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In vitro and *in vivo* anticandidal activity of *Swietenia mahogani* methanolic seed extract

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Abstract. *Swietenia mahogani* crude methanolic (SMCM) seed extract was investigated for the antifungal activity against *Candida albicans* which has not been evaluated previously. The antifungal activity was evaluated against *C. albicans* via disk diffusion, minimum inhibition concentration (MIC), scanning electron microscope (SEM), transmission electron microscope (TEM) and time killing profile. The MIC value of SMCM seed extract is 12.5 mg/ml. The SEM and TEM findings showed there is morphological changes and cytological destruction of *C. albicans* at the MIC value. Animal model was used to evaluate the *in vivo* antifungal activity of SMCM seed extract. The colony forming unit (CFU) were calculated per gram of kidney sample and per ml of blood sample respectively for control, curative and ketaconazole treated groups. There was significant reduction for the CFU/ml of blood and CFU/g of kidney. This indicated that the extract was observed to be effective against *C. albicans in vitro* and *in vivo* conditions.

INTRODUCTION

Candida albicans is an opportunistic pathogen that can cause local and systemic infections in predisposed persons, commonly affecting immunologically compromised patients and those undergoing prolonged antibiotic treatment (Zhang *et al.*, 2002; Duarte *et al.*, 2005). Treatments for these infections are still limited to a few agents. However, the clinical values of these agents have been limited by their relatively high risks of toxicity, the emergence of drug resistance, pharmacokinetic deficiencies, and/or insufficiencies in their antifungal activities (Fan-Havard *et al.*, 1991; Hay, 1991; Law *et al.*, 1994; Yotsuji *et al.*, 1997). Due to this emergence of antibiotic resistant human pathogenic fungi, it is important to develop new antifungal agents. With the increasing acceptance of herbal medicine as an alternative form of health care, the screening of medicinal plants for active compounds was focused.

Swietenia mahogani (Linn.) Jacq. (Meliaceae) is a large, deciduous, and economically important timber tree native to the West Indies. The seeds of this tree have been reported to have medicinal value for treatment of cancer, amoebiasis, coughs, chest pains and intestinal parasitism (Bacsal *et al.*, 1997). The biologically active ingredients, tetranortriterpenoids and fatty acids are considered to be responsible for these therapeutic effects (Bacsal *et al.*, 1997). Maiti *et al.* (2007) reported that the seeds of plant *Swietenia macrophylla* which is from the same genus possess significant antibacterial and antifungal activity. Various pharmacological activities of this plant were reported in the literature such as cytotoxic properties of the crude ethanolic

extracts of seed, bark and leaf and antimicrobial activity of oil extracted from seed (Majid *et al.*, 2004; Akbar *et al.*, 2009). Besides this, Sahgal *et al.* (2009) reported the antioxidant activity of the methanol extract of *S. mahagoni* seeds.

Candida albicans was obtained from Microbiology Laboratory, School of Biological Science, Universiti Sains Malaysia. The *C. albicans* culture was reconfirmed by conducting lactophenol cotton blue staining assay. The stock yeast cultured was maintained on Sabouraud dextrose (SD) agar slants at 4ºC.

The *S. mahogani* seeds were collected from the state of Penang, Malaysia in December 2007. The authenticity was carried out by botanist from School of Biological Sciences, Universiti Sains Malaysia where the plant material (number of voucher: BSM1207) was deposited.

The seed was washed and dried at 40ºC for four days to remove the moisture content before ground to fine powder using blender (New Deluhe, Suruchi, India). The powdered seed was extracted with methanol by means of maceration method. The *S. mahogani* crude methanolic (SMCM) seed extract was filtered through filter paper (Whatman No. 1); the filtrate was collected and concentrated in a rotary evaporator (RII0 Buchi) at 40°C. The concentrated SMCM seed extract was dried in an oven at 40ºC for three days to obtain consistent weight and stored at -20ºC for further analysis.

