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In vitro antiproliferative and antioxidant activity of three fungal strains from the White sea

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ABSTRACT

Fungi have been recognized as a prolific source of bioactive compounds with a wide spectrum of bioactivities, nevertheless, the information about marine polar fungi is scarce. Due to this fact, the purpose of this study was to investigate the antiproliferative activity and antioxidant capacity of the extracts of three fungal strains isolated from the White Sea (*Coprinellus diseminatus*, *Simplicillium lamellicola* and *Akanthomyces muscarius*). The antiproliferative activity test was carried out by the SRB method and the cell lines tested were A549, HBL-100, HeLa, SW1573, T-47D and WiDr. The antioxidant capacity was measured employing the ABTS and galvinoxyl methods. The strain that showed the most antiproliferative activity was *A. muscarius*; it demonstrated activity in all the extracts tested, at least against two of the six tested cell lines, also the biomass ethanolic extract and the culture broth ethyl acetate extract of *Coprinellus diseminatus* showed antiproliferative activity against some of the tested cell lines. In the antioxidant capacity test, the ethyl acetate extracts of the culture broth of the three strains showed more activity. This indicates that arctic marine-derived fungi are capable to produce bioactive metabolites with antiproliferative activity and antioxidant capacity, comparable to other marine-derived fungi, and in some cases even more active than known medicinal mushrooms.

1. Introduction

Fungi has been recognized as a prolific source of bioactive compounds with a wide spectrum of bioactivities like antibiotics, hypolipidemic, cytotoxic and immunomodulating, antioxidants, among others. However, an enormous number of that compounds were isolated mainly from terrestrial environments and even nowadays, when the research on marine fungi is constantly increasing, there is still a large number of studies to be done (Carroll et al., 2019).

Regarding marine fungi, most of the reports are mainly focused on environments like mangroves or tropical to temperate areas, but little is known about arctic areas, the marine fungi in those environments and the bioactivities that they could exhibit. This could be explained, among other reasons, due to the expensive costs and difficulty to access such areas of study. Nevertheless, some reports suggest that certain fungal species or strains are specifically adapted to the arctic environments, that is supported by the high frequency of appearance of certain publications (Comeau et al., 2016; Hagestad et al., 2020; Hassett et al., 2019; Pang et al., 2011).

Due to the shortage of information on the bioactivity of arctic marine fungi, the purpose of this study was to investigate the bioactivity of the extracts of three fungal strains isolated from the White Sea. For this reason, the antiproliferative activity and antioxidant capacity of the ethanol and ethyl acetate extracts from the strains identified as *Coprinellus diseminatus*, *Simplicillium lamellicola* and *Akanthomyces muscarius* were tested.

2. Methods

2.1. Collection and identification of fungal material

The sea sediment samples were collected from deep grounds of Kandalaksha Bay, the White Sea on depths of 10, 20 and 30 m during the summer seasons of years 2014–2016 (Khusnullina et al., 2018). For

