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Screening of antimicrobial potential of methanolic, acetone and quencher extracts from *Cladonia rangiformis* Hoffm. and *Cladonia pocillum* Ach.

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ABSTRACT: This study compared the efficacy of two species of lichen located in Tunisia belonging to *Cladonia rangiformis* and *Cladonia pocillum* species. The antibacterial, antifungal behavior of methanol, acetone and quencher extracts of *C. rangiformis* and *C. pocillum* and the lysozyme activity of both methanol extracts were investigated. The obtained results illustrated that the examined extracts gave antimicrobial properties against clinical bacteria species and against *Candida* species and that they also limited the spore germination of *Penicillium* and *Aspergillus*. Further results showed that the largest inhibition zone was obtained by the methanolic extract of *C. pocillum* with 31 mm and 27.5 mm against *E. cloacae* and *E. coli,* respectively. MIC values of bactericidal and fungicidal activities of both *Cladonia* extracts varied from 0.25 mg/ml to 2 mg/ml. *C. pocillum* possess superior lysozyme activity against *Staphylococcus aureus* and *Enterococcus faecalis*. Furthermore, the methanol extract of both *Cladonia* showed a remarkable destructive effect on the morphology of fungal hyphae.

1. INTRODUCTION

It is well known that the emergence of multiple resistances in clinically bacterial strains is a growing severe threat to human health. Resistance to currently conventional drugs presents an increasing global health threat concerning major clinical pathogens and currently antimicrobial agents (Levy & Marshall, 2004) . Bacterial infections with multidrug-resistant bacteria are hard to treat due to the absence of effective treatment, and in some cases, health care provides the necessity of using more toxic antibiotics or antifungal drugs for the patient like *Candida* infections. Because of these findings, there have been considerable efforts to develop new antimicrobial agents by screening natural products, enhancing existing antibiotics, and synthesizing new antimicrobial peptides. In this context, here we focused on using lichen as natural sources of new antimicrobial drugs since lichen compounds have different biological activities related to the species of lichen, type of solvent and microbial strains tested.

Lichen is a symbiotic organism composed of a mycobiont (a fungal species) and a photobiont algal species) with a stable and unique structure (Rascio & Rocca, 2013). Lichens have been used in industrial and medical fields (Money, 2016). They produce various chemical substances known as lichen acids. In addition, to other secondary metabolites owing important biological and pharmacological properties (Ranković, 2015). In particular, antimicrobial, antioxidant, anticancer, anti-inflammatory, analgesic and antipyretic potentialities were described for lichens. The antimicrobial efficacy of numerous lichen genus has been reported in the literature, for example *Cladonia*, , *Parmelia*, *Peltigera*, etc. (Ranković, 2015) .

In addition, among the multiple secondary metabolites synthesized by *Cladonia* species, we appointed atranorin, hypoprotocetraric acid, fumarprotocetraric acid and usnic acid (Kosanić et al., 2018). We have recently described the superior antimicrobial activities of some lichen species in Tunisia (Mendili, Essghaier, et al., 2021) . Thus, this work presents a continuation of our previous research, so that we aim to research other species of lichens located in Tunisia (*Cladonia* species). No previous reports have demonstrated the activity of Quencher extracts from these species, as well as methanolic or acetone extracts. Therefore, this study pioneered to elucidate the anti-microbial activities of methanolic, acetone and quencher lichen extracts from *Cladonia rangiformis* and *Cladonia pocillum* located in Tunisia.

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2. MATERIALS AND METHODS

2.1. Lichen sampling

Cladonia rangiformis Hoffm. and *Cladonia pocillum* Ach. were obtained in February 2016 in Tunisia's Bazina region (36°.95′05.80N, 09°29′73.84″E). Voucher specimens have been placed in Tunisia's Faculty of Sciences, Department of Biology's Lichenological Herbarium.

2.2. Extraction with organic solvents

Cladonia species (20 g each) were dried at room temperature in the dark, and chemicals extracted using acetone and methanol (200 mL). At room temperature, the ultrasonic extraction was carried out for two hours. Filtered extracts were concentrated on a rotary evaporator. The crude extracts were stored at a temperature of +4 °C until analysis.

2.3. QUENCHER approach extraction

The QUENCHER approach present a new, quick, simple, cheap and reproducible method used to quantifying phenolic compounds and measuring total antioxidant activity (Gökmen et al., 2009) . In this study, we have used this method to elucidate the antimicrobial activity of *Cladonia* species as previously described by Mendili, Essghaier, et al. (2021) .

