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In vitro and *in situ* antiyeast activity of *Gracilaria changii* methanol extract against *Candida albicans*

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Abstract. – *Background and Objectives:* **The *Gracilaria (G.) sp* are widely used in the traditional medicine in Malaysia. The methanol extract of *Gracilaria changii* B.M. Xia & I.A. Abbott (Gracilariaciae) was evaluated for antiyeast activity against *Candida albicans* (Berkhout).**

Materials and Methods: **The antiyeast activities were studied by using disc diffusion method and broth dilution method. The effect of the extract on the growth profile of the yeast was also examined via time-kill assay. In addition, the *in situ* antiyeast activity was studied by microscopic observations using scanning electron microscopy (SEM), transmission electron microscopy (TEM) to determine the major alterations in the microstructure of *Candida (C.) albicans*.**

Results and Discussion: **The extract showed a favourable antimicrobial activity against *C. albicans* with a minimum inhibitory concentration (MIC) value of 1.56 mg/mL. The main abnormalities noted from the SEM and TEM studies were the internal shrinkage of cell, disorganization within the cell cytoplasm and complete collapse of the yeast cells after 36 h of exposure to the extract. The time-kill assay suggested that the *G. changii* extract significantly inhibited *C. albicans* growth and it also exhibited prolonged antiyeast activity against the *C. albicans*.**

Conclusion: **The extract has shown *in vitro* fungicidal properties against *C. albicans* and should be investigated for its therapeutic potential.**

Key Words:

Candida albicans, Gracilaria changii, Electron microscopy, Marine algae.

Introduction

Malaysia is gifted naturally with a very rich algae life in its marine environment. The marine environment represents a treasure of useful products awaiting for discovery for the treatment of infectious diseases. Ecological pressures, including competition for space, the fouling of the surface, predation, and successful reproduction have led to the evolution of unique secondary metabolites with various biological activities[1]. The importance that these secondary metabolites play in the control of infectious microorganisms was for many years largely unnoticed. Among the algae with therapeutic properties in Malaysia, the *Gracilaria (G.) changii* B.M. Xia & I.A. Abbott (Gracilariaciae) has yet to gain importance and popularity. The *G. changii* was found predominantly in the mangrove areas of Malaysia. The *Gracilaria sp* are widely used in the traditional medicine in Malaysia. The Malay people administer the agar derived from *Gracilaria*, internally to treat coughs and also for consumption[2]. Besides that, *Gracilaria sp.* boiled in vinegar is used as a poultice to treat swollen knees and unhealthy sores[2].

Clinically, antimicrobial therapy is going through a crisis due to the rapid development of resistance to existing antimicrobial agents[3]. Twenty years ago, *Candida albicans* (Berkhout) was commonly regarded as little more than culture contaminat. However, because of developed antimicrobial resistance, in less than two decades this organism has become major pathogen[4,5]. Hence, the current study was carried out to determine the antiyeast activity of methanol extract of *G. changii* against *C. albicans*.

Materials and Methods

Algae Sample

Fresh *G. changii* sample was collected from Pantai Morib, Selangor, Malaysia, in January

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2003 and authenticated by Prof. Phang Siew Moi (Institute of Biological Sciences, Faculty of Science, University Malaya, Malaysia). Morphological examination and random amplified polymorphic DNA (RAPD) assay were used in the classification of seaweeds at the genus and species levels of *Gracilaria*[6, 7].

Extraction Procedure

At the field, the fresh algae were rinsed with seawater to remove debris and epiphytes before being brought it to the laboratory. In the laboratory, the algae were further washed with freshwater and brushed with a soft brush before sun drying. The sun-dried algae were cut into small pieces. Approximately 100 g dried algae was added to 400 mL methanol and soaked for 4 days. Removal of the algae from solvents was done by filtration through cheesecloth, and the filtrate was concentrated using a rotary evaporator.

Microorganism

Candida (C.) albicans (Clinical isolate) 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.

