Anti-Candida activity and brine shrimp toxicity assay of *Ganoderma boninense*

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**Abstract.** – Objectives: *Ganoderma (G.) boninense* is a white rot fungus, which can be found in the palm oil tree. Several studies have shown that *G. boninense* has antimicrobial and antagonistic properties. However, there is limited information reported on antifungal properties especially on *Candida (C.) albicans*. Hence, this study was conducted to determine the anti-Candida activity of *G. boninense* against *C. albicans*.

**Materials and Methods:** Crude methanolic extracts of *G. boninense* was obtained by maceration method with 70% methanol. Anti-Candida test was carried out using disc diffusion assay, broth dilution method, time killing profile and brine shrimp toxicity assay.

**Results and Conclusions:** Anti-Candida activity indicated that the mean zone of inhibition was 12.5 ± 0.6 mm. The MIC value for *C. albicans* found to be 3.125 mg/ml. The result from time-killing profile showed that the growth of *C. albicans* was inhibited hence decreases its exponential phase. For brine shrimp toxicity assay, the LC50 value was 3.59 mg/ml which proved that the extract of *G. boninense* is not toxic.

**Key Words:** *Ganoderma boninense*, Antifungal, *Candida albicans*, Brine shrimp assay.

**Introduction**

*Candida (C.) albicans* is a member of the normal flora of the gastrointestinal tract that often causes serious oral and vaginal mucosa invasion and systemic disease in hosts with impaired immune defences. *C. albicans* is a causative agent associated with serious fungal infection, accounting for more than 90% of candidiasis cases\(^1,2\). Candidiasis is known as the most common invasive fungal infections in critically ill non-ntropenic patients\(^3\). The management of Candida infections faces a number of problems including limited number of effective antifungal drugs, toxicity of the available antifungal drugs, resistance of Candida to commonly used antifungal drugs, relapse of Candida infections and the high cost of antifungal drugs\(^4,7\).

*Ganoderma (G.) boninense* is a white rot fungus which can be found on the palm oil tree. For thousands of years, *Ganoderma* (also known as *Lingzhi* or *Reishi*) has been considered by the Chinese to be of high quality herbal medication. This genus of fungi possesses valuable bioactive compounds which have promising prospects in the medical arena. Various reports suggest its uses as antitumour\(^8\), antibacterial\(^9\), anti-inflammatory\(^10\) and antiviral\(^11\) agent.

To date, most of the researches on *G. boninense* is concentrated on its pathogenicity against palm oil but there is still inadequate data on its health benefits. Previous investigations on the medicinal properties of this species make it commendable for further studies. A research conducted by Ofodile et al\(^12\), showed antimicrobial activity of *G. boninense* against *Pseudomonas syringae* and *Bacillus subtilis*. Since the species does exhibit some antimicrobial properties, it is thus also worth enquiring its anticanidial activities which may perhaps provide positive ramifications for candidiasis. Hence, this current study was carried out to determine the antifungal activity of crude extract of *Ganoderma boninense* against *Candida albicans*.

**Materials and Methods**

**Methanolic Extraction of G. boninense**

Fresh samples of *Ganoderma boninense* were collected from USM (University Science
Time-Killing Profile

The time-killing profile of C. albicans with 0.5 MIC, MIC and 2 MIC concentration over time was plotted to assess the fungicidal effect. The crude extract were added to an aliquot of 25 ml of Mueller-Hinton broth (MHB) at 37°C in an amount which would achieve the concentration of 0 mg/ml (control) and the above mentioned MIC concentrations after the addition of the inoculums. Later, a solution of 1ml inoculums was added to all MIC concentrations. Immediately after the addition of the inoculums, 100 µl of culture from each was inoculated onto a Potato Dextrose agar plate and incubated at 37°C for 24 hours. The growth of C. albicans was monitored by counting the number of colonies (CFU) after incubation. The growth of C. albicans was measured every 6 hours for 48 hours.

Brine Shrimp Assay

The procedure for brine shrimp lethality test (BSLT) was modified from the assay described by Finney14. Brine shrimp eggs, Artemia salina (SandersTM Great Salt Lake, Brine Shrimp Company L.C., U.S.A.), were hatched in artificial sea water prepared from commercial sea salt (38 g sea salt/ litre deionized water) with constant light source and oxygen supply after 24 hours of incubation. 300 mg of crude extract was used to prepare serial diluted working concentrations of 2-2000 µg/ml in sea water respectively. Each concentration had three replicates and control [phosphate buffered saline (PBS) and sea water] in 2.4 ml sea water. Ten 48-hour-old Nauplii were added into each concentration and adjusted to 4.8 ml sea water. Brine shrimp were then incubated for 24 hours under a constant light source and the number of living Nauplii was counted the next day. Lethal concentration (LC50) for Artemia salina with 95% confidence level was determined by Probit analysis14 on a Finney computer program (BioStats 2009). Percentage mortalities were corrected for the natural mortality observed in the negative controls using Abbott’s formula, p=(P-C)/(1-C), where p denotes the observed mortality rate and C means the natural mortality.

Statistical Analysis

The result of this study was analyzed using Student’s t-test, SPSS version 17, (SPSS Inc, Chicago, IL, USA).
Results

The antifungal activity of crude extract against *C. albicans* was assessed by the presence or absence of inhibition zones as showed in Table I. The antifungal activity by disc diffusion method showed that the methanolic crude extract was effective against *C. albicans*. The zone of clearance produced by the crude extract was 12.8±0.25 mm, whereas the zone of inhibition produced by commercial antibiotic (Nystatin) disk was 19.9±1.89 mm. The zone of inhibition is significant (*p* = 0.003) between the crude extract and commercial antibiotic. There was no zone of inhibition observed in the disc impregnated with methanol.