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isolation, a dilution of the sediments was plated on malt agar prepared on seawater with antibiotics (rifampicin, streptomycin, penicillin, laevomycetin) to suppress bacterial growth (Pivkin et al., 2006). Pure fungi cultures were stored in the tubes on Malt agar (MA) slants at +5 °C. The more than 250 strains were identified by morphologic and cultural features on MA and Czapek agar using taxonomic keys (Bissett, 1983; Crous et al., 2007; Domsh et al., 1993; Gams, 1971; Kepler et al., 2017; Klich, 2002; Kohlmeyer and Kohlmeyer, 1979; Rice and Currah, 2005; Seifert and Gams, 2011; Zare and Gams, 2001, etc) and by sequencing of the ribosomal DNA ITS region. The mycelium was obtained through the cultivation of pure cultures on MA or liquid wort for 7–20 days at +25 °C depending on the fungal growth rate, then separated from the medium, and crumbled with liquid nitrogen in a sterile ceramic mortar. The DNA was isolated with CTAB extraction buffer (0.5 M NaCl, 10 mM Tris-HCl [pH 7.5], 10 mM EDTA, and 2% [w/v] CTAB) using the standard extraction protocol (Rogers and Bendich, 1985). Universal primers ITS1 and ITS4 (TCCGTAGGTGAACCTGCGG/TCCTCCGCTTATTGATATGC) and standard PCR protocol were used for rDNA amplification (White et al., 1990). The PCR diagnostics were performed using HS Taq DNA polymerase produced by Evrogen (Russia). Amplified DNA fragments were separated through the standard electrophoresis in 1.2% agarose gel with EtBr. The amplicon was extracted from the gel using a CleanUp Mini kit produced by Evrogen (Russia). The DNA was sequenced using BigDye®Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, United States) by Evrogen. Newly obtained sequences were compared against nucleotide entries in the National Center for Biotechnology Information GenBank (www.ncbi.nlm.nih.gov) and in MycoBank (www.mycobank.org), using search tool blast to confirm species identity. Pairwise and multiple alignments were conducted using the Clustal W algorithm. A phylogenetic tree was constructed according to maximum likelihood criteria and the Juke-Cantor model using MEGA7 software (Kumar et al., 2016). Bootstrap analysis was performed with 1000 replications.

2.2. Preparation of the extracts

Each studied strain was cultured in Malt broth extract (1 L of medium each) at 25 ± 2 °C in constant agitation. After 14 days the culture was vacuum filtered, the obtained fungal biomass was frozen and lyophilized. When dry, the biomass was first extracted with hexane, then with ethyl acetate, and finally with ethanol. The culture broth was extracted liquid/liquid with ethyl acetate in a separation funnel. The excess of solvent in the extracts was removed by vacuum drying in a rotatory evaporator.

2.3. Antiproliferative activity assay

The cell lines employed in this study correspond to the solid tumor cells A549 (lung), HBL-100 (breast), HeLa (cervix), SW1573 (lung), T-47D (breast) and WiDr (colon). The cells were inoculated according to their doubling time at concentrations of 2500 (A549, HBL-100, SW1573 and HeLa), 5000 (T-47D and WiDr) cells per well in a volume of 100 µL.

For the assay, the dry extracts were dissolved in DMSO at concentration of 400 times the desired final maximum test a concentration (250 µL). The extract exposition was started on the first day after plating, then was incubated for 48 h, and after that, cells were precipitated with 25 µL ice-cold TCA (50% w/v) and fixed for 60 min at 4 °C. The control consists of cells exposed to an equivalent concentration of DMSO but without the extract.

The SRB assay was performed as described by (Skehan et al., 1990) The optical density (OD) of each well was measured at 530 nm using BioTek's Power Wave XS Absorbance Microplate Reader (BioTek, Winooski, VT, USA). The percentage growth was calculated as the OD difference between the start and end of each treatment level corrected for background OD of the control and compared with untreated cells. The results were expressed as the concentration of extract causing 50% reduction in the proliferation of cancer cells (GI_{50}).

2.4. Antioxidant assay

2.4.1. ABTS [2,2′-azino-bis(3-ethylbenzothioazoline-6-sulfonic) acid] assay

The test was carried out according to the method described by (Thaipong et al., 2006)with some modifications. A working solution made of equal amounts of ABTS 7.4 mM and potassium persulfate ($K_2S_2O_8$) 2.6 mM was used. Once the two solutions were mixed, the mixture was allowed to react for 12–16 h to form the $ABTS^{\cdot+}$ radical. The working solution was diluted 1:60 in methanol to be used in the assay. For each sample, 2.85 mL of working solution was mixed with 0.15 mL of fungi extract or Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) standard and left to react for 5 min. After that time, the decrease in absorbance was measured at 734 nm. For the calibration curve a standard Trolox was used for the following concentrations: 6.25, 12.5, 25, 37.5, 50, 75 and 100 µM. The extracts were tested at a concentration of 1 mg/mL. The results were obtained from the linear equation and were expressed as µM Trolox/mg extract.

