

Research Article

Antimicrobial Activity of *Cladonia verticillaris* Lichen Preparations on Bacteria and Fungi of Medical Importance

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Cladonia verticillaris lichen lectin (ClaveLL) was purified using a previously established protocol and then evaluated for its potential antimicrobial activity. Initially, the autochthonous lichen was submitted to extraction with sodium phosphate buffer pH 7.0, followed by filtration and centrifugation to obtain crude extract. A salt fractionation was performed with 30% ammonium sulfate. After centrifugation, the protein fraction was loaded onto molecular exclusion chromatography using Sephadex G-100 matrix to purify active lectin. ClaveLL showed antibacterial activity against Gram-positive (*Bacillus subtilis*, *Staphylococcus aureus*, and *Enterococcus faecalis*) and Gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*) assayed strains, with greater inhibitory effect on growth of *E. coli* (MIC of 7.18 $\mu\text{g mL}^{-1}$). The lowest minimum bactericidal concentration (MBC, 57.4 $\mu\text{g mL}^{-1}$) was detected against *E. faecalis*. The antifungal assay performed with *Trichophyton mentagrophytes*, *Microsporium gypseum*, *Trichophyton rubrum*, *Trichosporon cutaneum*, and *Trichosporon asahi* evaluated crude extract, fraction, and ClaveLL preparations. ClaveLL was the most active against *T. rubrum* with an inhibition percentage of 35% compared to negative control (phosphate buffer). Extract and fraction showed better activity on growth inhibition of *T. mentagrophytes* (35%). The results indicate the potential of ClaveLL and other *C. verticillaris* preparations as antimicrobial agents useful for applications focusing on human health.

1. Introduction

The treatment of diseases caused by bacteria and fungi is becoming an issue of concern, due to the growing emergence of microorganism strains resistant to drugs and opportunistic fungi that cause serious infections in humans [1, 2]. Microbial resistance is a genetic phenomenon, in which the microorganisms have genes that encode biochemical mechanisms which prevent drug actions. It may be caused by mutations in the reproductive process of the microorganisms or by imported genes acquired through transduction, conjugation, and transformation mechanisms [3]. Microbial resistance is sometimes due to natural evolution of microorganisms;

however, it gains importance through the excessive use of antimicrobial substances in medical, agricultural, and veterinary practices [4]. Therefore, the search for new antibiotics from natural sources has increased as an alternative to commercial drugs.

Lichens are symbiotic associations between fungus and one or more algae and/or cyanobacteria [5]. Species of the genus *Cladonia* have been used in folk medicine to treat respiratory diseases such as throat irritation, cough, asthma, and tuberculosis [6, 7]. The use of lichens in medicine is based on the fact that they contain unique and various biologically active substances, mainly with antimicrobial actions [8, 9]. *Cladonia verticillaris* is a lichen of cosmopolitan distribution

and represents a rich source of lectin (carbohydrate-binding protein). The protocol established for isolation of *C. verticillaris* lichen lectin (ClaveLL) is simple and yields milligram quantities of protein (18 mg from 20 g of lichen flour) free of secondary metabolites. ClaveLL showed insecticidal activity on termite *Nasutitermes corniger* [10].

The lichen *C. verticillaris* produces bioactive secondary metabolites, such as fumarprotocetraric and protocetraric acids. The fumarprotocetraric acid has demonstrated biological actions, such as expectorant, antioxidant, and allelopathic effect [11, 12]. However, little has been done with respect to lichen lectins and their biological properties and potential biotechnological applications.

Lectins with potential for biotechnological applications as antibiotics have been isolated and their structures have been characterized [13–15]. Lectins are a heterogeneous group of proteins from non-immune origin, containing two or more binding sites for mono or oligosaccharides [16]. Gomes et al. [17] isolated a lectin from leaves of *Schinus terebinthifolius* that showed antibacterial activity against human pathogenic bacteria and the fungus *Candida albicans*. Besides being applied to control bacteria and fungi growth, lectins can be used for detection and typing of microorganisms due to the selective interaction between lectins and microorganisms [18].