Fungicidal Activity

The fungicidal activity of the extract was determined following the method described by NCCLS[8] with slight modifications. The modification of the National Committee for Clinical Laboratory Standards (NCCLS) protocol was the replacement of pour plate method to streaking plate method to obtained an evenly growth culture.

Disk Diffusion Technique

The test microbe was removed aseptically with an inoculating loop and was transferred to a test tube containing 5 mL sterile distilled water. Sufficient inoculums were added until the turbidity was equivalent to 0.5 McFarland (10^8 colony-forming unit's mL^{-1}) standard (BioMerieux, Marcy Petoile, Craponne**,** France). The yeast suspension was then streaked evenly with a cotton swab on Sabouraud dextrose agar plate (9 cm in diameter). 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% dymethyl sulfoxide (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.

Statistical Analysis

The data were analyzed by Student's *t*-test for comparing the *G. changii* methanolic extract on the *C. albicans* vs miconazole nitrate, using Statistical Package for the Social Sciences (SPSS Version 12.0) software (SPSS Inc., Chicago, IL, USA). Statistical significance was assumed at the 0.05 levels ($p<0.05$).

Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) of the extract was determined by broth dilution assay. A range of concentrations (0.78 to 100.00 mg extract per ml) of extract was prepared in yeast extract-peptone-dextrose (YPD) broth medium in flasks. Tween 80 was included at a final concentration of 0.001% (v/v) to enhance extract solubility. A 16-h culture of *C. albicans* was diluted with a sterile physiologic saline solution [PS; 0.85% (w/v) sodium chloride] with reference to the 0.5 McFarland standards to achieve inoculums of approximately 5×10^5 colony-forming unit (CFU) ml^{-1}. Subsequently, each flask was inoculated with 20 µl of *C. albicans* suspension. Flasks containing Tween 20 without the extract were used as control. The flasks were incubated at 30 ± 2°C, in an orbital shaking incubator (100 rpm) for 48 h. From each flask, 20 µl of culture was inoculated on YPD agar plates and incubated at 30 ± 2°C for 48 h. The plates were observed and MIC was determined as the lowest concentration of the extract which completely inhibits the growth of *C. albicans*.

Scanning (SEM) and Transmission (TEM) Electron Microscope Observations

Scanning electron microscope observations were carried out on *C. albicans* cells. One milliliter of the *C. albicans* cell suspension at a concentration of 1×10^6 cells per milliliter was inoculated on a Sabouraud dextrose agar plates and then were incubated at 37°C for 12 h. The extract (2 mL), at a concentration of 100 mg per milliliter, was then dropped onto the inoculated agar and was further incubated for another 36 h at the same incubation temperature. A 10% DMSO-treated culture was used as a control. A small

block of yeast containing agar was withdrawn from the inoculated plate at 0, 12, 24, and 36 h and were fixed for scanning[9]. The remaining part was used for transmission[10] electron microscopy works. SEM analyses were performed on samples by placed on double-stick adhesive tabs on a planchette and the planchette placed in a Petri plate. In a fume hood, a vial cap containing 2% osmium tetroxide in water was placed in an unoccupied quadrant of the plate. After being covered, the plate was sealed with parafilm, and vapor fixation of the sample proceeded for 1 h. Once the sample was vapor fixed, the planchette was plunged into slushy nitrogen (–210°C) and transferred on to the "peltier-cooled" stage of the Freeze Dryer (Emitech K750), and freeze drying of the specimen proceeded for 10 h. Finally, the freeze dried specimen was sputter coated with 5-10 nm gold before viewing in the SEM (Fesem Leo Supra 50 VP, Carl Zeiss, Germany) operating at 15 kV at various levels of magnification. TEM analyses were performed on samples by fixed in 3% glutaraldehyde, rinsed in buffer, postfixed in 1% osmium tetroxide in 0.1 M potassium phosphate for 2h at 4°C, dehydrated in ethanol and embedded in Epon-Araldite resin. Ultra thin sections were stained with uranyl acetate and lead citrate and observed under a TEM (Philips CM12, Eindhoven, Netherlands).

