BIOLOGICAL ACTIVITY OF MACROMYCETES ISOLATED FROM CHILEAN SUBANTARCTIC ECOSYSTEMS

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ABSTRACT

Twenty six compounds were identified by GC-MS analysis from culture broth of six Macromycetes growing in subantarctic forests in southern Chile: *Mycena hialinotricha*, *Collybia butyracea*, *Inocybe geophylla*, *Entoloma nubigenum*, *Stropharia semiglobata* and *Psathyrella* sp. Antifungal and antibacterial activity were evaluated through agar diffusion test. This assay showed bioactivity against *Penicillium notatum*, *Fusarium oxysporum*, *Rhyzoctonia solani*, *Ceratocistys pilifera*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Escherichia faecalis* and *Staphilococcus aureus*.

Keywords: Fungi, subantarctic ecosystems, antibacterial, and antifungal activity, secondary metabolites.

INTRODUCTION

Microorganisms are recognized as prolific producers of bioactive natural products, many of them useful as clinical drugs[1]. In recent years, a large percentage of those have been isolated and identified from Basidiomycetes and the enormous potential of this group of organisms is now widely accepted in the scientific and industrial community[2]. Interesting compounds of different biogenetic origins have been isolated from mycelial fermentation with antibacterial, antifungal, cytostatic, antiviral, and other activity[3,4,5].

Currently, efforts to discover new bioactive compounds have been focused on fungi inhabiting Chilean subantarctic ecosystems, considering that this territory is not enough studied in terms of biological and chemical diversity of Macromycetes. In fact, the land stretching from 42º S to the southernmost end of the continent is the least studied in terms of biodiversity of fungi. Therefore, it is important to extend the study of our mycological heritage and evaluate their potential biological activity[6].

This study has investigated the bioactive extracts and its chemical constituents obtained from fermentation of six strains of saprophytes fungi: *Mycena hialinotricha*, *Collybia butyracea*, *Inocybe geophylla*, *Entoloma nubigenum*, *Stropharia semiglobata,* and *Psathyrella* sp. (Agaricales, Basidimycota), isolated from forests near to Concepción and Punta Arenas. It's reported the presence of nitrogenous compounds, 3-(2-methylpropyl)-2,3,6,7,8,8a-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione, 1H-Indole-3 methyl, 1H-indole-3-carboxylic acid methyl ester, 3-benzyl-6-isopropyl-piperazine-2,5-dione, 1H-indole-6-methyl, phenolic compounds, and terpenes, like β-selinene, and Valencene related to the activity of these compounds against to pathogenic bacteria and fungi.

EXPERIMENTAL

The samples of *Collybia butyracea* (TUDEC050), *Mycena hialinotricha* (TUDEC212), *Entoloma nubigenum* (TUDEC105), *Inocybe geophylla* (TUDEC101), and *Psathyrella sp.* (TUDEC030) were obtained from forests near to Concepción (36º 49' 52"S – 72º 54' 40"W), VIII Región, and sample of *Stropharia semiglobata* (RM047) was obtained at Punta Arenas (56º 06' 20''S – 71º 01' 50''W), XII Región, Chile. All mycelia were isolated from spore prints of fruiting bodies of fungi, which were then grown in a YMG medium (yeast extract 4 g, malt extract 10 g, glucose 4 g and 20 g agar L^{-1} distilled water) augmented with 200 mg L^{-1} of streptomycin sulphate[7]. Cultures were incubated to 22º C. The strains were deposited at the cepario de la Universidad de Concepción and were maintained at 4º C, subculturing every 12 months.

Small sections of the each stock culture were transferred to a 5 L Erlenmeyer flask containing 3 L of YMG medium (pH 5.8). This flask was incubated at 22° C on a rotary shaker (120 rpm). After 20 days of fermentation, the mixture was filtered and culture broth was extracted exhaustively with EtOAc (500 mL)[8]. The organic phase was dried on anhydrous Na_2SO_4, filtered, and concentrated with a rotary evaporator (120 rpm, 40º C)[9], yielding a crude extract (*S. semiglobata* (973 mg), *Psathyrella* sp. (408 mg), *I. geophylla* (891 mg), *C. butyracea* (550 mg), *M. hialinotricha* (993 mg), *E. nubigenum* (350 mg)[10].

