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Full Length Research Paper

In vitro antifungal activity of *Calocybe gambosa* extracts against yeasts and filamentous fungi

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Three strains of *Calocybe gambosa* (Fr.) Donk were investigated as possible sources of biologically active substances with antifungal activity. After submerged cultivation of *C. gambosa* mycelium, biologically active materials were isolated as ethyl acetate extracts from culture liquids. The *in vitro* activity of these *C. gambosa* extracts (Cg1, Cg2, Cg3) against yeasts and filamentous fungi was evaluated using the Clinical and Laboratory Standards Institute (CLSI) M27-A2 and M38-A2. The minimum inhibitory concentration (MIC) against most of the tested clinical fungal strains for Cg1 and Cg2 extracts ranges from 12.5 to 50 µg/ml while Cg3 ranged from 1.56 to 12.5 µg/ml. *Candida albicans* (DBVPG 4268) were the most sensitive fungal strain to *C. gambosa* extracts, with MIC ranges of 1.56 to 12.5 µg/mL, while the strains of *Aspergillus tubingensis* showed the least sensitivity to the extracts. The high performance liquid chromatography (HPLC) fingerprint of the isolates shows four principal compounds produced by the cultured mycelium. Considering the potential use of natural antifungal compounds in medicine, we are currently working on a small-scale extraction, isolation and structural characterization of compounds produced from the *C. gambosa*.

Key words: Anti-fungal, fungal metabolites, minimal inhibitory concentration (MIC), high performance liquid chromatography (HPLC), thin layer chromatography (TLC).

INTRODUCTION

During the last two decades, there has been a dramatic increase in the incidence of life-threatening systemic fungal infections. The challenge has been to develop effective strategies for treating fungal diseases, to treat opportunistic fungal infections in human immunodeficiency virus-positive patients and others who are immunocompromised due to cancer chemotherapy or the indiscriminate use of antibiotics (Pfaller and Diekema, 2004; Singh, 2001). Most clinically-used antifungal drugs have various drawbacks in terms of toxicity, efficacy and cost, and their frequent use has led to the emergence of resistant strains (Fridkin, 2005). Hence, there is a great need for novel antifungals that belong to a wide range of structural classes, that can selectively act on new targets with fewer side effects.

Macromycetes used in traditional medicine usually constitute an important source of new biologically active products (Liu, 2005). *Calocybe gambosa* (Fr.) Donk (Lyophyllaceae, Agaricales, Basidiomycota), commonly known as St. Georges mushroom, is widespread and found in temperate regions throughout much of Europe including parts of Russia, and Asia, including Korea and Japan. It is quite common in various grassy habitats, on roadsides and wood edges or in pastureland (Hall et al., 2010). *C. gambosa* has been reported to have important medicinal properties including antioxidant activity (Palacios et al., 2011), antibacterial activity towards *Bacillus subtilis* and *Escherichia coli* (Keller et al., 2002) and an ability to reduce blood sugar levels (Brachvogel, 1986). Earlier studies on the secondary metabolites produced by *C. gambosa*, demonstrated that in culture, this fungal species synthesized an orange crystalline metabolite which was shown to be the phenoxazine

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Figure 1. Basidiocarps of *C. gambosa* (Fr.) Donk (Giancarlo Bistocchi photographer).

Table 1. Mycelial dry weights and yields of ethyl acetate extracts (EtOAc) from six-weeks-old *C. gambosa* culture liquid.

C. gambosa strains	Mycelial dry weight* (mg)	EtOAc extract* (mg)
1	47.2±0.6	1.1±0.3
2	31.8±0.2	0.7±0.3
3	56.6±0.8	1.6±0.1

*The values are the means of three repetitions ± standard error.

derivatives (phenoxazone and a-amino-phenoxazone) (Clemencon, 1987; Moncalvo et al., 1991; Schlunegger et al., 1976). These derivatives are used extensively in medicine, biology and chemistry (Eregowda et al., 2000; Kalpana et al., 1999; Shimizu et al., 2004; Sridhar et al., 1999).

Considering the potential use of natural antifungal compounds in medicine, the aim of the present work was to evaluate the antifungal potential of ethyl acetate extract of *C. gambosa* cultural liquid on several fungal strains of medical importance.

MATERIALS AND METHODS

Mushroom

Three fruiting bodies of *C. gambosa* were collected in nature during field trips between 2009 and 2010, in the regions of Umbria and Abruzzo (Italy). The specimens were identified according to their macroscopic and microscopic features and the related literature (Watling, 1979; Moser, 1978) (Figure 1). Voucher cultures (Cg1, Cg2 and Cg3) are maintained in the DBA (Department of Applied Biology) culture collection (University of Perugia, Italy) and are accessible.

