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

Essghaier Badiaa [1,*], Mendili Mohamed [2], Khadri Ayda [2]

[1]Laboratory Mycology Pathology & Biomarkers, Department of Biology, Faculty of Sciences of Tunis, University Tunis ELMANAR II, Tunisia

[2]Plant, Soil, Environment Interactions Laboratory, Department of Biology, Faculty of Sciences of Tunis, University Tunis ELMANARII, Tunisia

ABSTRACT: This study compared the efficacy of two species of lichen located in Tunisia belonging to *Cladonia rangiformis* and *Cladonia pocillum* species. The antibacterial and antifungal potentials of methanol, acetone and quencher extracts of *C. rangiformis* and *C. pocillum* and the lysozyme activity of both methanol extracts were investigated. The results showed that the examined extracts had antimicrobial properties against gram-positive and gram-negative bacteria and anti-Candida properties and that they also limited the spore germination of Penicillium and Aspergillus. Further results showed that the largest diameter of the 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 ranged 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. Drug resistance presents an increasing global public health threat concerning all significant microbial pathogens and currently antimicrobial drugs (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 antifungals drugs for the patient like Candida infections. Because of these findings, there have been considerable efforts to develop new antimicrobials 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 depending on the species of lichen, type of solvent and microbial strains tested.

Lichen is a symbiotic organism composed of a fungal partner (mycobiont) and an algal partner (photobiont) with a stable and unique structure (Rascio & Rocca, 2013). Lichens have been used as dyes, food, and in traditional medicine (Money, 2016). They produce various chemical substances known as lichen acids. In addition, other secondary metabolites of lichens, including aliphatic, cycloaliphatic, aromatic and terpene chemicals, have 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, such as Cladonia, Evernia, Lobaria, Parmelia, Peltigera, Pertusaria, Physcia Roccella, Usnea and Xanthoria (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 reported 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). To the best of our knowledge, no previous reports have demonstrated the activity of Quencher extracts from these species, as well as methanolic or acetone extracts.

* Corresponding author.
E-mail address: badiaaessghaier@gmail.com (Essghaier Badiaa)

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.

2. MATERIALS AND METHODS

2.1. Lichen sampling

Cladonia rangiformis Hoffm. Furthermore, *C. pocillum* Ach. were collected from the Bazina region of Tunisia (36°.95′05.80^N, 09°29′73.84″E) in February 2016. Voucher specimens have been deposited in the Lichenological Herbarium of the Department of Biology, Faculty of Sciences of Tunisia.

2.2. Extraction with organic solvents

20 g of each Cladonia species were dried in the dark at room temperature, and compounds were extracted using acetone and methanol (200 mL). The ultrasonic extraction was conducted for 2 hours at room temperature. The extracts were filtered and then concentrated in a rotary evaporator. The crude extracts were kept at 4 °C until analyses.

2.3. QUENCHER approach extraction

The QUENCHER approach (Quick, Easy, New, Cheap, Reproducible) is a method for 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 the powdered Cladonia species. After dilution with an insert material, microcrystalline cellulose, the obtained powered material was analyzed as lichen extracts.

2.4. **Antimicrobial potentialities evaluation**

2.4.1. **Microorganisms**

In order to evaluate the antimicrobial activities of the *C. rangiformis* and *C. pocillum* extracts, a list of bacteria and fungi human clinical strains were kindly supplied from a Tunisian clinical laboratory. The anti-bacterial and antifungal detections were performed using the agar well diffusion method against gram-negative bacteria species (*Enterobacter cloacae*; *Escherichia coli*) and gram-positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*), three yeasts species (*Candida albicans*, *C. sake* and *C. parapsilosis*) and filamentous fungi species (*Penicillium spp*., *Aspergillus spp*., *Alternaria alternata*). Cultures were prepared as previously described by Mendili, Essghaier, et al. (2021) .

2.3.1 Agar diffusion method

Before use, each lichen extract was diluted in distilled water and adjusted to 2000 μg/mL, then sterilized by filtration through a 0.22 μm pore size filter. For antibacterial strains, the Mueller-Hinton media (BioRad, France) was used. The potato dextrose agar plates were used for antifungal assays. A pathogen inoculum of (0.1 mL) was adjusted to 10^7CFU/ml for bacterial strains, and 10^5spores /mL for fungi was transferred separately into the surface of appropriate agar plates. Then after 6 mm wells were punched in plate. A 50 μL aliquot of the filtered extracts was placed into the wells. After overnight pre-diffusion at 4°C, the plates were incubated at 37 °C for at least 24 h for antibacterial and anti-Candida assays and from 2 to 5 days for antifungal ones. The visualization of a clear zone around the well indicates the inhibitory activity against the tested pathogen, and the diameter of the inhibition zone was measured in mm. Each test was repeated three times. Ceftazidime CAZ30 and the fungicide Voriconazole VCZ were used as standard.

