Growth profile and SEM analyses of *Candida albicans* and *Escherichia coli* with *Hymenocallis littoralis* (Jacq.) Salisb leaf extract

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**Abstract.** *Hymenocallis littoralis* (Jacq.) Salisb (Melong kecil) commonly known as 'Spider Lily' is an herbaceous plant from the family Amaryllidaceae. Study was carried out to determine the effect of *H. littoralis* leaf extract on the growth and morphogenesis of two pathogenic microbes, *Candida albicans* and *Escherichia coli*. The leaf extract displayed favourable anticandidal and antibacterial activity with a minimum inhibition concentration (MIC) of 6.25 mg/mL. Time kill study showed both microbes were completely killed after treated with leaf extract at 20 h. Both microbes' cell walls were heavily ruptured based on scanning electron microscopy (SEM) analysis. The significant anticandidal and antibacterial activities showed by *H. littoralis* leaf extract suggested the potential antimicrobial agent against *C. albicans* and *E. coli*.

**INTRODUCTION**

Natural products have long been providing important drug leads for infectious diseases (Tempone et al., 2008). Naturally occurring substances of microbial origin have provided a continuing source of antibiotics and medicines since the origin of man (Ogunmwonyi et al., 2010). Nevertheless, the side effects experienced with available drugs, the misuse and over use of antibiotics have led to a wide spread resistance of antibiotics among human, animal and plant pathogens (Mahyudin, 2008).

Due to the outbreak of infectious diseases caused by different pathogenic bacteria and the development of antibiotic resistance, the pharmaceutical companies and the researchers are now searching for new antibacterial agents (Petrus et al., 2011). This has prompted researchers to explore for novel drugs with little or no side effects that could be used to treat infections caused by resistant bacteria pathogens (Ogunmwonyi et al., 2010).

Generally, time kill study will be determined after the disc diffusion and minimum inhibitory concentration (MIC) analysis but prior to SEM analysis. Aiyegoro et al. (2009) reported on the *in vitro* antibacterial time kill studies of *Helichrysum longifolium* extracts by using twenty-three bacteria species of eleven Gram positive and twelve Gram negative strains. It was observed that the minimum inhibitory concentrations (MICs) ranged between 0.5 and 5.0 mg/mL, for acetone and methanol extracts; 0.1-5.0 mg/mL for chloroform extract and 5.0 mg/mL for the ethyl acetate extract; while minimum bactericidal concentrations (MBCs) ranged between 1.0 and >5 mg/ml for all the extracts. In addition,
most of the extracts were rapidly bactericidal at 2 × MIC achieving complete elimination of most of the test organisms within 12 h exposure time.

Olajuyigbe & Afolayan (2012) reported in vitro time kill assessment of crude methanolic stem bark extract of *Acacia mearnsii* against *Shigellosis*. The extract exhibited a varied degree of antibacterial activity against all the tested isolates. The MIC values for Gram negative (0.0391–0.3125) mg/mL and those of Gram positive bacteria (0.0781–0.625) mg/mL indicated that the Gram negative bacteria were inhibited more by the extract than the Gram positive bacteria. Average log reduction in viable cell count in time-kill assay ranged between −2.456 Log10 to 2.230 Log10 cfu/mL after 4 h of interaction, and between −2.921 Log10 and 1.447 Log10 cfu/mL after 8 h interaction in 1× MIC and 2× MIC of the extract. Meanwhile, Ogunmwonyi et al. (2010) reported on in vitro time kill study of marine *Streptomyces* species isolated from the Nahoon beach, South Africa. They reported that between 3 to 4 MIC strength could eliminate more than 50% of the bacteria infection within a period of maximum 6 hours after interaction.

The scanning electron microscope (SEM) is one of the most versatile instruments available for the examination and analysis of microstructural characteristics of solid objects (Kamran, 1997). In addition, scanning electron microscopy (SEM) is an ideal technique for examining plant surfaces at high resolution (Pathan et al., 2008). They found that this technique can alleviate some of the problems encountered in conventional methods of pea leaf processing and visualization. In this study, the whole structure of *Candida albicans* before and after can be seen in detail by using SEM.

*Hymenocallis littoralis* (Jacq.) Salisb is a bulbous, herbaceous plant from the family of Amaryllidaceae (Chai et al., 2010). Its common name is Spider Lily (Rafael & Michael, 2009). It is also known as *Hymenocallis panamensis* Lindl., *Pancratium americanum* Mill., *Pancratium littorale* Jacq. (Ioset et al., 2001; Ocampo & Balick, 2009). For decades, many plants from the Amaryllidaceae plant family had been used as remedy for innumerable illnesses (as been reviewed by Jeevandran et al., 2012). Phytochemical analysis carried out on the bulbs and flowers of *H. littoralis* in Egypt resulted in the isolation of four alkaloids, lycorine (1), hippeastrine (2), 11-hydroxyvittatine (3), and (+)-8-O-demethylmaritidine (4), and of two flavonoids, quercetin 3′-O-glucoside (5), and rutin (6) (Abou-Donia et al., 2008). In addition, new alkaloid, named littoraline, together with 13 known lycorine alkaloids and one lignan, were isolated from *H. littoralis* by Lin et al. (1995). Accordingly, littoraline showed an inhibitory effect on HIV reverse transcriptase while lycorine and haemanthamine produced potent in vitro cytotoxicity. Based on the research by Lamoral-Theys et al. (2010), it stated that lycorine from Amaryllidaceae alkaloids displays very promising anti-tumor properties.