The disk diffusion (Kirby-Baurer) technique, which is of the recommended standards of the National Committee for Clinical Laboratory Standards (NCCLS), was used for antifungal test. The disc was impregnated with the SMCM seed extract at 100 mg/ml. Methanol was used as negative control and standard antifungal miconzole (30 µg/ml) as positive reference to determine the sensitivity of the strain. The inoculated plates were incubated at 37ºC for 48 h (Karaman *et al.*, 2003). The antifungal activity was evaluated by measuring diameter of the inhibition zone (mm) around the disc. The Minimum Inhibition Concentration (MIC) and Minimum Fungicidal Concentration (MFC) values were determined by using the technique recommended by the National Committee for Clinical Laboratory Standards (NCCLS).

The growth profile of *C. albicans* with half, one and two fold MIC concentration (6.25; 12.5 and 25.0 mg/ml) was plotted over time to assess the fungicidal effect. The SMCM seed extract was added to an aliquot of 25 ml SD broth in an amount which would achieve the concentration 1/2, 1 and 2 fold MIC. An 18 h *C. albicans* culture was harvested and adjusted using no. 0.5 McFarland standard. One milliter of adjusted culture transferred into curative (SMCM seed extract and SD broth) and control (SD broth alone). The samples were incubated on the water bath at 37ºC. After the addition of the adjusted culture, 1 ml of the test sample was diluted and transferred onto SD agar on spread plates. The plates were incubated at 37ºC for 24 h and CFU/ml was determined. The growth of *C. albicans* was measured every 4 h for 48 h (Sangetha *et al.*, 2008).

The methanol treated *C. albicans* (served as control) and the *C. albicans* cells exposed to SMCM seed extract were observed under SEM according to the method described by Sangetha *et al.* (2008, 2009).

The mice were divided in three per group and fed commercial rodent pellets and given water *ad libitum* throughout the experiment. The protocols were approved by the Institutional Animal Ethics Committee (IAEC) of School of Pharmaceutical Sciences, Universiti Sains Malaysia. The positive control mice group was given ketaconazole (10 mg/ml). The curative group of mice were challenged with 2.5 g/kg of SMCM seed extract. All groups of mice were infected with 100 µl of *C. albicans* (1×10^7 CFU/ml) prepared suspension via lateral tail vein injection technique. As for treatment intraperitoneal injection (i.p.) technique was used. The treatment was initiated 24 h after infection and continued for 7 days. On the day 8, the mice were sacrificed by exposing to diethyl ether and followed by cervical

dislocation. Hundred microliter of blood was withdrawn from renal artery into eppendoff tube containing 0.1 ml of heparin (25 U/ml). An aliquot from homogenised kidney and blood were serially diluted and 100 ml of diluted suspensions was transferred to SD agar before incubated at 37°C for 24 h. The colonies were enumerated and the colony forming units (CFU) were calculated per gram of organs (CFU/g) and per mililiter of blood sample (CFU/ml) (Sasidharan *et al.*, 2008). The kidney samples for histopathology evaluation were stained using Periodic Acid Schiff (PAS) reagent and also haematoxylin to identify the *C. albicans* infection in kidney cells. Statistical analysis (ANOVA) was performed using GraphPad Prism Software version 4.0. $P \leq 0.05$ was considered statistically significant for all comparisons.

The extract showed a clear inhibition zone at 100 mg ml^{-1} in disk diffusion assay. The MIC value of the extract against *C. albicans* was 12.5 mg/ml. The MFC of SMCM seed extract was 25 mg ml^{-1}. A time-killing plot of SMCM seed extract against *C. albicans* CFU ml^{-1} over period of 48 h was performed. *Candida albicans* growth in time killing profile was significantly inhibited when treated with 6.25 mg ml^{-1} extract concentration onwards to 25mg/ml concentration where the number of colonies in treatments was much lower than in control group. While in the control group number of cells increased, treatments group maintained the same level or even decreased the number of CFU. The log phase of *C. albicans* started at hour 4 and continues to hour 24 h in control group. However for the extract treated samples, log phase was vary. The log phase for 12.5 and 25.0 mg/ml was up to 28 h and the numbers of *C. albicans* colonies were significantly reduced compared to control during the growth phase. Thus it showed that the SMCM extract inhibit the viability of *C. albicans* and the rate of inhibition is improved with increasing concentration of the extract. SEM studies showed remarkable changes in *C. albicans* morphology in SMCM seed extract treated samples (Figure 1b-1d). The *C. albicans* micrographs growth in both control and treatment samples with MIC concentration of the extract is shown in Figure 1. Morphological changes were observed on the *C. albicans* cells during the treatment hour (4, 12 and 36 h). The cells started to shrink at 4 h after exposure to the extract. At 12 h exposure there was mucus like substances surrounding the *C. albicans* cells and the cells were clumped together (Figure 1c). Finally at 36 h of the treatment the *C. albicans* cells were completely destroyed by the extract. At 36 h only few single colonies were visualised throughout the media (Figure 1d).

