Lipid constituents of the edible mushroom, Pleurotus giganteus demonstrate anti-candida activity

Phan, Chia-Wei; Lee, Guan-Sern; Macreadie, Ian; Malek, Sri Nurestri Abd; Pamela, David; Sabaratnam, Vikineswary


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Different solvent extracts of *Pleurotus giganteus* fruiting bodies were tested for antifungal activities against *Candida* species responsible for human infections. The lipids extracted from the ethyl acetate fraction significantly inhibited the growth of all the *Candida* species tested. Analysis by GC/MS revealed lipid components such as fatty acids, fatty acid methyl esters, ergosterol, and ergosterol derivatives. The sample with high amounts of fatty acid methyl esters was the most effective antifungal agent. The samples were not cytotoxic to a mammalian cell line, mouse embryonic fibroblasts BALB/c 3T3 clone A31. To our knowledge, this is the first report of antifungal activity of the lipid components of *Pleurotus giganteus* against *Candida* species.

**Keywords:** *Pleurotus giganteus*, *Candida*, yeast, Antifungal, Medicinal mushroom, Fatty acid, Fatty ester methyl ester, Ergosterol.

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**Table 1: Activity of different extracts of *Pleurotus giganteus* against *Candida* species.**

<table>
<thead>
<tr>
<th>Candida strains</th>
<th>Untreated</th>
<th>Solvent extracts (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
</tr>
<tr>
<td>Candida albicans WM1172</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Candida albicans ATCC90028</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Candida dubliniensis</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Candida glabrata CBS138</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Candida glabrata ATCC90030</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Candida krusei ATCC6258</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Candida pseudotropicalis</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Candida tropicalis WM30</td>
<td>++++</td>
<td>++++</td>
</tr>
</tbody>
</table>

Strains were grown with different mushroom extracts at the concentrations shown for two days on YEPD media. Growth was scored from “-” to “++++”, indicating no growth to strong growth.

Fungal infections are problematic for human health and are responsible for high rates of morbidity and mortality worldwide. Species of *Candida* are the dominant cause of opportunistic mycoses and among them, *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei* account for 95–97% of all *Candida* infections [1,2]. *C. albicans* and *C. tropicalis* are susceptible to polyenes, flucytosine, azoles and echinocandins, while *C. glabrata* is either susceptible or resistant to fluconazole[3]. Furthermore, *C. krusei* displays decreased susceptibility to amphotericin B, as well as fluconazole. Considering the increasing incidence of drug-resistant *Candida* infections, the search for more effective anti-*Candida* agents as an alternative to synthetic ones is needed. The aqueous extract had minimum or no inhibitory activity against *C. albicans* activity.

Medicinal mushrooms are relatively less researched for their antifungal properties. However, in the last five years, there has been a renewed interest in using mushrooms as antimicrobial agents. *Lentinula edodes* (shitake), *Boletus edulis* (Penny bun), *Pleurotus ostreatus* (oyster mushroom), *Coprinus comatus* (shaggy mane), *Astraeus hygrometricus* (earthstar mushroom), and *Cordyceps militaris* were shown to exhibit antifungal activity against *C. albicans* [5–8]. *Pleurotus giganteus* (Zhudugu, Dabeijun, morning glory mushroom), a saprobiotic mushroom, is one of the largest fruiting bodies tested for its culinary properties. The medicinal properties of this mushroom are less known. We have previously reported the hepatoprotective and neuronal stimulating effects of *P. giganteus* [10,11]. In this study the antifungal activities of different solvent extracts of this mushroom were evaluated. The extracts prepared with different solvents had different profiles of fatty acids, and fatty acids have been shown to demonstrate antimicrobial activities [12].The main metabolites / components in the extracts were analysed by GC-MS. As a preliminary in vitro toxicity assessment, the *P. giganteus* extracts were also investigated for cytotoxicity to mouse embryonic 3T3 fibroblast cells.

