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Antifungal activity of eukaryotic microalgae extracts in dermatophytes

Atividade antifúngica de extratos de microalgas eucarióticas em dermatófitos

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Abstract

Introduction: *Microalgae are considered a promising source of biologically active secondary metabolites, with several biotechnological properties already recorded, including antifungal activity.* ***Objective:*** *thus, the properties of ethanol extracts of microalgae Ankistrodesmus falcatus, Chaetoceros neogracilis, Desmodesmus brasiliensis, Dunaliella tertiolecta, Kirchneriella lunaris and Tetraselmis gracilis were investigated in vitro for antifungal activity against dermatophytes Nannizzia gypsea, Nannizzia nana, Trichophyton mentagrophytes and Trichophyton tonsurans.* ***Methods:*** *the extracts were evaluated using broth microdilution methodology, with a test interval of 11.5 to 6.000 µg mL^{-1}. The antifungal itraconazole was tested at concentrations 0.0313 to 16 µg mL^{-1}.* ***Results:*** *all microalgae extracts showed antifungal activity, especially extracts from C. neogracilis, D. brasiliensis, and K. lunaris that inhibited the growth of all species of dermatophytes evaluated. The lowest MIC values recorded were for the extracts of D. brasiliensis and K. lunaris (MIC 188 µg mL^{-1}) against T. tonsurans e N. nana.* ***Conclusion:*** *this was a pioneering work about the antifungal activity of these microalgae against dermatophytes. It is expected that, from this study, further research will be carried out to identify and isolate the active biomolecules responsible for the antifungal activity of these microalgae species.*

Keywords: *Microalgae; chlorophycea; diatoms; antifungal potential; dermatophytosis.*

Resumo

Introdução: as microalgas são consideradas uma fonte promissora de metabólitos secundários biologicamente ativos, com diversas propriedades biotecnológicas já registradas, incluindo atividade antifúngica. **Objetivo:** assim, as propriedades dos extratos etanólicos das microalgas *Ankistrodesmus falcatus*, *Chaetoceros neogracilis*, *Desmodesmus brasiliensis*, *Dunaliella tertiolecta*, *Kirchneriella lunaris* e *Tetraselmis gracilis* foram investigadas *in vitro* quanto à atividade antifúngica frente aos dermatófitos *Nannizzia gypsea, Nannizzia nana, Trichophyton mentagrophytes* e *Trichophyton tonsurans.* **Metodologia:** os extratos foram avaliados através da metodologia de microdiluição em caldo, com intervalo teste de 11.5 a 6.000 µg mL^{-1}. O antifúngico itraconazol foi testado nas concentrações 0.0313 a 16 µg mL^{-1}. **Resultados:** todos os extratos microalgais apresentaram atividade antifúngica, com destaque para os extratos das microalgas *C. neogracilis, D. brasiliensis* e *K. lunaris*, que inibiram totalmente o crescimento de todas as espécies de dermatófitos avaliadas. Os menores valores de CIM registrados foram para os extratos de *D. brasiliensis* e *K. lunaris* (CIM 188 µg mL^{-1}), contra *T. tonsurans* e *N. nana*. **Conclusão:** este foi um trabalho pioneiro acerca da atividade antifúngica destas microalgas frente à dermatófitos. Espera-se que, a partir deste estudo, novas pesquisas sejam realizadas a fim de identificar e isolar as biomoléculas ativas responsáveis pela atividade antifúngica destas espécies de microalgas.

Palavras-chave: Microalga; clorofícea; diatomácea; potencial antifúngico; dermatofitoses.

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INTRODUCTION

Microalgae are photosynthesizing organisms that combine water and atmospheric carbon dioxide with

sunlight to produce various forms of energy and biomass[1]. Like plants, they produce a wide variety of compounds, some of which are collectively referred to as secondary metabolites, which are compounds synthesized by the body at the end of the first growth phase and in the stationary phase[2].