2.4.2. Galvinoxyl assay

This technique was carried out as described by (Palanisamy et al., 2008) with some modifications. Where 2.7 mL galvinoxyl 10 µM solution (methanolic) was mixed with 0.27 mL of fungi extract or Trolox standard and allowed to react for 20 min at room temperature and darkness. After this time, the decrease in absorbance was measured at 432 nm. The standard curve was made with Trolox concentrations of 5, 10, 15, 20, and 30 µM. The samples were tested at 1 mg/mL, the results were expressed as µM Trolox/mg extract.

3. Results

3.1. Strain identification

In total, 70 species were identified in the White Sea bottom soils. 90% of those were Ascomycota anamorphs (orders: Capnodiales, Eurotiales, Hypocreales, Pleosporales, Saccharomycetales, and Incertae sedis); Mucoromycota (orders: Mucorales and Umbelopsidales) constituted 8%, and the rest (2%) were of Basidiomycota (order: Agaricales) (Khusnullina et al., 2018). From all the isolates obtained, three strains of 3 species were chosen for the analysis of antiproliferative and antioxidant activities. The strains were identified as *Coprinellus disseminatus* (Pers.) J.E. Lange [family Psathyrellaceae, order Agaricales, phylum Basidiomycota], *Simpicillium lamellicola* (F. E. V. Sm.) Zare& W. Gams [family Cordicipitaceae, order Hypocreales, phylum Ascomycota] and *Akanthomyces muscarius* (Petch) Spatafora, Kepler & B. Shrestha [family Cordicipitaceae, order Hypocreales, phylum Ascomycota]. The species' identification was confirmed by the phylogenetic analyses (Fig. 1). These species are known to produce biologically active compounds against other microorganisms as well as to produce a set of extracellular enzymes, but there is a few or no reports on their antiproliferative activity (Table 1).

3.2. Antiproliferative activity

For each of the fungal strains studied, four extracts were obtained, the biomass hexanoic extract (BHE), the biomass ethyl acetate extract (BEA), the biomass ethanolic extract (BEt) and the culture broth ethyl acetate extract (CEA). For this assay, $GI_{50} < 50$ µg/mL were considered as active according to Couttolenc et al., (2016). In this way, the strain that manifested the most antiproliferative activity was *A. muscarius*; it demonstrated activity in all the extracts tested, at least against two of the six tested cell lines, with $GI_{50} < 50$ µg/mL (BEA and Bet extracts against four of the six cell lines, and CEA extract against two of the cell lines). The strain identified as *C. disseminates* demonstrated antiproliferative