Figure 2a shows the control mice kidney which is free from *C. albicans* infection and *C. albicans* infected mice kidney is depicted in Figure 2b. Figure 2c discloses the pseudohyphae of *C. albicans* in infected mice at higher magnification. There was a significant reduction ($P<0.05$) in CFU for kidney (Control = $1.7 \times 10^{11} \pm 8.8 \times 10^{9}$, Ketaconazole = $1.9 \times 10^{10} \pm 7.2 \times 10^{8}$, SMCM extract = $1.0 \times 10^{10} \pm 7.9 \times 10^{8}$) and blood (Control = $8.6 \times 10^{11} \pm 2.7 \times 10^{10}$, Ketaconazole = $2.4 \times 10^{11} \pm 9.0 \times 10^{9}$, SMCM extract = $1.0 \times 10^{11} \pm 1.1 \times 10^{10}$) samples in the curative group when compared to control. The SMCM seed extract has comparable activity with the commercial antifungal ketaconazole.

The SMCM seed extract showed remarkable anticandidal activity against *C. albicans* in both *in vitro* and *in vivo* studies. A strain is considered to be susceptible or tolerant by determining the MBC/MIC ratio (Canilac & Mourey, 2001). If the MBC/MIC ratio is found to be less than or equal to four, the strain is considered to be susceptible to the drug. On the other hand, if this ratio is greater than 4, the strain is considered to be tolerant (Mayachiew & Devahastin, 2008). The result showed the MFC/MIC ratio of SMCM seed extract was less than four. Therefore *C. albicans* was considered to be sensitive to the extract. Gas chromatography analysis of the SMCM seed extract showed the presences of palmitic, stearic and oleic acid in the extract (data not presented