There extracts were analyzed by GC-MS on a Hewlett Packard gas chromatograph 5890A, coupled to Hewlett Packard 5975 mass spectrometer system equipped with a 30 m long, 0.25 mm id, 0.25 µm film thickness HP5-MS capillary column. The temperature was programmed from 100° C to 275° C at rate of 10° C min^{-1} with a 10 min hold. Helium was used as a carrier gas with a constant flow at 1.4 mL min^{-1}. The ionization voltage was 70 eV. The compounds identification was performed by comparison the obtained spectra with those reported in the literature and mass spectra with authentic samples. When such samples were not available tentative structures were proposed on the basis of the mass spectral fragmentation with comparison spectral library NIST 2005 (NIST /EPA/NIH MASS 2005 spectral Library)[11]. A volume of 1 µL of the sample at the concentration of 10 mg/mL was injected. Spectra were considered coincident if the similarity index was higher than 95%. In addition, the spectra were compared with Kovats retention index. The chemical characterization of obtained extracts was performed with these compounds.

Antibacterial activity of the crude extracts was qualitatively evaluated by diffusion test in agar. Plates with Mueller-Hinton agar were inoculated with a concentration 10^8 CFU/mL of *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Escherichia faecalis,* and *Staphilococcus aureus.* Paper disks Whatmann Nº 1 (6 mm) were impregnated with 100 µg/disc of the fraction obtained. The kanamycin antibiotic was used as control. Cultures were incubated at 37º C by 24 h[12].

The phytopathogens *Penicillium notatum*, *Fusarium oxysporum*, *Rhyzoctonia solani*, *Pythium debaryanum,* and *Ceratocistys pilifera* were used as target species for the antifungal assay. Plates with YMG agar were inoculated with a concentration 10^6 spores/mL of these microorganisms. Paper disks Whatmann Nº 1 (6 mm) were impregnated with 100 µg/disc of the fraction obtained, benomyl was used as control. Petri dishes were incubated at 22º C by 21 days[13]. Antifungal activity as well as antibacterial activity was determined by displaying an inhibitory halo around disk. All assays were performed in triplicate.

RESULTS AND DISCUSSION

Six culture broth extracts from Chilean Macromycetes isolated from subantarctic forests were assessed for antibacterial and antifungal activities. The extracts exhibited considerable activity, demonstrating the existence of interesting bioactive compounds (see Table 1). Antibacterial assay showed activity against *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Escherichia faecalis* and *Staphilococcus aureus*. Extracts of *Psathyrella* sp.,

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I. geophylla, *M. hialinotricha* and *E. nubigenum* exhibits higher antibacterial activities towards *B. subtilis*, however, extracts of *S. semiglobata* and *C. butyracea* showed a moderate activity. *S. aureus* showed a moderate susceptibility to the antibacterial activity of the extracts of *S. semiglobata*, *I. geophylla*, *C. butyracea*, *M. Hialinotricha,* and *E. nubigenum*. Whereas, *I. geophylla* and *S. semiglobata* extracts exhibits low and diffuse antibacterial effect against *E. coli* and *P. aeruginosa*. *E. faecalis* was susceptible to the obtained extracts of *Psathyrella* sp. and *E. nubigenum*.

Table 1. Antimicrobial activity of Chilean macromycetes in agar diffusion test.

Organisms	Diameter of inhibition zone (mm)*							
	TUDEC030	TUDEC050	TUDEC101	TUDEC105	TUDEC212	RM047	kanamycin	benomyl
Bacillus subtilis	19	8	14	17	15	8	14	x
Pseudomonas aeruginosa	-	7di	7di	-	-	7di	-	x
Escherichia coli	-	-	7	-	-	9	12	x
Escherichia faecalis	16	-	-	9	-	9	10	x
Staphilococcus aureus	-	7	7	9	8	8	11	x
Penicillium notatum	9	-	12	11	17	7	x	15
Fusarium oxysporum	9	-	6	-	-	-	x	19
Rhyzoctonia solani	8	8	6	-	9	7	x	17
Pythium debaryanum	-	-	-	-	-	-	x	15
Ceratocistys pilifera	15	13	-	7	13	8	x	18

* diameter disk = 6 mm, concentration = 100 µg/disc; - = No inhibition zone; di = diffuse inhibition zone; x = No tested.

