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Research Article

ASSESSMENT OF ANTIMICROBIAL ACTIVITY IN *DIPLOSTEPHIUM PHYLICOIDES* AND *DIPLOSTEPHIUM REVOLUTUM* EXTRACTS BY PLATES AND WELLS METHOD

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ABSTRACT

Objective: The evaluation of the antimicrobial activity of *Diplostephium phylicoides* and *Diplostephium revolutum* on different microorganisms was carried out on using bacteria such as *Escherichia coli* (CMPUJ:034), *Staphylococcus aureus* (CMPUJ:370), *Salmonella typhi* (CMPUJ:045), and *Pseudomonas aeruginosa* (CMPUJ:065); yeasts such as *Saccharomyces cerevisiae* (CMPUJ: H042) and *Candida albicans* (CMPUJ: H022); and filamentous fungi such as *Penicillium chrysogenum* (CMPUJ: H061) and *Aspergillus niger* (CMPUJ: H002).

Methods: This assessment was made by the method of plates and wells using extracts from the leaves of the previously mentioned plants. The extracts were made with different solvents, ethanol, ethyl acetate, dichloromethane, and petroleum ether.

Results: The results showed that the ethyl acetate extract of *D. phylicoides* has antimicrobial activity against *S. aureus* and *C. albicans*; furthermore, the dichloromethane extract showed an inhibitory effect against *S. cerevisiae*.

Conclusions: When comparing the extracts of the two plants, under the evaluated conditions, the extracts presented antimicrobial activity and the ethyl acetate extract of *D. revolutum* the one that showed better activity against all the microorganisms.

Keywords: Antimicrobial, *Diplostephium phylicoides*, *Diplostephium revolutum*, Extracts, Solvents, Plates and wells.

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INTRODUCTION

According to the World Health Organization, antibiotic resistance is increased by the misuse and abuse of drugs, as well as with the deficiencies in the prevention and control of infections [1]. However, an alternative solution is the development of drugs that are not only effective in their antimicrobial activity but is also accessible to the entire population.

Phytomedicines are identified as a viable option, because plants, in their survival nature, produce secondary metabolites such as terpenes, phenols, flavonoids, quinines, tannins, and alkaloids [2]. These are obtained by making plant extracts, which have been identified to possess antimicrobial activity and have been previously used for the production of phytomedicines, which like synthetic drugs, are able to treat, cure, or alleviate diseases. Furthermore, phytomedicines represent a great opportunity since it is possible to develop them with endemic plants, which are exclusive to a region. For this reason, it is important to identify the plants that produce the secondary metabolites required to guarantee effective phytomedicines. Some native plants that may exhibit these properties belong to the genus *Diplostephium* [3-5].

Diplostephium phylicoides (H. B. K.) Wedd. and *Diplostephium revolutum* are species exclusive to the *páramos* (high Andean moors) of the departments of Cundinamarca and Boyacá, at altitudes between 3000 and 4000 m [6,7]. They are low bushes that have dense woolly foliar branches and dense leaves with oval and oblong shapes. They have showy capitula with violet or white ligules [6,7]. Secondary metabolites such as triterpenes (uvaol, bauerenol acetate, and friedelin) and flavonoids (genkwanin, quercetin, and sorbifolin) have been found from extracts of *D. phylicoides,* in which antioxidant and antimicrobial activity has been reported [8]. In the present study, we evaluated the antimicrobial activity of leaf extracts from *D. phylicoides* and *D. revolutum* plants against *Escherichia coli*, *S. aureus*, *Salmonella typhi,* and *Pseudomonas aeruginosa* bacteria; *Saccharomyces cerevisiae* and *Candida albicans* yeasts; and the filamentous fungi *Penicillium chrysogenum* and *Aspergillus niger*.

METHODS

Microorganism

E. coli (CMPUJ:034), *S. aureus* (CMPUJ:370), *S. typhi* (CMPUJ:045), *P. aeruginosa* (CMPUJ:065), *S. cerevisiae* (CMPUJ: H042), *C. albicans* (CMPUJ: H022), *P. chrysogenum* (CMPUJ: H061), and *A. niger* (CMPUJ: H002) used in the study were obtained from the microorganism bank of the Pontificia Universidad Javeriana.