The anti-*Candida* activity of methanol, ethyl acetate and aqueous extracts of *P. giganteus* against all yeast species tested are summarised in Table 1. *Candida* species showed strong growth (denoted as “++++”) when extracts were not added to the medium. The aqueous extract had minimum or no inhibitory activity against all *Candida* spp. The ethyl acetate extract completely inhibited the growth of all *Candida* spp. when tested at 50 and 100 µg/mL. Thus, the ethyl acetate extract was fractionated to identify the active component/s responsible for the antifungal activity. Sub-fractions A to H were obtained and the minimum inhibitory concentration (MIC) values against all the tested yeasts are given in Table 2. The MIC values for all the *Candida* spp. tested, ranged from 2.0 ± 1.0 to 10.3 ± 2.5 µg/mL for sub-fraction A; and 9.3 ± 2.3 to 34.3 ± 10.8 µg/mL for sub-fraction B, respectively. The MIC values of...
The methanol, ethyl acetate, and aqueous extracts were not toxic to 3T3 fibroblasts cells and the IC50 values were more than 2 mg/mL. (Fig. 1). Meanwhile, cell viability (%) decreased steadily with increasing concentrations of sub-fr actions A and B at levels up to 100 µg/mL. The sub-fractions A and B were shown to contain several bioactive components. Since they are blends of fatty acids and fatty acid methyl esters, they do not act on specific targets in the fungal cells, and fungal resistance may be unlikely to occur. Furthermore, fatty acids and their methyl esters were reported to have fungicidal activity to C. albicans, C. krusei, C. tropicalis and C. parapsilosis [13]. The entities might play crucial roles in lipophilic or hydrophilic effects on the cell wall and membrane, hence affecting the distribution of the lipids in the cells [14]. Moreover, ergosterol present in the sample could disrupt the ergosterol biosynthesis pathway in the yeast, causing growth inhibition or cell death. This was further supported by a study of Irshad et al. [15], who reported that ergosterol-rich Cassia fistula oil significantly decreased the in vivo ergosterol content in the Candida cell wall.

In this study, the sub-fractions A and B were not cytotoxic to mouse fibroblasts at the concentrations tested (Fig. 1). Animal testing is becoming less popular and is gradually being replaced by in vitro methods for toxicity assessment of pharmaceutical products. In conclusion, P. giganteus lipids are promising natural products to be further explored as antifungal agents against Candida species.

### Experimental

**Mushroom:** The fruiting bodies of *Pleurotus giganteus* (Berk) Karunarathna & K.D. Hyde were obtained from Nas Agro Farm, Selangor, Malaysia. A voucher specimen (KLUM-1227) was deposited in the Herbarium in the University of Malaya.

**Chemicals:** Fluconazole and amphotericin B were purchased from Sigma Co. (St. Louis, MO, USA). The stocks were prepared in dimethyl sulfoxide (DMSO) prior to bioassays. [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), was also obtained from Sigma. Methanol (MeOH), ethyl acetate (EtOAc), n-hexane and acetone were from Merck (Darmstadt, Germany).

**Extracts preparation:** The fresh fruiting bodies of *P. giganteus* were sliced, freeze-dried and ground to a fine powder (500 g). The mushroom powder was extracted with 80% MeOH to yield a MeOH extract (115 g, 23.0%). This (125 g) was further partitioned in EtOAc-H2O (100 mL: 100 mL) to give an EtOAc-soluble extract (6.96 g, 6.05%) and a H2O extract (74.2 g, 64.5%).

**Fractionation of extract:** The EtOAc extract (5.00 g) was further fractionated by CC over silica gel. The extract was eluted with n-hexane containing increasing concentrations of acetone to obtain 8 fractions (A to H) based on similarity of spots on TLC.