The present investigation was carried out to further substantiate the antimicrobial activity at ultrastructural level. The objective of this research is to determine the effect of the flowering old leaf extract of *H. littoralis* wild plant on the growth and morphogenesis of two selected microbes namely *Candida albicans* and *Escherichia coli* by SEM observations at 6.25 mg/mL concentration. *Candida albicans* and *E. coli* strains was obtained from the School of Biological Sciences, Universiti Sains Malaysia (USM). The yeast was cultured on Sabouraud dextrose agar and bacteria were cultured on nutrient agar at 30ºC for 24 h. The stock culture was maintained on Sabouraud dextrose agar and nutrient agar slants at 4ºC in a refrigerator.

*Hymenocallis littoralis* (Jacq.) Salisb leaf from flowering stage was collected from the Penang Botanical Gardens, Malaysia (Figure 1). The leaves were harvested, washed and dried in the oven at 45ºC for 48 h before grounding using electronic blender. Extraction of the leaves was performed with some modifications from Choo & Chan (2001). One (1) gram of the powdered leaves was extracted with 20 mL of CH$_3$OH: CHCl$_3$ (3:1). The extract was then sonicated for 15
min and the mixture was filtered through four layers of muslin cloth and centrifuged at 12,000 × g for 10 min at 4°C. The extract was stored at 4°C until further use.

Time kill study was performed to evaluate the antimicrobial activity of the leaf extract at ½, 1 and 2 times MIC from 0 h until 32 h and growth curve was plotted. A 16 h culture was harvested by centrifugation, washed twice with sterile phosphate saline buffer (SPSB) and resuspended in SPSB. The suspension was then adjusted using McFarland standard to know the turbidity of the initial suspension and further diluted in SPSB to obtain approximately 10^6 CFU/mL. Reconstituted leaf extract was added to 25 mL Muller Hinton broth (MHB) tubes to achieve a final concentration of ½, 1 and 2 times MIC value (6.25 mg/mL). One (1) mL of the microbes was transferred into nutrient agar for E. coli and potato dextrose agar for C. albicans. Microbes colonies were observed after 24 h of incubated at 37°C, accordingly.

Scanning electron microscopy (SEM) observations were carried out on both C. albicans and E. coli cells. One (1) milliliter of C. albicans and E. coli cell suspension at the concentration of 1 × 10^6 cell/mL was inoculated on a Sabouraud dextrose agar and nutrient agar plate and incubated at 37°C for 24 h and 12 h respectively. About 2 mL of extract at the concentration of 6.25 mg/mL was dropped onto the inoculated agar and further incubated for 0, 8, 16 and 20 h at the same temperature. Culture treated with 10% DMSO was used as control treatment. Small segments (5-10mm) of C. albicans and E. coli was withdrawn from each incubated plate and placed on a planchette. The samples were fixed with 2% osmium tetroxide for 1 h. Finally, samples were freeze dried (Emitech K750) for 10 h before coating with gold for viewing under SEM (FESEM LEO Supra 50 VP, Carl Zeiss, Germany) at different magnifications (Borgers et al., 1989). SEM study was done under the following analytical condition: L = SE2, WD = 7 mm and EHT = 5.00 kV. Growth profile curve in Figure 2 shows the fungicidal activity exhibited by H. littoralis leaf extract against C. albicans. The increase in OD value for control group was evidenced in the plotted growth curve as the diploid fungus was actively growing from 4 to 32 h. The ½ MIC (3.125 mg/mL) extract treated C. albicans showed fast growing of the cells within 8 h to 12 h which dropped at 16 h and continued growing until 36 h. The presence of anti candidal compound

Figure 1. Hymenocallis littoralis (Jacq.) Salisb (Scale bar 1cm : 5cm)
in the extract showed weak activity towards the cells with a slow growth being observed after 16 h until 32 h. At both 1 MIC (6.25 mg/mL) and 2 MIC (12.5 mg/mL), the leaf extract exhibited fungicidal activity at 20 h with large drop in OD values compared to the control and starting inoculums. No cell growth was observed after 20 h which might be due to the cell death caused by the leaf extract. Constant growth of *E. coli* was observed in the control group from 0 h until 32 h (Figure 3). Slightly lower and moderate *E. coli* growth was observed at ½ MIC when compared to control group at 0 until 32 h. At 1 MIC (6.25 mg/mL) there was a drop in OD value at 4 h followed by stationary phase until 12 h. After 20 h, no growth was observed in the growth curve. The leaf extract caused complete eradication of the cells after 20 h at 2 MIC (12.5 mg/mL).