Preparation of extracts

The plants used in the study were collected along the road between Bogotá and Guasca in a place known as the *páramo de Chingaza* (Cundinamarca, Colombia). The extraction process was carried out with the leaves of each plant separately, by hot extraction method in Soxhlet extraction and using as solvents petroleum ether, dichloromethane, ethyl acetate, and ethanol. These extracts were concentrated under reduced pressure using a rotary evaporator at a temperature of 40°C [9-11] and they were solubilized and brought to a concentration of 100 mg/mL in dimethyl sulfoxide (DMSO) for later use.

Preparation of inoculums

From the microorganisms obtained in Potato Dextrose Agar (PDA) and Nutrient Agar for fungi and bacteria, respectively, isolated colonies (approximately ¼ from the box) were taken and added to a tube with sterile peptone water, which is used for the recovery of the microorganisms, until obtaining a turbidity corresponding to a transmittance of 25%. The fungi inoculums were diluted to a concentration of 10^6 cells/ml.

Preparation of culture media
For the preparation of Nutrient Agar (composition) used for bacterial growth, 14.38 g of commercial NA was weighed and added to an Erlenmeyer with 626 mL of distilled water under constant agitation and temperature. In the case of PDA (composition) used for fungi and yeast growth, 24.38 g of commercial PDA was weighed and added to an Erlenmeyer with 626 mL of distilled water under constant agitation and temperature. Both Erlenmeyer were agitated until the media were completely homogenized with the water and the media were sterilized in an autoclave at 121°C.

Preparation of controls
DMSO was used as negative control in each microorganism and as positive control, an antibiotic (Gibco™ – Thermo Fisher scientific) and a commercial antifungal (Quirucidal® – QUIRUMEDICAS) were used for bacteria and fungi, respectively. The antibiotic was brought to a concentration of 5X and the antifungal to a concentration of 2% (w/v). In each control, a volume of 20 µl was used.

Preparation and identification of Petri dishes
From the inoculums made, an aliquot of 62.5 mL was taken and added in 562.5 mL of sterile heated NA or PDA. Subsequently, approximately 25 mL of medium inoculated with the strain was added to the Petri dish and the agar layer was left to solidify. With the medium already solid, five perforations were made in the agar, from the surface of the agar to the base of the plate with an inverted pipette. Finally, the extracts were added in different volumes in four wells (10 µl, 20 µl, 30 µl, and 50 µl) and in the last well, 20 µl of the positive control was added.

Relative percentage of inhibition (RPI)
At the end of the incubation time, the inhibition halos obtained in each well were measured. The inhibition halos were expressed in millimeters (mm) and the antimicrobial activity was determined as an RPI using the following formula [11]:

$$RPI=\frac{Di\text{-}Dn}{Dp\text{-}Dn}\times 100$$

Where, Di is the diameter of the inhibition zone of the extract, Dn is the inhibition zone diameter of the negative control, and Dp is the diameter of the inhibition zone of the positive control, which were antibiotic (Gibco™ – Thermo Fisher scientific) and antifungal (Quirucidal® – QUIRUMEDICAS) commercial for bacteria and fungi, respectively.

Statistical analysis
All experiments were performed in triplicates (n=3). A design was developed for which the assumptions of compliance with the parametric statistics were determined by performing tests of normality (Shapiro–Wilk) and variance (Levene). An analysis of variance was performed together with Tukey and *post hoc* tests for comparison of means to determine significant differences. The test was performed in the R statistical software.

RESULTS

In this study, the antimicrobial activity of leaf extracts of *D. phylicoides* and *D. revolutum* was evaluated using the plate and well method on a set of microorganisms: Bacteria (*E. coli*, *S. aureus*, *S. typhi,* and *P. aeruginosa*), filamentous fungi (*P. chrysogenum* and *A. niger*), and yeasts (*S. cerevisiae* and *C. albicans*), considering the solvent (petroleum ether, dichloromethane, ethyl acetate, and ethanol) and volumes used (10 µl, 20 µl, 30 µl, and 50 µl) as factors of variation in the inhibition response.