2.4. Antimicrobial potentialities evaluation

2.4.1 Microorganisms

In order to evaluate the antimicrobial activities of the *Cladonia rangiformis* and *Cladonia pocillum* extracts, a list of bacteria and fungi human clinical strains were used as following: *Enterobacter cloacae; Escherchia coli, Staphylococcua aueurs, Enterococcus faecalis , Candida albicans, C sake; C parapsilosis . Penicillium digitattum; Aspergillus niger Alternaria alternata.* Cultures were prepared as previously described by Mendili, Essghaier, et al. (2021).

2.4.2 Agar diffusion method

The agar diffusion method was used as recently described by Essghaier et al. (2021). Prior to use, each lichen extract was diluted with sterile water and the concentration was adjusted to 2000 μg/mL. To sterilise the extract solution, it was filtered through a 0.22 m pore size filter. The pathogen concentration was increased to 107 CFU/mL for bacterial strains and 105 spores/mL for fungal strains, and 100 μL of each concentration was applied individually to the surface of the appropriate agar plates (the Mueller-Hinton media and the potato dextrose agar were used for the antibacterial and the antifungal assays, respectively).

A clear zone surrounding the well indicates the presence of activity against the pathogen under investigation, and the diameter of the inhibition zone was measured in millimetres. Three times were conducted per exam. Ceftazidime *CAZ30* and the fungicide Voriconazole VCZ were used as standard.

2.4.3 Minimum Inhibitory Concentration (MIC determination

The MIC values given in μg/mL were determined using the microdilution broth method in 96 well flat microliter plates.The MIC value, which denotes the lowest antimicrobial inhibition concentration, was visually determined by the absence of turbidity in the well, with three independent replicates (Thakur et al., 2015).

2.4.4 Bactericidal and fungicidal activity determination

The previous method described by Graciela et al. (1995) was used to determine the bactericide activity of tested extracts expressed in arbitrary units per ml (AU/mL). We have transferred 50 μL onto an agar culture medium surface previously inoculated with 10^5 CFU/mL of the tested pathogen from a serial twofold dilution of the Cladonia extracts. It was determined that AU/mL was equal to 1000X D/A (where A is the volume of the extract spotted on agar (50 μL in this example) and D is the reciprocal of the greatest dilution providing an inhibition zone for the indicator strain) (Mendili, Essghaier, et al., 2021).

2.4.5 Lysozyme activity

To demonstrate the lysozyme activity of each lichen methanolic extract against gram-positive bacteria cells by measuring the absorbance at 660 nm of gram positive cell bacteria. we have applied the turbidimetrically method of Ryazanova et al. (2005) as detailed in Sehimi et al. (2019) .

2.5. Antifungal activity

2.5.1 Spore germination inhibition

The conidial suspensions from each tested fungi (*Penicillium* sp., *Aspergillus* sp.) were prepared as recently detailed by Mendili, Essghaier, et al. (2021). An incubation at 21°C for 24 h was followed by the addition of the conidial suspensions, the tested extract, and 1 mL of 5 percent glucose to the mixture. Microscopic inspection of each extract (E) using the Sarangi et al. (2010) method yielded the percentage of extracts that inhibited spore germination (I percent).

2.5.2 Mycelial hyphae destruction and microscopic observation

The effect of each lichen extract was tested on the mycelial hyphae morphology of *Alternaria* pathogen. The preparation of hyphae mycelial suspension was done as recently reported by Mendili, Essghaier, et al. (2021). Briefly, a mycelial solution expressed in mg/mL was prepared in Tris-HCl buffer (0.01 M, pH 8) after three lavages of fungal culture with sterile water and recuperation of mycelial pellet centrifugation at 8000 rpm for 10 min. In an Eppendorf tube, we added 500 μL of 5000 μg/mL of the methanol extracts to 500 μL of mycelial hyphae solution and incubated at 37°C for 14 h. Optical density (OD) was measured at 540 nm. An increase of OD compared to the control tube (containing only a mycelial suspension) suggested the destruction of fungal hyphae by the presence of

the methanol extracts Ryazanova et al. (2005) .