Time-Kill Study

In order to assess the antiyeast activity with MIC, 1/2MIC, and 2MIC concentration over time, growth profile curves were plotted. A 16-h culture was harvested by centrifugation, washed twice with phosphate saline, and resuspended in phosphate saline. The suspension was adjusted using the McFarland standard and was then further diluted in phosphate saline to achieve approximately 10^7 CFU mL^{-1}. *G. changii* extract was added to aliquots of 25 mL Mueller-Hinton broth (MHB) in a 50-mL Erlenmeyer flask (37°C) to achieve a concentration of 0 (control) and 1.56 mg/mL (MIC), 0.78 mg/mL (1/2 MIC), and 3.13 mg/mL (2 MIC), after addition of the inocula. Then, 1 mL inoculas were added to all Erlenmeyer flasks. Finally, 1 mL portion was removed and the growth of *C. albicans* was monitored using this portion by measuring optical density at 540 nm (UV-9100; Ruili Co., Beijing, China)[11]. The growth of *C. albicans* was measured every 4 h for 48 h continuously by the above method.

Results

Antiyeast Activity

The result of fungicidal activity of the extract against *C. albicans* is given in Table I. The extract exhibited a favorable activity against the yeast tested. The zone of clearance produced by the commercial antibiotic disk was larger than that produced by the extract disk (Figure 1) (p=0.0001). There are colonies within the zone of inhibition were also observed. The agar dilution method recorded the MIC value of 1.56 mg/mL.

Scanning (SEM) and Transmission (TEM) Electron Microscope Observations

Figure 2 shows the SEM photomicrographs of the untreated and extract treated cells of *C. albicans* at various time of exposure to the crude extract of *G. changii*. Untreated cells (Figure 2a) show many oval and smooth cells in appearance and some at a budding stage. After 12 hours of exposure (Figure 2b), a significant effect of the extract was observed. The 12 hours treated cells showed a rough cells appearance with holes compared to the untreated control cells. The 24 hour

Table II. Antiyeast activity (zone of inhibition and MIC[a]) of *Gracilaria changii* extract compared with commercial antibiotic miconazole nitrate.

	Methanol extract		
	Zone of Inhibition (mm)[b]		
Yeast	*Crude extract*	*Miconazole nitrate*	MIC (mg/ml) of the extract
Candida albicans	16.00	21.00	1.56

[a]Agar dilution method, mean value n = 3. [b]The values (average of triplicate) are diameter of zone of inhibition at 100 mg/ml crude extract and 30 µm/ml miconazole nitrate.

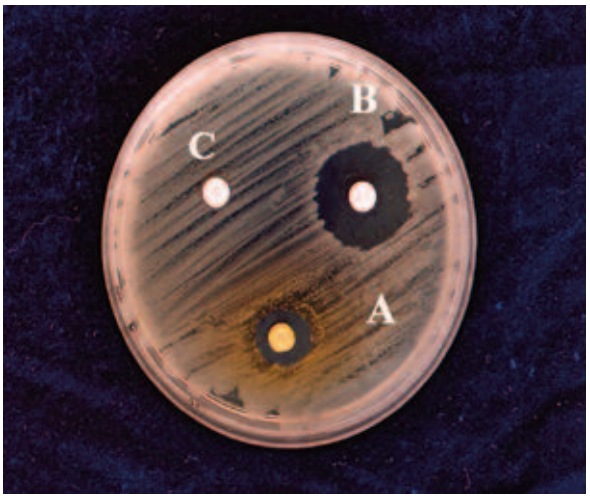

Figure 1. Agar plate showing inhibitory zones: ***(A)*** crude extract (100 mg/mL), ***(B)*** miconazole nitrate (30 µg/mL), and ***(C)*** 10% DMSO (v/v).

treated cells (Figure 2c) showed severe alterations of the cell wall with the formation of invaginations and followed by the collapsed cells. After 36 hours of exposure (Figure 2d), completely collapsed cells were seen. It was believed that at this stage, the cells had lost its metabolic functions completely.