The negative control showed no antimicrobial activity against the study microorganisms, while the positive controls showed antimicrobial activity, which was variable depending on the microorganism. In addition, for each experiment, the RPI was calculated.

Diplostephium phylicoides

Bacteria

Leaf extract from ethyl acetate of *D. phylicoides* showed significant antibacterial activity, as shown in Table 1, as it recorded inhibitory activity in three of the four bacteria used in this study, presenting an inhibition percentage of 32.015% on the bacterium *S. aureus* (Figs. 1a and 2), being this bacterium the one that presented sensitivity to all extracts. Regarding the other extracts, no antimicrobial activity was recorded in *E. coli*, *S. typhi,* and *P. aeruginosa*. In addition, no halo of inhibition was recorded on *E. coli bacteria*, demonstrating their resistance to the different extracts.

Fungus

Table 2 shows the antimicrobial activity on fungi and is observed that, in general, leaf extracts of *D. phylicoides* presented antifungal activity on the set of microorganisms, presenting greater activity on *S. cerevisiae*. Yeasts were more sensitive to extracts, *S. cerevisiae* being the most sensitive, while *A. niger* was the least sensitive filamentous fungus.

Dichloromethane extract showed inhibition against the fungi and yeasts used. In addition, this extract presented an elevated inhibition

Table 1: Average inhibition percentage of *Diplostephium phylicoides* leaf extracts on bacteria

Bacteria	Extract	Mean percentage inhibition	Percentage inhibition variance
Escherichia coli	Petroleum ether		
	Dichloromethane		
	Ethyl acetate		
	Ethanol		
Staphylococcus aureus	Petroleum ether	3.945	0.509
	Dichloromethane	11.403	0.948
	Ethyl acetate	32.015	2.995
	Ethanol	4.320	0.429
Salmonella typhi	Petroleum ether		
	Dichloromethane		
	Ethyl acetate	3.573	0.418
	Ethanol		
Pseudomonas aeruginosa	Petroleum ether		
	Dichloromethane		
	Ethyl acetate	3.333	0.606
	Ethanol		

Table 2: Average inhibition percentage of *Diplostephium phylicoides* leaf extracts on fungi

Fungi	Extract	Mean percentage inhibition	Percentage inhibition variance
Candida albicans	Petroleum ether		
	Dichloromethane	8.333	2.778
	Ethyl acetate	15.278	10.838
	Ethanol	12.500	5.619
Saccharomyces cerevisiae	Petroleum ether		
	Dichloromethane	43.333	11.515
	Ethyl acetate	23.333	12.242
	Ethanol	11.667	7.606
Penicillium chrysogenum	Petroleum ether		
	Dichloromethane	6.944	2.757
	Ethyl acetate	15.278	5.787
	Ethanol		
Aspergillus niger	Petroleum ether		
	Dichloromethane	11.111	4.209
	Ethyl acetate		
	Ethanol		

percentage on *S. cerevisiae* of 43.33% (Table 2 and Fig. 1b). The yeasts *S. cerevisiae* and *C. albicans* presented sensitivity to the different extracts, so defined inhibition halos could be observed (Figs. 3 and 4). On *S. cerevisiae*, an antimicrobial effect is evidenced with the ethyl acetate and dichloromethane extracts, the other extracts do not present significant activity. Petroleum ether extract has no significant effect on *C. albicans*, while ethanol has the highest inhibition percentage (Table 2 and Fig. 1c).