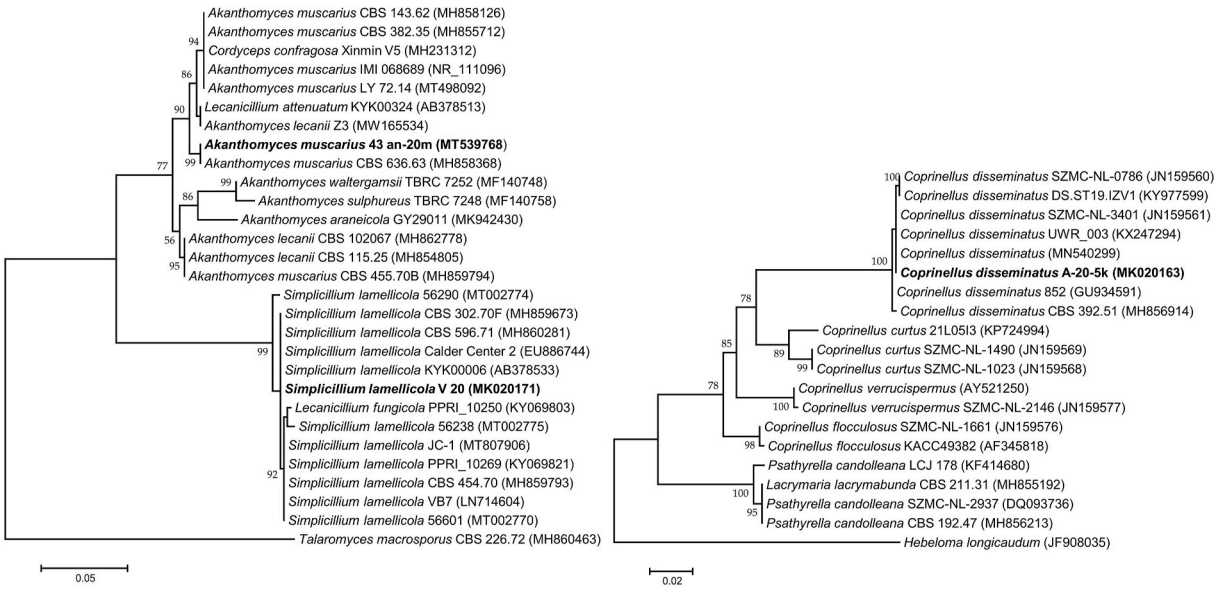

Fig. 1. Molecular Phylogenetic analysis by Maximum Likelihood method. Strains marked in boldface are those that were sequenced in this study. Codes indicate accession numbers from the National center for Biotechnology Information's GenBank. The trees were constructed by using a maximum likelihood method based on the Jukes-Cantor model, with bootstrap analysis (1000 replications). Bootstrap values are shown at the point of branching. The scale bar defines the number of nucleotide substitutions per site. Phylogenetic analyses were conducted in MEGA7.

Table 1
Strain identification and accession number.

Strain	Species	GenBank accession number
1	*Coprinellus disseminatus* (Pers.) J.E. Lange	MK020163
2	*Simplicillium lamellicola* (F.E.V. Sm.) Zare& W. Gams	MK020171
3	*Akanthomyces muscarius* (Petch) Spatafora, Kepler & B. Shrestha	MT539768

activity (GI_{50} < 50 μg/mL) in the BEA extract against all the cell lines tested except for SW1573, in which presented a GI_{50} value of 52 μg/mL that is quite close to the reference value. For the strain identified as *S. lamellicola* none of the assayed extracts show antiproliferative activity. All the results are summarized in Table 2 and Fig. 2. The hexanoic extracts were not tested due to lack of solubility.

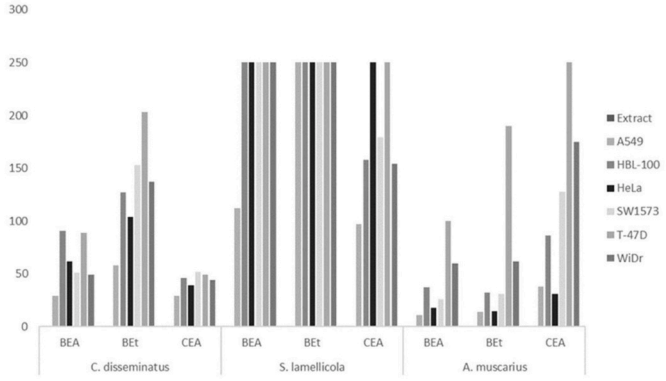

Fig. 2. Antiproliferative activity of the tested extracts expressed as GI_{50}values. All the results under 50 μg/mL are considered as active.

3.3. Antioxidant capacity

In both antioxidant capacity assays, the well-known antioxidant Trolox was used as standard and the results were expressed as μM Trolox equivalents antioxidant capacity (TEAC) for mg of dry fungal extract. For the galvinoxyl radical assay, the calibration curve obtained present a $R^2 = 0.9931$, and for the ABTS assay, the coefficient R^2 was 0.9909.

In the galvinoxyl radical test, the most active extract was *A. muscarius* CEA, followed by *C. disseminatus* CEA with values of 20.306 ± 0.249 and 10.281 ± 0.237 μM TEAC respectively. Furthermore, for the three fungal species, the culture broth extract exhibits a higher TEAC value than any of the biomass extracts. On the other hand, in the ABTS assay, the *C. disseminatus* BEA extract shows the highest TEAC among all the extracts (126.67 ± 7.69 μM), followed by *A. muscarius* CEA and

Table 2
Antiproliferative activity of the assayed extracts. All results are expressed as μg/mL.