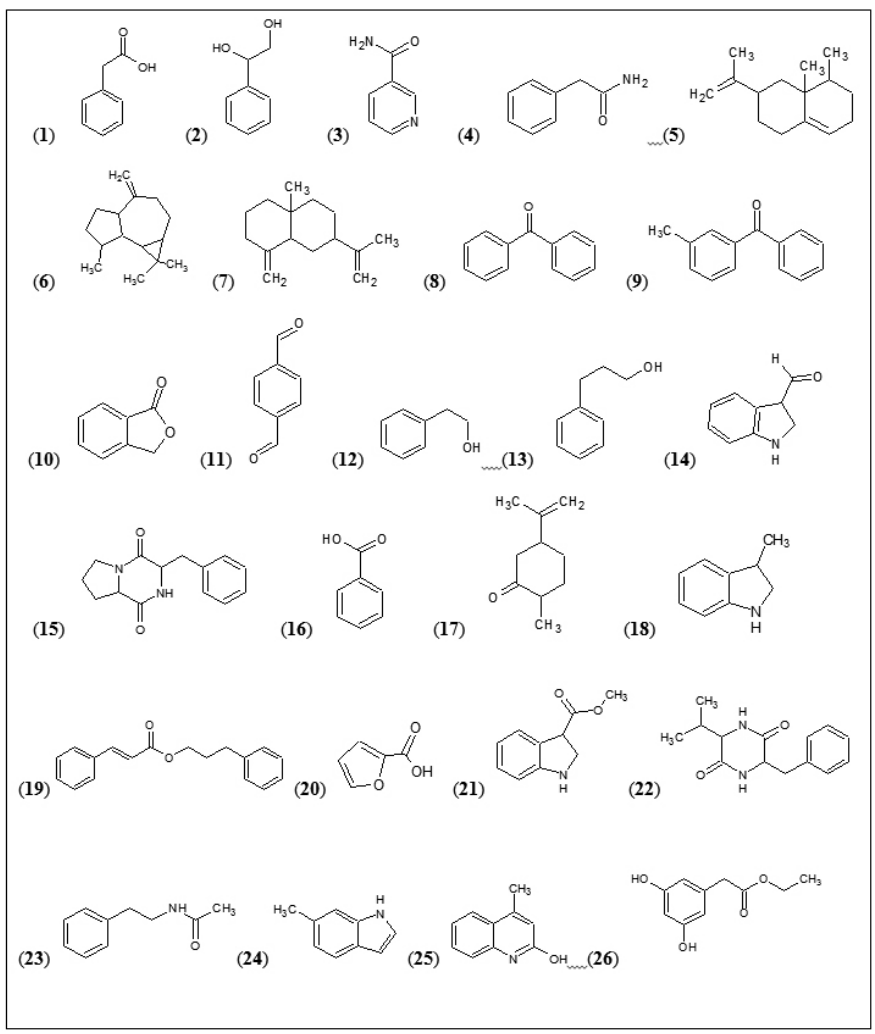

Fig. 1. Compounds structures obtained by comparison spectral library NIST 2005.

Table 2. GC-MS data of the compounds in extracts from liquid cultures.

