 ± 0.5	35.0 ± 0.5	35.3 ± 0.5
<i>C. tropicalis</i>	45.0 ± 1.0	45.5 ± 0.5	45.0 ± 1.5
<i>C. neoformans</i>	50.0 ± 0.5	50.4 ± 1.0	50.0 ± 0.5

Values are mean inhibition zones ± SD (in mm) of three replicates. The analysis of ANOVA reflected p > 0.05 there aren't statistical difference between the three diffusion methods.

Table 4. MIC and MFC of ethanol and dichloromethane extracts of *Cladonia* aff. *rappii* against yeasts pathogens used in the present study.

Yeasts	MIC		MFC	
	EtOH	DCM	EtOH	DCM
<i>C. albicans</i>	11.9 ± 0.0*	11.9 ± 0.0*	17.9 ± 2.2	17.9 ± 2.2
<i>C. glabrata</i>	2.2 ± 0.7*	5.9 ± 0.0*	11.9 ± 0.0	8.9 ± 2.2*
<i>C. krusei</i>	11.9 ± 0.0	11.9 ± 0.0	11.9 ± 0.0	11.9 ± 0.0
<i>C. parapsilosis</i>	11.9 ± 0.0	23.8 ± 0.0*	11.9 ± 0.0	23.8 ± 0.0*
<i>C. tropicalis</i>	11.9 ± 0.0	11.9 ± 0.0	11.9 ± 0.0	11.9 ± 0.0
<i>C. neoformans</i>	5.9 ± 0.0*	2.9 ± 0.0*	17.9 ± 2.2	7.4 ± 3.3*

Values are means ± SD (in mg/mL) of two replicates. EtOH: ethanol extract. DCM: dichloromethane extract. MIC: Minimum Inhibitory Concentration. MFC: Minimum Fungicidal Concentration. Student's t-test analysis reflected *p < 0.05 represents the statistical difference between species of yeast.

Table 5. MIC and MFC of fluconazole.

Yeasts	MIC	MFC
<i>C. albicans</i>	8 ± 0.0	16 ± 0.0
<i>C. glabrata</i>	8 ± 0.0*	16 ± 0.0*
<i>C. krusei</i>	16 ± 0.0*	32 ± 0.0*
<i>C. parapsilosis</i>	4 ± 0.0*	8 ± 0.0*
<i>C. tropicalis</i>	4 ± 0.0	8 ± 0.0
<i>C. neoformans</i>	4 ± 0.0	8 ± 0.0

Values are means ± SD (in µg/mL) of two replicates. MIC: Minimum Inhibitory Concentration. MFC: Minimum Fungicidal Concentration. Student's t-test analysis reflected *p < 0.05 represents the statistical difference between species of yeast.

DISCUSSION

Lichens are self-supporting symbiotic associations of a fungus and one or several algal or cyanobacterial components. Since the fungal constituent is unique in that symbiosis and usually dominates the association, lichens traditionally have been considered a type of fungus (Kumar et al., 2010). Lichens are well known for the diversity of secondary compounds they produce. These compounds are isolated from various lichen species have been reported to display diverse biological activities. Most studies have focused on the activities of crude lichen extracts (Ranković et al., 2009; Santiago et al., 2010; Açıkgöz et al., 2013). Compounds in *Cladonia* spp. that have previously been tested for antimicrobial activity include usnic, perlatolic, ursolic, and didymic acids, as well as strepsilin and atranorine (Yilmaz et al., 2004; Stark et al., 2007).

In the present work, summarizing the results obtained with the three different techniques applied to evaluate the antifungal activity, it was observed that ethanolic extract of *Cladonia* aff. *rappii* at 20 mg/mL, showed better activity against *Candida albicans* followed by *C. tropicalis*, *C. krusei*, *C. parapsilosis*, *Cryptococcus neoformans* and *Candida glabrata*. While with dichloromethane extract at 20 mg/mL, it demonstrated activity against *Cryptococcus neoformans*, *Candida albicans* and *C. krusei*. No activity was observed at the 100 mg/mL evaluated. Some researchers have found antimicrobial activity in extracts of lichens *Cladonia mitis* and *Cladonia foliacea* at 100 and 200 mg/mL respectively, using just a diffusion method (Yilmaz et al., 2004; Sinha and Biswas, 2011). In this study, no activity was obtained at 100 mg/mL by any of the diffusion methods used. This could be explained by the difficulty of the extract to diffuse into the medium because of its high

concentration and low amount (3 μ L), while better results were observed at a lower concentration and higher volume (15 μ L).