Species	Extract	Cell lines					
		A549	HBL-100	HeLa	SW1573	T-47D	WiDr
C. disseminatus	BEA	29	91	62	51	89	49
	BEt	58	127	104	153	203	137
	CEA	29	46	39	52	49	44
S. lamellicola	BEA	112	250	250	250	250	250
	BEt	250	250	250	250	250	250
	CEA	97	158	250	179	250	154
A. muscarius	BEA	11	37	18	26	100	60
	BEt	14	32	15	31	190	62
	CEA	38	86	31	128	250	175

C. disseminatus CEA with TEAC of 119.385 ± 2.19 and 70.902 ± 9.62 respectively (Figs. 3 and 4). All the results have a statistically significant $p < 0.05$ in ANOVA test.

4. Discussion

The research presented here was focused on marine fungi isolated from sediments of the White See with the aim to evaluate its bio-resources' potential. All fungal strains isolated were deposited in the local fungal collection of extremophiles at the Department of Mycology and Algology, Moscow State University. For the initial analysis, we were chosen the species that can be considered as relatively rare for the White Sea, such as *C. diseminatus, S. lamellicola* and *A. muscarius*. Although these species are rather common for terrestrial soil, the research on their possible bioactivity is scarce, not to mention microscopic fungi from the arctic regions. In the first place, the basidiomycete fungus *C. disseminates* was mainly studied by its enzymatic activity, specifically xylanases (Singh et al., 2009). Nevertheless, (Han et al., 1999) proposed that the culture broth of a terrestrial strain of *C. disseminatus* induced apoptosis in human cervical carcinoma cells (HeLa, SiHa and Caski cell lines). Similarly, we observed an antiproliferative effect of the culture broth extract derived from *C. disseminatus* against five of the six tested cell lines including one of the cervical carcinoma (HeLa).

The cordycipitoid species *S. lamellicola* is known to be an endoparasite of other fungi such as *Botrytis cinerea*, a phytopathogenic fungus of economic and scientific relevance. Because of the mentioned above, a biofungicide against the gray mold was developed from this fungus (Shin et al., 2017). Likewise, *S. lamellicola* is reported to produce compounds with antimicrobial activity against phytopathogenic bacteria (Le Dang et al., 2014). In the same way, another species of this genera, a marine derived *S. obclavatum* produce secondary metabolites that manifested both antiviral activity against HSV-1 and antifungal activity against *Aspergillus versicolor* and *Curvularia australiensis* (Liang et al., 2017).

The closest relative species of *A. muscarius* is *Lecanicilluim muscarium.* The strains of *L. muscarium* have the well-studied entomopathogenic activity against some insects considered as pests like *Bemisa tabaci*. In fact, there are some commercial products for whitefly and aphid control that are made from *L. muscarium* (Cuthbertson and Walters, 2005; Lazreg et al., 2009). Also, *L. muscarium* strain isolated from Antarctic waters produces a cold-tolerant chitinolytic enzyme that could be related to the strong mycoparasitic action that the fungus can exert against various other fungi and oomycetes at low temperatures (Fenice, 2016).

Apart from the previously mentioned activity of the culture broth extract of *C. disseminatus*, it is not of the knowledge of the authors that there are previous reports on the antiproliferative activity nor the antioxidant capacity of the studied species, making this work a novel report on the antiproliferative activity of the biomass ethyl acetate and

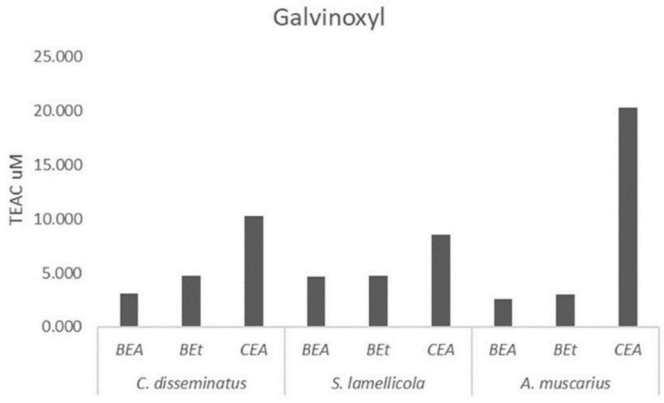

Fig. 3. Antioxidant capacity by galvinoxyl assay.

