Tropical Biomedicine 31(4): 871–879 (2014)

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

Rosli, N.[1,4], Sumathy, V.[2], Vikneswaran, M.[3] and Sreeramanan, S.[1*]
[1]School of Biological Sciences, Universiti Sains Malaysia, Minden 11800, Penang, Malaysia
[2]School of Distance Education, Universiti Sains Malaysia, Minden 11800, Penang, Malaysia
[3]School of Pharmaceutical Sciences, Universiti Sains Malaysia, Minden 11800, Penang, Malaysia
[4]Faculty of Applied Sciences UiTM Negeri Sembilan Branch, Kuala Pilah Campus, 72000 Kuala Pilah, Negeri Sembilan, Malaysia
[*]Corresponding author e-mail: sreeramanan@gmail.com / sreeramanan@usm.my
Received 9 May 2013; received in revised form 17 January 2014; accepted 19 March 2014

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* anti-bacterial 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* spesies 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 30°C for 24 h. The stock culture was maintained on Sabouraud dextrose agar and nutrient agar slants at 4°C 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 45°C 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_3OH: $CHCl_3$ (3:1). The extract was then sonicated for 15

Figure 1. *Hymenocallis littoralis* (Jacq.) Salisb (Scale bar 1cm : 5cm)

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 McFarland standard adjusted suspension each was contained of yeast and bacteria previously prepared as inoculums was added to each solution tube. Extract-free medium served as a control. The growth of *C. albicans* in Sabouraud dextrose liquid containing leaf extract at ½, 1 and 2 times MIC was monitored at predetermined time points at 4 fold time series during 32 h by measuring the optical density (OD) at 540 nm. All solutions were incubated at 37°C in water bath. The growth profile curve was plotted using Microsoft Excel (Basma *et al.*, 2010).

Striating method was performed on solid media using the procedure described by Okoli & Iroegbu (2005). At first, overnight microbe cultures of *E. coli* and *C. albicans* were adjusted to a turbidity equivalent to that of a 0.5 Mc Farland (10^6 CFU/mL). Then 0.5 mL of the microbes was transferred into 4.5 mL extract with a concentration of 6.25 mg/mL. The prepared samples were streaked triplicate at 0, 4, 8, 12, 16, 20, 24, 28 and 32 h 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) mililiter 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

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 striating 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

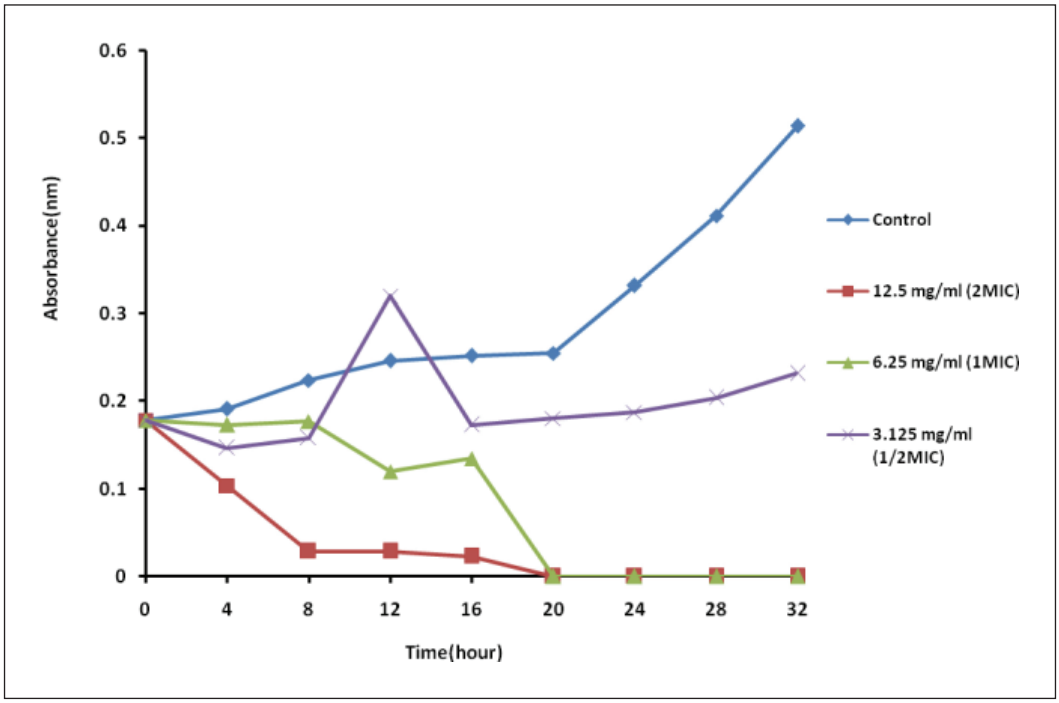

Figure 2. Representative time-kill curve plots for *Candida albicans* at the concentrations: 12.5 mg/mL (2 MIC), 6.25 mg/mL (1 MIC) and 3.125 mg/mL (1/2 MIC)

