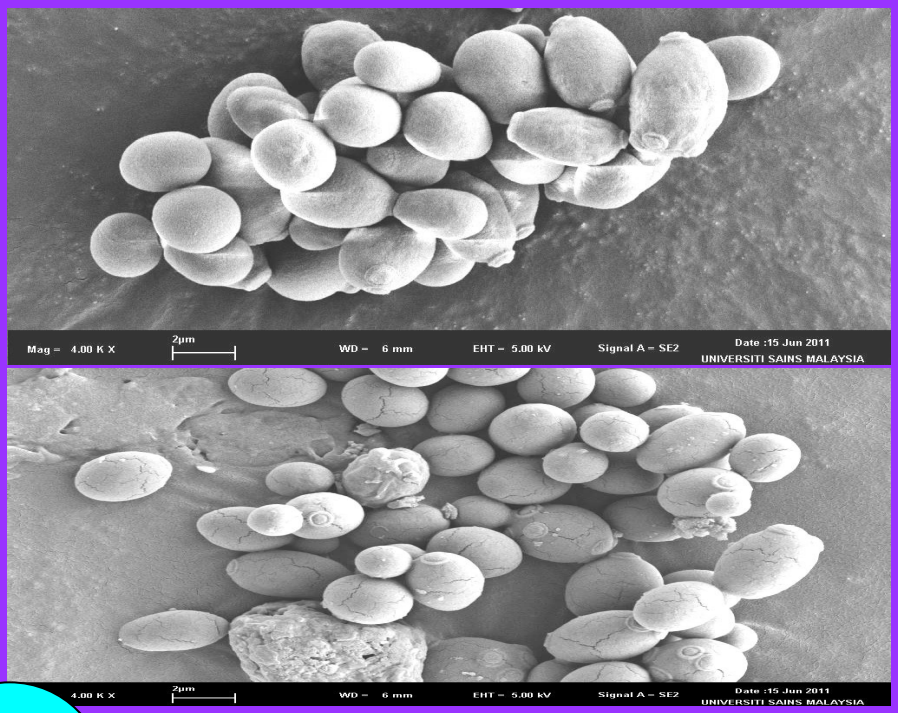

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Anti-candida activity by *Hymenocallis littoralis* extracts for opportunistic oral and genital infection *Candida albicans*

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Abstract

Candida albicans is a unicellular fungus that causes an opportunistic infection in the oral cavity and vagina. The present article reports the inhibitory effects of *Hymenocallis littoralis* methanol sonication extracts against *Candida albicans*. This plant has been used widely in traditional medicine and has proven to possess anti-cancer properties for numerous cell lines. Various plant parts such as bulb, flower, anther, root, leaves and stem were tested against this opportunistic organism and found that the flower and anther were effective at 6.3 mg/mL. This is the first report on anti-candida activity of *H. littoralis* methanol extract. Transmission (TEM) and scanning electron microscope (SEM) observations were used to observe the cytological and morphological expression of the extract treated *C. albicans*. TEM reveal the complete destruction of nucleus and internal organelles in the yeast like fungus cell. SEM reveals the fissure on the membrane cells. The methanol crude extract has anti-candida activity for tested concentration.

Introduction

Opportunistic pathogen *Candida albicans* is very difficult to treat with conventional antifungal compounds (Tyagi et al., 2010). *C. albicans* is yeast like fungus which cause local and systemic infections in predisposed persons, especially in immunocompromised patients (Najafi et al., 2011) and those undergoing prolonged antibiotic treatment (Zhang et al., 2002; Duarte 2005). Most of the infections can be treated with topical antifungal drugs such as clotrimazole, miconazole, nystatin or oral drug such as fluconazole and amphotericine B (Najafi et al., 2011). Nevertheless the clinical usages of these drugs are inadequate by their relatively high risks of toxicity, the emergence of drug resistance, pharmacokinetic deficiencies, and/or insufficiencies in their antifungal activities for the *C. albicans* strain (Fan-Havard et al., 1991; Hay, 1991; Law et al., 1994; Yotsuji et al., 1997). To overcome this problem, here we searched for potential anti-candida therapeutic herbal extract from *Hymenocallis littoralis*.