Table 3: Average inhibition percentage of *Diplostephium revolutum* leaf extracts on bacteria

Bacteria	Extract	Mean percentage inhibition	Percentage inhibition variance
Escherichia coli	Ethyl acetate	16.668	6.182
Staphylococcus aureus	Ethyl acetate	25.440	0.646
Salmonella typhi	Ethyl acetate	18.253	0.612
Pseudomonas aeruginosa	Ethyl acetate	43.889	3.593

Table 4: Average inhibition percentage of *Diplostephium revolutum* leaf extracts on fungi

Fungi	Extract	Mean percentage inhibition	Percentage inhibition variance
Candida albicans	Petroleum ether	8.333	2.778
	Dichloromethane	6.667	2.424
	Ethyl acetate	11.112	6.735
	Ethanol	12.500	5.619
Saccharomyces cerevisiae	Petroleum ether		
	Dichloromethane		
	Ethyl acetate	11.112	6.735
	Ethanol	11.667	7.606
Penicillium chrysogenum	Petroleum ether	8.333	2.778
	Dichloromethane	20.000	9.455
	Ethyl acetate	4.444	1.158
	Ethanol		
Aspergillus niger	Petroleum ether		
	Dichloromethane		
	Ethyl acetate	41.667	19.697
	Ethanol		

Diplostephium revolutum

Bacteria

Leaf extracts from ethyl acetyl of *D. revolutum* were the only ones that presented antibacterial activity on the study microorganisms (Fig. 5). The highest percentage of inhibition obtained was 44% in *P. aeruginosa* followed by 25.4% in *S. aureus* and 18.3% in *S. typhi*. Most of the samples recorded low percentages of inhibition in *E. coli* (Table 3).

Fungus

In general, the extracts of *D. revolutum* presented antimicrobial activity on the set of fungi. Table 4 shows that all extracts had inhibitory activity against *C. albicans*, it can also be observed that *A. niger* is sensitive only to ethyl acetate extract and that this is the extract with antimicrobial activity against all fungi (Fig. 6).

DISCUSSION

The increase in microbial resistance specifically in treatment failure is directly responsible for the current increase morbidity and mortality associated with microbial infections. The use of antibiotics that once regarded as one of the biggest discoveries of the 20th century is effective on saving many lives against bacterial infection. Unfortunately, uncontrolled use of antibiotics, caused from either patients or prescriptions made without cell culture analyses, increased the resistance of bacteria [9]. The medicinal plants are very important to the health of individuals and communities worldwide; this is mainly due to that, most of the drugs derived from herbs are free of side effects or reactions. The herbs have medicinal quality provide rational means for the treatment of many diseases, which are considered of difficult cure [10]. The assessment of the extracts made with the four solvents (petroleum ether, ethyl acetate, ethanol, and dichloromethane) from *D. phylicoides* on *E. coli*, *S. aureus*, *S. typhi*, and *P. aeruginosa*, as evidenced in Table 1 and Fig. 1a, allowed to determine the sensitivity of these microorganisms to the extracts evaluated. *S. typhi* and *P. aeruginosa* obtained an inhibition percentage of 3.573% and 3.333%, respectively; however, the microorganism that obtained greater sensitivity with respect to the other evaluated was *S. aureus* since it presented inhibition percentages of 3.945%, 11.403%, 32.015%, and 4.320% for the extracts of petroleum ether, ethyl acetate, ethanol, and dichloromethane, respectively. These results are similar to those obtained by Ávila *et al*., in 2006 [5], in which the inhibition of *S. aureus* but not of *E. coli* by flavonoids extracted in *Diplostephium tolimense* was reported.