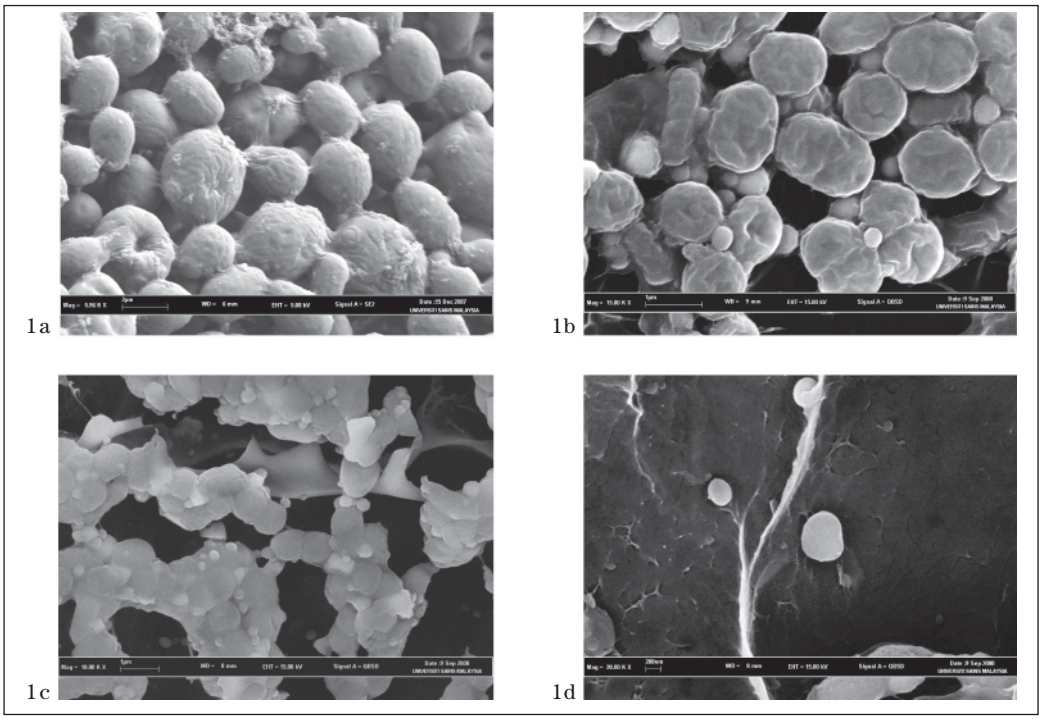

Figure 1. SEM micrograph of the control (1a) and *Swietenia mahogani* crude methanolic extract (12.5 mg/ml) treated *Candida albicans* cells at various exposure times. 1b: 4h of treatment; 1c: 12h of treatment; 1d: 36 h of treatment

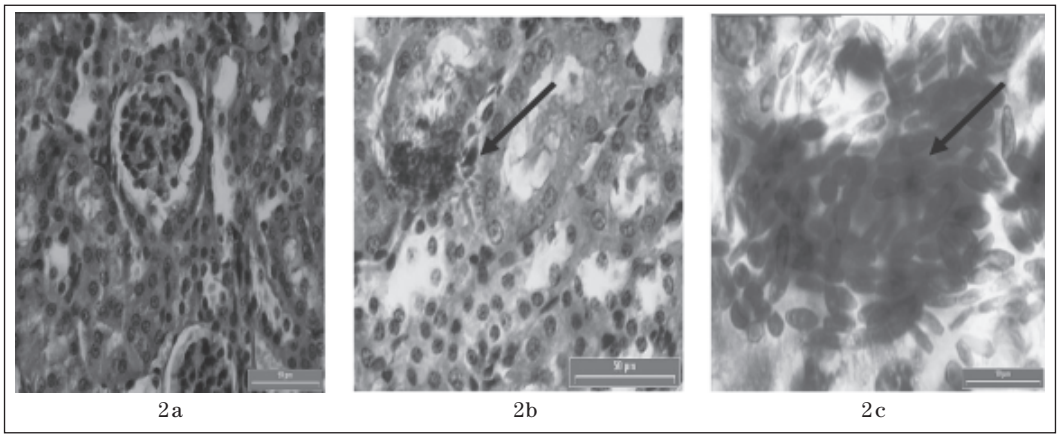

Figure 2. Histopathology image of control mice kidney (2a) and *C. albicans* infected kidney mice 2b (low magnification) and 2c (100x magnification). Figure 2c shows the pseudohyphae of *C. albicans*

here). Phytochemical analysis of SMCM seed extract showed presence of saponins, phenols, volatile oils, alkaloids, antraquinones and terpenoids (Sahgal *et al.*, 2009). The antimicrobial activities of SMCM seed extract could be attributed to these phytochemical substances which need further studies for confirmation. Fatty acids can act as anionic surfactants and have antibacterial and antifungal properties at low pH by targeting the structure, cell wall and membrane (Nalina & Rahim,

2007). The acid production inhibition is related to the energy producing process or glycolysis. It was found that the reducing effect on acid production is comparable to the reduction in its growth rate (Sheu & Freese, 1972; Nalina & Rahim, 2007). SMCM seed extract contains palmatic, oleic and stearic acid hence could be the bioactives responsible for diminished *C. albicans* growth rate (Figure 2d).

As for *in vivo* study, infected mice challenged with SMCM seed extract also showed significant reduction of both blood and kidney CFU counts when compared to control group. The treated kidney failed to demonstrate the presence of either *C. albicans* or pseudomycelia. Khan *et al.* (2003), stated that the candidacidal pathway in murine neutrophils is nitric oxide (NO) dependent. NO is responsible for defense against pathogens that survive and proliferate in the intracellular environment of many somatic cells (Fierro & Fidalgo, 1996). There is possibility for the plant extract to contain bioactive substances which directly or indirectly stimulate the granulocytes and monocytes to generate NO and eradicate *C. albicans*. The seven days i.p administration of SMCM seed extract to mice was highly effectual in the prevention and treatment of candidiasis. On the whole SMCM seed extract is effective against *C. albicans* in both *in vitro* and *in vivo* studies.

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