H. littoralis from Amaryllidaceae family is pharmacologically well known plant. Besides *H. littoralis*, other *Hymenocallis* species also have been used widely as traditional remedies. The bulbs of *H. americana* were made into poultice for varicose veins, sores and swellings. It is commonly employed as an ornamental plant and used in cosmetic preparations also (Yew et al., 2010). The genus was first phytochemically studied in 1920 and resulted in isolation of lycorine (1) (Abou-Donia et al., 2008). Since 1920, there are several alkaloids that have been discovered from *H. littoralis* bulbs (Yew et al., 2010); such as littoraline, trazettine, o-methyllycorinine, pretazettine, macronine, lycorine, homolycorine, lycorenine, hippeastrine, lycoramine,

demethylmaritidine, haemanthamine, vittatine, and 5,6-dihydrobicolorine (Lweis, 1998), hippeastrine, 11-hydroxyvittatine and two flavonoids quercetin3'-O-glucoside and rutin were isolated (Abou-Donia et al., 2008). In addition, Abou-Donia and coworkers (2008) identified 26 known volatile constituents from *H. littoralis* flowers (Abou-Donia et al., 2008). The primarily isolated lycorine alkaloid from *H. littoralis* was proven to have antineoplastic, cytotoxicity and antiviral properties (Ioset et al., 2001; Yew et al., 2010). Pancratistatin from *H. littoralis* has been proven to be effective against 60 human cancer cell lines including melanoma, brain, colon, lung and renal cancers by U.S. National Cancer Institute's panel (Backhaus et al., 1992; Pettit et al., 1993; Yew et al., 2010). The littoraline alkaloid was demonstrated to have inhibitory activity on HIV reverse transcriptase (Lin et al., 1995). Even though various group of phytochemical constituents were identified from this plant, the scientific database for the biological properties are less. Therefore, we investigate on the anti-candida activity of the *H. littoralis* for leaves, stem, bulbs, anther, flower and root using sonication technique.

Materials and Methods

Plant materials

H. littoralis plants were collected in the Penang state, Malaysia and the authenticity work was carried out by botanist from School of Biological Sciences, University Sains Malaysia.

Sample preparation

Each plant parts were carefully cut and washed with running tap water to remove dirt prior to the drying process. Each of the plant parts (leaves, stem, root, bulbs, flowers, and anther) were cut into small pieces and dried at 40°C for a week to remove the moisture content. The samples were powdered using blender (Panasonic). The powdered plant materials were extracted using methanol solvent by maceration technique to obtain the crude extracts. The extracts were stored under refrigeration (-20°C) condition for further analysis.

Anti-candida assay

H. littoralis's leaves (HL), stem (HS), roots (HR), flowers (HF), bulbs (HB) and anther (HA) crude extracts was subjected for anti-candida activity using disc diffusion, micro-dilution broth and in situ microscopy analysis.

Microorganism

C. albicans was grown in potato dextrose broth (PDB) at 37°C for 24 hours and maintained in potato dextrose agar (PDA) slant at 4°C. However the anti-candida susceptibility tests were carried out using Muller Hinton broth (MHB) and agar (MHA).

Agar disc diffusion assay

H. littoralis's extracts were subjected to disc diffusion technique (Kirby-Baurer) as recommended by National Committee for Clinical Laboratory Standards (NCCLS 2002). No. 0.5 McFarland standardized *C. albicans* culture was spread on the media and sterile discs were placed on the surface of inoculated agar plates. All the techniques were carried out in aseptic condition. The extracts were prepared using 25% of methanol (AR grade, Merck, Germany). Appropriate concentration of the extracts in methanol were applied onto the discs, 50 mg/mL final concentration was obtained for each discs. Standard antibiotic disc miconazole nitrate (30 µg/mL) was served as positive control. Negative control was 25% of methanol. The plates were incubated at 37°C for 48 hours (Hofling et al., 2010). The experiments were repeated in triplicate and the anti-candida activity was evaluated by measuring diameter of the inhibition zone (mm) around the discs.