3. RESULTS

3.1. Detection of antibacterial and anti-Candida activities

The antimicrobial activities of the methanol, acetone as well as QUENCHER extracts of *Cladonia pocillum* and *Cladonia rangiformis* were analyzed against the pathogens: *Enterobacter cloacae; Escherichia coli, Staphylococcus aureus, Enterococcus faecalis* and three yeasts species (*Candida albicans, C. sake and C. parapsilosis*). The results obtained revealed that the methanolic extract of *C. pocillum* provided the highest diameter of the inhibitory zone with 31 mm and 27.5 mm against *E. cloacae* and *E. coli*, respectively (**Figure 1**).

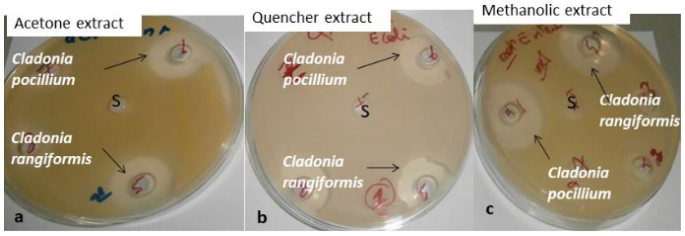

Figure 1. Observation of zone inhibition onagar plate obtained by the acetone, quencher and methanolic extracts of both *Cladonia* species. a: *Enterobacter cloaceae*, b: *Escherichia coli* and c: *Enterococcus faecalis* and S present the corresponding organic solvent used for acetone or methanol extraction and quencher extract the distilled water was used as a solvent.

For the *C. rangiformis*, a higher inhibition diameter (22.5 mm) was observed with the methanol and quencher extracts. Based on this finding, the methanol extract from *C. pocillum* was the most effective against all tested gram-positive and gram-negative bacteria, compared to *C. rangiformis* extracts (**Table 1**).

The methanol and acetone extract of *C. rangiformis* exhibited high inhibition diameters against all tested yeast Candida species for anti-yeast activity. The methanol extract of *C. rangiformis* showed high activity against *C. parapsilosis*, with an 18.5 mm inhibition zone. Methanolic and quencher extracts of *C. pocillum* were only active against *Candida albicans*, followed by the acetone extract against *Candida parapsilosis.*

In addition, only methanolic extract of *C. pocillum* was more effective against *E. coli*, *E. cloaceae* and *Candida albicans* than the Ceftazidime *CAZ30* and the fungicide Voriconazole VCZ, respectively (**Table 1) .**

Table 2 shows the variations in MIC (minimum inhibitory concentration) values between the different extracts utilized. MIC values ranged from 250 μg/mL to 2000 μg/mL. Methanol extracts of *C. pocillum* were more active against *E. cloacae* at only 250 μg/mL. The methanol and acetone extracts of *C. rangiformis were active* against *S. aureus* at only 500 μg/mL. However, the quencher extracts of *C. rangiformis* and *C. pocillum* could inhibit all bacterial species at the same MIC value (2000 μg/mL), unlike *C. pocillum* was more active against *E. faecalis* at 500 μg/mL. The acetone and methanolic extracts of *C. rangiformis* have the best MIC against *Candida* spp. studied. It is noted that the quencher extracts of *C. pocillum* inhibited *Candida albicans* (500 μg/mL) (**Table 2).**

3.2. Bactericide and lysozyme activities

In order to evaluate the antibacterial potential of methanol extract of *C. rangiformis* and *C. pocillum*, other parameters were determined as lysozyme effect. The lysozyme activity was tested against two tested gram-positive bacteria species, *E. faecalis* and *S. aureus* (**Figure 2**), and the bactericide activity expressed in UA/mL (**Table 3**). As a result, *C. pocillum* has a superior lysozyme potential against *S. aureus*, with 160 AU/mL, and against *E. faecalis*, with 80 AU/mL values. In contrast, *C. rangiformis* have a lower lysozyme potential against *S. aureus*, not exceeding 15AU/mL, also being not effective against *E. faecalis* (**Figure 2**).

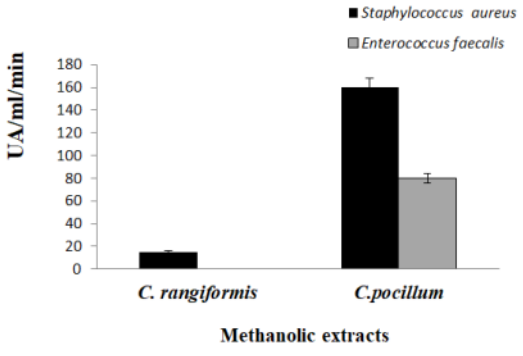

Figure 2. Lysozyme activity effect of both methanolicextracts from *Cladonia rangiformis* and *Cladonia pocillum* species. Error bars represent the SE of themeans.