### Table 2: Activity of the sub-fractions of ethyl acetate extracts against Candida species.

<table>
<thead>
<tr>
<th>Candida strains</th>
<th>Sub-fractions from ethyl acetate extract (MIC)</th>
<th>IC50 (mM)</th>
<th>Amphotericin B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>Candida albicans WM1172</td>
<td>7.3 ± 16.0 ± 26.6 ±</td>
<td>0.9 ± 10.0 ± 14.0 ±</td>
<td>1.0 ± 14.0 ± 16.0 ±</td>
</tr>
<tr>
<td>Candida albicans ATCC90028</td>
<td>7.0 ± 22.6 ± 40.6 ±</td>
<td>0.9 ± 10.0 ± 14.0 ±</td>
<td>1.0 ± 14.0 ± 16.0 ±</td>
</tr>
<tr>
<td>Candida dubliniensis</td>
<td>3.7 ± 7.0 ± 9.0 ±</td>
<td>0.9 ± 10.0 ± 14.0 ±</td>
<td>1.0 ± 14.0 ± 16.0 ±</td>
</tr>
<tr>
<td>Candida glabrata CBS138</td>
<td>8.1 ± 12.5 ± 28.3 ±</td>
<td>0.9 ± 10.0 ± 14.0 ±</td>
<td>1.0 ± 14.0 ± 16.0 ±</td>
</tr>
<tr>
<td>Candida glabrata ATCC90030</td>
<td>1.5 ± 2.4 ± 6.6 ±</td>
<td>0.9 ± 10.0 ± 14.0 ±</td>
<td>1.0 ± 14.0 ± 16.0 ±</td>
</tr>
<tr>
<td>Candida krusei ATCC658</td>
<td>10.3 ± 37.2 ± 90.0 ±</td>
<td>0.9 ± 10.0 ± 14.0 ±</td>
<td>1.0 ± 14.0 ± 16.0 ±</td>
</tr>
<tr>
<td>Candida pseudotropicalis</td>
<td>3.8 ± 9.4 ± 24.0 ±</td>
<td>0.9 ± 10.0 ± 14.0 ±</td>
<td>1.0 ± 14.0 ± 16.0 ±</td>
</tr>
<tr>
<td>Candida tropicalis WM30</td>
<td>2.0 ± 9.4 ± 24.0 ±</td>
<td>0.9 ± 10.0 ± 14.0 ±</td>
<td>1.0 ± 14.0 ± 16.0 ±</td>
</tr>
</tbody>
</table>

Results were from three independent experiments performed in triplicate. *MIC is expressed in µg/mL.* :

- sub-fraction C varied from 23.0 ± 11.0 to >50 µg/mL; whereas the MIC values for sub-fractions D-H were all >50 µg/mL. Overall, sub-fraction A showed the lowest MIC value for all Candida spp.

- Sub-fractions A and B were further analysed by GC-MS. Both samples were pale yellow-colored oils with a distinct odor. Constituents of sub-fractions A and B are listed in Table 3. Twelve compounds were identified in sub-fractions A and B. Sample A was characterized by high amounts of fatty acid methyl esters, namely: methyl palmitate, ethyl palmitate, methyl linoleate, methyl oleate, methyl stearate, and ethyl oleate. Sample B contained fatty acids (palmitic acid and oleic acid), fatty acid methyl esters (methyl linoleate and methyl oleate), ergosterol, ergost-5,8(14)-dien-3-ol, bet-ergostenol.