Determination of minimum inhibitory concentration (MIC) value

Minimum inhibitory concentrations (MIC) of the *H. littoralis's* extracts were carried out using broth dilution technique. MIC is considered as the lowest concentration of the sample, which inhibits the visible growth of fungus (fungistatic concentration) (Sahgal et al., 2009). The inoculums of *C. albicans* was prepared from an overnight culture and standardized to No. 0.5 McFarland to obtain a density of 1x 10^8 CFU/mL. the extracts initially dissolved in 25% of methanol and subsequently serially diluted in MHB, to reach a final concentration in between 0.2 and 10 mg/mL. The positive control was the standard antibiotic in MHB with inoculums and negative control was the MHB and inoculums. The test tubes were gently mixed using vortex and incubated. After 24 hours of incubation at 37°C, the test tubes were observed for turbidity changes. The least concentration which showed no visible growth (turbidity) was denoted as the MIC value for the particular extracts (Kuete et al., 2008 and Sahgal et al., 2009).

Determination of minimum fungicidal concentration (MFC) value

The lowest concentration where no fungal growth is observed on plates is referred as minimum fungicidal concentration (MFC) of a plant extract (Sahgal et al., 2009). This assay is followed from the broth dilution test. The test tubes which showed no visible growth (MIC value) and no turbidity were used for sub-culturing on MHA. One loop of sterile wire loop of inoculums was streak on the MHA media and incubated at 37°C for 48 hours. The lowest concentration which showed the absence of *C. albicans* growth was

recoded as MFC value.

Scanning electron microscopy (SEM) observation

HF and HR extracts were used for SEM observation. A thin film of *C. albicans* cells were smeared on top of a silver stub and coated with gold particles (Polaran (Fisons) SC515 Sputter Coater, U.K.). The gold coated samples observed under SEM instrument (LEO SUPRA 50 VP-SEM, Oxford INCA 400, U.K.). The morphology of the control (*C. albicans* cell without any treatment), HF and HR extracts treated samples at 24 hours were observed under SEM instrument.

Transmission electron microscopy (TEM) observation

The cytological destruction of control and treated *C. albicans* cell (at 24 hours) were inspected under transmission electron microscopy instrument (Libra® 120, ZEISS). The pellet of control, HF and HR treated *C. albicans* was fixed with fixative and post fixed with 1% osmium tetraoxide. Thereafter, the pellet was washed with phosphate buffer solution (pH 7.2), serially dehydrated in ethanol and embedded in resin for making the block of cells pellet. The embedded blocks were trimmed and cut into semi thin sections (90 nm) using ultramicrotome (Sorvall Ultra Microtome MT 5000, Du Pont, USA) with the help of glass knives. The ultra-thin serial sections were stained with uranyl acetate and lead citrate and observed under TEM.

Results

Table I represents the antimicrobial activity of *H. littoralis* for disc diffusion, MIC and MFC against *C. albicans*. The tabulated data shows that, all the extracts show a moderate inhibition zone against the tested strain. However the root and flower extract (HR and HF respectively) shows promising results in MIC and MFC evaluation. The root of *H. littoralis* shows 10.5 ± 0.6 mm of inhibition zone, 6.3 mg/mL of MIC and 25 mg/mL MFC value. The *H. littoralis's* flower extract shows 11.8 ± 1.3 mm, 6.3 mg/mL of MIC and 25 mg/mL MFC value. The inhibition zone value for bulb, anther, leaves and stem are 10.0 ± 1.3, 11.5 ± 1.0, 12.3 ± 1.3 and 12.3 ± 3.0 mm respectively. The MIC and MFC value for bulb, anther, leaves and stem were 12.5 mg/mL and 25 mg/mL respectively. The MIC concentration of the extract shows reduction on the growth of the *C. albicans* and at MFC value the yeast like fungus is fully destroyed at 24 hours in broth dilution technique. To ensure the effect of the extract on *C. albicans*, SEM and TEM microscopy analysis was followed. At the MIC value the extract shows effects on the treated *C. albicans* cells. The HF and HR treated *C. albicans* cells showed cracking effect on the cell membrane compare to control sample. The TEM analysis exhibits the damage on the *C. albicans* cells for both extract. The cell membrane and organelle were distorted for HF and HR treated groups. There were lack of uniformity for the cell membrane and organelles like nucleus. The cell membrane of HF treated *C. albicans* cell was ruptured and could not see any intact cell wall in *C. albicans* cell. The HR treated sample showed lack of uniformity in cell wall and membrane and disappearance of the nucleus. The cell wall of HR treated *C. albicans* was transparent and not dense as the control cells. This shows the HF and HR sample was effective on the *C. albicans* cell.

