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Assessment of Anticandidal Activity and Cytotoxicity of Root Extract from *Curculigo latifolia* on Pathogenic *Candida albicans*

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Curculigo latifolia Dryand has been widely used as a traditional medicine, with beneficial effects in various diseases, including to ease joint pains and prevention of diabetes, obesity and cardiovascular. In this study, we demonstrated anticandidal activity and cytotoxicity activities of the methanolic root extract of *C. latifolia* Dryand. The solvent used for the process of extraction was methanol. The crude extract from *C. latifolia* Dryand root showed a favourable anti-*Candida* activity against different strains of pathogenic *C. albicans* cell by using disc diffusion method and broth dilution method. Brine shrimps assay was used to determine the cytotoxicity of methanolic root extract of *C. latifolia* Dryand. The Minimum Inhibitory Concentration (MIC) for the extract against *C. albicans* had determined. Time-kill assay demonstrated that *C. latifolia* Dryand root extract had inhibited the growth profile of *C. albicans*. The microscopic studies showed the extract acts by caused the alterations in morphology and complete collapse of the yeast cells after 36 h of exposure time. The result of *in vivo* cytotoxicity test revealed that the value of LC_{50} of the root extract from *C. latifolia* Dryand was 2.25 mg mL^{-1} . Our data indicate that methanolic root extract of *C. latifolia* Dryand exerts significant anticandidal activity. Thus, the findings are demonstrating its relevant therapeutic potential in clinical treatment of candidiasis.

Key words: *Curculigo latifolia*, antimicrobial activity, *Candida albicans*, minimum inhibition concentration, cytotoxicity

INTRODUCTION

Infection induced by pathogenic fungi are increasingly recognized as emerging threat to public health and incidence of opportunistic fungal infections in immune compromised host especially in AIDS patients has increased dramatically in recent years. Although, there are several synthetic and natural product based drugs available for treating infections, they are not consistently effective against potentially pathogenic fungal infection. Furthermore, the development of resistance in fungi and yeast against most of the antifungal drugs (Sanglard, 2002; Gaurav *et al.*, 2010; Mohandas *et al.*, 2012) and the toxicity during prolonged treatment with several antifungal drugs (Giordani *et al.*, 2001) have been reported. Therefore, there is need to identify new compounds which would be exploited for developing new antifungal formulation.

Many herbal plants have been used for medicinal purposes and play a fundamental role in traditional medicine in some country. Recent research, many researchers have focused on extraction of natural active compounds against pathogenic microorganisms (Papadopoulou *et al.*, 2005; Erina *et al.*, 2012; Wendakoon *et al.*, 2012). Wide varieties of plant extracts have play an importance role in searching the new sources of antimicrobial agents (Taweekhaisupapong *et al.*, 2005; Pawar and Thaker, 2006). According to the World Health Organization as 65% of world population are depended on bioactive compounds as their medicines (Fabricant and Farnsworth, 2001).

C. latifolia Dryand is a herbal plant and belong to family Hypoxidaceae (Kocyan, 2007). It is widely distributed in Malaysia. This plant is commonly used as traditional medicine to cure the knee joint pain by local Malaysian. Interest in *C. latifolia* Dryand has increased owing to their roles as alternative sweeteners and the possible beneficial implications in prevention of diabetes, obesity and cardiovascular (Abdullah *et al.*, 2010).

Previous investigations had demonstrated that the extract of *C. latifolia* Dryand exhibited significant antimicrobial activities (Akpanabiatu, 2001). Thus, this study further evaluated anticandida activity of the crude methanolic extract from the root of this plant. We described the effects of the *C. latifolia* Dryand crude extract against five clinical isolate *C. albicans*. Besides, with the aid of microscopy we would like to look at the closer view of the morphological and ultrastructural alterations of the *C. albicans* cells after exposure to the extract.

To our knowledge, no data have been published on the cytotoxicity of *C. latifolia* Dryand root extract. According to Ramachandran *et al.* (2011), toxicity studies are an important step for identification and isolation of some compounds from plant extracts. Therefore, current study also determined the lethality concentrations (LC_{50}) of *C. latifolia* Dryand root extract which can be used as important preliminary data for selecting the natural remedies with potential antimicrobial properties for future work.