From the SEM findings, we suggested that the cells had undergone some distinct morphological and cytological alterations. Further evidence of these changes was obtained from TEM studies. Figure 3a shows the TEM photomicrographs of the untreated cells of *C. albicans*. It showed a typically structured nucleus and vacuoles. The cytoplasm contains several element of endomembrane system and enveloped by a typical structure of cell wall. After 12 hours of exposure to the extract (Figure 3b), the cell was very densed with the vesicles and membranous bodies dispositioned within the cell. After 24 hours of exposure (Figure 3c), the cells exhibited notable alterations in the cell membrane and the cell wall. The cytoplasmic volume decreased, and the cell membrane started to invaginate leaving a state of structural disorganization within the cell cytoplasm. It seems that the extract induced dysfunc-

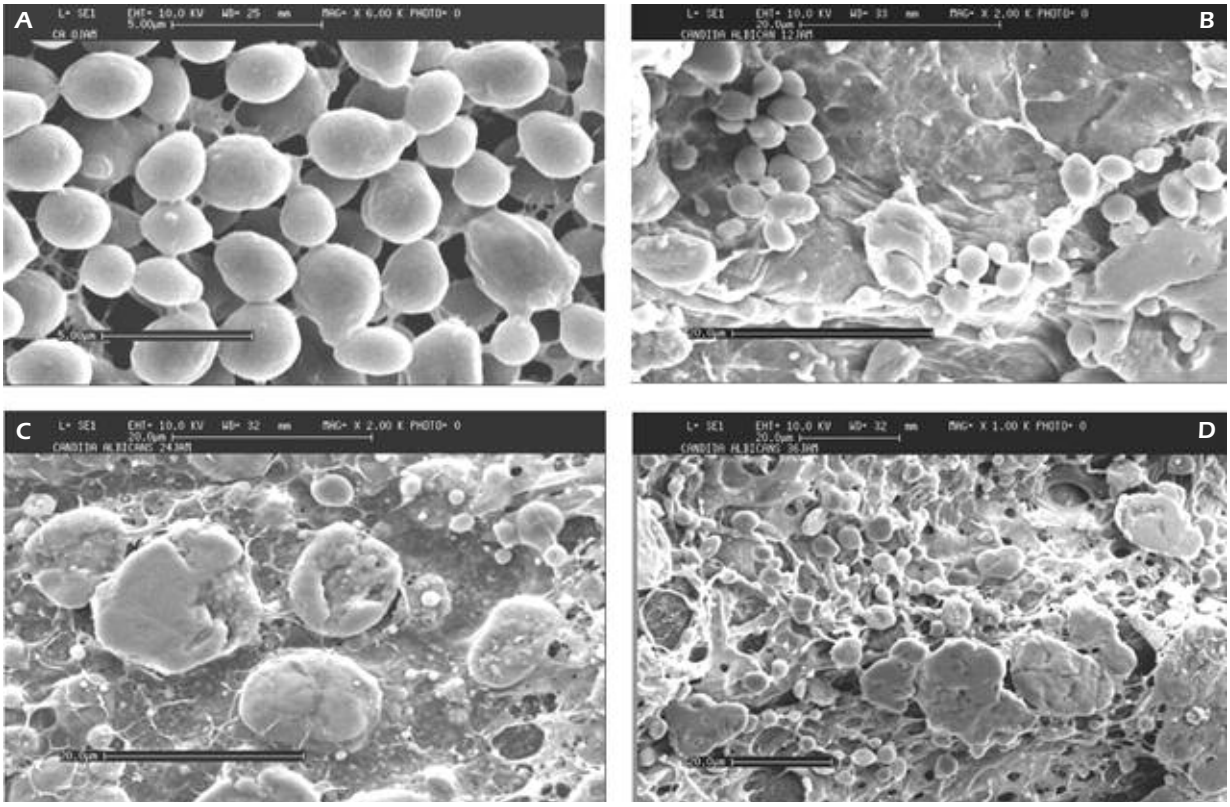

Figure 2. SEM micrographs of *C. albicans* treated with 1.56 mg/ml of crude extract of *G. changii*. ***A,*** Control, untreated cells. ***B,*** Cells treated for 12 hr showing a rough cells appearance with holes. ***C,*** Cells treated for 24 hr showing severe alterations of the cell wall with the formation of invaginations. ***D,*** Cells treated for 36 hr showing completely collapsed and cavitated cells. Bars represent 5 µm (for a) and 20 µm (for b, c and d). (×6,000(a), 2,000(b), 2,000(c), and 1,000(d) Magnifications).