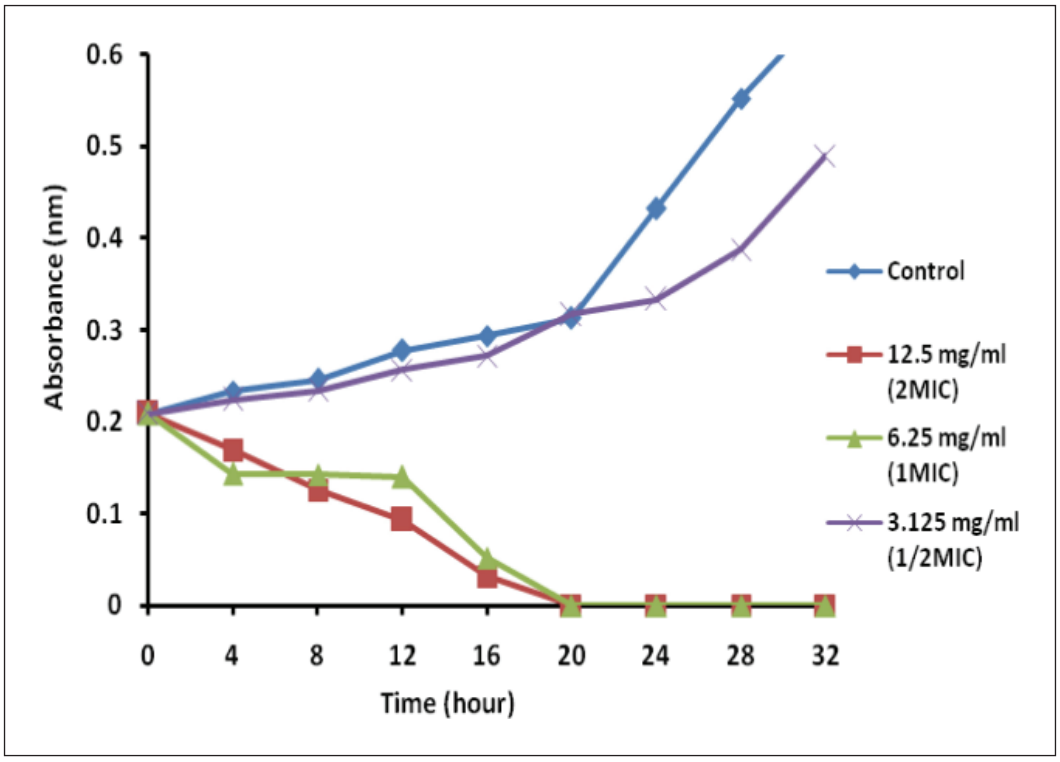

Figure 3. Representative time-kill curve plots for *E. coli* at the concentrations : 12.5 mg/mL (2 MIC), 6.25 mg/mL (1 MIC) and 3.125 mg/mL (1/2 MIC)