MATERIALS AND METHODS

Preparation of the plant extract: The plants of *C. albicans* Dryand were obtained from several locations around Penang Island in Malaysia. The roots of plants were then washed thoroughly with running tap water and oven dried at 50°C for 2 days. The dried and finely ground (0.5 mm) of root samples were extracted with methanol by using the modified method described previously by Darah and Annie-Clara (1992). About 100 g of finely ground root sample was extracted by 500 mL of methanol for 3 days with frequent agitation at ambient temperature. The mixture was then filtered through the muslin cloth followed by filtration with filter paper (Whatman No. 1). The entire extract was evaporated to dryness in a rotary evaporator with vacuum condition at 50°C. The concentrated methanolic extract then poured in glass Petri dishes and put into oven at 60°C for dryness purpose. The percentage yield of the crude extract was about 5.1%. The obtained paste was then stored in parafilm sealed Petri dishes in dark container at 4°C. The extracts were reconstituted by dissolving in methanol for the required concentration and maintained at 4-8°C.

Candida albicans isolate: The five *C. albicans* isolates used throughout this study were obtained from the Medical Microbiology and Parasitology Department, School of Medical Sciences, Healthy Campus, Universiti Sains Malaysia, Kelantan, Malaysia. It was grown and maintained on Sabouraud Dextrose Agar (SDA) slants at 30°C for 24-48 h. The cultures of yeast stock were kept in SDA and stored at 4°C for further use.

Antimicrobial activity tests: Paper disc agar diffusion method according to Clinical and Laboratory Standards Institute (NCCLS, 2006) was used. One milliliter of the suspension containing 4×10^6 yeast cells were added to 20 mL of SDA before setting aside the seeded agar plate to solidify for 15 min. Each sterile disc was impregnated with 20 μ L of crude root extract with concentration of

100 mg mL⁻¹; methanol (negative control) and 30 µg mL⁻¹ of miconazole nitrate (positive control). The discs were placed on the surface of the seeded plates, incubated at 30°C overnight and examined for zones of growth inhibition.

Determination of the minimum inhibitory concentrations (MIC):

The MIC value was determined by the liquid dilution method. A modified tube method Clinical and Laboratory Standards Institute (CLSI, 2006) was followed to determine the MIC of *C. latifolia* Dryand root extract. About 1.0 mL of Sabouraud dextrose broth containing 4×10⁶ cells mL⁻¹ for yeast cells was mixed with the extract to give final concentrations between 0.78-100 mg mL⁻¹ and incubated at 30°C for 48 h. The lowest concentration which did not show any growth of the test microorganism after macroscopic evaluation was determined as the MIC value (mg mL⁻¹).

Times-kill study: One milliliter of standardized *C. albicans* cells suspension (4×10⁶ cells mL⁻¹) was added into SDB medium containing extract to give a final concentration of at MIC level (1.56 mg mL⁻¹), ½ MIC level (0.78 mg mL⁻¹) and 2MIC level (3.13 mg mL⁻¹). The mixtures were then incubated at 30°C for 48 h at agitation speed of 150 rpm. Finally, 1 mL portion was removed and the growth of *C. albicans* was determined by measuring optical density at 540 nm (Genesys 10 UV, Spectronic Unicam, USA). The growth of *C. albicans* was measured every 4 h for 48 h continuously by the above method.

The effect of *C. latifolia* Dryand root extract on the morphology of *C. albicans* cells were observed for the above method by light microscopy. The morphology of the *C. albicans* cells were viewed at various incubation time intervals (4, 24 and 36 h). One drop of the cell suspension was placed on a clean slide and subjected to Lactophenol Cotton Blue staining before been observed under the light microscope. The use of Lactophenol Cotton Blue Stain facilitates the examination of cells for morphological examination after treated with *C. latifolia* extract.