Due to the diversity and richness of bioactive substances, microalgae have become promising in biotechnology, mainly due to their biological activities in the pharmacological area[3]. Several compounds extracted from microalgae revealed potent biological activities, such as antioxidant[4], anticancer[5], antibacterial[6], antiprotozoal[7], antiviral[8] and antifungal[9].

Dermatophytosis, or tinea, is a disease caused by filamentous hyaline keratinophilic fungi collectively called dermatophytes[10]. These microorganisms have a special biotropism by tissues of keratinized structures, infecting mainly the skin, hair and nails[11]. It is one of the most common zoonosis and has a wide potential for contamination, so it is considered a public health problem[12].

The emergence of resistant dermatophytes in recent years has become alarming worldwide. Consequently, this fact led to a renewed interest in research directed at these parasites, mainly in the search for new drugs and alternative treatments10.

Despite the tremendous pharmacological potential of microalgae, there are still few studies on the antifungal activity of these microorganisms in dermatophytes, and many species have not yet been contemplated13. Thus, this study aimed to evaluate the in vitro antifungal activity of eukaryotic microalgae Ankistrodesmus falcatus, Chaetoceros neogracilis, Desmodesmus brasiliensis, Dunaliella tertiolecta, Kirchneriella lunaris and Tetraselmis gracilis against dermatophyte strains.

MATERIALS AND METHODS

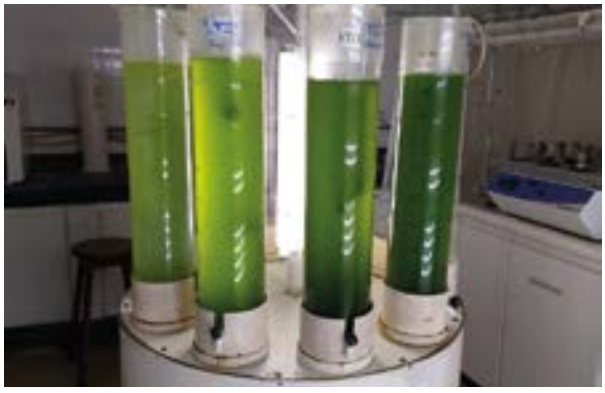
Cultivation and biomass production

The strains of microalgae A. falcatus (ALCB137416), C. neogracilis (ALCB137425), D. brasiliensis (ALCB137414), D. tertiolecta (ALCB137436), K. lunaris (ALCB137418) e T. gracilis (ALCB137433) were provided by the Microalgae Bank of the Bioprospection and Biotechnology Laboratory (LABBIOTEC), located at the Institute of Biology of the Federal University of Bahia (UFBA) in Salvador, Bahia. The use of these strains is recorded in the National System of Management of Genetic Heritage and Associated Traditional Knowledge (SisGen), with registration n° A653955.

These microalgae were cultivated according to the standards proposed by Nascimento et al.[14] (2015). The cultivation conditions used were photoperiod 12h (light/dark cycles), luminosity of 35 µmol of photons m^{-2} s^{-1}, temperature of 22°C, pH 7.0 and constant aeration. Oligo[15] and Conway[16] culture media were used for freshwater and marine microalgae, respectively. For diatom C. neogracilis, 2 mL L^{-1} to 0.32 mM of sodium silicate (Na_2SiO_3) was added to the culture medium.

To obtain biomass, the crop volume was gradually increased until reaching the final stage, which was in a tubular photobioreactor with a capacity of 10 L (Figure 1). The microalgae culture's growth was monitored to determine the appropriate time for biomass collection. For this, cells were counted by optical microscopy with a Neubauer Haemocytometer[16]. The number of cells present was monitored daily for 20 days, and, from the following equation[14], it was possible to determine it: Cells/mL = Number of cells counted/Nº of quadrants x Dilution factor x 10.000.