Compounds	M^+ and characteristic ions (%)	RT (min.s)	Estimated Kovats RI
***Psathyrella* sp.**			
Phenylacetic acid (**1**)	136(30), 118(5), 92(20), 91(100), 65(20), 51(7)	8.20	1262
(1S)-1-Phenyl-1,2-ethanediol (**2**)	138(8), 107(100), 79(73), 77(48), 51(77)	9.21	1298
pyridine-3-carboxamide (**3**)	122(100), 106(64), 78(87), 51(50)	10.30	1426
2-phenylacetamide (**4**)	135(17), 92(84), 91(100), 65(31), 51(12)	10.45	1302
Valencene (**5**)	204(20), 189(18), 162(100), 107(54), 93(34), 81(33), 51(27)	13.36	1491
Collybia butyracea			
Aromadendrene (**6**)	204(18), 161(30), 147(25), 133(30), 119(32), 150(50), 91(100), 79(60), 67(40), 55(35)	11.17	1474
β-selinene (**7**)	204(60), 189(50), 161(55), 147(25), 133(55), 121(65), 105(100), 98(10), 81(95), 67(70), 55(63)	12.61	1530
Diphenylmethanone (**8**)	182(55), 152(5), 126(3), 105(100), 77(60), 51(26)	13.30	1603
(3-methylphenyl)-phenylmethanone (**9**)	196(60), 181(9), 165(3), 119(100), 105(27), 91(30), 77(21), 51(10)	14.50	1716
3H-2-benzofuran-1-one (**10**)	134(100), 105(90), 77(58), 63(7), 51(23), 50(20)	15.37	1207
p-benzenedicarboxaldehyde (**11**)	134(100), 133(89), 105(60), 77(50), 51(92)	16.71	1284
Inocybe geophylla			
2-phenylethanol (**12**)	122(23), 103(5), 91(100), 77(56), 65(23), 51(9)	5.59	1121
3-phenyl-1-propanol (**13**)	136(20), 117(80), 91(100), 79(16), 77(25), 65(25)	7.83	1219
1H-Indole-3-carboxaldehyde (**14**)	145(100), 144(67), 117(20), 116(65), 90(13), 63(12), 62(6)	14.99	1476
3-(phenylmethyl)-2,3,6,7,8,8a-hexahydropyrrolo[1,2-a]pirazine-1,4-dione (**15**)	244(45), 200(9), 153(37), 125(100), 120(12), 91(50)	19.60	2138
Entoloma nubigenum			
Benzoic acid (**16**)	105(100), 122(83), 94(15), 77(66), 51(33)	6.68	1180
2-methyl-5-(prop-1-en-2-yl)-cyclohexanone (**17**)	152(12), 138(6), 122(100), 106(60), 78(90), 55(80), 51(75)	10.40	1600
3-methyl-1H-indole (**18**)	131(100), 103(10), 95(40), 77(20), 51(10)	10.74	1264
3-phenylprop-2-enyl 3-phenylprop-2-enoate (**19**)	266(45), 262(5), 131(100), 117(20), 101(30), 81(20), 65(35), 55(30)	21.85	1583
Mycena hialinotricha			
Furan-2-carboxylic acid (**20**)	112(100), 95(59), 83(4), 66(7)	4.30	999
2-phenylethanol (**12**)	122(23), 103(5), 91(100), 77(56), 65(23), 51(9)	5.60	1136
3-phenyl-1-propanol (**13**)	136(21), 117(88), 91(100), 79(16), 77(26), 65(25)	7.90	1235
Phenylacetic acid (**1**)	136(30), 118(5), 92(20), 91(100), 65(20), 51(7)	8.17	1262
1H-indole-3-carboxylic acid methyl ester (**21**)	175(53), 167(7), 144(100), 116(18), 89(16), 63(7)	17.57	1531
3-benzyl-6-isopropyl-piperazine-2,5-dione (**22**)	246(45), 208(20), 127(50), 91(100), 70(60), 55(90)	18.66	1976
3-(phenylmethyl)-2,3,6,7,8,8a-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione (**15**)	244(45), 200(9), 153(37), 125(100), 120(12), 91(50)	19.60	2138
Stropharia semiglobata			
N-(2-phenylethyl)acetamide (**23**)	163(12), 104(80), 103(5), 91(15), 72(10), 65(4)	9.20	1492
1H-Indole-6 methyl (**24**)	131(100), 130(90), 115(5), 103(6), 91(6), 65(15), 77(10)	9.63	1288
3-methyl-2(1H)-Quinolinone (**25**)	159((75), 131(25), 130(100), 103(10), 77(13), 51(10)	11.01	1401
Ethyl(3,5-dihydroxyphenyl)acetate (**26**)	196(51), 124(37), 123(100), 69(85), 67(10), 57(10), 55(11)	16.21	1700

The agar diffusion test showed that the extract of native fungi were active against phytopathogenic fungi *Penicillium notatum*, *Fusarium oxysporum*, *Rhyzoctonia solani,* and *Ceratocistys pilifera* producing inhibition halos of different intensities (see Table 1). *F. oxysporum* showed a diffuse susceptibility to the antifungal activity of the extracts of *Psathyrella* sp. and *I. geophylla*. *S. semiglobata* showed moderate activity against *P. notatum*, *R. solani* and *C. pilifera*. The obtained extracts from liquid cultures of *M. hialinotricha* exhibit higher antifungal activities towards *P. notatum*, *R. solani* and *C. pilifera*. It is interesting to note that the extract produced by *M. hialinotricha* strain TUDEC212 was active against pathogenic fungi, this effect could be result of presence the active substances, probably ecologically significant in nature, where this specie produces antifungal compounds that gives competitive advantages against potential competitors[8].

Psathyrella sp. showed low effect towards *P. notatum*, *F. oxysporum*, *R. solani* and *C. pilifera*. Finally, it was observed that *E. nubigenum* extract showed antifungal activity against *P. notatum* and *C. pilifera*; these fungi causing the "blue stain" of wood and actually their control is not possible.