Scanning (SEM) and transmission (TEM) electron microscope observations:

One milliliter of the yeast cell suspension of the concentration of 4×10⁶ cells mL⁻¹ was inoculated on SDA plate and then incubated at 30°C for 12 h. Two milliliters of the *C. latifolia* root extract at the concentration of 1.56 mg mL⁻¹ was then dropped onto the inoculated agar and was further incubated for another 36 h at the same incubation temperature. Methanol

treated culture was used as control. A small block of yeast containing agars was withdrawn from the inoculated plate at 0 and after 36 h. Then fixed the withdraw blocks for scanning electron microscopy (FESEM LEO Supra 50 VP field, Carl Zeiss, Germany) studies. The remaining part was used for transmission electron microscopy (Philips CM12, Eindhoven, Netherlands) works (Mades, 1989).

In vivo cytotoxicity test against the brine shrimp nauplii:

The cytotoxicity test was carried out by brine shrimp lethality test (Ramachandran *et al.*, 2011; Hong *et al.*, 2011). The artificial sea water prepared by dissolving 38 g of sea salt in 1.0 L of distilled water. The *C. latifolia* Dryand root extract was then dissolved in 1% DMSO and then further diluted to obtain final concentrations of 1.0-6.0 mg mL⁻¹ in artificial sea water.

Brine shrimp eggs, *Artemia salina* eggs were hatched in artificial sea water after 48 h incubation at room temperature (25-30°C), the larvae (nauplii) were then collected with pipette. The nauplii were transferred into universal bottle which containing 5.0 mL of various concentration of root extract. The number of survivor nauplii was counted after 24 h of exposure to the *C. latifolia* Dryand root extract. The universal bottle with only contain artificial sea salt water served as a drug-free control or negative control. The surviving shrimps were counted and the concentration that could kill 50% of larvae (LC₅₀) was assessed (Geran *et al.*, 1972).

Statistical analysis: The data were analyzed by student t-test for comparing the root extract of *C. latifolia* Dryand on the *C. albicans* vs miconazole nitrate using Statistical Package for the Social Sciences (SPSS version 12.0) software (SPSS, Chicago, IL, USA). Statistical significance was assumed at the 0.05 levels (p<0.05).

RESULTS

Anticandidal activity: The methanolic root extract of *C. latifolia* Dryand revealed that the extract yielded positive anticandidal activity against all the tested pathogenic yeasts *C. albicans* (Table 1). The commercial antibiotics miconazole nitrate has shown significantly (p<0.05) larger inhibition zone compared to the *C. latifolia* Dryand root extract.

From our study, it shows that the root extract exhibits a good antiyeast activity against *C. albicans*. The broth dilution method recorded the MIC value obtained in the range of 1.56-6.25 mg mL⁻¹ for all the yeast tested

Table 1: Anticandidal activity of methanolic *Curculigo latifolia* Dryand root extract compared with commercial antibiotic miconazole nitrate

Yeast	Inhibition zone (mm) ¹		Root extract (mg mL ⁻¹)
	Root extract (100 mg mL ⁻¹)	Miconazole nitrate (30 µg mL ⁻¹)	MIC
<i>Candida albicans</i> B3095	14.0±1.7	19.0±1.3	6.25
<i>Candida albicans</i> B3228	16.0±0.9	22.0±1.2	3.13
<i>Candida albicans</i> B3440	13.0±1.5	20.0±0.8	6.25
<i>Candida albicans</i> B3648	19.0±1.1	22.0±1.9	3.13
<i>Candida albicans</i> B3751	21.0±1.8	23.0±1.5	1.56

¹The values (average of triplicate) are diameter of zone of inhibition at 100 mg mL⁻¹ crude extract and 30 µg mL⁻¹ miconazole nitrate

(Table 1). However, further investigation was concentrated on *C. albicans* B3751 since it's showed the lowest MIC value in this study with only 1.56 mg mL⁻¹.