2.3.2 Minimum Inhibitory Concentration (MIC determination

The MIC in (μg/mL) was determined using the microdilution broth method in 96 well flat bottomed microliter plates. For that, the lichen extracts were diluted in the appropriate growth media for clinical pathogens. 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).

2.3.3 Bactericidal and fungicidal activity determination

The agar diffusion assay 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 105 CFU/mL of the tested pathogen from a serial twofold dilution of the Cladonia extracts. The AU/mL was calculated as AU/mL = $1000X$ D/A (where: A is the volume of the extract, aliquot spotted on an agar plate (50 μL in this case); D is the reciprocal of the highest dilution giving an inhibition zone of the indicator strain) (Mendili, Essghaier, et al., 2021) .

2.3.4 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 a suspension of *Staphylococcus aureus* and *Enterococcus faecalis*, we have applied the turbidimetrically method (Ryazanova et al., 2005). Brief, bacterial cells obtained from 24h culture at 37°C, were washed twice with distilled water and then suspended in 50 mM phosphate buffer (pH 6.5). The mix reaction consisting of 100 μL of the bacterial cell wall suspension and 50 μL of the tested extracts adjusted at 2000 μg/mL and incubated at 37°C for 60 min. Arbitrary Unit of lysozyme activity was determined as the decrease in OD at 660nm with 0.001per mL of the extract solution per min compared to the untreated one (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) . The reaction containing 20 μL of conidial suspensions (10^4spores/mL) and 20 μL of the tested extract (5000 μg/mL) and 1mL of 5% glucose, and then incubated at 21°C for 24 h. The percentage of spore

germination inhibition **(I %)** was determined by microscopic examination for each extract (E), compared to the control tube containing only the conidia suspensions with three repetitions tests. As follow: I (%) = (C-E)/C *X* 100 (where: C: the number of spores present in the control tube (without extract) and E is the number of spores in the tube containing the suspension of spores in the presence of the extract separately (Sarangi et al., 2010).

2.3.5 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. Antibacterial and anti-Candida activity

The antimicrobial activities of the methanol, acetone and QUENCHER extracts of *C. pocillum* and *C. rangiformis* were analyzed against the microorganisms: *E. cloacae*; *E. coli*, *S. aureus*, *E. 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**).

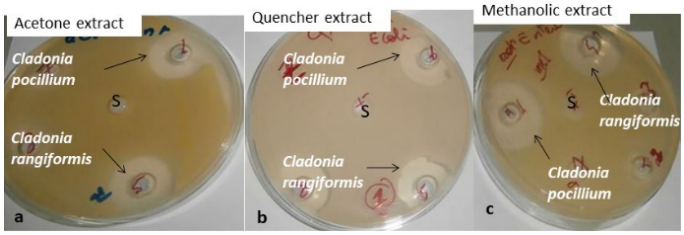

Figure 1. Observation of zone inhibition on agar plate obtained by the acetone, quencher and methanolic extracts of both Cladonia species. **a:** *Enterobacter cloaceae*, **b:** *Escherichia coli* and **c:** *Enterococcus faecalis* and **S** present the corresponding organic solvent used for acetone 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 *C. albicans*, followed by the acetone extract against *C. parapsilosis*.

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

Table 1 shows the variations in MIC (minimum inhibitory concentration) values between the different extracts utilized. MIC values ranged from 250μg/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 *C.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**).

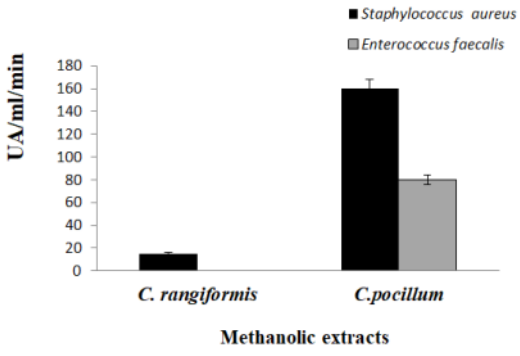

Figure 2. Lysozyme activity effect of both methanolic extracts from *Cladonia rangiformis* and *Cladonia pocillum* species. The values shown are the average from triplicate experiments. Error bars represent the SE of the means.