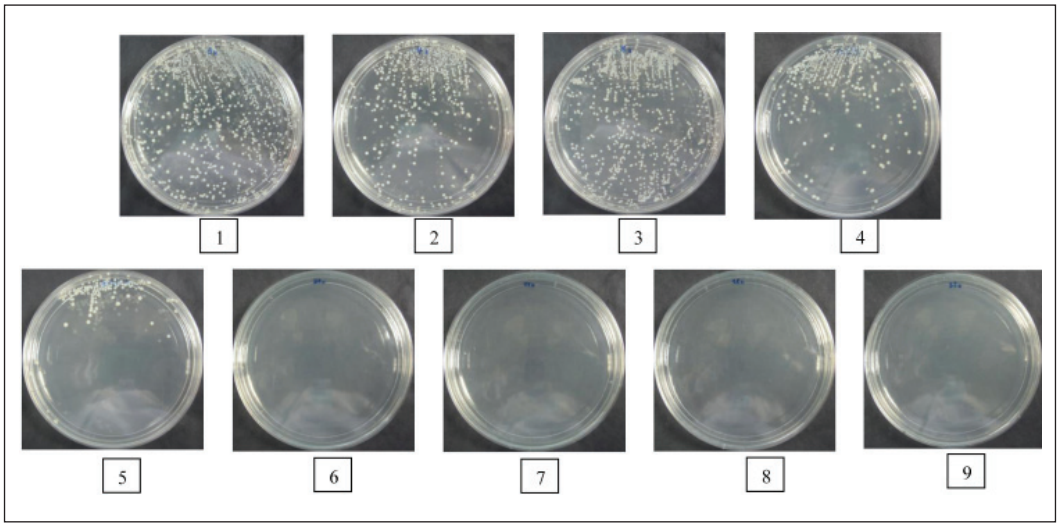

Figure 4. Growth of *Candida albicans* against *Hymenocallis littoralis* leaf extract by striating method (6.25 mg/mL) incubated for 1 (0 h), 2 (4 h), 3 (8 h), 4 (12 h), 5 (16 h), 6 (20 h), 7 (24 h), 8 (28 h), 9 (32 h) at 30°C. The scale (1 cm = 0.2 cm) representing the plates above

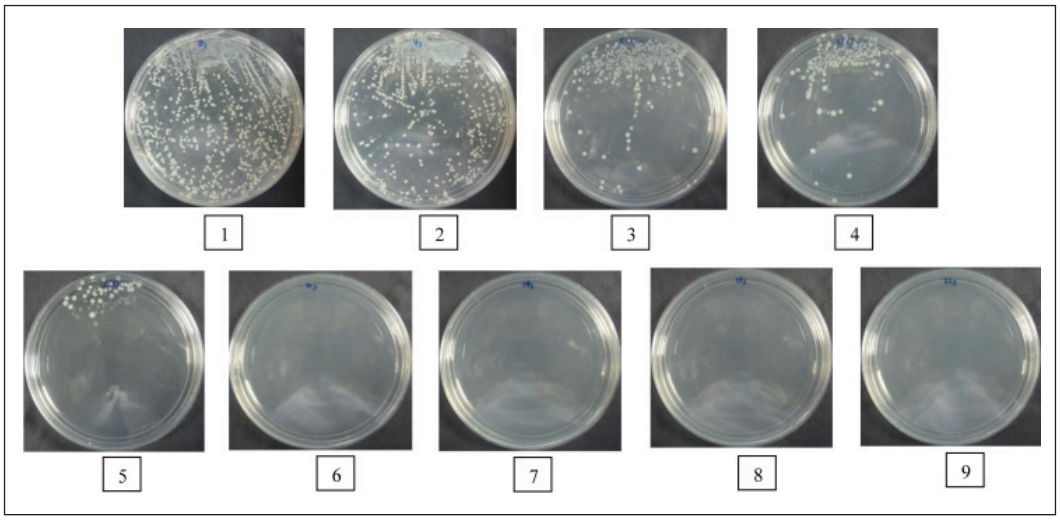

Figure 5. Growth of *Escherichia coli* against *Hymenocallis littoralis* leaf extract by striating method (6.25 mg/mL) incubated for 1 (0 h), 2 (4 h), 3 (8 h), 4 (12 h), 5 (16 h), 6 (20 h), 7 (24 h), 8 (28 h), 9 (32 h) at 30°C. The scale (1 cm = 0.2 cm) representing the plates above