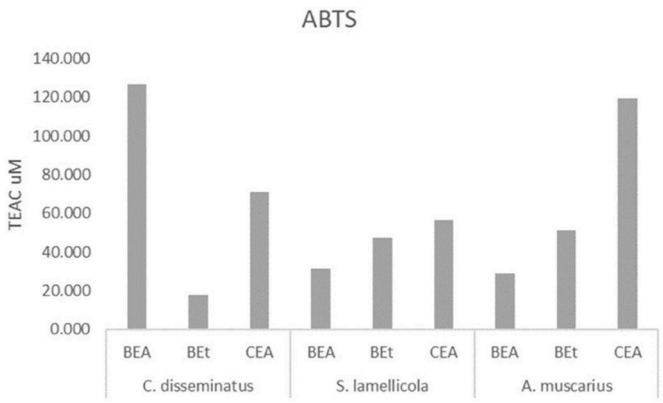

Fig. 4. Antioxidant capacity by ABTS assay.

ethanolic extracts as well as the culture broth ethyl acetate extract of *L. muscarium*. Also, an antiproliferative activity was observed in the culture broth ethyl acetate extract of *C. disseminatus* against practically all the cell lines assayed (SW1573 GI_{50} value of 52 μg/mL was slightly above of the standard value); and, in the biomass ethyl acetate extract of the same fungus, the antiproliferative activity was observed against two of the six cell lines tested (A549 and WiDr cell lines). On the other hand, the isolated strain of *S. lamellicola* did not manifest antiproliferative activity. As for the antioxidant capacity of the extracts, the culture broth extracts of the three fungal species studied demonstrated significant free radicals scavenging activity, as well the antioxidant capacity of the ethyl acetate extract of *C. disseminatus* was relatively high.

The antiproliferative activity of *C. disseminatus* and *A. muscarius* extracts is comparable to the previously observed in fungal strains isolated from a coral reef from Mexico, in which the biomass extract of the isolated strains of *Curvularia*, *Cladisporium* and *Fusarium* presented antiproliferative activity against the cancer cell lines HBL-100, SW1573, HeLa, T-47D and WiDr at a concentration of 250 μg/mL (Couttolenc et al., 2016).

In the same manner, the BEA and BEt extracts of *A. muscarius* showed a higher antiproliferative activity against the cancer cell lines A549, HBL-100, SW1573, HeLa, and WiDr if compared with the extracts from bioactive mangrove fungal strains (three from the genus *Aspergillus* and one *Fusarium*); moreover, *A. muscarius* extracts were active as a compound derived from *Acremonium persicinum* strain isolated from an alkaline lake, which was tested on the same cancer cell lines (Franceschy et al., 2019; Lumbreras Martínez et al., 2018).

On the other hand, the bioactive extracts of *C. disseminatus* and *A. muscarius* manifested much more higher antiproliferative activity against the tested cell lines than the mycelial extracts of a group of basidiomycete fungi known as medicinal mushrooms, such as *Inonotus obliquus, Fomes fomentarius*, *Trichaptum biforme*, and *Coniophora puteana* (Shnyreva et al., 2018).

5. Conclusion

Thus, the arctic marine derived fungi are capable to produce bioactive metabolites with antiproliferative activity and antioxidant capacity, comparable to other marine derived fungi, and in some cases even more active than known medicinal mushrooms.

These results point out the relevance to continue the research of the fungi that are inhabited and reproduced in polar environments with the aim of searching new bioactive compounds that can be useful to medicine, pharmacology and other areas. It would be also of interest to compare bioactivity of the species studied that derived from marine and terrestrial ecosystems.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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