Previous studies have reported antifungal activity in species of the genus *Cladonia*, exhibiting usually slight to moderate activity against *Candida* yeasts and filamentous fungi, even against phytopathogenic fungi depending on the concentration used (Halama and Van-Haluwin, 2004 taken from Molnár and Farkas, 2010; Ribero et al., 2006; Ranković et al., 2010; Mitrović et al., 2011; Verma et al., 2011). Ranković et al. (2009) reported antifungal activity in acetone and ethanol extracts from *Cladonia furcata* to 50 mg/mL against *Candida albicans*, obtaining inhibition zones of 10 mm for both extracts. On the present study, a greater inhibition zone (between 7.0 ± 1.0 and 24.6 ± 0.5 mm diameter) was observed at a lower concentration (Table 1). Opposite case, Verma et al. (2011) did not find activity with acetone and methanol extract from *Cladonia ochrochlora* against *Candida albicans* at a lower concentration (10 μ g/mL). Probably this variation among these results is due to the compounds concentration used and the different substances obtained from the used solvents.

Different researches (Ranković et al., 2009; Kosanić and Ranković, 2010), affirm that there are differences in the antifungal activity between extracts and indicate that bioactive components have different solubility in different extracting solvents. Aqueous extracts showed no activity in relation to the yeasts tested. Some literature data reported that aqueous extracts of lichens have no antifungal effects (Baral and Maharjan, 2011; Kosanić and Ranković 2011). In fact, one of the major secondary substances in the genus *Cladonia* is usnic acid, and it is poorly water-soluble (Ingólfssdóttir, 2002; Madamombe and Afolajan, 2003). This explains the reason why aqueous extracts show poor or no antifungal activity, despite the extraction time that could be devoted to obtaining it.

After obtaining the results with the three diffusion methods in this study used, were observe differences between them, despite having the same principal, which is the diffusion of a sample on the medium. These differences could be explained by diffusion variations of the lichen extract on the surface medium, such as from a drop or filter paper until a hole in the surface. These generate different results according to the diffusion method used.

However, the results obtained using spot-on-a-lawn and well diffusion methods were reproducible.

In this research, there were antifungal significant effects on MIC using ethanol extract compared with the dichloromethane extract of *Cladonia* aff. *rappii* to *Candida glabrata* and *Cryptococcus neoformans*, obtaining the lowest concentrations with these yeast species. In addition, the same results were obtained with MFC in dichloromethane extract (Tabla 4). There are not previous studies evaluating extracts with this specie of lichen (*Cladonia* aff. *rappii*), to compare the results obtained. Ranković et al. (2009), examined the antifungal activity of the acetone extract of *Cladonia furcata* and obtained a MIC of 6.25 mg/mL against *Candida albicans*, a concentration lower than that obtained in the present study.

Mitrović et al. (2011) evaluated the methanol extracts of *Cladonia foliaceae* and found that the MIC was 5 mg/mL, obtaining a lower MIC than *Cladonia* aff. *rappii*. But, observed a MFC at 20 mg/mL against *Candida albicans*, being better with *C. aff. rappii* as obtained in the present study. These differences between the species of lichens could be by the chemical diversity of bioactive compounds that interact with the proteins targets of microorganisms or their low quantities, probably lower than their MIC. Hence, detailed studies on the role of individual phytochemicals involved in the antifungal activity of specific lichens are required for their use in the pharmaceutical industry.

A variety of common biological active substances isolated from divers species of *Cladonia* have been reported with antifungal activity, such as: usnic acid, atranorina, fumarprotocetraric, hipoprotactraric and protocetraric acid and others. Probably, the antifungal activity of the lichen extract may be due to the result of a synergistic effect of several compounds (Yilmaz et al., 2004; Ranković & Mišić 2008; Açıkgöz et al., 2013) and that surely vary according to the species of *Cladonia* in study. Compounds with potential biological activity of *Cladonia* aff. *rappii* are to be defined.

CONCLUSIONS

After evaluating the antifungal potential of *Cladonia* aff. *rappii*, it was observed that this species of lichen possesses antifungal activity at 20 mg/mL,

mainly with ethanolic extract, followed by dichloromethane extract. Also, it showed fungicidal activity in both extracts, because inhibition of microbial growth was observed. The results depended on several factors: different extraction solvents, concentration of the lichen extract, amount of active compounds present in the extract, species of yeasts used and diffusion method implemented.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENT

Authors thank to the staff of Department of Microbiology at Facultad de Farmacia y Bioanálisis of the Universidad de los Andes (ULA) to allow the development of this research. The authors also thank to staff from Mycology Laboratory at Facultad de Farmacia y Bioanálisis-ULA, especially Alexander Moreno and Oduar Salazar, for their support and cooperation on this work and in others previous scientific projects.