Pieces of the fruiting bodies were isolated and placed on solid medium in test tubes. After being cultivated for 3 weeks at room temperature, the mycelium was removed from the agar tubes and transferred into a liquid medium (Angelini et al., 2008). The culture was then incubated for 6 weeks at 25°C. The evaluation of growth by dry weights determination of fungal mycelia from liquid culture was examined (Lilly and Barnett, 1951).

Extraction and isolation

After submerged cultivation, the culture liquids were separated from the mycelia by filtration and extracted with ethyl acetate using a separating funnel. The ethyl acetate fractions were evaporated to dryness in vacuo.

The dried extracts were analysed with a high-performance liquid chromatography (HPLC) Jasco 880-PU using a C18 column (250 x 4 mm) eluent water/methanol in a gradient from 60 to 0% water in 30 min. The chromatographic profiles were recorded at 254 nm. The chromatographic analysis of the culture media extract of before inoculation did not show any peaks. The dried extracts were also subjected to thin layer chromatography (TLC) analysis (chloroform/toluene 1:1) on silicagel G.

Antifungal assay

Thirty-three clinical strains, five of yeasts (obtained from Industrial Yeast Collection DBVPG) and twenty-eight filamentous fungi (moulds), were used in this study (Table 2) while two ATCC (American Type Culture Collection) referente strains [Candida parapsilosis (ATCC 22019) and *C. albicans* (ATCC 90028)] served as controls as recommended by Clinical and Laboratory Standards Institute, CLSI (formerly the National Committee for Clinical Laboratory Standards). The filamentous fungi (provided by the Clinical Microbiology and Parasitology Section, R. Silvestrini Hospital, Perugia, Italy), were identified by morphological and molecular analyses (Pagiotti et al., 2011). Stock cultures were maintained on Sabouraud Dextrose Agar (SDA; Oxoid, Milan, Italy) slants at 4°C.

Antifungal agents

The dried extracts of *C. gambosa* strains (Cg 1, Cg 2 and Cg 3) were dissolved in 10% methanol and diluted with sterile double-distilled water. Amphotericin B (Sigma-Aldrich, Milan, Italy) were used as reference drug.

Antifungal susceptibility tests

Evaluation of the susceptibility of yeasts and filamentous fungi was performed using the broth microdilution method (BMM) according to CLSI M38-A2 (for filamentous fungi) and M27-A2 (for yeast) guidelines (NCCLS, 2002; CLSI, 2008). Yeast strains were grown aerobically overnight at 35°C on SDA plates. Yeasts were harvested and suspended in 1% sterile saline and the turbidity of the supernatants measured spectrophotometrically at 625 nm with an absorbance of 0.08-0.1 equivalent to the No. 0.5 McFarland standard following the NCCLS M27-A2 guideline (NCCLS, 2002). The working suspension was diluted 1:20 in a mixture containing RPMI 1640 medium (with L-glutamine, without bicarbonate; Cambrex Bio Science, Verviers, Belgium) and 0.165 M morpholinepropanesulfonic acid (MOPS; Sigma-Aldrich, Milan, Italy) buffered to pH 7.0. The working suspension was further diluted with the medium (1:50) to obtain the final test inoculum (1-5x103 CFU ⁄ ml). The working inoculum suspension (100 µl) were added to each well of 96-well flat-bottom microdilution plates containing 100 µl of drug dilution incubated in an aerobic environment at 35°C for 24 h. Growth was observed visually with the aid of a concave mirror; MICs (Minimal Inhibitory

Table 2. Minimal inhibitory concentrations (MICs) of *C. gambosa* extracts against yeasts and filamentous fungi.

Yeasts and filamentous fungi	MIC (µg/mL)			
	Cg1*	Cg2*	Cg3*	Amphotericin B
Candida albicans (DBVPG 6133)	25	12.5	3.12	> 8.0
Candida albicans (DBVPG 4268)	12.5	12.5	1.56	4.0-8.0
Candida albicans (DBVPG 3908)	12.5	12.5	3.12	2.0-8.0
Filobasidiella neoformans var. neoformans (DBVPG 6010)	25	25	6.25	>8.0
Filobasidiella neoformans var. bacillospora (DBVPG 6225)	25	25	6.25	>8.0
Penicillium chrysogenum (3)**	50-100	25-50	12.5-50	>8.0
Verticillium sp. (3)**	25-50	25-50	6.25-12.5	>8.0
Aspergillus tubingensis (6)**	100-200	100-200	25-50	>8.0
Aspergillus minutus (4)**	12.5-50	12.5-50	6.25-12.5	4.0-8.0
Beauveria bassiana (5)**	25-50	25-50	12.5-50	>8.0
Microsporum gypseum (7)**	50-100	25-50	12.5-50	>8.0

*ethyl acetate extracts from culture liquids of *C. gambosa* strains. **Values in brackets indicate number of strains that were screened.