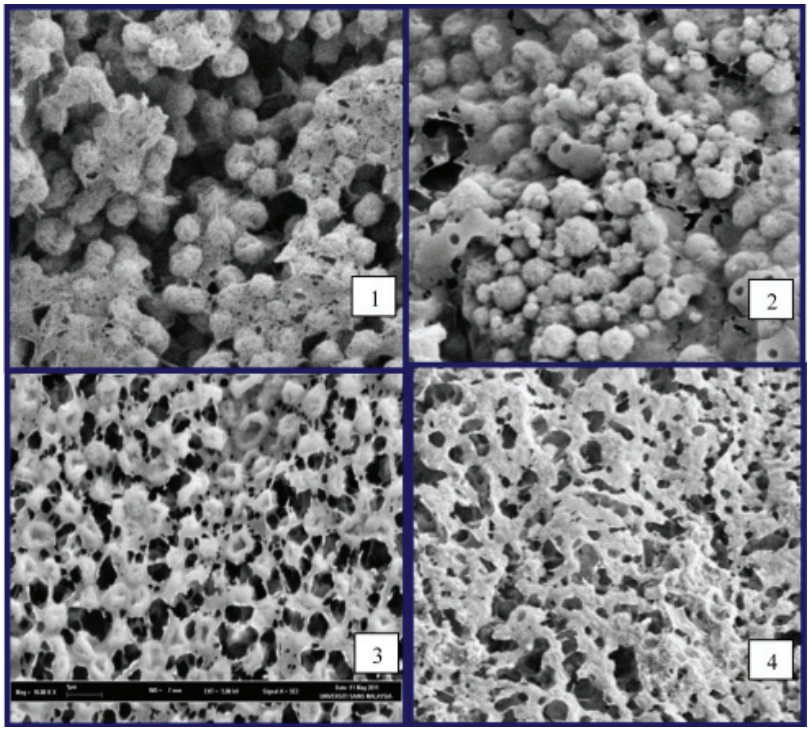

Figure 6. Scanning electron micrographs (SEM) for *Candida albicans* treated with *Hymenocallis littoralis* leaf extract incubated for 1 (0h-Control), 2 (8h), 3 (16h), 4 (20h) at 30°C. The scale (1 cm = 3 cm) representing the plates above. 10,000 x magnification

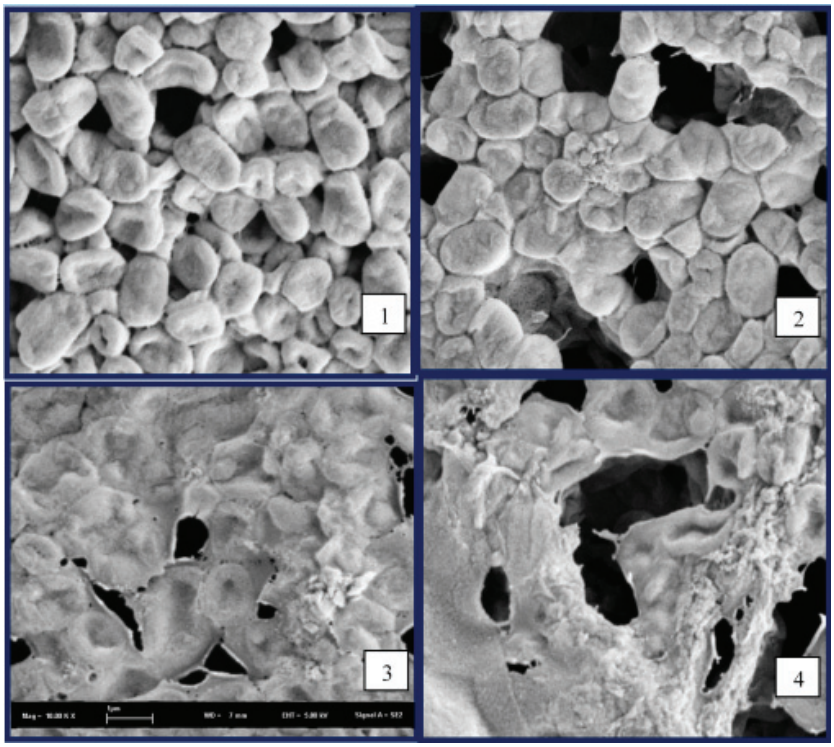

Figure 7. Scanning electron micrographs (SEM) view for *Escherichia coli* treated with *Hymenocallis littoralis* leaf extract incubated for 1 (0 h-Control), 2 (8 h), 3 (16 h), 4 (20 h) at 30ºC. The scale (1 cm = 3 cm) representing the plates above. 10,000 x magnification

E. 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.

Acknowledgements. This research was supported by Universiti Sains Malaysia (USM), Ministry of Higher Education (MOHE) and Universiti Teknologi MARA (UiTM) Scholarship.

REFERENCES

Abou-Donia, A.H., Toaima, S.M., Hammoda, H.M., Kinoshita, E. & Takayama, H. (2008). Pytochemical and biological investigation of *Hymenocallis littoralis SALISB*. *Chemistry and Biodiversity* **5**: 332-40.

Aiyegoro, O.A., Afolayan, A.J. & Okoh, A. (2009). *In vitro* antibacterial time kill studies of leaves extracts *of Helichrysum longifolium*. *Journal of Medicinal Plants Research* **3**: 462-467.

Basma Rajeh, M.A., Zuraini, Z., Sasidharan, S., Yoga, Latha, L. & Amutha, S. (2010). Assessment of *Euphorbia hirta* L. Leaf, flower, stem and root extracts for their antibacterial and antifungal activity and brine shrimp lethality. *Journal of Molecules* **15**: 6008-6018.