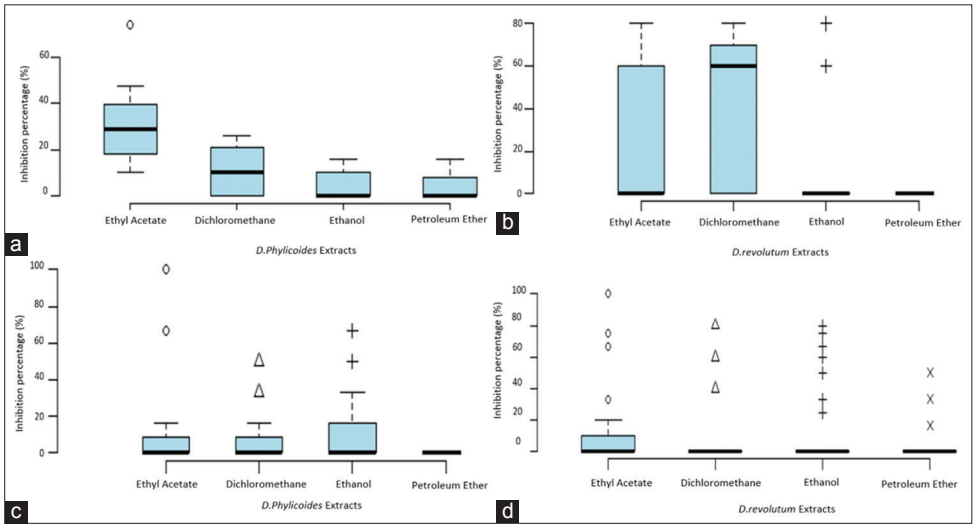

Fig. 1: Distribution of inhibition percentage of *Diplostephium phylicoides* extracts on *Staphylococcus aureus* (a) *Saccharomyces cerevisiae* (b) *Candida albicans* (c) and *Diplostephium revolutum* extracts on fungi (d). Extreme, atypical values and median of specific data are determined

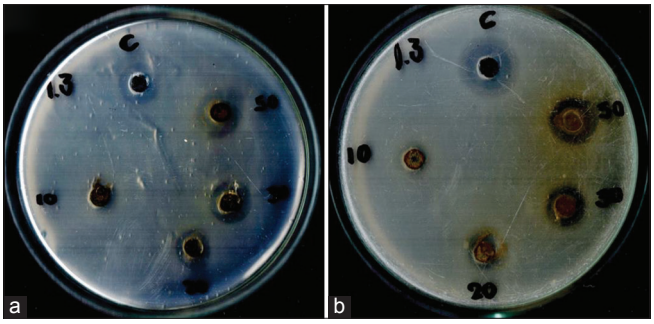

Fig. 2: (a and b) Replicates of inhibition halos of positive control and *Diplostephium phylicoides* leaf extract obtained from ethyl acetate in *Staphylococcus aureus*

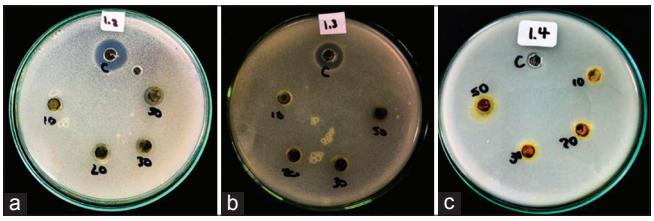

Fig. 3: Inhibition halos of *Diplostephium phylicoides* leaf extracts obtained from (a) dichloromethane, (b) ethyl acetate, and (c) ethanol in *Saccharomyces cerevisiae*

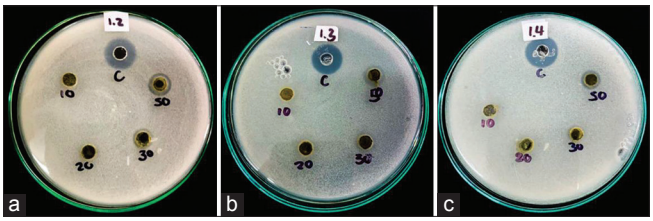

Fig. 4: Inhibition halos of *Diplostephium phylicoides* leaf extracts obtained from (a) dichloromethane, (b) ethyl acetate, and (c) ethanol in *Candida albicans*

This antimicrobial activity is due to the presence of the secondary metabolites of the plant in each of the extracts, where ethyl acetate presents a greater inhibition compared to the other extracts since it contains flavonoids such as sorbifolin, genkwanin, and quercetin [8], which have the ability to alter the permeability of microbial membranes [14]. According to Wang *et al*., in 2017 [14], quercetin is one of the flavonoid compounds with greater antibacterial activity, significantly inhibiting *E. coli*, *S. aureus*, *P. aeruginosa,* and *S. enterica*, but presenting a stronger bacteriostatic effect in *S. aureus* due to the differences, it presents with Gram-negative bacteria in structure and composition of the cell wall and membrane. A possible explanation that in our results this effect has not occurred in all bacteria is due to low concentrations of this compound. It is important to highlight the results of *S. aureus* against the four extracts since this microorganism is involved in most nosocomial infections. In addition, the resistance to many antibiotics by this microorganism has been pointed out in numerous cases [15], which at some point could cause the results obtained to be applied.