Time-kill study: Further investigation was performed to determine the action of the extract on the growth of yeast cells. Time-kill study was carried out in this study to verify MIC findings and to evaluate the ability of root extract of *C. latifolia* Dryand for its yeastostatic and yeastocidal activity. In the present study time-kill study was performed over a period of 48 h with *C. albicans* B3751 being exposed to MIC, ½MIC and 2MIC of the *C. latifolia* Dryand root extract. The result of the time-kill curves for *C. albicans* was shown in Fig. 1. At ½MIC, root extract of *C. latifolia* Dryand demonstrated a critical drop in OD after 16 h which leads to the stationary phase of yeast growth compared with the control. However, at MIC level of *C. latifolia* Dryand root extract produced absolute yeast eradication after 8 h exposure. No growth can be detected after the yeast cell exposed with 2MIC level. It seems that the growths of the yeast cells were correspondingly decreased by increasing the concentration of extract and the growth was inhibited at the MIC values (1.56 mg mL⁻¹). The results revealed that the extract exhibited yeastostatic activity at lower concentration (1.56 mg mL⁻¹ or below the MIC level) and yeastocidal activity at higher concentration (>1.56 mg mL⁻¹ or above the MIC level).

Microscopic observation on the effect of the *C. latifolia* Dryand root extract on yeast cells was performed by light microscopy study revealed that the extract turned effect on morphological changes in the test yeast. The yeast cells were destroyed and collapsed as the result of exposure to *C. latifolia* Dryand root extract. Figure 2 shows the damaged yeast cells such as formation of hole and distorted cell membrane compare with control.

Scanning (SEM) and transmission (TEM) electron microscope observations: The SEM photomicrographs of the untreated and extract treated cells of *C. albicans* to the root extract of *C. latifolia* Dryand were shown in Fig. 3. Untreated cells (Fig. 3a) showed oval in shape and

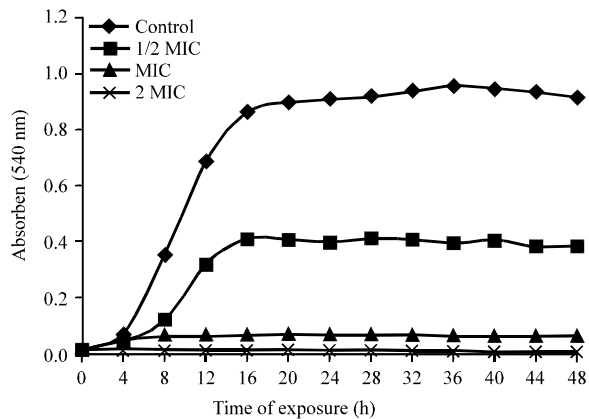


Fig. 1: Growth profile for *Candida albicans* in Sabouraud dextrose broth with 0 (c, control), 1.56 mg mL⁻¹ (MIC), 0.78 mg mL⁻¹ (1/2 MIC) and 3.13 mg mL⁻¹ (2 MIC) concentration of *Curculigo latifolia* Dryand root extract

smooth cells in appearance and some at a budding stage. After 36 h of exposure (Fig. 3b), the treated cells exhibited notable alterations with hole formation, distorted cell membrane due to cell leakage and some cells were collapsed and cavitated cells were seen. It was believed that at this stage, the cells had lost its metabolic functions completely. Therefore, the SEM findings suggested the cells had encountered some distinct morphological and cytological alterations. Further evidence of these changes was observed from TEM studies. TEM observation indicated some form of disorganization of *C. albicans* and destruction of its organelles. The TEM photomicrograph of the untreated cells of *C. albicans* was shown in Fig. 4a. It showed a typically structured of nucleus and vacuoles. The cytoplasm contains several organelles and enveloped by a typical structure of cell wall. The effect of the root extract of *C. latifolia* Dryand on the yeast cells after 36 h of exposure is shown in Fig. 4b. It was noted that unorganized masses of cytoplasmic granules were located near the cell wall. It shows that the yeast cells collapsed and lysed.