Fungi known as dermatophytes of the genus *Trichophyton* and *Microsporum* cause superficial mycoses affecting the hair, nails, and skin. *Trichosporon* species have contributed to increased mortality in immunocompromised individuals [19]. Thus, the aim of this study was to evaluate the antimicrobial activity of purified *C. verticillaris* lichen lectin (ClaveLL) against human pathogenic bacteria Gram-positive (*Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and *Klebsiella pneumoniae*) as well as against dermatophyte fungi (*Trichophyton mentagrophytes*, *Microsporum gypseum*, *Trichophyton rubrum*, *Trichosporon cutaneum*, and *Trichosporon asahi*).

2. Materials and Methods

2.1. Purification of ClaveLL. *C. verticillaris* lichen was collected at Alhandra City, State of Paraíba in the Northeastern of Brazil, and gently identified by Dr. Eugênia Cristina Gonçalves Pereira (Departamento de Ciências Geográficas, Centro de Filosofia e Ciências Humanas, UFPE). *C. verticillaris* lichen lectin (ClaveLL) was obtained through a sequential purification protocol according to Silva et al. [10]. Lichen was dried at 28°C and an overnight extraction (10%, w/v) for 16 h at 28°C was performed in 0.15 M sodium phosphate buffer pH 7.0 containing 0.15 M NaCl (PBS), followed by filtration and centrifugation (3000 g, 4°C, 15 min). The lichen extract was treated with 30% (w/v) ammonium sulphate for 4 h at 28°C. The 0–30% precipitate fraction was obtained by centrifugation (3000 g, 4°C, 15 min), dialyzed for salt removal, submitted to protein quantification, and loaded (18 mg of protein) onto a gel filtration chromatography containing 100 mL of Sephadex G-100 (1.4 × 63 cm). The molecular exclusion column was performed using with PBS at a flow

rate of 20 mL/h. The collected fractions were monitored for absorbance at 280 nm and hemagglutinating activity.

2.2. Hemagglutinating Activity. Hemagglutinating activity was determined according to method described by Correia et al. [20]. The sample (50 µL) was twofold serially diluted in 0.15 M NaCl on microplate wells. Next, a suspension of 2.5% glutaraldehyde-treated rabbit erythrocytes [21] was added (50 µL) to each well. One hemagglutination unit (titer⁻¹) was defined as the reciprocal of the highest sample dilution which promoted full erythrocyte agglutination. Specific hemagglutinating activity was defined as the ratio between the titer and protein concentration (mg·mL⁻¹).

2.3. Protein Content. Protein concentration was determined according to Lowry et al. [22] using bovine serum albumin as standard (31.25–500 µg mL⁻¹).

2.4. Antibacterial Activity. Gram-positive (*Bacillus subtilis* ATCC-6633, *Staphylococcus aureus* ATCC-6538 and *Enterococcus faecalis* ATCC-6057), and Gram-negative (*Escherichia coli* ATCC-25922 and *Klebsiella pneumoniae* ATCC-29665) bacterial strains were provided by the *Departamento de Antibióticos, Universidade Federal de Pernambuco, Brazil*. Stationary cultures were maintained in nutrient agar (NA) and stored at 4°C. Bacteria were cultured in nutrient broth (NB) and incubated under permanent shaking at 37°C overnight. The culture concentrations were adjusted turbidimetrically at a wavelength of 490 nm to 10⁵–10⁶ colony forming units (CFU)·mL⁻¹.

An aliquot of ClaveLL (100 µL; 0.184 µg) was diluted 1:1 in NB and then submitted to a series of ten double dilutions, until a final ratio of 1:2048. Next, a 180 µL aliquot of each dilution was dispensed into each well of a 96-well microplate. All wells were inoculated with 20 µL of the bacterial culture and incubated at 37°C for 24 h. Assay for each concentration was performed in triplicate. Control wells contained only NB medium and microorganism. After incubation, the optical density at 490 nm (OD₄₉₀) was measured using a microplate reader. Minimal inhibitory concentration (MIC) was determined as the lowest lectin concentration at which there was ≥50% reduction in OD₄₉₀ in comparison with the control [23].

To determine the minimal bactericidal concentration (MBC), inoculations (10 µL) from the sample wells at which inhibition of bacterial growth was detected were transferred to NA plates and incubated at 37°C for 24 h. The MBC corresponded to the lowest sample concentration at which no bacterial growth was observed. The assay was performed in triplicate.