When evaluating the extracts elaborated with the four solvents from *D. phylicoides* on the fungus, the four fungi evaluated to show sensitivity to the extract elaborated with dichloromethane. As shown in Table 2, *S. cerevisiae* showed an inhibition percentage of 43.333% in the extract made from dichloromethane, this being the highest inhibition percentage obtained in the test made for fungi with *D. phylicoides*. However, it is not possible to describe the antimicrobial activity

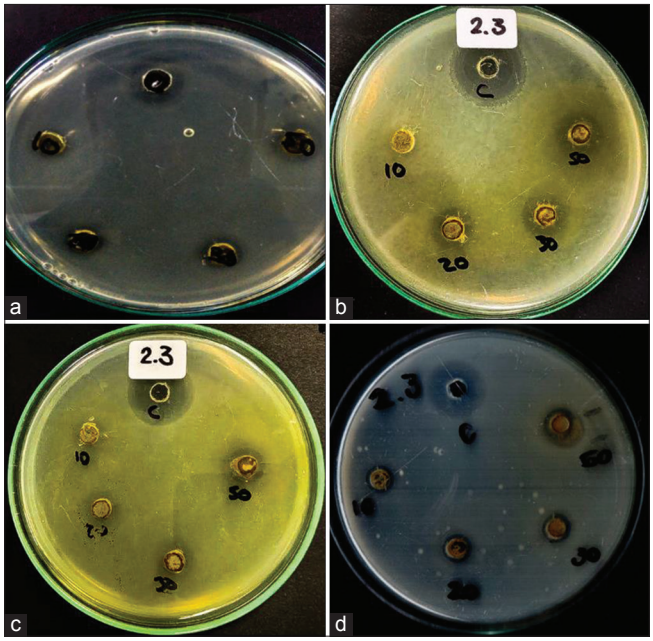

Fig. 5: Inhibition halos of *Diplostephium revolutum* leaf extract obtained from ethyl acetate in (a) *Escherichia coli*, (b) *Pseudomonas aeruginosa*, (c) *Salmonella typhi,* and (d) *Staphylococcus aureus*

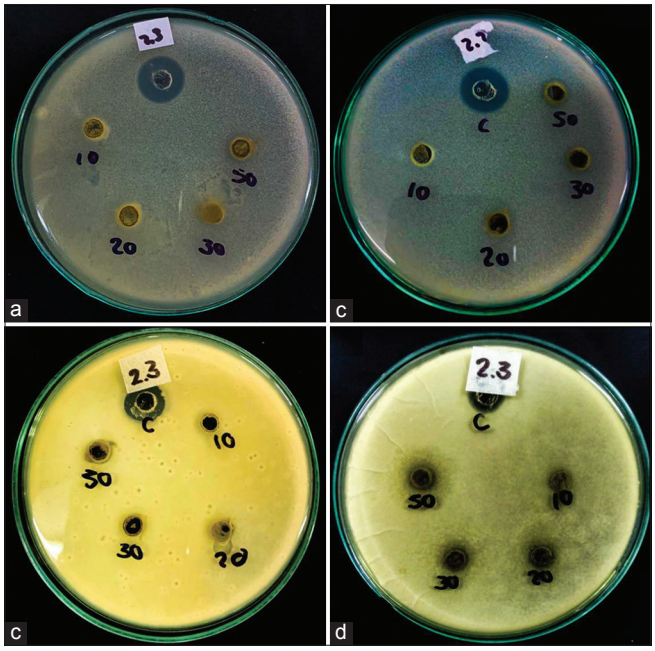

Fig. 6: Inhibition halos of *Diplostephium revolutum* leaf extracts obtained from ethyl acetate in (a) *Candida albicans*, (b) *Saccharomyces cerevisiae*, (c) *Penicillium chrysogenum,* and (d) *Aspergillus niger*

performed by dichloromethane extract because no compounds extracted by this solvent have been previously described.