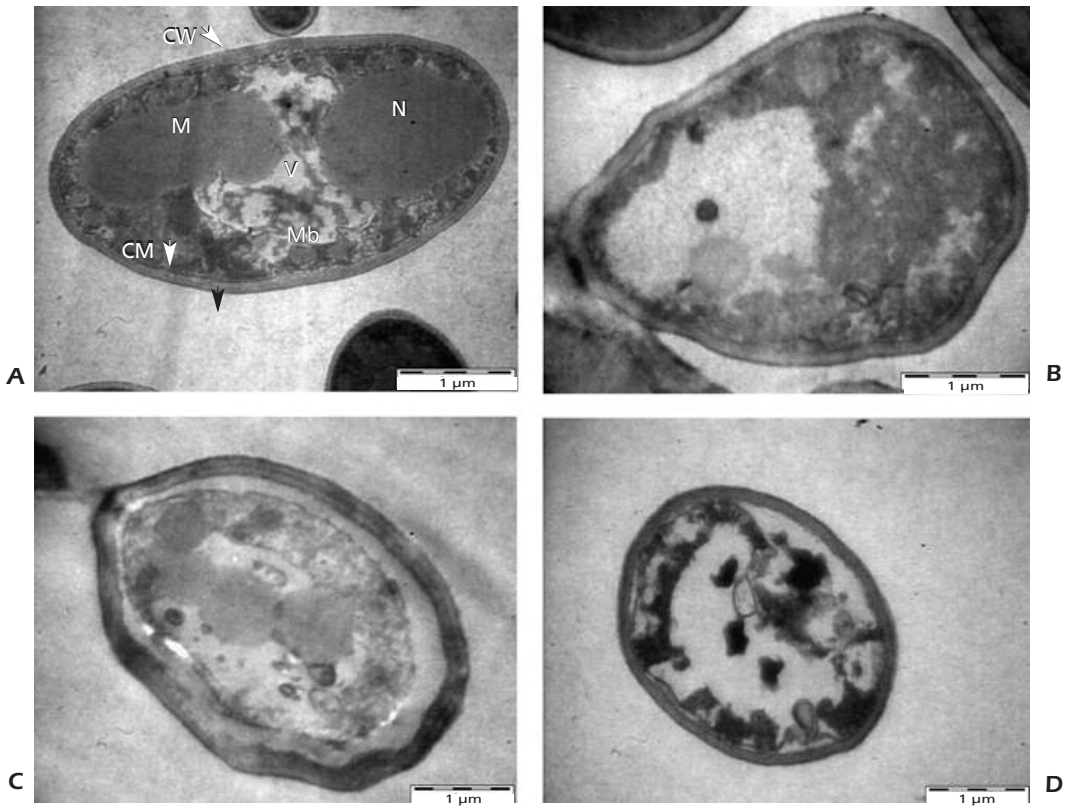

Figure 3. TEM micrographs of *C. albicans* treated with 1.56 mg/ml of crude extract of *G. changii*. ***A,*** Control, untreated cell. ***B,*** Cell treated for 12 hr showing a very dense cell with the vesicles and membranous bodies dispositioned within the cell. ***C,*** Cell treated for 24 hr showing internal shrinkage of cell and leaving a state of structural disorganization within the cell cytoplasm. ***D,*** Cell treated for 36 hr showing completely collapsed cell. Abbreviations: CM, cell membrane, CW, cell wall, M, mitochondrion, Mb, microbody (peroxisome), N, nucleus and V, vacuole. Bars represent 1 µm. (×30,000 Magnifications).

tions of the cell membrane, which followed by internal shrinkage of cells. Figure 3d shows the effect of the extract on the yeast cells after 36 hour of exposure. It shows that yeast cells were collapsed and lysed.