2.5. Antifungal Activity. *Trichophyton mentagrophytes* (URM5541), *Microsporum gypseum* (URM6199), *Trichophyton rubrum* (URM5145), *Trichosporon cutaneum* (URM5743), and *Trichosporon asahi* (URM5781) were obtained from the Cultures Collection of University Recife Mycologia (URM) from the *Departamento de Micologia* of the *Universidade*

TABLE 1: Summary of ClaveLL purification.

Sample	Volume (mL)	Protein (mg/mL)	HA ^a	SHA ^b (HA/mg)	Purification fold ^c
Lichen extract	430	9.4	512	54.3	1.0
0–30% fraction	18	40.4	16384	405	7.5
ClaveLL	396	0.1	64	711	13.1

^aHA: hemagglutinating activity; ^bSHA: specific HA; ^cpurification fold: relation between SHA of crude extract and SHA of lectin.

TABLE 2: MIC and MBC of ClaveLL against bacteria species.

Microorganisms	MIC ($\mu\text{g mL}^{-1}$)	MBC ($\mu\text{g mL}^{-1}$)
<i>Staphylococcus aureus</i> (+)	229.9	459.9
<i>Bacillus subtilis</i> (+)	28	57.4
<i>Enterococcus faecalis</i> (+)	28	57.4
<i>Klebsiella pneumoniae</i> (–)	114.9	919.8
<i>Escherichia coli</i> (–)	7.18	229.9

Gram-positive (+) and Gram-negative (–) bacteria; MIC: minimum inhibitory concentration. MBC: minimum bactericidal concentration.

Federal of Pernambuco (UFPE), Brazil. Antifungal activity was performed according to Wong and Ng [24] with modifications. Fungi were grown at 28°C on potato dextrose agar (PDA) in Petri plates for an 8–15-day period until the surfaces of the plates were completely covered by them. Fungal mycelium discs (0.625 cm in diameter) were removed from the peripheral part of the colonies. ClaveLL (100 μL ; 1.67 mg mL⁻¹), lichen extract (100 μL ; 9.43 mg mL⁻¹), and 0–30% fraction (100 μL ; 40.45 mg mL⁻¹) were spread on solidified PDA medium in Petri plates (100 × 15 mm²) and a fungal mycelium disk was disposed in the center of the Petri plates. All assays were carried out in triplicate. PBS and nystatin (1 mg/mL) were used as negative and positive controls, respectively. The plates were incubated at 28°C for 72 h. Antifungal activity was indicated by reduction of the fungal growth zone (diameter) in the plates.

2.6. Statistical Analysis. Standard deviations (SD) were calculated using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA) and data were expressed as a mean of replicates \pm SD. Significant differences between treatment groups from the antifungal assays were analysed by Student's *t*-test (significance at $P < 0.05$) using the Origin 6.0 program.

3. Results and Discussion

Extraction and isolation of pharmacologically active compounds with medicinal importance have received attention in the search for new economically viable alternatives for treating human infections. ClaveLL was purified through a preestablished protocol by Silva et al. [10] using molecular exclusion chromatography on Sephadex G-100 matrix; the column is a viable approach since the same array can be reused efficiently at least 30 times to purify ClaveLL.

Treatment of lichen extract with ammonium sulphate yielded a precipitated protein fraction (0–30% fraction). Chromatography of 0–30% fraction on Sephadex G-100 separated two peaks (Figure 1): the first (corresponding to fractions 7–22) was colorless and showed hemagglutinating

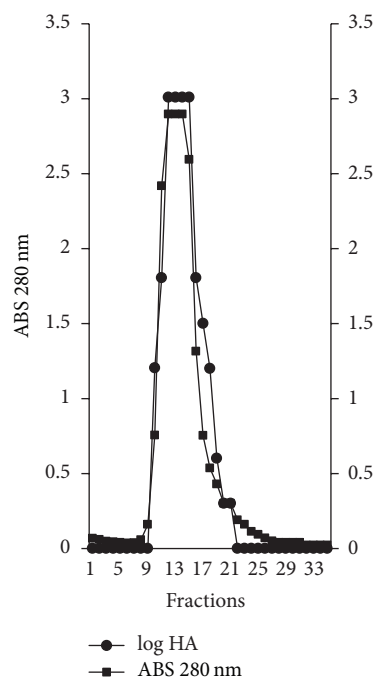


FIGURE 1: ClaveLL isolated from 0–30% fraction through molecular exclusion chromatography using Sephadex G-100 column (HA in log scale).

activity of 64, corresponding to ClaveLL, with a purification factor of 13.1 times (Table 1). A total of 8.28 mg of ClaveLL were purified from 10 g of *C. verticillaris*, yield similar to that obtained by Silva et al. [10]. The second peak showed a strong color similar to the fraction and did not show hemagglutinating activity.