Figure 1 – *Cultivation of microalgae to obtain biomass in a tubular photobioreactor.*



Fonte: Autoria propria

The microalgal biomass was collected in the stationary growth phase by centrifugation in an MPW®-351 centrifuge at 4732 x g for 20 min[17]. The precipitate from the centrifugation was stored in sterile glass containers in the freezer at -20ºC while the supernatant was discarded. To obtain the dry biomass, the frozen material was lyophilized for 48 h, at a vacuum of 0137µHg and -30°C in a lyophilizer model Enterprise II, Terroni®. The dry biomass obtained was weighed and stored at -20ºC until the extract was prepared.

Extraction procedures

After the lyophilization process, each species' aliquot of dry biomass was submitted to the extraction process. In the Erlenmeyer flask, 1g of dry biomass was added to 100 mL of absolute ethyl alcohol (99.9%) from Merck®. This material was homogenized in a vortex and subject to sonication on ice to promote cell lysis in two cycles of 6 min at 10% power, using Sonicator model Sonopuls HD 2070 from Bandelin®. After sonication, samples went to agitation in a pendular shaker table for 72 h. After this period, the material was centrifuged at 4732 x g for 10 min in 15 mL Falcon tubes. The supernatant was placed in sterile glass flasks to start drying at room temperature until the complete evaporation of the solvent. The extraction process was repeated for three cycles, and the precipitates were discarded at the end.

Fungi cultivation

The microalgal extracts were tested against dermatophytes Nannizzia gypsea (ALCB072421), Nannizzia nana (ALCB072422), Trichophyton mentagrophytes (ALCB072423) and Trichophyton tonsurans (ALCB072424) strains deposited at the Alexandre Leal Costa Herbarium (ALCB) of Institute of Biology, Federal University of Bahia (UFBA), Salvador, Bahia. The isolates were registered in SISGEN (National System for the Management of Genetic Heritage and Associated Traditional Knowledge) under AB07806. All species were cultivated in Potato Dextrose Agar (PDA) medium at 32°C, with maintenance repeats every seven days.

Susceptibility Tests

To determine the Minimum Inhibitory Concentration (MIC) and Minimum Fungicide Concentration (MFC), the broth microdilution technique was used, according to the norms proposed by the National Committee for Clinical Laboratory Standards[18] (2002), with modifications suggested by Curatolo et al.[11] (2020). The ethanolic microalgae extracts were evaluated in the concentration range from 11.5 to 6.000 µg mL^{-1} (4% DMSO). Itraconazole (Infinity®) at concentrations from 0.03125 to 16 µg mL^{-1} (4% DMSO). TM MEDIA® Broth Sabouraud Dextrose culture medium was used. The fungal suspensions were adjusted to the 0.5 McFarland scale and diluted 1:20 in 2X Sabouraud Dextrose Broth, which produces the inoculum with a final concentration of 1.5 x 10^8 CFU mL^{-1}.

In 96-well plates, 50 µL of each serial dilution of extracts and itraconazole were added to 50 µL of the inoculum. The lowest concentration of extract or itraconazole that limited visible fungal growth after seven days of incubation at 32°C was considered the Minimum Inhibitory Concentration (MIC).

The contents of the wells without visible mycelial growth were seeded in a Petri dish containing Potato Dextrose Agar (PDA) medium[19]. These plates were incubated under standardized conditions (32°C for seven days). At the end of the defined period, it was possible to determine whether the inoculum was completely inhibited, determining the MFC value. The experiments were performed under sterile conditions and in triplicates.