Table 3 Illustrates the bactericide activity and the methanolic extract of *C. pocillum*, owing to the highest efficacy against *E. cloaceae* with 160 UA/mL, following by methanol and acetone extract of *C. rangiformis* and quencher extract of *C. pocillum* against *S. aureus* and *E. feacalis*, with 80 UA/mL, respectively.

3.3. Antifungal activity

The probably inhibitory activity of Methanol , acetone and Quencher extracts obtained from both *Cladonia* species (*C. rangiformis* ; *C. pocillium)* on the spore germination of the test filamentous fungi *Aspergillus niger* and *Penicillium digitatum* were investigated in **Figure 3**.

The results show that the methanolic extract had a high negatively effect on the germination of spores. The methanolic extract of *C. rangiformis* had 60.7% inhibition against *Penicillium* spores. The methanolic extract of *C. pocillum* had 46.4% and 41% inhibition of *Penicillium* and *Aspergillus* spores, respectively. In turn, *C. rangiformis* specie (Quencher extract) mentioned a less effect on the germination of *Aspergillus* spores with a percentage inhibition of 30.7% (**Figure 3**).

Furthermore, the methanolic extracts of *C. rangiformis* and *C. pocillum* were able to destroy *Alternaria* hyphae so that the methanolic extract of *C. pocillum* presented 2.7 times more destruction than the methanolic extract of C. rangiformis (**Figure 4**).

Table 1
Antimicrobial activity of *Cladonia rangiformis* and *Cladonia pocillum* extracts evaluated against pathogen strains species tested at 2000 μg/mL, as detected in the agar well diffusion test, values are expressed in mm

Zone of inhhibition (mm)							
Microorganisms	***Cladonia rangiformis* extract**			***Cladonia pocillum* extract**			**Control**
	Methanol	**Quencher**	**Acetone**	**Methnaol**	**Quencher**	**Acetone**	
Bacteria							CAZ30
E. coli	16.5 ± 0.7	11.5 ± 0.7	12.5 ± 0	27.5 ± 0.7	24 ± 0.7	22.5 ± 0.7	24 ± 0.2
S. aureus	22.5 ± 0.7	22.5 ± 0.7	11.5 ± 0.7	23 ± 0	13.5 ± 0.7	12.5 ± 0.7	24 ± 0.7
E. faecalis	19.5 ± 0.7	15.5 ± 0.7	11.5 ± 0.5	21 ± 0.7	19.5 ± 0	29 ± 0	27 ± 0.5
E. cloacae	16.5 ± 0	13.5 ± 0	-	31± 0.5	-	-	23.1 ± 0.5
Yeasts							**VCZ**
C. albicans	13 ± 0	-	13.5 ± 1.2	12.5 ± 0.5	14.5 ± 0	-	13 ± 1.4
C. parapsilosis	18.5 ± 0.7	-	16 ± 0	-	-	14.5	30 ± 1.2
C. sake	14.5 ± 0.7	-	16 ± 0	-	-	-	35 ± 0.5
					-	-	

(-) Negative means absence of activity. Control means the commercialized antibiotic Ceftazidime CAZ30 and the fungicide Voriconazole VCZ.

Table 2
MIC of the extracts of *C. rangiformis* and *C. pocillum* against the pathogen strains. Values were expressed in ug/mL

Minimum inhibitory concentration (MIC)						
Microorganisms	***Cladonia rangiformis* extract**			***Cladonia pocillum* extract**		
	Methanol	**Quencher**	**Acetone**	**Methanol**	**Quencher**	**Acetone**
Bacteria						
E. coli	2000	2000	2000	2000	2000	2000
S. aureus	500	2000	500	2000	2000	2000
E. faecalis	2000	2000	2000	2000	500	-
E. cloacae	2000	2000	-	250	2000	2000
Yeasts						
C. albicans	2000	-	500	2000	500	-
C. parapsilosis	2000	-	2000	-	-	2000
C. sake	500	-	2000	-	-	-