ClaveLL revealed antibacterial activity against all tested species, both Gram-positive and Gram-negative. The values for MIC and MBC are shown in Table 2. The antibacterial activity of lectins may occur through the interaction with N-acetylglucosamine (GlcNAc), N-acetylmuramic acid (MurNAc), and tetrapeptides linked to MurNAc present in

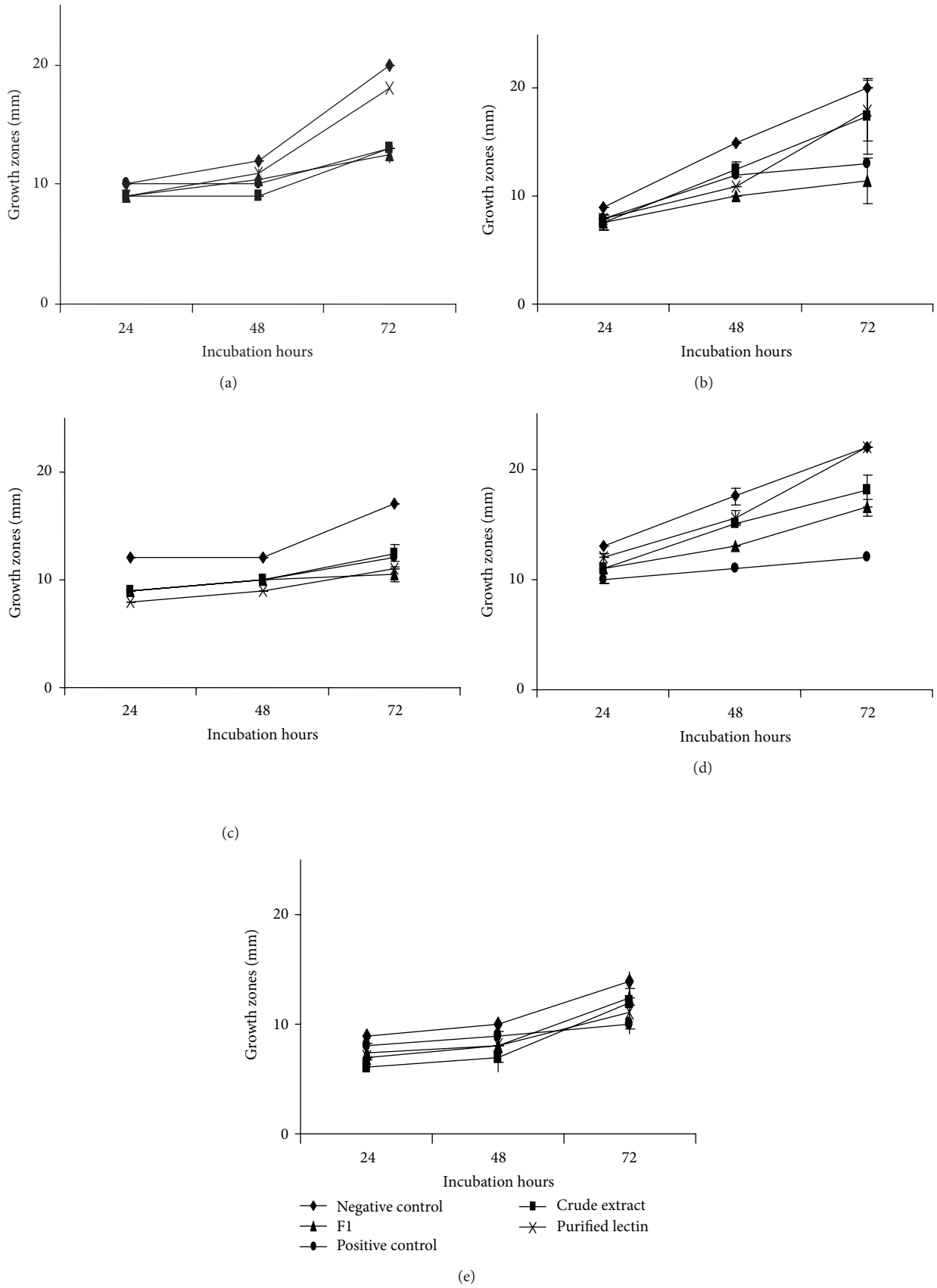


FIGURE 2: Growth zones (mm) of the fungi *T. mentagrophytes* (a), *M. gypseum* (b), *T. rubrum* (c), *T. cutaneum* (d), and *T. asahi* (e) in PDA medium (y-axis) throughout the incubation hours. Each point represents the mean \pm SD of three experiments.