In vivo cytotoxicity test against the brine shrimp nauplii: *In vivo* cytotoxicity test alive brine shrimp has showed

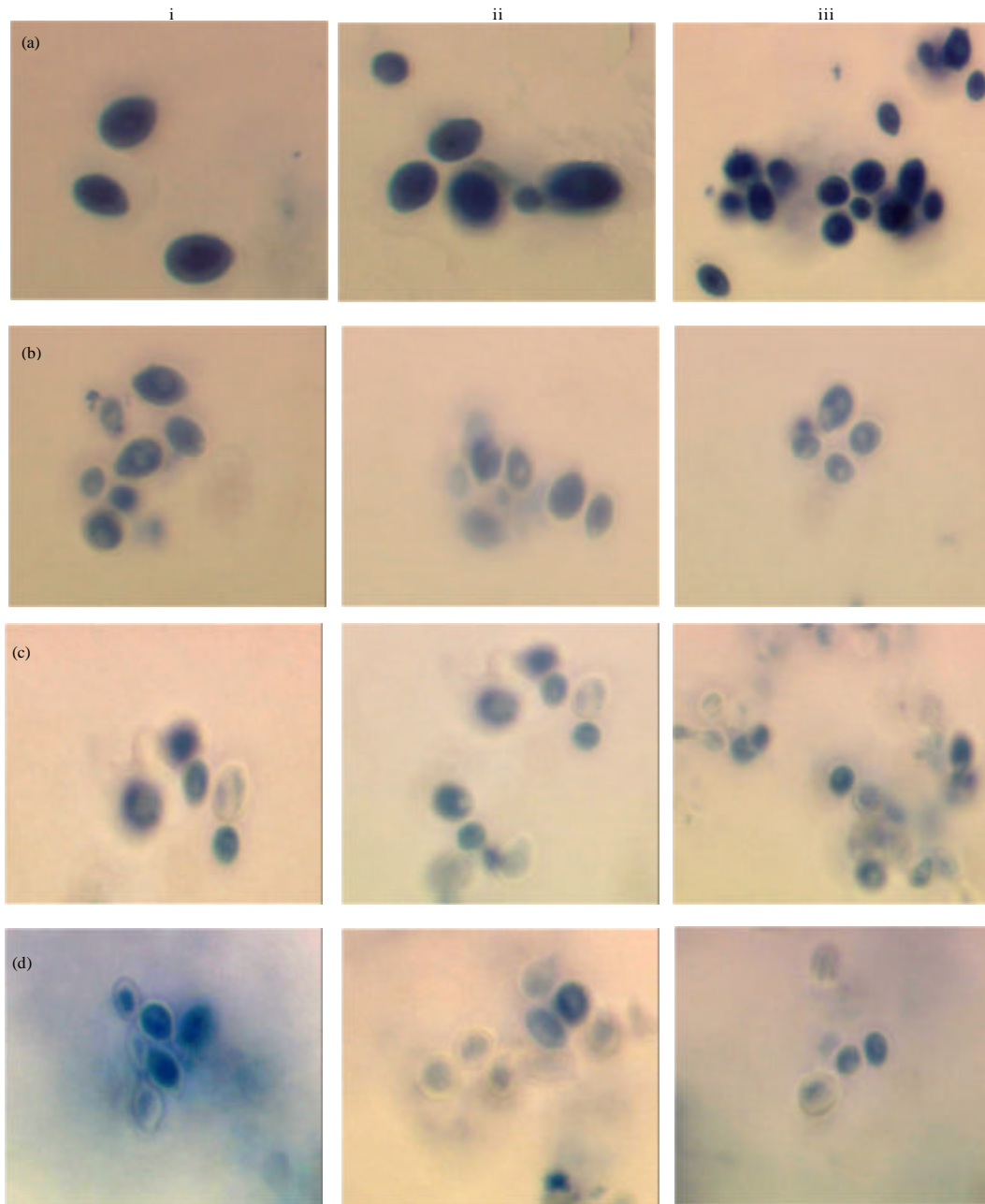


Fig. 2(a-d): Antimicrobial effects in growth profile study of *Curculigo latifolia* Dryand root extract on *Candida albicans* cell morphology (a) Control, (b) 0.78 mg mL^{-1} ($1/2 \text{ MIC}$), (c) 1.56 mg mL^{-1} (MIC) and (d) 3.13 mg mL^{-1} (2 MIC) and (i) 4, (ii) 24 and (iii) 36 h incubation. ($\times 1,000$ Magnifications)

internal and external movement. The dead nauphii however showed no movement at all when seen under the light microscope. In this study the LC_{50} value obtained from *in vivo* cytotoxicity assay of the *C. latifolia* Dryand root extract against brine shrimp was 2.25 mg mL^{-1} .