### Table 3: Chemical composition of lipids in sub-fractions A and B of *P. giganteus*.

<table>
<thead>
<tr>
<th>Constituents</th>
<th>RT (min)</th>
<th>Percentage (%)</th>
<th>Quality</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sub-fraction A</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methyl palmitate</td>
<td>20.50</td>
<td>14.8</td>
<td>99</td>
</tr>
<tr>
<td>Ethyl palmitate</td>
<td>37.12</td>
<td>1.2</td>
<td>98</td>
</tr>
<tr>
<td>Methyl linoleate</td>
<td>23.70</td>
<td>19.8</td>
<td>99</td>
</tr>
<tr>
<td>Methyl oleate</td>
<td>23.80</td>
<td>39.3</td>
<td>99</td>
</tr>
<tr>
<td>Methyl stearate</td>
<td>24.26</td>
<td>3.3</td>
<td>99</td>
</tr>
<tr>
<td>Ethyl oleate</td>
<td>24.99</td>
<td>12.3</td>
<td>99</td>
</tr>
<tr>
<td><strong>Sub-fraction B</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methyl palmitate</td>
<td>20.49</td>
<td>0.2</td>
<td>95</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>23.18</td>
<td>14.4</td>
<td>99</td>
</tr>
<tr>
<td>Methyl linoleate</td>
<td>23.68</td>
<td>0.4</td>
<td>93</td>
</tr>
<tr>
<td>Methyl oleate</td>
<td>23.79</td>
<td>1.0</td>
<td>93</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>24.61</td>
<td>31.7</td>
<td>99</td>
</tr>
<tr>
<td>Ergosta-5,7,9(11),22-tetraen-3β-ol</td>
<td>39.83</td>
<td>2.2</td>
<td>90</td>
</tr>
<tr>
<td>Ergosterol</td>
<td>40.33</td>
<td>24.4</td>
<td>98</td>
</tr>
<tr>
<td>Ergost-5,8(14)-dien-3-ol</td>
<td>40.51</td>
<td>10.2</td>
<td>87</td>
</tr>
<tr>
<td>γ-ergostenol</td>
<td>41.32</td>
<td>3.7</td>
<td>94</td>
</tr>
</tbody>
</table>
**Cell culture:** Mouse embryonic fibroblasts were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10%, v/v, heat-inactivated fetal bovine serum (PAA), 100 U/mL penicillin and 100 µg/mL streptomycin. The cells were routinely passaged every 2-3 days and incubated at 37°C and 5%, v/v, CO₂ in a humidified atmosphere.

**Cytotoxicity:** The crude MeOH and fractionated EtOAc extracts were dissolved in DMSO (10 mg/mL) as stock solutions. The H₂O extract (10 mg/mL) was stocked in a sterile, distilled water. The cytotoxic effects of varying concentrations of MeOH, EtOAc and H₂O extracts, as well as the fractions A-H in DMSO to 3T3 fibroblast cells were tested by the established colorimetric MTT assay [16]. The absorbance was measured at 550 nm using a microplate reader. The IC₅₀ is the concentration of extract or fraction that reduced fibroblast cell growth by 50%.

**Anti-yeast activity:** Candida albicans WM1172, C. albicans ATCC90028, C. dubliniensis, C. glabrata CBS138, C. glabrata ATCC90030, C. krusei ATCC6258, C. pseudotropicalis, and C. tropicalis WM30 were used in these studies. In the yeast inhibition assay, the method was performed according to the method of Macreadie et al. [17]. The yeast strains were grown in YEPD (1% yeast extract, 2% peptone, 2% glucose). If required, media were solidified by the addition of 1.5% agar. Yeast inocula (100 µL) were added to Microlabels, providing initial starting optical density at A₅₉₅ of 0.02-0.04 were added to each well of a 96-well microplate (Orange Scientific, Braine-l’Alleud, Belgium). Mushroom extracts were then added as two-fold serial dilutions commencing with a 100 µg/mL concentration. Fluconazole (0.1 mM) and amphotericin B (1.0 mM) were used as positive controls. A growth control DMSO solvent alone was also included. The microplate was incubated in a microplate shaker at 35°C. After 2 h and 4 h incubation, the A₅₉₅ was recorded using a microplate reader (Sunrise™, Tecan, Austria). Each sample was assayed in triplicate. The lowest concentration of extracts that inhibited growth of *Candida* spp. is the minimum inhibitory concentration (MIC).

**Gas chromatography-mass spectrometry (GCMS):** GCMS analysis was performed on sub-fractions A and B using Network Gas Chromatography system (Agilent Technologies 6890N) equipped with an Inert Mass Selective Detector (Agilent Technologies 5975) (70eV direct inlet) on a HP-5ms (5% phenyl methyl siloxane) capillary column (30 m x 250 µm x 0.25 µm) initially set at 150°C, then increased at 5°C per min to 300°C and held for 10 min. Helium was used as carrier gas at a flow rate of 1 mL per min. The total ion chromatogram obtained was auto-integrated by chemstation and the constituents were identified by comparison with the accompanying mass-spectra database (Wiley 9th edition with NIST 11 Mass Spectral Library, USA) wherever possible.

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