In vitro and In vivo antifungal activity of the methanol extract from Gracilaria changii
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Citation

Abstract
Background: There is currently an enormous surge of interest in the use of marine algae as antifungal agent. The aims of this study were to determine the in vitro and in vivo antifungal activity of the methanol extract of G. changii against systemic candidiasis

Study design: The effect of Gracilaria changii methanol extract of was studied by disc diffusion method, broth dilution method and candidiasis in mice. Histopathological examination of control and extract treated mice was done.

Results: The extract showed a favorable antimicrobial activity against Candida albicans with a Minimum Inhibition Concentration (MIC) value of 3.12 mg/mL. An intravenous inoculums of Candida albicans (Berkhout) produced colonies of the organism in the kidneys. Histopathological examination of the respective organs confirmed these findings. Treatment of the C. albicans infected mice with the G. changii extract (2.5 g/kg body weight) exhibited a considerable decline of mortality (%) and a significant CFU reduction in the animals tested. In addition, the reduction of mortality (%) by the G. changii extract was comparable with commercial antibiotic ketoconazole.

Conclusion: These results indicate that the methanol extract of G. changii exhibits inhibitory effect against candidiasis.

INTRODUCTION
There is currently an enormous surge of interest in the use, development and conservation of marine algae throughout the world. Malaysia is endowed naturally with a very rich algae life and the use of some of these in traditional medicines needs to be well documented. Among the algae with therapeutic properties in Malaysia, the Gracilaria changii B.M. Xia & I.A. Abbott has yet to gain importance and popularity. The G. changii (Gracilariaciae family) found predominantly in the mangrove areas of Malaysia and Thailand. The Gracilaria sp are widely used in the traditional medicine in Malaysia. The Malay people administer the agar derived from Gracilaria, internally for coughs and in consumption. Beside that, Gracilaria sp boiled in vinegar used to treat swollen knees and unhealthy sores.

There is still much that is not known about the G. changii, and research will be required on many levels as data are deficient on status, extent and utilization. Therefore the current study was carried out to determine the antifungal activity of extract of Gracilaria changii. Candida albicans (Berkhout) and related species pathogenic for man become resistant to antifungal agents. The clinical consequences of antifungal resistance can be seen in treatment failures in patients and in changes in the prevalences of Candida species causing disease. Hence, in this investigation we describe the in vitro and in vivo antifungal activity of the methanol extract of G. changii against systemic candidiasis.

MATERIALS AND METHODS
SAMPLES AND EXTRACTION
G. changii were obtained from a Pantai Morib, Selangor, authenticated by by Prof. Phang Siew Moi (Institute of Biological Sciences, Faculty of Science, University Malaya, Malaysia). The sun-dried algae were cut into small pieces. Approximately 100 g of dried algae was added to 400 mL of methanol and soaked for 4 days. Removal of the algae from solvents was done by filtration through cheesecloth, and
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refiltered through a Whatman filter no 4. The filtrate was concentrated using a rotary evaporator and stored at 4°C in a sterile tube until use.

MICROORGANISM
Candida albicans (B3648) was used as the test organism and was obtained from a laboratory stock culture. The yeast was cultured on Sabouraud dextrose agar at 30 °C for 24 h. The stock culture was maintained on Sabouraud dextrose agar slants at 4 °C.

LABORATORY ANIMALS
Swiss albino Mice (male) weighing between 25 and 35 g were used. The cages with the mice were placed in a room (temperature 26 ± 2 °C) with controlled cycles of 12 h of light and 12 h of darkness; light went on at 7 am and relative humidity 45-55%. Water and food were provided to animals ad libitum. The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of School of Biological Sciences, Universiti Sains Malaysia. Experiments were conducted in accordance with the internationally accepted principles for laboratory animal use and care (EEC Directive of 1986; 86/609/EEC).

IN VITRO ANTICANDUDAL ACTIVITY
The fungicidal activity of the extract was determined following the method described by NCCLS with slight modifications.

DISK DIFFUSION TECHNIQUE
The test microbe was removed aseptically with an inoculating loop and transferred to a test tube containing 5mL sterile distilled water. Sufficient inoculums were added until the turbidity was equal to 0.5 McFarland (10^8 colony-forming units mL^-1) standard (bioMerieux, Marcy Petoile, France). One milliliter of the test tube suspension was added to 15–20 mL of Sabouraud dextrose agar before setting aside the seeded agar plate (9 cm in diameter) to solidify for 15 min. Nine Whatman's filter paper no. 1 disks of 6 mm diameter were used to screen the fungicidal activity. Each sterile disk was impregnated with 20 µL of extract (corresponding to 100 mg/mL of crude extract); miconazole nitrate (30 µg/mL, as positive control); 10% DMSO (v/v) (as negative control). The disks were placed on the surface of the seeded plates, incubated at 37 °C overnight, and examined for zones of growth inhibition.

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)
A 16-h culture was diluted with a sterile physiologic saline solution [PS; 0.85% (w/v) sodium chloride] with reference to the 0.5 McFarland standard to achieve inoculums of approximately 106 CFU mL^-1. A serial dilution was carried out to give final concentrations between 1.563 and 200.00 mg crude extract per milliliter. The tubes were inoculated with 20 µL yeast suspension per milliliter nutrient broth, homogenized, and incubated at 37 °C. After incubation, 50 µL was withdrawn from each tube, inoculated on agar plates, and incubated at 37 °C for 24 h. The MIC value was determined as the lowest concentration of the crude extract in the broth medium that inhibited the visible growth of the test microorganism.

IN VIVO ANTIFUNGAL ASSAY
The standard intravenous (i.v.) inoculation of C. albicans used in this study was 1x10^7 viable cells/mL PBS, of which 0.1 mL was injected into the lateral tail vein of mice. Animals were divided into three groups of 10 mice each and received treatment as described in Table 1. All mice were killed by cervical dislocation on day 7 after i.v. C. albicans inoculation.