Concentrations) were taken on a growth or no-growth (100% visible-growth inhibition) scale.

The activity of the ethyl acetate extracts of *C. gambosa* strains against filamentous fungi was also determined using the BMM, according to the CLSI M38-A2 guideline (CLSI, 2008). Cultures were grown on Potato Dextrose Agar (PDA; Oxoid, Milan, Italy) at 35°C until sporulation (48 h to 7 days). Spores were harvested and suspended in 1% sterile saline, allowed to settle and the upper layer aspirated. The turbidity was measured spectrophotometrically at 625 nm, and optical density was adjusted to yield a stock suspension of 0.4-5x106 sporangiospores per millilitre. A working suspension was prepared by diluting 1:50 of the conidia stock suspension in a standard medium (RPMI 1640, MOPS). The fungal inocula (100 µl) were added to each well of 96-well flat-bottom microdilution plates containing 100 µl of drug dilution and incubated for 48 h in an aerobic incubator at 35°C. After incubation, potential antimicrobial activity (MICs) was assessed as described previously.

RESULTS

Analysis of *C. gambosa* extract by high-performance liquid chromatography (HPLC) and thin layer chromatography (TLC)

The mycelial dry weights and the yields of ethyl acetate extracts from 6 week old *C. gambosa* culture liquids are shown in Table 1.

The biosynthetic activity of the dried extract obtained from *C. gambosa* culture medium involves at least twenty chemical compounds that absorb at 254 nm (Figure 2). The TLC analysis showed that the coloured fraction of the culture media is due to four components.

Antifungal activity

The MIC values of ethyl acetate extracts from C. gambosa relative to the yeasts and filamentous fungi tested are reported in Table 2. *C. albicans* (DBVPG 4268) were the most sensitive fungal strain to *C. gambosa* extracts, with MIC ranges of 1.56 to 12.5 µg/ml, while the strains of *A. tubingensis* showed the least sensitivity to the extracts.

The Cg1 and Cg2 extracts displayed MIC in the concentration range of 15.5 to 50 µg/ml against some of the fungal strains while Cg1 extract was found to possess a MIC in a concentration range of 1.56 to 12.5 µg/ml against most of the tested fungal strains. The Cg3 extract showed MIC in a range of 1.56 to 6.25 µg/ml against all *Candida albicans* and *Filobasidiella neoformans* strains. Of the three extracts employed in this study, Cg3 was the most potent against majority of fungal strains.

Amphotericin B showed a low activity *in vitro* against the fungal species tested. *C. albicans* (DBVPG 4268 and 3908) and *Aspergillus minutus* had an amphotericin MIC range of 2.0 to 8.0 µg/mL, whereas the other fungal species exhibited amphotericin MIC values >8 µg/ml.

The MIC values of amphotericin B, for the strains Candida parapsilosis (ATCC 22019) and *C. albicans* (ATCC 90028) were within the established ranges (NCCLS, 2002; CLSI, 2008).

DISCUSSION

Five clinical strains of yeast and twenty-eight clinical strains of filamentous fungi were employed in evaluating the antifungal potency of the ethyl acetate extracts from C. gambosa strains (Cg1, Cg2 and Cg3). Most of the filamentous fungi strains tested in this study were resistant to amphotericin B, according to the recently proposed *in vitro* breakpoints; susceptible: MIC ≤1 µg/ml, intermediate, MIC = 2 µg/ml, and resistant, MIC ≥4 µg/ml (CLSI, 2008). Breakpoints with proven clinical relevance are not available for yeasts vs. amphotericin B due to methodology problems (Espinel-Ingroff, 2008).