Table 3
Bactericidal and fungicidal activities of the *Cladonia* extracts expressed in UA/mL

Microorganisms	*Cladonia rangiformis* extract			*Cladonia pocillum* extract		
	Methanol	**Quencher**	**Acetone**	**Methanol**	**Quencher**	**Acetone**
Bactericidal						
E. coli	20	20	20	20	20	20
S.aureus	80	nd	80	20	20	20
E. faecalis	20	20	20	20	80	nd
E. cloacae	20	nd	nd	160	20	20
Fungicidal						
C. albicans	20	nd	80	80	20	nd
C. parapsilosis	20	nd	20	20	nd	20
C. sake	80	nd	20	20	nd	nd

nd means not determined by the absence of activity

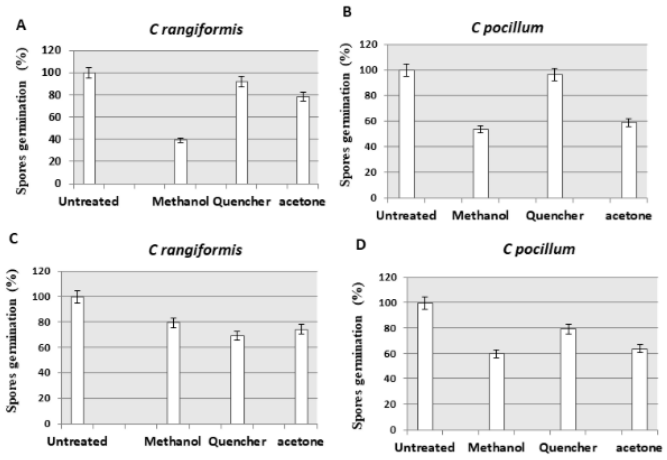

Figure 3. Effect of Various extracts produced from *Cladonia rangiformis* and *Cladonia pocillum* on spore germination of *Penicillium* (A, B) and *Aspergillus* (C, D) Values show the spore germination in percentage

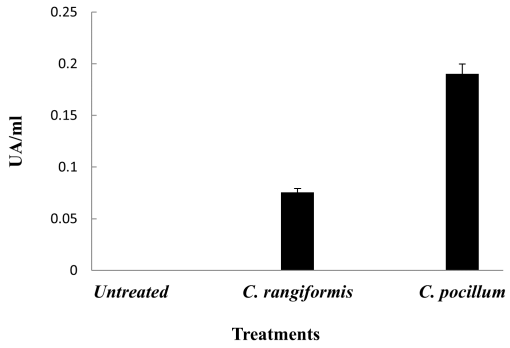

Figure 4. The methanol extracts from *Cladonia rangiformis* and *Cladonia pocillum* on the mycelial morphology of *Alternaria alternata* compared to the untreated culture tube containing the mycelium suspension in the absence of the methanol extract. Values were expressed in UA per volume.

4. DISCUSSION

At present, owing to the infection's pathogens and the opportunists' microorganisms that treat human health, the uncontrolled use of antibiotics and the increasing of the multi-resistance mechanisms, great attention was directed to the discovery of a safe alternative approach to harmful synthetic drugs. Thus, Lichens are natural sources for therapeutic compounds by producing secondary metabolites exhibiting biological potentialities like antimicrobial activity (Barnes, 2000; Plaza et al., 2017). Numerous publications have revealed the biological actions of crude extracts and purified lichen components, including antioxidant, antibacterial, antiviral, cytotoxic, insecticidal, and anti-inflammatory characteristics (Mendili, Bannour, et al., 2021; Mendili, Essghaier, et al., 2021; Ranković, 2015). In this context, the *Cladonia* genus was previously described as secondary metabolites source (Kosanić et al., 2018). Thus, this was the first study in Tunisia to examine the antibacterial and antifungal properties of methanolic, acetone, and quencher extracts of C. rangiformis and C. pocillum species. They have exhibited antibacterial and anti-*Candida* effects, as well as an inhibiting spore germination of filamentous fungi. Therefore, the antimicrobial potential depend on the species, the nature of the extract, and the extraction methods. As a result, the differences are species-specific, extract-specific, and extraction-method-specific. It was noted by Mendili, Essghaier, et al. (2021) that the antimicrobial activity was influenced by the extraction method as well as the type of solvent and confirmed by Plaza et al. (2017). Our results corroborate other studies indicating that *Cladonia* species exhibiting high antimicrobial activity against bacteria species belonging to gram positive and gram negative groups (Açıkgöza et al., 2013; Kosanić et al., 2018).