The extract made with ethyl acetate presented antimicrobial activity in *S. cerevisiae* (Fig. 3), *C. albicans* (Fig. 4). and *P. chrysogenum*, this is because this solvent is able to retain flavonoids as mentioned above, which have the ability to inhibit the germination of plant pathogen spores [16]. This is a possible explanation to the antimicrobial activity obtained from this extract against the previously mentioned fungi. In

addition, the antimicrobial activity against these fungi is consistent with what was proposed by Cushnie and Lamb, in 2005 [16], which indicates that flavonoids can be used against fungal pathogens of human [16]. Structural requirements for antifungal activity have not yet been well defined; however, there is agreement that at least one OH group and some degree of lipophilicity must be present.

The cell wall of filamentous fungi is composed of glucans (30%–80%), chitin and chitosan (1%–15%), mannan, and glycoproteins, while the cell wall of yeasts differs in the amounts of these compounds, especially chitin [17,18]. This difference in composition may be a possible cause of yeast sensitivity to plant extracts, as chitin allows greater resistance to antifungals because its production increases when the cell wall is being affected by some compound, so a cell with high levels of chitin is less susceptible to an antifungal [19]. This matches with our results since a higher percentage of inhibition was observed in yeasts than in filamentous fungi.

In the case of *D. revolutum* (Table 3 and Fig. 1c), antimicrobial activity was evidenced only in the ethyl acetate extract; however, this inhibition was presented in the four bacteria evaluated (Fig. 5). However, for *D. revolutum,* no research has been carried out on the compounds to which the previously mentioned antimicrobial activity can be attributed. Although, in preliminary studies of the genus *Diplostephium* [5-12], compounds such as flavonoids and triterpenes have been determined, which have been described as antimicrobial activity due to the alteration of the lipid bilayer in the plasma membrane, which leads to an alteration of permeability. The differences between the biological activities of terpenes against microorganisms can be explained by differences in the composition of the bacterial wall [20]. According to Castaño *et al*., in 2010 [21], the greater sensitivity of Gram-negative bacteria with respect to Gram-positive bacteria against the terpenes of *Rosmarinus officinalis* L. is due to the fact that these interact with the molecules of the membrane, generating destabilization of the lipid bilayer, which leads to increased permeability and alteration of the structure of the membrane. This explains the antimicrobial activity against all evaluated bacteria.

In the evaluation of the fungi against the four extracts of *D. revolutum,* it was determined that the ethyl acetate extract was the most efficient since it presented antimicrobial activity against the four microorganisms evaluated, obtaining percentages of inhibition from 4.444% to 41.667% (Table 4 and Fig. 1d) where its greater inhibitory activity was presented against *A. niger* for what it is deduced that the concentrations of the compounds produced in *D. revolutum* are in high concentrations since they were able to inhibit both yeasts and filamentous fungi (Fig. 6).

CONCLUSIONS

D. revolutum and *D. phylicoides* presented inhibition in yeasts and filamentous fungi, additionally, *D. revolutum* generated inhibition against all bacteria; therefore, it is considered that the extracts of this plant are more effective to have a broad spectrum of inhibition, being the extract made with the ethyl acetate solvent the most effective to obtain significant percentages of inhibition in the tests performed. It should be noted that dichloromethane leaf extract presented high percentages of inhibition against *S. cerevisiae*, while petroleum ether extract presented the lowest antimicrobial activity against the microorganisms evaluated.

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AUTHORS' CONTRIBUTIONS

All authors contribute to design the experiments; Janeth Del Carmen Arias Palacios, Leonardo Peña Carranza, and Natalia Paola Torres Niño performed the experiments. Wrote the paper: Janeth Del Carmen Arias Palacios, Leonardo Peña Carranza, and Natalia Paola Torres Niño. All authors revised the article critically for important intellectual content.

CONFLICTS OF INTEREST

We declare that we have no conflicts of interest.

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