The findings of our results showed that the extracts of *I. geophylla* (TUDEC101) and *Psathyrella* sp. (TUDEC030) had significant antifungal activity against a *F. oxysporum* pathogenic strain; this fungus is causing lesions at basal level of the stem similar to cortical cankers and disease known as damping-off[14]. This pathogen causes considerable economic losses in agroforestry industry, for this reason, our results suggest the use of compounds biosynthesized by *Inocybe* and *Psathyrella* as natural products for their control.

When the extracts were subjected to GC-MS analysis to find out the components produced by the fungus, it was observed peaks in agree to fungal bioactive compounds. Interpretation of mass spectrum GC-MS was conducted using the database of National Institute Standard and Technique (NIST). The spectrum of the component was compared with the data stored in the NIST library. The name, molecular weight, ionic fragmentation patterns, structure of the compounds was ascertained (see Figure 1, Table 2).

Six compounds were identified in extract of *Collybia butyracea* (TUDEC050) by GC-MS analysis. This extract containing β-selinene (**7**), diphenylmethanone (**8**), (3-methylphenyl)-phenylmethanone (**9**), 3H-2-benzofuran-1-one (**10**), p-benzenedicarboxaldehyde (**11**) showed activity against *S. aureus*, *B. subtilis*, *P. aeruginosa*, *C. pilifera* and *R. solani*. β-selinene (**7**) was isolated from *Plectranthus amboinicus* and antitumor, antibacterial, anti inflammatory and fungicide activities were reported[15].

Seven compounds were identified in liquid culture extract of *Mycena hialinotricha* (TUDEC212), furan-2-carboxylic acid (**20**), 2-phenylethanol (**12**), 3-phenyl-1-propanol (**13**), phenylacetic acid (**1**), 1H-indole-3-carboxylic acid methyl ester (**21**), 3-benzyl-6-isopropyl-piperazine-2,5-dione (**22**), 3-(phenylmethyl)-2,3,6,7,8,8a-hexahydropyrrolo[1,2-a]pirazine-1,4-dione (**15**). The bioactivity of these compounds is consistent with previous studies. *Mycena* species uses the shikimic acid pathway thereby antifungal compounds strobilurins[8]; the compounds identified by GC-MS analysis have similar structure to fungicide strobilurin A isolated from *Strobilurus tenacellus*, *Oudesmansiella mucida,* and *Mycena* sp.[16], suggesting that were synthesized by the same pathway.

In extract of *Stropharia semiglobata* (RM47) were determined 4 compounds: acetamida, N-(2-phenylethyl) acetamide (**23**), 1H-Indole-6 methyl (**24**), 3-methyl-2(1H)-quinolinone (**25**), and ethyl (3,5-dihydroxyphenyl)acetate (**26**). Four compounds were detected in *Inocybe geophylla* (TUDEC101), 2-phenylethanol (**12**), 3-phenyl-1-propanol (**13**), 1H-Indole-3-carboxaldehyde (**14**) and 3-(phenylmethyl)-2,3,6,7,8,8a-hexahydropyrrolo[1,2-a]pirazine-1,4-dione (**15**). These molecules were isolated previously from fungi *Aporpium caryae*[17], *Agrocybe cylindracea*[18], and *Lactarius subplinthogalus*[19], antifungal and bactericidal activities were reported.

Chromatographic analysis determined 5 compounds in *Psathyrella* sp. (TUDEC030), Phenylacetic acid (**1**), (1S)-1-Phenyl-1,2-ethanediol (**2**), pyridine-3-carboxamide (**3**), 2-phenylacetamide (**4**) and Valencene (**5**). Whereas, in extract of *Entoloma nubigenum* (TUDEC105) were detected four compounds, benzoic acid (**16**), 2-methyl-5-(prop-1-en-2-yl)-cyclohexanone (**17**), 3-methyl-1H-indole (**18**) and 3-phenylprop-2-enyl 3-phenylprop-2-enoate (**19**), and antimicrobial was reported in this studies (see Table 1).

CONCLUSIONS

The obtained results in antimicrobial assays crude extracts of macromycetes isolated from subantarctic forests indicate their potential generation of bioactive metabolites. These fungi are therefore good sources for exploring the possibility of new antimicrobial drugs discovery. It is suggested the need to further investigation the potential application of the bioactive secondary metabolites in agroforestry industry as control of phytopathogenic agents.

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