Time-Kill Study

The time-kill studies were performed over a period of 48 h with yeast being exposed to 1, 1/2 or 2×MIC of the *G. changii* extract. Figure 4 shows the results of the time-kill curves for *C. albicans*. At 1/2×MIC, *G. changii* extract demonstrated a large drop in OD after 16 h, which leads to the stationary phase of yeast growth compared with the control. At MIC, *G. changii* extract caused complete yeast eradication after only 4 h. At 2×MIC, *G. changii* extract exhibited complete eradication within 2 h. The time-kill curves described above confirm the potency of *G. changii* extract as an anticandidal agent against *C. albicans*.

Discussion

The inhibitory effect of *G. changii* methanolic extract on pathogenic yeasts *C. albicans* has been studied. In this study, we particularly concentrated on *C. albicans* for the reason that the management of *Candida* infections faces a number of problems including: limited number of effective antifungal agents, toxicity of the available antifungal agents, resistance of *Candida* to commonly used antifungals, relapse of *Candida* infections, and the high cost of antifungal agents[12]. The difficulties associated with the management of *Candida* infections necessitate the discovery of new antifungal agents, in order to widen the spectrum of activity against *Candida* and combat strains expressing resistance to the available antifungal agents.

From this investigation, it appears that the extract of *G. changii* exhibits a favourable antiyeast activity against *C. albicans* with the MIC