Table 1
Antimicrobial activity of *Cladonia rangiformis* and *Cladonia pocillum* extracts evaluated against microorganisms 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
Minimum inhibitory concentration (MIC) of the extracts of *C. rangiformis* and *C. pocillum* against the test organisms. 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

Table 3 Illustrates the bactericide activityand 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 effects of extracts from *C. rangiformis* and *C. pocillum* on the spore germination of the fungi *Aspergillus niger* and *Penicillium digitatum* were investigated in **Figure 3**.

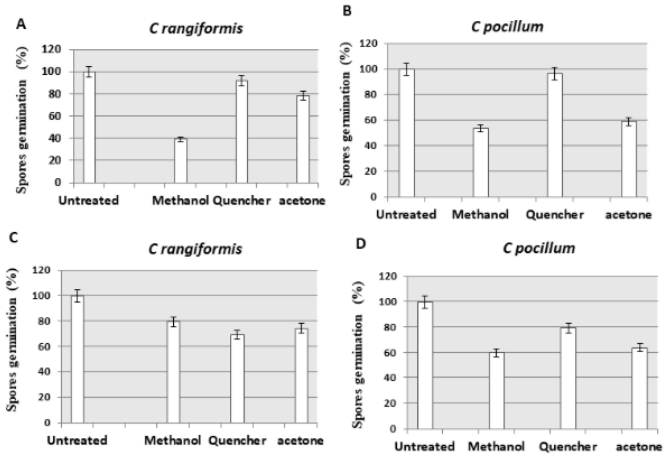

Figure 3. Effect of Various extracts produced from *Cladonia rangiformis* and *Cladonia pocillum* on spore germination of Penicillium (A and B) and Aspergillus (C and D). Values present the percentage of spore germination.

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, the quencher extract of *C. rangiformis* showed a moderate 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**).

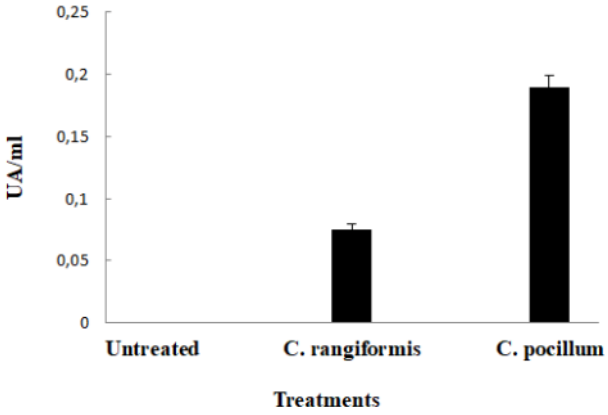

Figure 4. The methanol extracts from *Cladonia rangiformis* and *Cladonia pocillum* on the mycelial morphology of *Alternaria alternata* compared to the control tube containing the mycelium suspension without the methanol extract. Values were expressed in UA /mL.

4. DISCUSSION

At present, due 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 were 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). Several studies have investigated the biological activities of crude extracts and purified compounds of lichen species, such as antioxidant, antimicrobial, antiviral, cytotoxic, insecticidal and anti-inflammatory properties (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). So, this study was the first to investigate the antibacterial and antifungal activities of the methanolic, acetone and quencher extracts from *C. rangiformis* and *C. pocillum* species in Tunisia. They have exhibited an antibacterial effect against gram-positive and gram-negative bacteria, Candida and fungal activities by inhibiting spore germination. Therefore, the differences depend on the species, the nature of the extract, and the extraction methods. 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 Gram-positive and Gram-negative bacteria (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 within the range of 0.25mg/mL to 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* species which ranged from 1.56 to 12 mg/mL for bacteria and from 12.5 to 25 g/mL concerning fungi (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 (Verma et al., 2011; Ranković et al., (2010) B. Ranković et al. (2010), 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 methanol, acetone and quencher extracts of *C. rangiformis* and *C.pocillum* species. Recently Mendili, Essghaier, et al. (2021) mentioned that the methanol, acetone, and quencher extracts from four lichens: *Diploschistes ocellatus*, *Flavoparmelia caperata*, *Squamarina cartilaginea*, and *Xanthoria parietina*, have antibacterial, lysozyme, and 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 associated with 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 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 gram-positive and gram-negative bacteria 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 declares that there is no conflict of interest associated with this research work.

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ORCID

Essghaier Badiaa 0000-0003-0818-7896
Mendili Mohamed 0000-0003-0234-5240
Khadri Ayda 0000-0003-2651-1602

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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