In view of the results obtained by the disc diffusion method, the MIC values of the crude extract were established for *C. albicans* and the results were shown in Table II. The MIC values confirmed the existence of inhibitory effects on *C. albicans* tested in the study, with MIC value of 3.125 mg/ml for the crude extract and 0.0625 mg/ml for standard antibiotic.

The growth profile of *Candida albicans* in peptone dextrose broth (PDB) at 0.5 MIC (1.56 mg/ml), MIC (3.125 mg/ml), 2 MIC (6.25 mg/ml) and control are shown in Figure 1. The growth profile of *C. albicans* in the presence of various MIC concentrations was studied to evaluate the ability of crude extract to eliminate *C. albicans* growth *in vitro*. In the case of 1 and 2 fold MIC Concentrations the extract inhibited the yeast growth within 6 h and subsequent regrowth was not seen. The finding confirmed the fungicidal effect of the crude extract on *Candida albicans* at the MIC concentration.

Figure 2 showed the toxic effect of the *G. boninense* methanol extract after 6 hours using brine shrimp lethality assay. Figure 2 comparing the percentages of mortality versus log10 concentration. According to15,16 LC50 value lower than 1000 µg/ml is considered bioactive in toxicity evaluation of plant extracts by BSL bioassay, being highly cytotoxic compound. As shown in Figure 2, the extract showed no significant toxicity against brine shrimp (LC50 = 3.59 mg/ml). In this experiment potassium dichromate was used as a positive control. Potassium dichromate is an oxidizing agent and it is highly toxic compound. Figure 3 showed a toxic effect of potassium dichromate tested against brine shrimp after 6 hours of treatment. From the graph LC50 was 0.76 mg/ml, hence potassium dichromate is highly toxic compound.

Discussion

To our knowledge, the present study is the first systematic positive report on the efficacy of *G. boninense* methanolic extracts against *C. albicans*. *G. boninense* is used in traditional medicine to increase the body’s healing ability, and maintaining a healthy body. So far, there have been no reports showing the anti-candidal activity of *G. boninense*. Hence, an attempt was made to study the anti-candidal activity of this fungus.

The present study was aimed at the investigation of a Malaysian fungi, *G. boninense* antifungal activity against *C. albicans*. The results obtained from the diffusion method showed that the extract exhibits a favorable antifungal activity against the tested organisms. The zone of inhibition obtained was significant (*p* = 0.003) between

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<tr>
<th>MIC [mg/ml] on <em>C. albicans</em> with</th>
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<tr>
<td>Crude extract</td>
<td>Nystatin</td>
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<td>3.125</td>
<td>0.0625</td>
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Table II. Anticandidal activity of methanolic extracts of the *G. boninense*.
The brine shrimp lethality assay represents a rapid, inexpensive and simple bioassay for testing plant extracts bioactivity which in most cases correlates reasonably well with cytotoxic and anti tumor properties. The degree of lethality was found to be directly proportional to the concentration of the extract. This extract may be used as an antifungal agent in known dosages, especially in rural communities where conventional drugs are unaffordable or unavailable and the health facilities are crude extract and commercial antibiotic. The MIC values obtained for the extract against the *C. albicans* also support the findings of the diffusion method. Furthermore, *C. albicans* infection could be treated by the extract, as the MIC for this fungal was found to be only 3.125 mg/ml. The finding was further confirmed by the alteration of the normal growth profile of *C. albicans* by the extract at the MIC concentration. Moreover, through brine shrimp lethality assay, the extract was found to be non-toxic as the LC₅₀ value was 3.59 mg/ml. The brine shrimp lethality assay represents a rapid, inexpensive and simple bioassay for testing plant extracts bioactivity which in most cases correlates reasonably well with cytotoxic and anti tumor properties. The degree of lethality was found to be directly proportional to the concentration of the extract.

Figure 1. The above figure shows the growth profile of *C. albicans* PDB in PDB with control (0), 1/2 MIC (1.56 mg/ml), MIC (3.125 mg/ml) and 2 MIC (6.5 mg/ml). CFU = colony forming unit.

Figure 2. The above figure shows the toxicity effects of *G. boninense* methanolic extract after 6 hr using brine shrimp lethality assay. The LC₅₀ value was 3.59 mg/ml.
Moreover, the search for safer and more effective antifungal agents has been propelled by the increasing evidence of systemic mycoses in immunocompromised patients and the incidence of certain strains of *C. albicans* becoming resistant to certain anti-fungal agents. Hence, there is a need for developing wider variety of antifungal agents for the treatment of fungal diseases. The results presented here indicate that the natural products analyzed seem to be a good choice for the development of new strategies to treat *Candida albicans*.

Further purification and identification of the crude extract should be done to identify the bioactive compound responsible for anti-Candida activity.

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**References**


13) NCCLS: 2001, PERFORMANCE STANDARDS FOR ANTIMICROBIAL SUSCEPTIBILITY TESTING; ELEVENTH INFORMATIONAL SUPPLEMENT. NCCLS document M100-S11. NCCLS, Wayne, PA.


16) PARRA AL, YHEBRA RS, SARDINAS IG, BUELA LI. Comparative study of the assay of Artemia salina L. and the estimate of the medium lethal dose (LD₅₀ value) in mice, to determine oral acute toxicity of plant extracts. Phytomedicine 2001;8:395-400.