DISCUSSION

In this study methanolic root extract of *C. latifolia* showed positive result in anticandidal activity test against all the strain of *Candida albicans*. Similarly

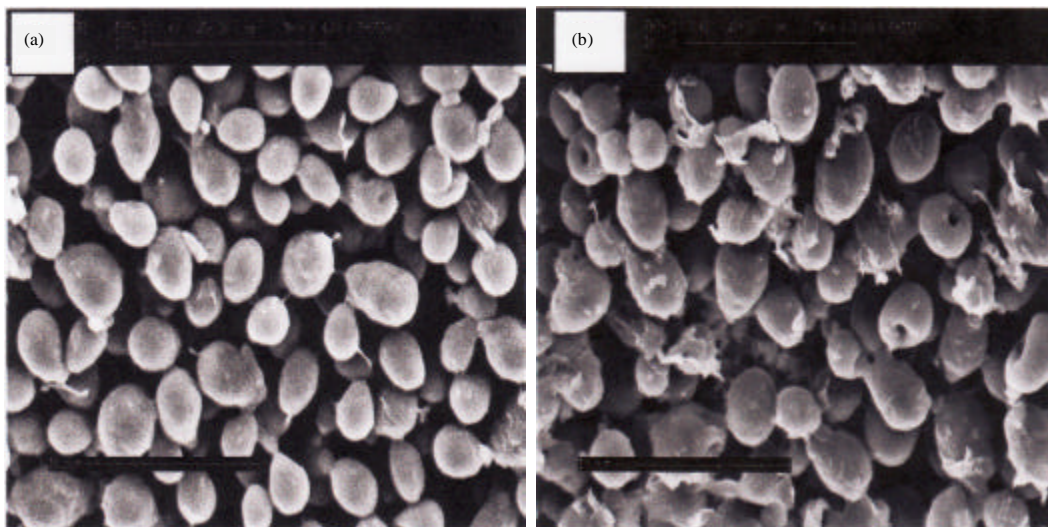


Fig. 3(a-b): SEM micrograph of the untreated and extract treated cells of *Candida albicans* ($\times 4,000$ magnification) (a) Control cells of *Candida albicans* and (b) 36 h *C. albicans* cells treated with 1.56 mg mL^{-1} (MIC) of root extract of *Curculigo latifolia* Dryand