REFERENCES

- Açıkgöz B, Karaltı İ, Ersöz M, Coşkun Z. M, Çobanoğlu G., Sesal C (2013) Screening of antimicrobial activity and cytotoxic effects of two *Cladonia* Species. *Z Naturforsch* 68c: 191-197.
- Ahti T (2000) *Cladoniaceae*. *Flora Neotrópica Monograph* 78: 1-363.
- Andrews J (2005) BSAC Working Party on Susceptibility Testing. BSAC standardized disc susceptibility testing method. *J Antimicrob Chemother* 56: 60-76.
- Aslan A, Güllüce M, Sökmen M, Adigüzel A, Sahin F, Özkan H (2006) Antioxidant and antimicrobial properties of the lichens *Cladonia foliacea*, *Dermatocarpon minutum*, *Everinia divaricata*, *Evernia prunastri*, and *Neofuscella pulla*. *Pharm Biol* 44(4): 247-252.
- Babiah P, Upreti D, John S (2014) An *in vitro* analysis of antifungal potential of lichen species *Parmotrema reticulatum* against phytopathogenic fungi. *Int J Curr Microbiol App Sci* 3(12): 511-518.
- Baral B, Maharjan B (2011) Assessment of antimicrobial and phytochemical potentials of high altitudinal Nepalese lichens. *J Microbiol Biotechnol Food Sci* 1(2): 98-112.
- Espinel-Ingroff A, Chaturvedi V, Fothergill A, Rinaldi M G (2002) Optimal testing conditions for determining MICs and minimum fungicidal concentrations of new and established antifungal agents for uncommon molds: NCCLS collaborative study. *J Clin Microbiol* 40: 3776-3781.
- Goodman-Gilman A, Rall T, Nies, Taylor P (1991) Agentes antimicrobianos. Consideraciones generales en: *Las bases farmacológicas de la terapéutica*. 8ª Ed. Mexico: Editorial Médica Panamericana, p. 1006.
- Hoda S, Vijayaraghavan P (2015) Evaluation of antimicrobial prospective of *Parmotrema perlatum* hexane extract. *Int J Pharm Res Allied Sci* 4(2): 47-53.
- Ingólfssdóttir K (2002) Usnic acid. *Phytochemistry* 61: 729-736.
- Karagöz A, Dođruöz N, Zeybek Z, Aslan A (2009) Antibacterial activity of some lichen extracts. *J Med Plants Res* 3(12): 1034-1039.
- Kosanić M, Ranković B (2010) Screening of antimicrobial activity of some lichen species *in vitro*. *Kragujevac J Sci* 32: 65-72.
- Kosanić M, Ranković B (2011) Antioxidant and antimicrobial properties of some lichens and their constituents. *J Med Food* 14(12): 1624-1630.
- Kumar P, Kekuda P, Vinayaka K, Sudharshan S, Mallikarjun N, Swathi D (2010) Studies on antibacterial, antihelmintic and antioxidant activities of a macrolichen *Parmotrema pseudotinctorum* (des. Abb.) Hale (*Parmeliaceae*) from Bhadra wildlife sanctuary, Karnataka. *Int J PharmTech Res* 2(2): 1207-1214.
- Madamombe I, Afolajan A (2003) Evaluation of antimicrobial activity of extracts from South African *Usnea barbata*. *Pharmaceut Biol* 41(3): 199-202.
- Mitrović T, Stamenković S, Cvetković V, Tošić S, Stanković M, Radojević I, Stefanović O, Čomić L, Đačić D, Čurčić M, Marković S (2011) Antioxidant, antimicrobial and antiproliferative activities of five lichen species. *Int J Mol Sci* 12: 5428-5448.
- Molnár K, Farkas E (2010) Current results on biological activities of lichen secondary metabolites: A Review. *Z Naturforsch* 65(3-4): 157-173.
- Nash T, Ryan BD, Diederich P, Gries C, Bungartz F (2002) *Lichen Flora of the greater Sonoran Desert Region, Vol. 1. Lichens unlimites*, Arizona State University, US, 532 p.
- Nash T, Ryan BD, Diederich P, Gries C, Bungartz F (2004) *Lichen Flora of the greater Sonoran Desert Region, Vol. 2. Lichens unlimites*, Arizona State University, US, 742 p.
- NCCLS (National Committee for Clinical Laboratory Standards) (2002) Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard—Second edition (M27-A2). Wayne, PA, USA, 29 pp.
- Ranković B, Kosanić M, Stanojković T (2011) Antioxidant, antimicrobial and anticancer activity of the lichens *Cladonia furcata*, *Lecanora atra* and *Lecanora muralis*. *BMC Complement Altern Med* 11: 97.
- Ranković B, Mišić M (2008) The antimicrobial activity of the lichen substances of the lichens *Cladonia furcata*, *Ochrolechia androgyna*, *Parmelia caperata* and *Parmelia consprespa*. *Pharm Biotechnol* 22(4): 1013-1016.
- Ranković B, Mišić M, Sukdolak S (2009) Antimicrobial activity of extracts of the lichens *Cladonia furcata*, *Parmelia caperata*, *Parmelia pertusa*, *Hypogymnia physodes* and *Umbilicaria polyphylla*. *Biologia* 64(1): 5-58.
- Ranković B, Ranković D, Maric D (2010) Antioxidant and antimicrobial activity of some lichen species. *Microbiology* 79(6): 809-815.
- Ribeiro S, Pereira E, Buarque-Guzmão N, Falcão E, Da-Silva N (2006) Produção de metabólitos bioativos pelo líquen *Cladonia substellata* Vainio. *Acta Bot Brasilica* 20(2): 265-272.
- Santiago K, Borricano J, Canal J, Marcelo D, Pérez M, De la Cruz T (2010) Antibacterial activities of fruticose lichens