Figure 1
Table 1: Details of experimental groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
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<tbody>
<tr>
<td>1</td>
<td>i.v. Candida, 24 h, followed by treatment with PBS (100 µL/week for 7 days)</td>
</tr>
<tr>
<td>2</td>
<td>i.v. Candida, 24 h, followed by treatment with ketoconazole, 10 mg/kg/day for 7 days</td>
</tr>
<tr>
<td>3</td>
<td>i.v. Candida, 24 h, followed by treatment with Gracilaria changii extract, 20 µL/kg/day for 1 week</td>
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i.v., intravenous; o.a., oral administration; PBS Phosphate buffer solution

The kidneys of each of these animals were removed aseptically, and 0.1 ml blood was withdrawn from renal artery before shipped to 0.1 ml heparin 25U/ml. The kidneys than, transferred into sterile centrifuge tubes and homogenized in 5 mL of sterile PBS. Aliquots from each homogenate and blood samples were serially diluted and plated on Sabouraud dextrose agar plates, and incubated at 37 oC for 24 h. All cultures were done in triplicate. The colonies were then enumerated and the colony forming units (CFU) were calculated per gram of each organ and per ml of blood sample respectively.

HISTOLOGY
Kidneys were processed for histopathological examination. Formalin fixed slides were prepared and stained with periodic acid–Schiff (PAS).
STATISTICAL ANALYSIS
The values are expressed as mean ± SEM (standard errors of means). The student's t-test for significance of two independent sample means was employed. The difference between means was considered significant when p was <0.05.

RESULTS
IN VITRO ANTICANDIDAL ACTIVITY
The results of antiyeast activity of the extract against C. albicans are given in Table 2. The extract exhibited a favorable activity against the yeast tested. The zone of clearance produce by the commercial antibiotic disk was larger than that produced by the extract disk. The agar dilution method recorded the MIC value of 3.13 mg/mL.

IN VIVO ANTICANDIDAL ACTIVITY
The intravenous inoculum of C. albicans (1x10⁷ viable organisms/mL) produced a colony of abscesses in different organs with a maximum concentration in the kidneys as expected (Figure 1, a).

Table 2: Antiyeast activity (zone of inhibition and MIC ) of extract compared with commercial antibiotic miconazole nitrate.

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Methanol extract</th>
</tr>
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<tr>
<td></td>
<td>Zone of Inhibition (mm)</td>
</tr>
<tr>
<td></td>
<td>Crude Extract</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>18.00</td>
</tr>
</tbody>
</table>

*Agar dilution method, mean value n = 3.

On gross examination of the specimens there were fewer abscesses present in the animals of group 2 and 3 compared with group 1. The histopathological examination showed that the kidney was massively infected by the C. albicans with the formation of blastospore and Clamydospore (Figure 1, d, e and f) compared to treated group 3 (Figure 1, c).

Figure 2 show the mean of CFU/g organ from the three groups. In group 2 and 3 animals, that received the plant extract (2.5 g/kg body weight) and ketoconazole, 10 mg/kg body weight, a significant (p<0.05) reduction in CFU was observed compared to the group 1 animal that received PBS. There was no C. albicans colony was formed by the blood sample. Figure 3 showed the mortality (%) of the animal in the group 1 to 3. The mortality (%) was decreased from 50 to 20% when the animals group received the treatment of G. changii extract or ketoconazole compared with PBS.
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**DISCUSSION**

In recent 20 years, the risk of opportunistic fungal infections has significantly increased in patients who are severely immunocompromised due to cancer chemotherapy, organ or bone marrow transplantation and human immunodeficiency virus infection. Despite advances in antifungal therapies, many problems remain to be solved for most antifungal drugs available. Algae provide rich resources of antifungal compounds and have been used for years to inhibit fungal growth. Hence, in this study we evaluate the in vivo antifungal activity of marine alga G. changii extract against C. albicans.

In this study the methanol extract of G. changii was shown to have a significant antifungal activity with the MIC values 3.12 mg/mL and in a mice model inoculated with C. albicans followed by the oral administration of the methanol extract of G. changii, indicating a strong antifungal activity. The extract gave a good effect on the reduction of the mortality when the treatment was given after infection by the C. albicans by decreased the mortality from 50% (group 1) to 20 % (group 2 and 3). In addition the effect of the extract was comparable to the commercial antibiotic ketoconazole. Furthermore, the CFU study showed corresponding result with this finding where the treatment with G. changii extract showed a significant (p<0.05) reduction in CFU amongst the animals groups tested. This suggests that the methanol extract might act by potentiating the immune system, which obviously becomes active after exposure with the infecting organism. This speculation gains further strength with the recent report, that immunopotentiating activity has also been observed in the plant extract. It is possible that the alga extract contains active ingredient(s), which may directly stimulate the granulocytes and monocytes to generate NO, which in turn kills C. albicans.

In the present report, we particularly concentrate on dermatophytic fungi C. albicans because the increasing prevalence of drug resistant C. albicans recovered from patients is a major concern worldwide. C. albicans is pathogenic yeast, which can cause vulvovaginitis, oral thrush, nosocomial infection, and candidiasis in humans. In views of this data, it appears that the extract of G. changii exhibited good antifungal activity and it could be use to in the treatment of C. albicans infection.

In conclusion, the methanol extract of G. changii possesses significant antifungal activity. This is the first report of an in vivo antifungal activity of the methanol extract of the G. changii. Although evidence on the antifungal efficacy of the aqueous extract of the alga has been obtained, further investigations are necessary prior to the recommendation for its use as a safe and effective antifungal remedy.

**References**

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