Table I

Antimicrobial activity of *H. littoralis* for disc diffusion, MIC and MFC against *C. albicans*

Extracts	Disc diffusion (mm)[a]	MIC (mg/mL)[a]	MFC (mg/mL)[a]
Bulb (HB)	10.0 ± 1.3	12.5	25
Anther (HA)	11.5 ± 1.0	12.5	25
Leaves (HL)	12.3 ± 1.3	12.5	25
Root (HR)	10.5 ± 0.6	6.25	12.5
Stem (HS)	12.3 ± 3.0	12.5	25
Flower (HF)	11.8 ± 1.3	6.25	12.5

Discussion

The effect of natural products upon antimicrobial activity has been recognized in many literatures. Plant materials such as extracts, essential oils and isolated compounds have been demonstrated to inhibit the growth of bacteria (Erturk, 2006), yeast (Duarte et al., 2005) and filamentous fungi (Fukai et al., 2003) (Furletti, 2011). *H. littoralis* is a well-known plant for its isolated alkaloids and flavonoid compounds. Due to the lack of scientific investigation data on its pharmacological activity, anti-Candida activity of the *H. littoralis* of various plant part crude extracts was undertaken.

Leaves, stem, bulb, anther, flower and root methanol extract of the *H. littoralis* showed anti-candida activity. The plant extracts showed a clear inhibition zone for all the tested samples (Table I) and the HF and HR extracts (flower and root extracts) exhibit more promising anti-candida activity at 12.5 mg/mL (MIC value) concentration. This findings show, *H. littoralis* plant extract is susceptible for *C. albicans*. Canilac and Mourey (2001) stated that, the susceptibility and tolerance of plant extract can be determined by using MIC/MFC ratio formula. If MIC/MFC ratio of a strain falls to be less than or equal to four, it is considered to be susceptible to the drug. Whereas, if the ratio is greater than four the strain is considered to be tolerant to the extract (Canilac and Mourey, 2001; Mayachiew and Devahastin, 2008). Based on the results, *C. albicans* is sensitive to the HF and HR extracts. The SEM and