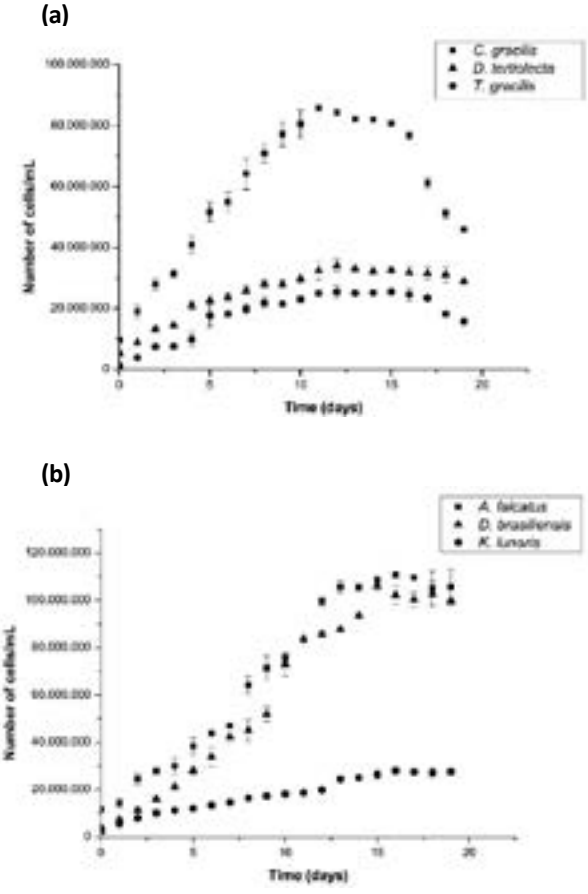
The MFC/MIC ratio[20] was calculated to determine whether a particular drug or extract tested had a fungicidal action when the ratio value was < 4 or fungistatic when the ratio value was ≥ 4.

RESULTS

Microalgal growth curve and biomass harvest

The microalgal biomass was collected in the stationary phase of the growth curve using a centrifugation method. This period was established from daily monitoring of microalgae growth for 20 days. The biomass of marine microalgae (C. neogracilis, D. tertiolecta and T. gracilis) was collected on the fourteenth day (Figure 2a). For freshwater microalgae (A. falcatus, D. brasiliensis and K. lunaris), harvesting was performed on the eighteenth day (Figure 2b). In the graphical representations, points indicate the average values obtained from duplicates (Figure 2).

Figure 2 – *Growth curve of marine microalgae (C. neogracilis, D. tertiolecta and T. gracilis) (a) and freshwater microalgae (A. falcatus, D. brasiliensis and K. lunaris) (b).*



Susceptibility tests

All ethanolic extracts of the tested microalgae species showed antifungal activity (Figure 3). The microalgal extracts inhibited the growth of the four dermatophyte species evaluated, with Minimum Inhibitory Concentration (MIC) values ranging from 188 to 6.000 µg mL^{-1} (Tables 1 and 2). Itraconazole inhibited three of the evaluated dermatophyte species, with MIC values ranging from 0.03125 to 0.0625 µg mL^{-1} (Table 3). The species N. nana was not inhibited by itraconazole.

The ethanolic extracts of C. neogracilis and K. lunaris showed fungicidal activity against the four dermatophyte species. The chlorophyte extract D. brasiliensis showed fungistatic activity against T. tonsurans and fungicidal activity against other dermatophyte species. The ethanolic extract of A. falcatus showed fungistatic activity against

T. tonsurans and fungicidal activity against N. nana. The marine microalgae D. tertiolecta and T. gracilis showed only partial inhibitory activity. Itraconazole showed fungicidal activity only against T. mentagrophytes. The ethanolic extracts of K. lunaris and D. brasiliensis registered the lowest MIC values (188 µg mL^{-1}) against the dermatophytes T. tonsurans and N. nana. T. tonsurans and T. mentagrophytes were the most sensitive species to itraconazole, with MIC value equal to 0.03125 µg mL^{-1} (Table 3).

Table 1 – *Values of MIC (µg mL^{-1}), MFC (µg mL^{-1}) and MFC/MIC ratio of marine microalgae extracts (C. neogracilis, D. tertiolecta and T. gracilis) against dermatophyte isolates. The extract evaluated was considered to have fungicidal (FC) or fungistatic (FT) activity according to the MFC/MIC ratio result. The cut-off point for itraconazole was 1 µg mL^{-1}. Each isolate was classified as resistant (R) or sensitive (S) according to pre-established cutoff values.*