*Hymenocallis littoralis*’s leaf extract inhibited growth of *C. albicans* and *E. coli* at different incubation time. Leaf extract displayed higher fungicidal effect against *C. albicans* compared to bactericidal against *E. coli* (Figures 2 and 3). At 2 MIC, the OD dropped significantly for the extract containing *C. albicans* compared to *E. coli*. Thus, the extract showed that the presence of 3.125 mg/mL extract was more efficient in killing *C. albicans*.

Figures 4 and 5 showed the *C. albicans* and *E. coli* growth obtained using stratified method with the presence of *H. littoralis* leaf extract in the solid media. The solid media was incubated at 37°C from 0 h (1), 4 h (2), 8 h (3), 12 h (4), 16 h (5), 20 h (6), 24 h (7), 28 h (8) and 32 h (9) to support the OD results above. The results obtained showed active growth of both the microbes at 0 h but after 4 h until 16 h slow growth were observed on the solid media. No growth was observed in both microbes between 20 to 32 h. The rate of *C. albicans* infections increased due to limited effective antifungal agents or from the toxic effects of currently available antifungal agents (Runyoro et al., 2006). Results from time kill study confirmed *H. littoralis* leaf extract exhibited antimicrobial activity towards *C. albicans* and *E. coli*.

Figures 6 and 7 showed the SEM observations for control and *H. littoralis* treated leaf extract against *C. albicans* and

![Figure 2](image.png)

Figure 2. Representative time-kill curve plots for *Candida albicans* at the concentrations: 12.5 mg/mL (2 MIC), 6.25 mg/mL (1 MIC) and 3.125 mg/mL (1/2 MIC)
Figure 3. Representative time-kill curve plots for *E. coli* at the concentrations: 12.5 mg/mL (2 MIC), 6.25 mg/mL (1 MIC) and 3.125 mg/mL (1/2 MIC).

Figure 4. Growth of *Candida albicans* against *Hymenocallis littoralis* leaf extract by striating method (6.25 mg/mL) incubated for 1 (0 h), 2 (4 h), 3 (8 h), 4 (12 h), 5 (16 h), 6 (20 h), 7 (24 h), 8 (28 h), 9 (32 h) at 30°C. The scale (1 cm = 0.2 cm) representing the plates above.
Figure 5. Growth of *Escherichia coli* against *Hymenocallis littoralis* leaf extract by striating method (6.25 mg/mL) incubated for 1 (0 h), 2 (4 h), 3 (8 h), 4 (12 h), 5 (16 h), 6 (20 h), 7 (24 h), 8 (28 h), 9 (32 h) at 30°C. The scale (1 cm = 0.2 cm) representing the plates above.

Figure 6. Scanning electron micrographs (SEM) for *Candida albicans* treated with *Hymenocallis littoralis* leaf extract incubated for 1 (0h-Control), 2 (8h), 3 (16h), 4 (20h) at 30°C. The scale (1 cm = 3 cm) representing the plates above. 10,000 x magnification.
Figure 7. Scanning electron micrographs (SEM) view for *Escherichia coli* treated with *Hymenocallis littoralis* leaf extract incubated for 1 (0 h-Control), 2 (8 h), 3 (16 h), 4 (20 h) at 30°C. The scale (1 cm = 3 cm) representing the plates above. 10,000 x magnification

*Escherichia coli* for 0 h, 8 h, 16 h and 20 h. *C. albicans* was seen as oval shape with smooth cell wall in the control treatment (0 h). Budding stage of the cells was observed in this group. The 8 h treated cells displayed rough cell wall. After 16 h treatment distorted cells with the presence of invaginations and convolutions were noted. As the treatment period continued until 20 h, collapsed cells were prominent. A similar finding was reported by Basma *et al.* (2010) on *C. albicans* treated with *Euphorbia hirta* leaf extract between 12 h to 36 h.

Referring to figure 7, rod shaped cells was obvious in the untreated *E. coli* (at 0 h). At 8 h, only mild effect of the leaf extract was seen on the treated *E. coli*. Disappearance of the smooth cell lining was seen in 16 h treated *E. coli*. Collapsed and broken cells were visible at 20 h. The major ultrastructural changes observed on both *C. albicans* and *E. coli* at 20 h suggested that the cells has lost its metabolic functions that resulted in cell necrosis due to the anticandidal and bactericidal activity of *H. littoralis* leaf extract. Therefore, *H. littoralis* leaf extract has the potential to become an anticandidal and antibacterial agent for infections caused by these strains.

*Candida albicans* and *E. coli* treated with the leaf extract at 6.25 mg/mL showed morphological alteration such as cell wall disruption and broken cells. Therefore, *H. littoralis* leaf extract could serve as a potential remedy against microbial infections especially *C. albicans* and *E. coli*. However, further studies on the isolation and identification of active compounds exhibiting antimicrobial activity would be more beneficial for researchers to develop novel antimicrobial agents to overcome the current microbial resistance problem.
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REFERENCES