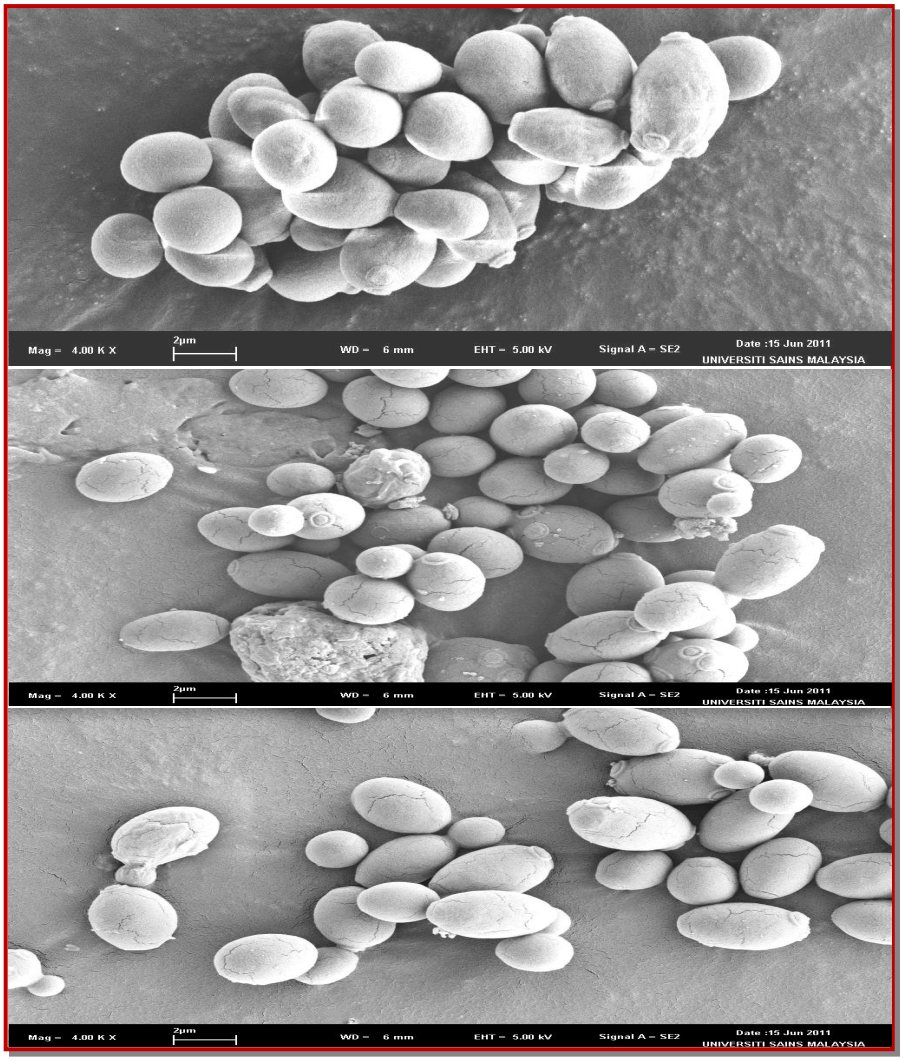

Figure I: SEM results for *C. albicans cells treated with H. littoralis*'s flower and root extract at MIC value. 1a: control; 1b and 1c: flower and root extract treated *C. albicans* at magnification of 4000x

TEM revealed the HF and HR effect on the *C. albicans* cell. The extracts showed morphological changes on SEM micrograph. The extracts treated cells were having cracks on the cell membrane compared to the control group whereby the control group has undisturbed cell membrane. This shows that, the extract is affecting the cell membrane of *C. albicans* and its may contribute for the inhibition of the cell growth.

As to date, one study reported for the antimicrobial activity of the *H. littoralis* plant (Abou-Donia et al., 2008). Conversely, he stated that, the petroleum extract of the *H. littoralis* flower extract does not show activity for *C. albicans* strain. Nevertheless, the HF and HR methanolic extract of *H. littoralis* in this study exhibited anti-candida activity. This is the first report on the anti-Candida activity for the *H. littoralis* extracts. The different solvent extraction may contribute for the various antimicrobial activities. Petroleum ether is a non-polar solvent whereas methanol is a highly polar solvent. This polarity variation might influence the extracted phytochemical constituents in these two different extracts. Polar solvent such as methanol generally extract more polar and non-polar phytochemical substances from natural product. In addition, methanol has proven to be the best high yield and antimicrobial compound extractant (Elof, 1998) compared to other organic solvents.

In further, SEM and TEM microscopy analysis was carried out. This microscopy analysis revealed the morphological changes of treated sample under high magnification and resolution. SEM micrograph exhibits disruption on the cell (Figure 1b and 1c) at 24 hours. The HF extract showed cracks on the treated *C. albicans* cells compared to control group. Besides, there is a mucus kind of secretion on the cells. This show the extracts have interacted with the cell membrane of treated *C. albicans* cells. The TEM micrographs are also exhibiting the similar changes as SEM micrograph. The