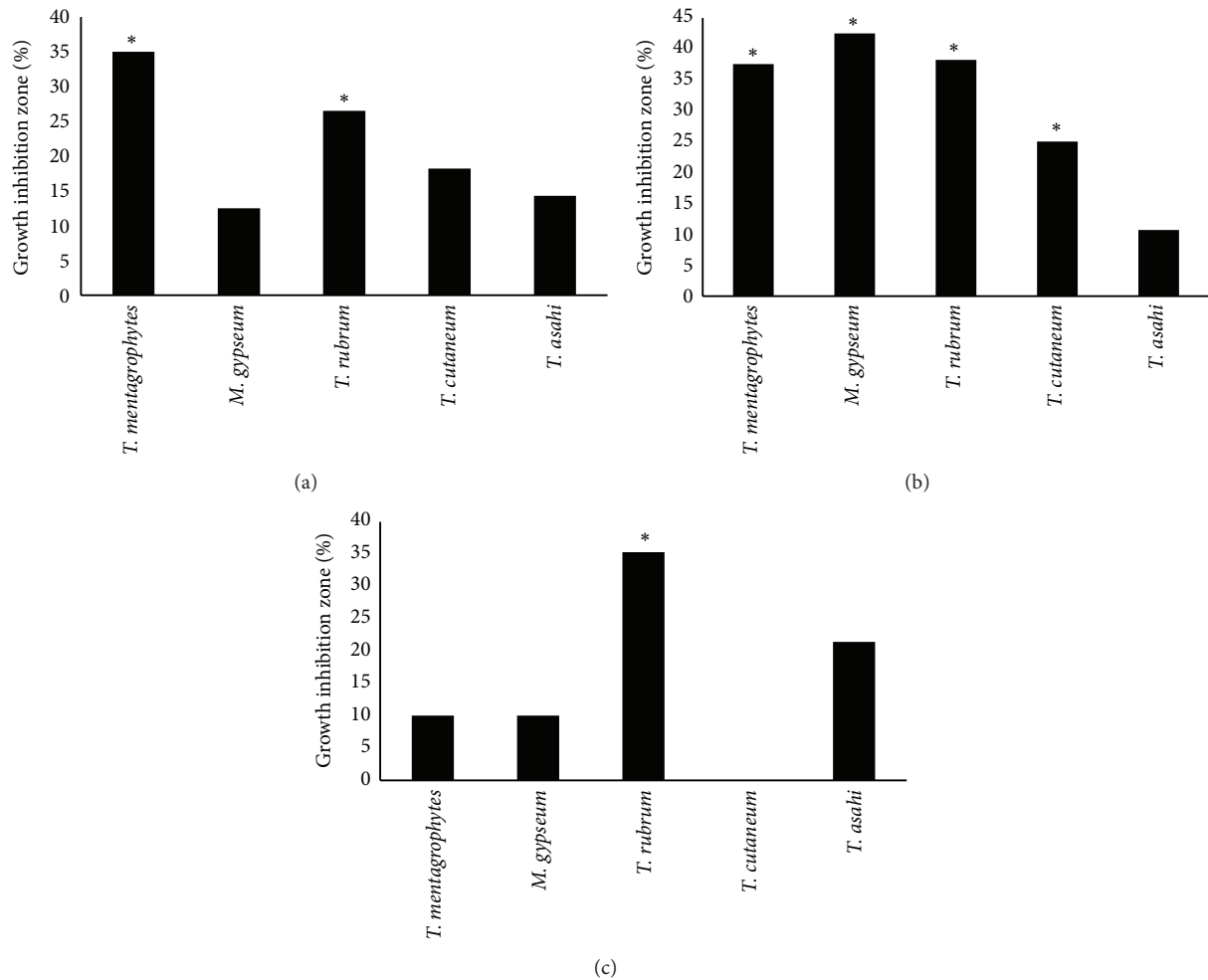


FIGURE 3: Antifungal activity of lichen extract (a), 0–30% fraction (b), and ClaveLL (c) observed as percentage of growth inhibition in comparison with growth in control. The histograms representing average values of tree measurement of inhibition zones. The * indicates significant differences between treatments and control.

the cell wall of Gram-positive bacteria or to lipopolysaccharide present in the cell walls of Gram-negative bacteria [25].