Dermatophyte	*Chaetoceros neogracilis*				*Dunaliella tertiolecta*				*Tetraselmis gracilis*				*Itraconazol*
	MIC	MFC	MFC/ MIC	Activity	MIC	MFC	MFC/ MIC	Activity	MIC	MFC	MFC/ MIC	Activity	Response
T. tonsurans	1.500	6.000	4	FC	750	ND	ND	ND	1.500	ND	ND	ND	S
T. mentagrophytes	6.000	6.000	1	FC	6.000	ND	ND	ND	NI	ND	ND	ND	S
N. gypsea	6.000	6.000	1	FC	NI	ND	ND	ND	NI	ND	ND	ND	S
N. nana	375	375	1	FC	NI	ND	ND	ND	NI	ND	ND	ND	R

NI: No inhibition; ND: Not determined.

Table 2 – *Values of MIC (µg mL^{-1}), MFC (µg mL^{-1}) and MFC/MIC ratio of freshwater microalgae extracts (A. falcatus, D. brasiliensis and K. lunaris) against dermatophyte isolates. The extract evaluated was considered to have fungicidal (FC) or fungistatic (FT) activity according to the MFC/MIC ratio result. The cut-off point for itraconazole was 1 µg mL^{-1}. Each isolate was classified as resistant (R) or sensitive (S) according to pre-established cutoff values.*

Dermatophyte	*Ankistrodesmus falcatus*				*Desmodesmus brasiliensis*				*Kirshnariella lunaris*				*Itraconazol*
	MIC	MFC	MFC/ MIC	Activity	MIC	MFC	MFC/ MIC	Activity	MIC	MFC	MFC/ MIC	Activity	Response
T. tonsurans	375	6.000	16	FT	188	1.500	8	FT	188	375	2	FC	S
T. mentagrophytes	6.000	ND	ND	ND	750	750	1	FC	750	750	1	FC	S
N. gypsea	NI	ND	ND	ND	750	750	1	FC	1.500	1.500	1	FC	S
N. nana	6.000	6.000	1	FC	750	750	1	FC	188	188	1	FC	R

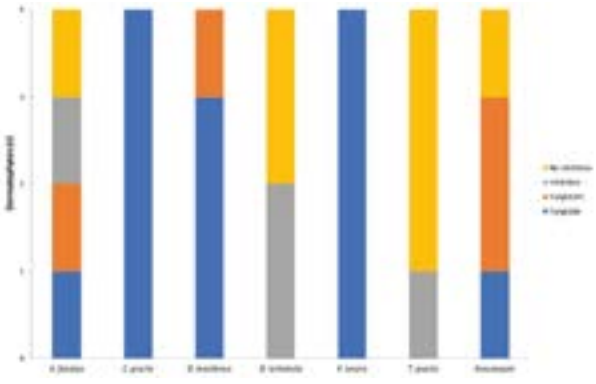
NI: No inhibition; ND: Not determined.

Table 3 – *MIC (µg mL^{-1}), MFC (µg mL^{-1}) and MFC/MIC ratio of itraconazole against dermatophytes. Fungicidal (FC) or fungistatic (FT) activity was determined according to the MFC/MIC ratio.*

Dermatophyte	Itraconazol			
	MIC	MFC	MFC/ MIC	Activity
T. tonsurans	0.03125	0.5	16	FT
T. mentagrophytes	0.03125	0.0625	2	FC
N. gypsea	0.0625	0.5	8	FT
N. nana	NI	ND	ND	ND

NI: No inhibition; ND: Not determined.

Figure 3 – *Responses of dermatophyte isolates to ethanolic extracts of microalgae and to the drug itraconazole.*



DISCUSSION

Growth curve and harvesting of microalgae biomass

For the microalgae C. neogracilis, the exponential phase occurred from day zero to day twelve, followed by the stationary phase from day thirteen to seventeen. These results were similar to other studies in which microalgae growth was followed for ten days, and only the exponential phase was obtained[21-22].

In the growth curve of D. tertiolecta, the exponential phase was obtained from day zero to thirteen, followed by the stationary phase from day fourteen to twenty. Chen et al.[23] (2011) followed the growth of D. tertiolecta for seven days and obtained the exponential phase in this curve period.