In this work, we described the behavior of the powdered material without organic solvent (Gökmen et al., 2009) as a new method used firstly for the measurement of antifungal and antibacterial behavior of extracts obtained from lichen species *C. pocillum* and *C. rangiformis*. As a result, they showed a high antibacterial action against gram-positive and gram-negative bacteria and antifungal activity against *Candida* and other species. The methanolic extracts of *C. rangiformis* and *C. pocillum* were more active against *C. albicans*, *C. parapsilosis* and *C. sake*, with the most significant zone inhibition exceeded 13mm. However, Plaza et al. (2017) indicated that *Cladonia affrappii* extracts showed antifungal effect against *C. albicans* at 20 mg/mL, with zone inhibition not exceed 11.9 mm. In the present study, we have described a successful extraction method since our various extracts were able to give the most potent antibacterial and antifungal effects compared to other lichen species extracts reported by comparing the MIC values varied from 0.25mg/mL and 2mg/mL concerning bacteria and fungi species. On the contrary, MIC values obtained by the acetone, the methanolic and the aqueous extracts from *Leacanora atra*, *Parmelia saxatilis* and *Parmeliopsis ambigua* speciesvaried from1.56 to 12 mg/mL for bacteria and from 12.5 to 25 g/mL concerning tested fungal species (B.R. Ranković & Kosanić, 2012).

The efficiency of the antimicrobial potential of our *C. rangiformis* and *C. pocillum* various extracts was confirmed by their MIC values, which ranged from 0.25 mg/mL to 2 mg/mL so that these findings were more important than those reported by *Cladonia affrappii* extracts against Candida species which varied from 2.2 mg/mL to 11.9mg/mL (Plaza et al., 2017).

In contrast to previous studies describing the moderate antifungal potential of *Cladonia* species (B. Ranković et al., 2010; Verma et al., 2011), here we mentioned the most potent antifungal effect these described *Cladonia* species. Moreover, the acetone extract of *C. rangiformis* possesses an inhibitory effect against *C. albicans*, *C. parapsilosis* and *C. sake*, which is opposite to the results described by Verma et al. (2011) who found no activity against *C. albicans* neither by acetone and or methanol extracts from *C. ochrochrola*.

Our study investigated the antimicrobial, lysozyme and antifungal effects of various extracts of *C. rangiformis* and *C.pocillum* species. Recently Mendili, Essghaier, et al. (2021) mentioned that the similar extraction method by methanol, acetone and quencher from four lichens: *Diploschistes ocellatus, Flavoparmelia caperata, Squamarina cartilaginea,* as well as *Xanthoria parietina,* have antibacterial, lysozyme, in addition to antifungal properties.

Ersoz et al. (2017) revealed that the extract of *C. pocillum* had cytotoxic, anti-proliferative, antioxidant, apoptotic, and antimicrobial activities. In addition, Mendili, Essghaier, et al. (2021) described the antimicrobial efficacy of some Tunisian lichen species and the antioxidant properties and phenolic compounds produced by the species *C. rangiformis*. They also showed a positive correlation between antioxidant activity and phenolic compounds Mendili, Bannour, et al. (2021) , which could prove that the antimicrobial capacity of lichens was positively correlated to their phenolic content. Moreover, Yucel et al. (2007) suggested a relation between antioxidant and antimicrobial activities of the chloroform extract from *C. rangiformis*. In addition, several works have evaluated a positive correlation between phenolic constituents of lichen and their antimicrobial activity Buçukoglu et al. (2012); Gulluce et al. (2006); Kosanić et al. (2018); Rankovic et al. (2007) .

5. CONCLUSION

The antimicrobial activity of ground material of *C. rangiformis* and *C. pocillum* showed an inhibitory effect against clinical bacterial pathogens and antifungal activities against *Candida* and filamentous species. According to these results, the chemical compounds related to the antibacterial and antifungal effects should be identified.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest in connection with this study activity, which they have conducted.

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AUTHOR CONTRIBUTIONS

EB, MM, KA - Research concept and design, EB, MM, KA - Collection and/or assembly of data, EB, MM, KA - Data analysis and interpretation, EA, KA - Writing the article, KA - Critical revision of the article, EB, KA - Final approval of the article.

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