Chai, K.Y., Bhavani, B., Jeevandran, S. & Sreeramanan, S. (2010). The effect of cytokinins on *in vitro* shoot length and multiplication of *Hymenocallis littoralis*. *Journal of Medicinal Plants Research* **4**: 2641-2646.

Choo, C-Y. & Chan, K.L. (2001). High performance liquid chromatography analysis of canthinone alkaloids from *Eurycoma longifolia*. *Planta Medica* **68**: 382-384.

Jeevandran, S., Jessica, J.J.A., Vickneswaran, M. & Sreeramanan, S. (2012). Preliminary responses of 2,4-D and BAP on callus initiation of an important medicinal-ornamental *Hymenocallis littoralis* plants. *Journal of Medicinal Plants Research* **6**: 2088-2093.

Kamran, M.N. (1997). Fracture analysis of concrete using scanning electron microscopy. *Scanning* **19**: 426-430.

Lamoral-Theys, D., Decaestecker, Mathieu, V., Dubois, J., Kornienko, A., Kiss, R., Evidente, A. & Pottier, L. (2010). Lycorine and its derivatives for anticancer drug design. *Medicinal Chemistry* **10**: 41-50.

Lin, L.Z., Hu, S.F., Chai, H.B., Pengsuparp, T., Pezzuto, J.M., Cordell, G.A. & Ruangrungsi, N. (1995). Lycorine Alkaloids From *Hymenocallis littoralis*. *Phytochemistry* **40**: 1295-1303.

Ioset, J.R., Marston, A., Gupta, M.P. & Hostettmann, K. (2001). A methyl flavanwith free radical scavenging properties from *Pancratiumlittorale*. *Fitoterapia* **72**: 35-39.

Mahyudin, N.A. (2008). Actinomycetes and fungi associated with marine invertebrates: a potential source of bioactive compounds. Doctor of Philosophy in Microbiology at the University of Canterbury. Ogunmwonyi INH. Actinomycetes diversity of Nahoon beach and Tyume River. Honours dissertation submitted to the department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa.

Ocampo, R. & Balick, M.J. (2009). *Hymenocallis littoralis* (Jacq.) Salisb., in Plants of Semillas Sagradas: An Ethnomedicinal Garden in Costa Rica, (1st Edition). Finca Luna Nueva Extractos de Costa Rica, S.A. pp. 54-55.

Ogunmwonyi, I.H., Mazomba, N., Mabinya, L., Ngwenya, E., Green, E., Akinpelu, D.A., Olaniran, A.O. & Okoh, A.I. (2010). *In vitro* time-kill studies of antibacterial agents from putative marine Streptomyces species isolated from the Nahoon beach, South Africa. *African Journal of Pharmacy and Pharmacology* **4**: 908-916.

Okoli, S. & Iroegbu, C.U. (2005). *In vitro* antibacterial activity of synclisascabrida whole root extracts. *African Journal of Biotechnology* **4**: 946-952.

Olajuyigbe, O.O. & Afolayan, A.J. (2012). *In vitro* antibacterial and time-kill assessment of crude methanolic stem bark extract of *Acacia mearnsii* de wild against bacteria in shigellosis. *Molecules* **17**: 2103-2118.

Pathan, A.K., Bond, J. & Gaskin, R.E. (2008). Sample preparation for scaning electron microscopy of plant surfaces-horses for courses. *Micron* **39**: 1049-1061.

Petrus, E.M., Tinakumari, S., Chai, L.C., Ubong, A., Tunung, R., Elexson, N., Chai, L.F. & Son, R. (2011). A study on the minimum inhibitory concentration and minimum bactericidal concentration of nano colloidal silver on food-borne pathogens. *International Food Research Journal* **18**: 55-66.

Rafael, O. & Michael, J. (2009). Plants of Semillas Sagradas: An Ethnomedical Garden in Costa Rica. *Balick Revista Cubana de Plantas Medicinales* **4**: 61-62.

Runyoro, D.K., Matee, M.I., Ngassapa, O.D., Joseph, C.C. & Mbwambo, Z.H. (2006). Screening of Tanzanian medicinal plants for anti-Candida activity. *BMC Complement Alternative Medicine* **30**: 6-11.

Tempone, A.G., Sartorelli, P., Teixeira, D., Prado, F.O., Calixto, I. A.R.L., Lorenzi, H. & Melhem, M.S.C. (2008). Brazilian flora extracts as source of novel antileishmanial and antifungal compounds. *Memoir Institut Oswaldo Cruz*, *Rio de Janeiro* **103**: 443-449.