The microalgae T. gracilis presented the exponential phase from day zero to the thirteenth of the curve and from the fourteenth to the eighteenth day the stationary phase. These results are similar to those obtained by Rigobello-Masini et al.[24] (2006), in which the T. gracilis curve was monitored until the tenth day and the exponential phase was obtained until this period. In Aidar et al.25 (1994) work, the T. gracilis curve was performed until the fourteenth day, with the exponential phase obtained from day zero to the eighth and the stationary phase from the eighth to the fourteenth.

In this work, the three species of marine microalgae had their stationary phase starting on the thirteenth day of the curve, ending between the seventeenth and the twentieth (Figure 2a). Other studies show that the secondary metabolites of marine microalgae are also concentrated in this period of the stationary phase, as well as a higher cell density[26-27]. The minor differences observed between the studies can be attributed to the intrinsic factors of each species evaluated and the volume and cultivation conditions since such elements directly influence the growth of these microorganisms[16].

For the microalgae A. falcatus, the exponential phase of the growth curve was from day zero to seventeen, followed by the stationary phase from day eighteen to twenty. This result is similar to Kalita et al.[28] (2011), where the exponential phase was obtained from day zero to eighteen and the stationary phase from nineteen to twenty-four.

In the growth curve of D. brasiliensis, the exponential phase was from day zero to sixteen, followed by the stationary phase from day seventeen to twenty. This result is similar to Ferreira et al.[29] (2021), where the exponential phase was from day zero to seventeen and the stationary phase from day eighteen to twenty-one.

For K. lunaris, the exponential phase was from day zero to seventeen, followed by the stationary phase from day eighteen to twenty. In the study by Ortega-Salas and Reyes-Bustamante[30] (2012), the growth curve of the microalgae Kirchneriella obese was followed for ten days, and the exponential phase was found from day zero to ten.

In this work, the three freshwater microalgae started their stationary phase between the seventeenth and eighteenth days and remained until the last reading day, the twentieth (Figure 2b). Other studies indicate that secondary metabolites of freshwater microalgae are also concentrated in this period of the stationary phase, as well as a higher cell density[3-31].

Susceptibility tests

The highest performances, with the lowest MIC values, were from extracts of the freshwater microalgae D. brasiliensis (MIC 188 µg mL^{-1}), K. lunaris (MIC 188 µg mL^{-1}) and A. falcatus (MIC 375 µg mL^{-1}). Among marine microalgae, C. neogracilis showed the highest activity (MIC 375 µg mL^{-1}), followed by D. tertiolecta (MIC 750 µg mL^{-1}) and T. gracilis (MIC 1.500 µg mL^{-1}).

T. tonsurans was sensitive to the six microalgae extracts evaluated (Tables 1 and 2), and T. mentagrophytes was not sensitive only to the T. gracilis extract (Tables 1 and 2). The sensitivity of species of the genus Trichophyton to macro and microalgae extracts and their isolated compounds has already been investigated[9-32-33].

Al-Rekabi[9] (2011) reported antifungal activity of the microalgae Oscillatoria irrigua and Scenedesmus quadricauda against different fungal species, including T. rubrum, with MIC of 150 µg mL^{-1} for cyanobacteria and MIC of 250 µg mL^{-1} for chlorophyte.

Guedes et al.[33] (2012) evaluated the antifungal potential of six macroalgae (Hypnea musciformis, Digenea simplex, Padina gymnospora, Dictyota dichotoma, Sargassum vulgare and Ulva lactuca) against dermatophytes, including T. rubrum, T. tonsurans and T. mentagrophytes. All algae were active against at least one of the evaluated species, the seaweed H. musciformis and P. gymnospora apresentaram MIC from 0.031 to 4 µg mL^{-1}; D. simplex MIC between 0.031 and 8 µg mL^{-1}; D. dichotoma MIC from 4 to 16 µg mL^{-1}; S. vulgare MIC from 2.5 to 8 µg mL^{-1} and U. lactuca MIC from 0.5 to 8 µg mL^{-1}.