The antifungal activity of the *C. gambosa* extracts (Cg1, Cg2 and Cg3) against fungal species varied markedly, probably due to differences in mycelial growth and metabolite yield but this activity was more effective

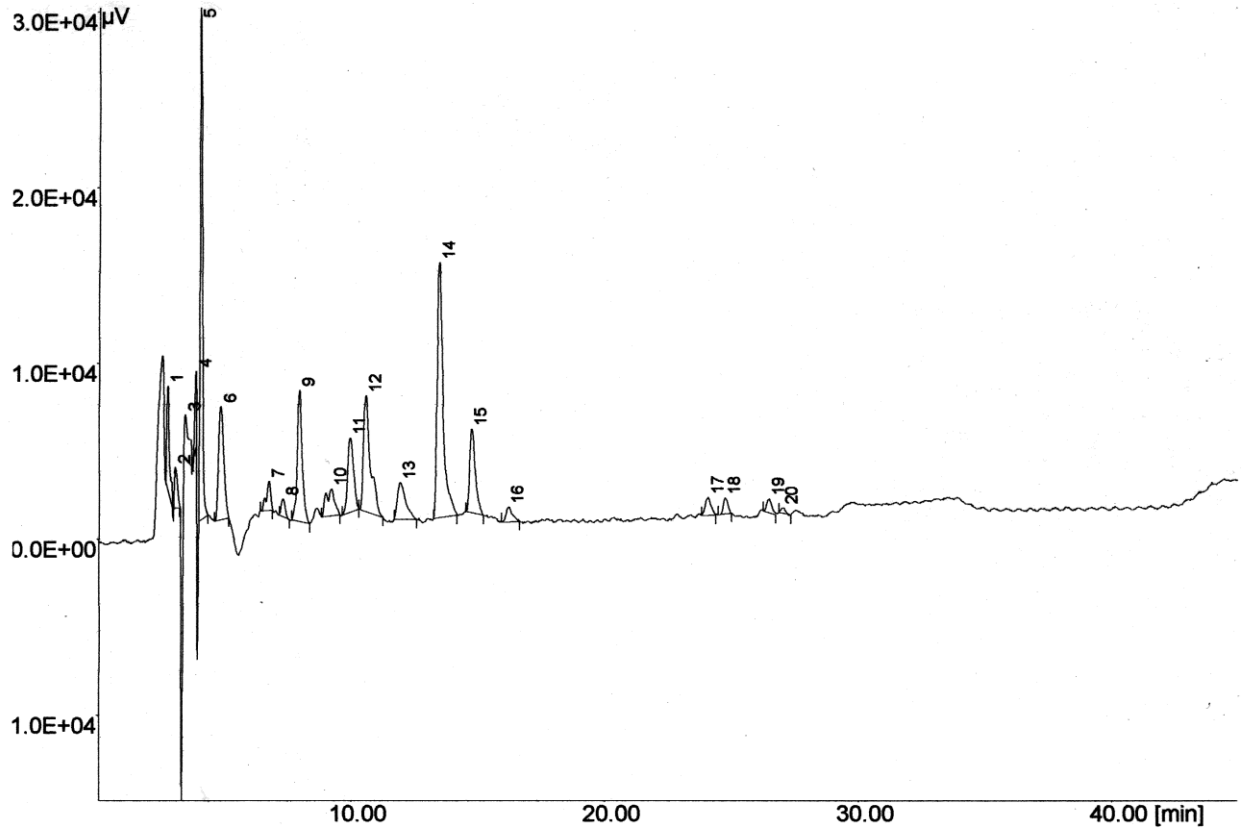

Figure 2. High-performance liquid chromatography (HPLC) of ethyl acetate extract of *C. gambosa* culture liquid.

than that of the commercial drug, amphotericin B. It is not clear why yeasts were more sensitive than filamentous fungi to the metabolites produced by *C. gambosa* mycelium culture. However, it is possible that the biosynthesis of fungal cell wall including pathways, regulation and assembly of cell wall components may differ among the various fungi, resulting in different sensitivities to the *C. gambosa* metabolites. In liquid medium, high levels of *C. gambosa* metabolites were produced after mycelium growth has occurred. The highest mycelial growth of *C. gambosa* strains in liquid culture, increased the yields of ethyl acetate extracts and their antifungal activity (Tables 1 and 2). The production of secondary metabolites can be delayed until the end of the trophophase (mycelium growth phase) by repressing the enzymes of secondary metabolism during growth by removing the sources of carbon, nitrogen and phosphorus (Demain, 1986).

In light of the results of this study, it can be concluded that *C. gambosa* is a prospective candidate from which new antifungal compounds or chemical substances can be isolated. Considering the potential use of natural antifungal compounds in medicine, we are currently working on a small-scale extraction, isolation and structural characterization of compounds produced from the *C. gambosa*.

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