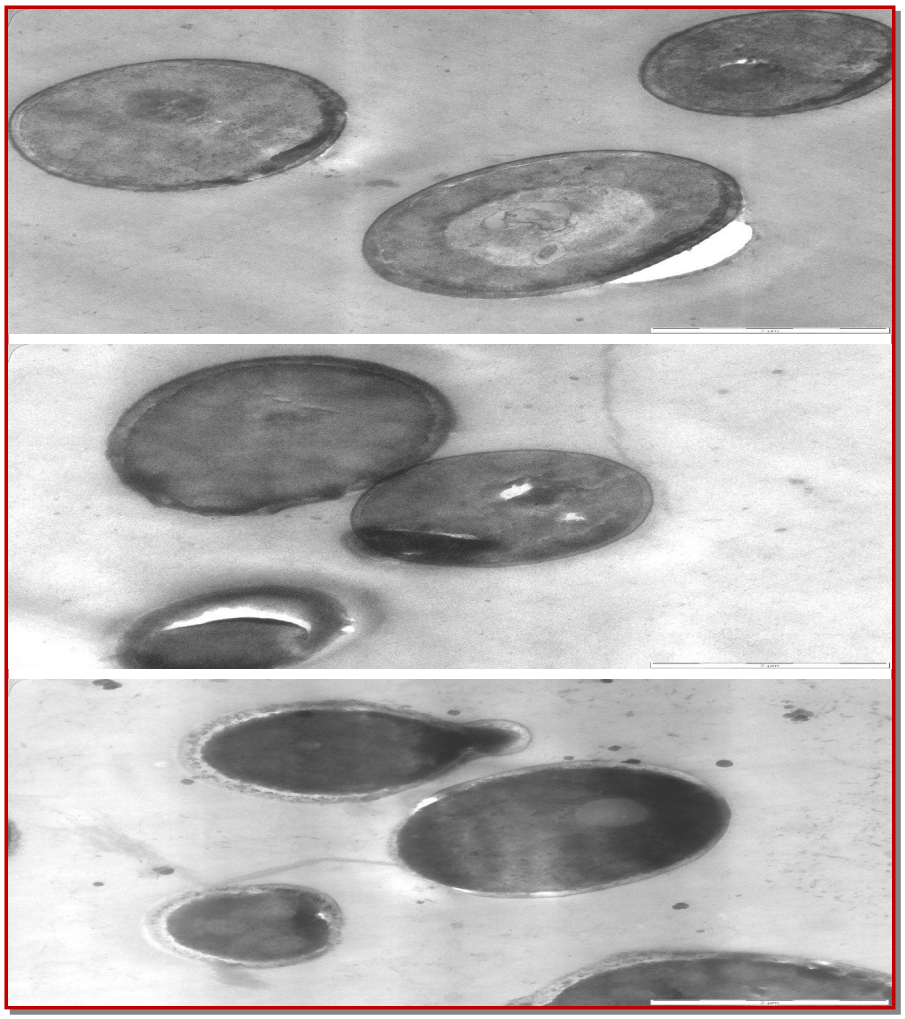

Figure 2: TEM results for *C. albicans* cells treated with *H. littoralis's* flower and root extract at MIC value. 1a: control; 1b and 1c: flower and root extract treated *C. albicans* at magnification of 4000 x

cell membrane of the *C. albicans* were disrupted (Figure 2b) and it lost its intact contact with the cytoplasm (Figure 2c). Moreover, the internal organelles and nucleus also could not be observed in the TEM micrograph. Although the cell structure in SEM and TEM micrograph was in complete form but there is disturbance in cell wall. The rupture of the cell wall is important in this treatment because the broken cell wall is capable to expose the soft lipoprotein membrane in the cell and give an approach for the destruction of the whole cell (http://www.yeastinfectionadvisor.com/structureofcandida.html). Even though the HF and HR extract does not directly burst or damage the cell membrane, but illustrates the changes in the cell membrane and nucleus. There is lack uniformity of the important organelles. Nucleus is the important organelle for a cell to regulate the cellular activity. Distortion in the nucleus will cause the interruption of a cell activity. The destruction caused in the *C. albicans* may be due to the activity of active bioactive(s) in the plant materials. The longer time exposure of the extracts may demonstrate more significant changes on the *C. albicans* cells.

As per literature search there are numbers of alkaloids, volatile oils and flavonoids compounds identified in *H. littoralis*. Generally phytochemical substances are claimed to be the major source of pharmacological effects of a plant extract. Cotoras reported that flavonoids, alkaloids, terpenoids and stilbenes are an important antifungal group in higher plants (Cotoras et al., 2001). Thus the presence of the alkaloids, flavonoids and volatile oils in this *H. littoralis* plant also may contribute for the anti-candida activity.

Conclusion

H. littoralis flower and root methanol extract exhibits anti-candia activity against *C. albicans* strain.

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