The chlorotannin, called "dieckol", isolated from the macroalgae Ecklonia cava, presented MIC equal to 200 µg mL^{-1} against T. rubrum. The image obtained by fluorescence microscopy showed that the compound exhibited a fungicidal activity due to the loss of integrity of the cytoplasmic membrane[32].

In the present work, itraconazole was active against T. mentagrophytes (MIC 0.03125 µg mL^{-1}) and T. tonsurans (MIC 0.03125 µg mL^{-1}). Maurya et al.[34] (2019) consider the cutoff point for itraconazole to be 1 µg mL^{-1}. The genus Trichophyton is generally sensitive to itraconazole[35-36].

The susceptibility of 59 dermatophytes, including the species T. mentagrophytes and T. tonsurans, against the drugs itraconazole, terbinafine and clotrimazole was evaluated by Esteban et al.[35] (2005). Itraconazole was active against the evaluated dermatophytes, T. mentagrophytes (MIC 0.68 µg mL^{-1}), and T. tonsurans (MIC 0.289 µg mL^{-1}), considered sensitive[34].

Araújo et al.[36] (2009) also found sensitivity of T. mentagrophytes and T. rubrum to itraconazole (MIC of

0.09 and 0.10 µg mL^{-1}, respectively), being considered sensitive[34].

In this study, D. brasiliensis (MIC 750 µg mL^{-1}), K. lunaris (MIC 1.500 µg mL^{-1}) and C. neogracilis (MIC 6.000 µg mL^{-1}) inhibited N. gypsea. For N. nana, the most active extract was that of K. lunaris (MIC 188 µg mL^{-1}), followed by C. neogracilis (MIC 375 µg mL^{-1}), D. brasiliensis (MIC 750 µg mL^{-1}) and A. falcatus (MIC 6.000 µg mL^{-1}).

Carvalho et al.[37] (2019) reported the activity of the macroalgae Bifurcaria bifurcata against seven dermatophytes, including N. gypsea (MIC 800 µg mL^{-1}). Colloidal silver nanoparticles isolated from the macroalgae Colpomenia sinuosa were shown to be effective against dermatophytes, including N. nana (MIC 30 µg mL^{-1})[38].

In the present study, itraconazole was active against the N. gypsea strain (MIC 0.0625 µg mL^{-1}), an activity similar to that reported by Krakhecke et al.[39] (2005) with MIC <0.03 µg mL^{-1} and by Méndez et al.[40] (2008) with MIC of 0.54 µg mL^{-1}.

The species N. nana showed resistance to itraconazole with MIC >16 µg mL^{-1}. Human isolates had a MIC between 0.25 and 0.78 µg mL^{-1} [41-42]. In the literature, no reports of resistance to azoles were found for the species.

Different factors related to microalgae can synergistically influence the results, such as the solvent used in the extraction, the growth phase at the time of biomass collection, the biomass collection method (centrifugation, electroflocculation or decantation), the place of origin and the cultivation conditions used (luminosity, temperature, pH, aeration, and culture medium)[27-43]. In addition, the influence of seasonality on the synthesis of antimicrobial compounds was also observed, which may be related to the production of single or multiple compounds[44].

CONCLUSION

All microalgae in this study showed in vitro antifungal activity against the evaluated dermatophyte species. The microalgae D. brasiliensis, C. neogracilis and K. lunaris stand out for their inhibition of the four dermatophyte assessed species and their fungicidal activity. Furthermore, the chlorophytes D. brasiliensis and K. lunaris registered the lowest MIC value (188 µg mL^{-1}), revealing the highest antifungal activity among the extracts. The ethanolic extracts of A. falcatus, C. neogracilis, D. brasiliensis and K. lunaris were able to inhibit the species N. nana, resistant to itraconazole. This work was a pioneer in the study of the antifungal activity of these microalgae species. Future studies are expected to identify and isolate the active biomolecules responsible for antifungal activity so that microalgal extracts or biomolecules derived from them can compose new phytotherapics.

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