- collected from selected sites in Luzon Island, Philippines. *Philipp Sci Lett* 3(2): 18–29.
- Sinha S, Biswas M (2011) Evaluation of antibacterial activity of some lichen from Ravangla, Sikkim, India. *Int J Pharma Bio Sci* 4(2): 23–28.
- Stark S, Kytoviita M, Neumann A (2007) The phenolic compounds in *Cladonia* lichens are not antimicrobial in soils. *Oecologia* 152: 299–306.
- Valgas C, Machado de Souza S, Smânia E, Smânia A (2007) Screening methods to determine antibacterial activity of natural products. *Braz J Microbiol* 38(2): 369–380
- Vera E, Salvucci E, Sesma F, Nader-Macías M (2007) Different strategies for purification of antimicrobial peptides from Lactic Acid Bacteria (LAB). *Communicating Current Research and Educational. Topics and Trends in Applied Microbiology* A. Méndez-Vilas (Ed.) Formatex 557–568.
- Verma N, Behera B, Parizadeh H, Sharma B (2011) Bactericidal activity of some lichen secondary compounds of *Cladonia ochrochlora*, *Parmotrema nilgherrensis* & *Parmotrema sancti-angelii*. *Int J Drug Dev* 3(3): 222–232.
- Vizcaya M, Pérez C, Rojas J, Rojas-Fermín L, Plaza C, Morales A, Pérez P (2014) Composición química y evaluación de la actividad antifúngica del aceite esencial de corteza de *Vismia baccifera* var. *dealbata*. *Rev Soc Ven Microbiol* 34(2): 86–90.
- Yılmaz M, Türk A, Tay T, Kivanc M (2004) The antimicrobial activity of extracts of the lichen *Cladonia foliacea* and its (-)-usnic acid, atranorin, and fumarprotocetraric acid constituents. *Z Naturforsch* 59c: 249–254.

Author contribution:

Contribution	Plaza CM	Pérez De Salazar C	Vizcaya M	Rodríguez-Castillo CG	Medina-Ramírez GE	Plaza RE
Concepts or ideas	X	X				
Design	X	X				
Definition of intellectual content	X	X		X		
Literature search	X		X	X		
Experimental studies	X	X	X		X	X
Data acquisition	X	X	X			
Data analysis	X	X	X	X		X
Statistical analysis	X			X		X
Manuscript preparation	X	X			X	X
Manuscript editing	X	X			X	X
Manuscript review	X	X	X	X	X	X

Citation Format: Plaza CM, Pérez De Salazar C, Vizcaya M, Rodríguez-Castillo CG, Medina-Ramírez GE, Plaza RE (2017) Potential antifungal activity of *Cladonia* aff. *rappii* A. Evans. *J Pharm Pharmacogn Res* 5(5): 301–309.