Among the bacteria tested, ClaveLL showed the best inhibitory activity against the Gram-negative bacteria *E. coli* with MIC value of $7.18 \mu\text{g mL}^{-1}$ and the best bactericidal activity against Gram-positive bacteria *B. subtilis* and *E. faecalis* with MBC value of $57.4 \mu\text{g mL}^{-1}$. Thus, ClaveLL was more efficient in killing Gram-positive than Gram-negative bacteria. Similarly, the isolated lectin from leaves of *S. terebinthifolius* was more efficient against the Gram-positive *Staph. aureus* than Gram-negative bacteria such as *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Salmonella enteritidis* [17]. The difference in the susceptibility of Gram-positive and Gram-negative bacteria may be linked to the difficulty of lectins to cross the Gram-negative bacteria outer cell wall to reach the periplasmic space [15]. Additionally, the high level of peptidoglycan (which contains GlcNAc) in the cell wall of Gram-positive bacteria may provide more interaction sites

for N-acetyl-glucosamine-binding lectins, such as ClaveLL [10].

Costa et al. [14] studied the leaf lectin of *Phthirusa pyriformis* (PpyLL) and found MBC of approximately 10 times higher ($500 \mu\text{g mL}^{-1}$) than ClaveLL for *B. subtilis*. Instead, Sá et al. [13] found MIC of $2.34 \mu\text{g mL}^{-1}$ of the *Myracrodruon urundeuva* heartwood lectin (MuHL) for this same bacterium, corresponding to a concentration 10 times lower than that of ClaveLL ($28 \mu\text{g mL}^{-1}$).

Although several lectins purified from various natural sources have shown antibacterial activity [14, 26–28], its mechanism of action was not elucidated in detail. It has been proposed that lectins with antibacterial activity form pores in the cell membrane and cell dies as a result of a outflowing of cellular contents [29, 30].

The lichen extract (Figure 2(a)) inhibited the growth of *T. mentagrophytes* (35%), fungus that was not inhibited by ClaveLL, revealing that the antimicrobial activity of

C. verticillaris involves more than one active principle which was removed during lectin purification protocol. Perry et al. [31], studying the antimicrobial activity of 69 species of lichens from New Zealand, found that *Cladonia fimbriata* extract also inhibited the growth of *T. mentagrophytes*.

The 0–30% fraction was the sample that showed the highest antifungal activity (Figures 2 and 3), inhibiting growth of *T. mentagrophytes* (37.5%), *M. gypseum* (42.5%), *T. rubrum* (38.2%), and *T. cutaneum* (25%).

ClaveLL showed highest antifungal activity against *T. rubrum* (35.3% of inhibition) (Figures 2 and 3). However, ClaveLL (170 µg) was not active against *T. mentagrophytes*, *M. gypseum*, and *T. cutaneum* (Figures 2 and 3). The inhibition of fungi growth can occur through lectin binding to hyphae resulting in poor absorption of nutrients as well as by interference on spore germination process [13]. Antifungal activity of lectins is related to their property to bind carbohydrates such as chitin, a large polymer of N-acetyl-glucosamine, and component of fungal cell wall [20]. The binding of N-acetyl-glucosamine-binding lectins can lead to rupture of the fungal cell wall [32].

The antibacterial and antifungal activities of extract, 0–30% fraction, and ClaveLL are not correlated with secondary metabolites since Silva et al. [10] showed that all these preparations were absent from this class of compounds. The antifungal activity detected for extract and fraction from *C. verticillaris* encourages further studies to isolate other lichen compounds to find the chemical constituents responsible for such activity.

4. Conclusion

In conclusion, the lectin purified from *C. verticillaris* showed antibacterial activity, particularly against Gram-positive bacteria; also, it was able to inhibit the growth of the dermatophyte fungus *T. rubrum*. ClaveLL and other *C. verticillaris* preparations are potential antimicrobial agents to be applied for treating human infections.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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