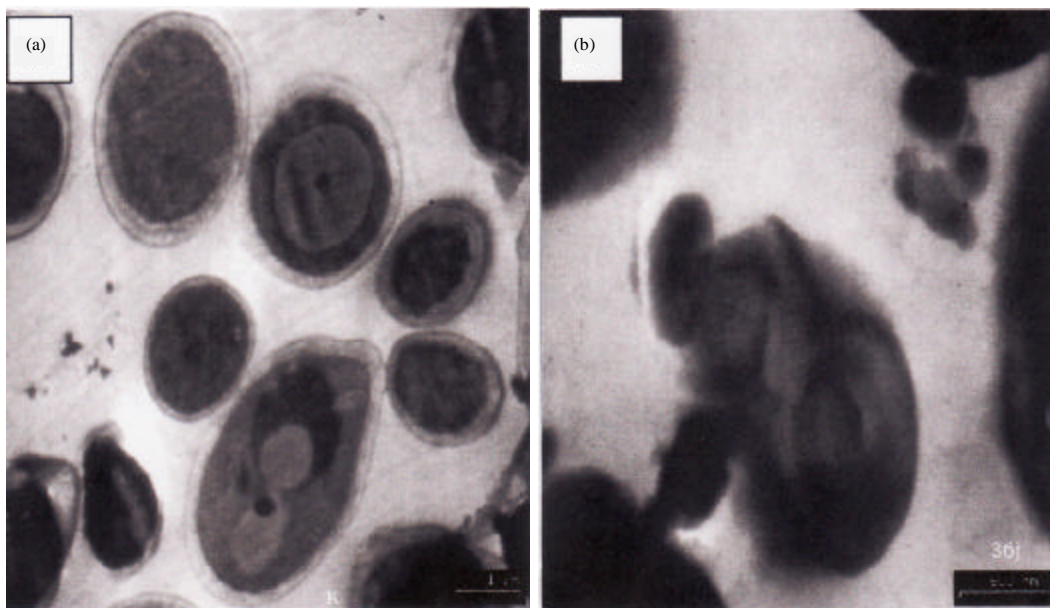


Fig. 4(a-b): TEM micrograph of a cross section of the untreated and extract treated cells of *Candida albicans* (a) Control cells of *Candida albicans* ($\times 18,000$ Magnifications) (b) 36 h *C. albicans* cells ($\times 20,000$ Magnification) treated with 1.56 mg mL^{-1} (MIC) of root extract of *Curculigo latifolia* Dryand

extract obtained from *Curculigo orchiodies* Gaertn was also reported to possess anticandidal activity (Swarnkar and Katewa, 2009). However, root extract of *C. latifolia* has shown smaller inhibition zone compared to the commercial antibiotics miconazole nitrate. This may

due to not all the compounds presence in the extract reacted as antimicrobial agent.

The MIC value defined as the lowest amount of extract necessary to completely inhibit the growth of *C. albicans*. Effectiveness of antiyeast activity

inversely correlated with their MIC values. According to Fabry *et al.* (1998) the extract with the MIC values below 8 mg mL⁻¹ are considered to have some antimicrobial activity. Our study showed that the root extract of *C. latifolia* Dryand was susceptible to all of tested strain of *C. albicans* with the MIC value less than 8 mg mL⁻¹. This result suggested that the extract may be effective in treating *C. albicans* which is the most frequently isolated yeast species from hospital patients.

To the best of our knowledge this is the first report wherein the inhibition of *C. albicans* by *C. latifolia* Dryand root extract is shown in detail. However, antimicrobial activity of *C. latifolia* Dryand has been reported by Akpanabiatu (2001) against some pathogenic bacteria. It possessed positive results against six microbial species, including *Bacillus subtilis*, *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. In this study, we particularly focused on *C. albicans* for the reason that the management of *Candidiasis* has faced various problems including limited number of effective antifungal agents, the development of resistance in known fungal pathogens and emergence of new fungal pathogens intrinsically resistant to the currently available antibiotics, relapse of *Candida* infections and the high cost of antifungal agents (Rukayadi *et al.*, 2006; Runyoro *et al.*, 2006).

Based on the microscopy study, our results have suggested that *C. latifolia* Dryand root extract completely inhibited *C. albicans* growth and it also exhibited prolonged antiyeast activity against the *C. albicans* as determined by time-kill curves. The inhibition or reduction of the growth possibly occurred due to interference by the active compound in the extract (Ibrahim and Osman, 1995). Similar observations were reported by Lim *et al.* (2010) in their study of exposure tannin on the yeast cells.

According to Venugopal *et al.* (2002) bioactive compounds which exhibit an LC₅₀ value more than 1.0 mg mL⁻¹ are considered not toxic to the nauplii of *Artemia salina*. Thus, the cytotoxicity result obtained in this study (2.25 mg mL⁻¹) indicated that the root extract showed no toxicity against brine shrimp. The brine shrimp assay is a useful tool for the isolation of bioactive compounds from plant extracts (Hong *et al.*, 2011). Hence, the results suggested that the methanolic root extract of *C. latifolia* Dryand could be a potential candidate to be used as an antimicrobial agent.

CONCLUSION

In summary, the present investigation confirms that the anti-*Candida* potential of root extract of *C. latifolia*

Dryand. Methanolic root extract of *C. latifolia* Dryand is proven to be anticandida agent for *C. albicans*. Furthermore, it may have potential for clinical treatment of candidiasis although this use will require additional investigation. However, it is important to point out that crude extract such as this needs to be further processed to obtain pure active compound(s) and its individual antiyeast activity study can be suggested on the basis of the present study. The study also re-emphasizes the importance of exploring the plant wealth of unexplored niches for novel bioactive molecules.

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