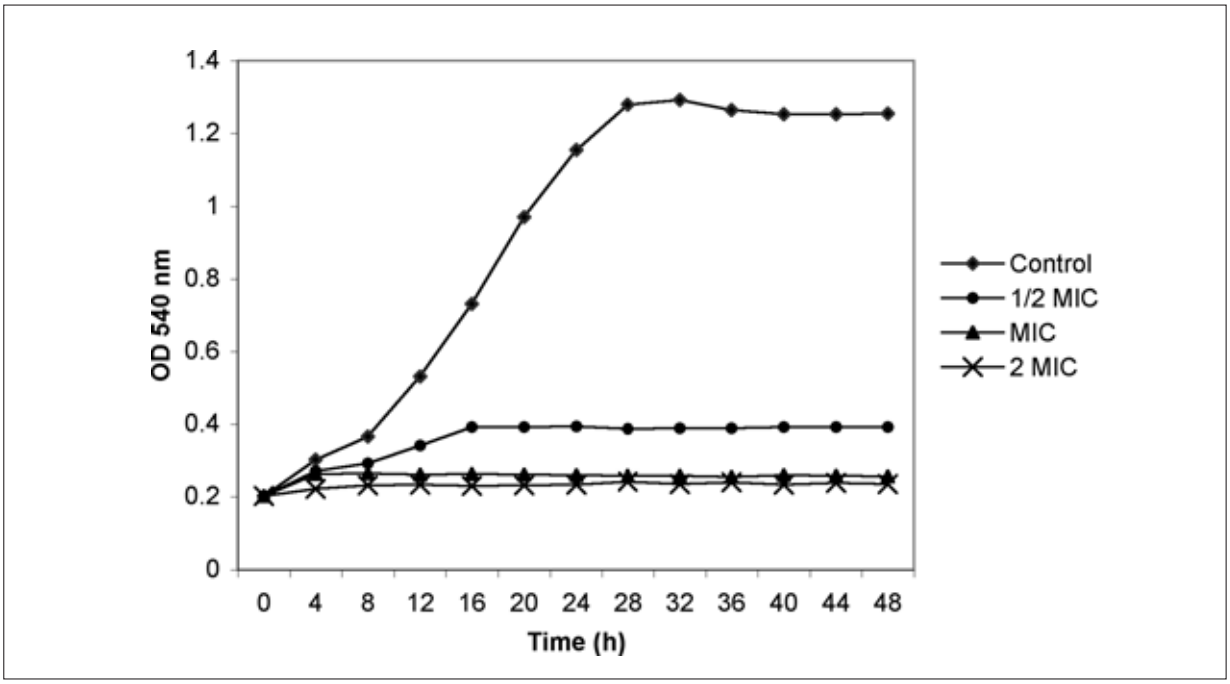

Figure 4. Time killing profile for *Candida albicans* in Mueller-Hinton broth with 0 (control) and 1.56 mg/mL (MIC), 0.78 mg/mL (1/2MIC), and 3.13 mg/mL (2MIC) concentration of *Gracilaria changii* extract.

value to be only 1.56 mg/mL. The Time-kill assays was utilised in this study to confirm MIC findings and to evaluate the ability of *G. changii* extract to eliminate *C. albicans* growth *in vitro.* In the case of 1 and 2 time MIC concentrations the extract was inhibited the yeast growth within 4 h and subsequent re-growth was not seen. This is because there is no increment in the OD values which, allied with the yeast growth, was observed for these two concentrations. The time-kill assay suggested that the *G. changii* extract significantly inhibited *C. albicans* growth and it also exhibited prolonged antiyeast activity against the *C. albicans* as determined by time-kill curves. Furthermore, the SEM and TEM studies showed that the extract could completely collapse the yeast cell and inhibit the growth of *C. albicans*. The commercial antibiotics Miconazole Nitrate showed significantly (p<0.05) larger zone of clearance compared to the *G. changii* extract because not all the compounds presence in the extract possess antimicrobial properties.

There are colonies observed within the zone of inhibition suggesting a resistant subpopulation. These data suggest that some *in vivo* conditions may exert a selective pressure on *C. albicans*, thus leading to emergence of subpopulations exhibiting reduced susceptibility and allowing their growth in the presence of the extract of *G. changii*. The conditions leading eventually to emergence of stable *G. changii* extract-resistant subpopulations are still unknown and deserve further investigations. Such studies would need to correlate fungal treatment failures with initial and post-treatment susceptibility data and antimicrobial drug exposure. Ultimately, they could find the emergence of resistance among commensal flora.

Our previous study shows that the *G. changii* extract was not toxic[13]. The methanol extract of *G. changii* was tested for *in vivo* brine shrimp lethality and *in vitro* anticancer cell line activity. Both toxicity tests [brine shrimp test with LC_{50} value 4.28 mg/mL (12 h), 3.13 mg/mL (24 h) and anticancer cell line activity with IC_{50} value 44.10 µg/mL] exhibited no significant toxicity result. In addition, in an acute toxicity study using mice, the median lethal dose (LD50) of the extract was greater than 2000 mg/kg, and we found no pathological changes in macroscopic examination by necropsy of mice treated with extract. We conclude that *G. changii* might be safely used as an antimicrobial agent[14]. Our previous study also showed that there are no differences or changes in the phytochemical con-

stituent from the algal sample collected from different month[15]. The *C. albicans* use in this study was local clinical isolate and we also tested many other *Candida* strains and bacteria[16] and the extract showed favorable activity against all the tested microbes. *G. changii* extract was showed a significant antimicrobial activity against seven bacterial and four yeast isolates tested. The MIC values of *C. albicans* strains were from 3.125 to 6.25 mg/mL. These findings prove that *G. changii* extract has a broad spectrum of antimicrobial activities. Hence, *C. albicans* infection could be treated with the extract, as the MIC for this yeast was found to be only 1.56 mg/mL. In addition, *G. changii* may be use as an antiyeast agent in known dosages, especially in rural communities where conventional drugs are unaffordable or unavailable and the health facilities inaccessible particularly in developing country. The alga has never been evaluated for anti-*Candida* activity before, and thus, it has been shown for the first time in this study to have anti-*Candida* activity. Therefore, further purification of active compound and its individual antimicrobial activity studies of the extracts from *G. changii* may be suggested on the basis of the present study.

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