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Evaluation of the effect of *Cassia surattensis* Burm. f., flower methanolic extract on the growth and morphology of *Aspergillus niger*

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Abstract. – BACKGROUND AND AIM: *Cassia (C.) surattensis* Burm. f. (Leguminosae), a medicinal herb native to tropical equatorial Asia, was commonly used in folk medicine to treat various diseases. The aim of the present study is to investigate the effects of methanolic flower extract of *C. surattensis* against *Aspergillus (A.) niger*.

MATERIALS AND METHODS: Antifungal activity of *C. surattensis* flower extract was studied by using agar disc diffusion method, broth dilution method, percentage of hyphal growth inhibition and scanning electron microscopy (SEM) observation.

RESULTS: The extract exhibited good antifungal activity with zone of inhibition 15 mm and minimum inhibitory concentration (MIC) 6.25 mg/ml. The flower extract exhibited considerable antifungal activity against *A. niger* with a IC_{50} of 2.49 mg/ml on the hyphal growth. In scanning electron microscopy (SEM) squashed, collapsed, empty and deformation of hyphae were the major changes observed. Shrunken conidiophores were the obvious alteration on the spores. Morphological alterations observed on *A. niger* caused by the flower extract could be the contribution of chemical compounds present in the *Cassia* flower. Phytochemical screening reveals the presence of carbohydrate, tannins, saponins and phenols in the extract. The amount of tannin, total phenolics and flavonoids were estimated to be 55.14 ± 3.11 mg/g, 349.87 ± 5.41 mg/g gallic acid equivalent and 89.64 ± 3.21 mg/g catechin equivalent respectively.

CONCLUSIONS: *C. surattensis* flower extract potently inhibited the growth of *A. niger* and are, therefore, excellent candidates for use as the lead compounds for the development of novel antifungal agents.

Key Words:
Cassia surattensis, Aspergillus niger, Antifungal, Phytochemical screening.

Introduction

In most of the Asian tropical countries like Malaysia and Indonesia floral diversity has been an important asset to mankind to be explored for various therapeutic purposes. Since ancient days, these medicnal plants and herbs were used for therapeutic purposes due to the presence of phytochemical with various medicinal properties. Literature has proven 65% of the world's population depended on medicines derived from natural product such as herbs and edible plants[1]. Natural resources are always favorable especially those which are edible because they are rich with phytochemicals, vitamins and minerals. On the contrary, it is not deniable that some medicinal plants can be toxic and poisonous to human if proper dose is not consumed.

Cassia surattensis Burm. f., shrub belongs to the family of Leguminosae and locally called as bushy cassia. It is commonly found along the roadside in Malaysia. The height of this shrub is normally about 6 m tall with bipinnate and broad leaves. *C. surattensis* is a very popular herb amongst practitioners of traditional medicine, widely used as a decoction or infusion to treat various ailments. In Chinese traditions, leaves are used to treat constipation problem, sore throat and cough by consuming the infusion of boiled leaves orally. A study on antioxidant assessment on *C. surattensis* flowers proved the flowers have good antioxidant property[2]. Bark and leaves of this shrub is believed to be antihemorrhagic. It is also believed that Balinese rub the leaves of *C. surattensis* into both external and internal cooling medicine[3]. Until today very little literature has been reported on the medicinal properties of

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C. surattensis. Therefore, the present study was conducted to evaluate the antifungal properties of *C. surattensis* flower against human pathogenic microorganism *A. niger*.

Materials and Methods

Plant Collection and Extraction

C. surattensis flowers were collected in Universiti Science Malaysia, Penang. The flowers were washed under the running water and dried in the oven at 50°C for 24 h. Dried flowers were milled to a fine powder using an electronic blender[1]. 200 g of the powdered plant material were macerated and percolated with 600 mL of methanol in a beaker for 4 days. The solvent was than filtered using No. 1 Whatman filter paper and evaporated using rotary evaporator. Crude extract obtained in a paste form was stored at room temperature for further use.

Bacterial and Fungal Test Organism

Aspergillus niger used in this study was obtained from the School of Biological Sciences, USM Penang. The fungus was maintained in Potato Dextrose Agar (PDA) at 4°C until further use.

Antimicrobial Assay

Agar disc diffusion method was used for antifungal susceptibility test. Inoculums of spore suspension spread uniformly on the plate. Sterile 6 mm discs were placed on the plate and loaded with 25 µl of methanolic extract (100 mg/ml). Similarly 25 µl of 100% methanol loaded for negative control and miconazole nitrate (30 µg/ml) for fungus positive control. The fungus plate was incubated at 28°C for 48 h. Zone of inhibition was measured and recorded. This experiment was performed in triplicates.

Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration of the plant extract was determined using broth dilution method. The test extract was dissolved in methanol to obtain 1000 mg/ml of stock solutions. 1 ml of the stock solution was pipetted into potato dextrose broth for fungus to obtain the concentration as 100 mg/ml. Two-fold serial dilution was performed and 500 µl of test culture were added into each tube. Turbidity of the test culture was adjusted using the 0.5 McFarland standard. Control tubes contained organisms, broth and extract individually. Tubes with fungus were incubated at 28°C for 48 h. MIC value indicate the tubes with the lowest concentration without any growth of the microorganisms[4].

Minimum Fungicidal Concentration (MFC)

This test was performed to determine the antifungal activity of *C. surattensis* flower extract against *A. niger* as percentage of hyphal growth inhibition. Serial dilutions were performed with the test solution and PDA to give the final concentrations as 100, 50, 25, 12.5, 6.25, 3.125, 1.562, 0.78, 0.39 and 0.195 mg/ml. The mixture was than mixed well and poured onto Petri plates. 1 mm plugs of fungal mycelium were cut from the actively growing culture and placed on the centre of each Petri plate after solidification of the PDA medium. Methanol was used as negative control and Miconazole nitrate as positive control. The plates were incubated at 28°C for 48 h. Diameter of the *A. niger* growth was measured and the results were expressed as percentage of hyphal growth inhibition. IC_{50} was calculated for the % inhibition of hyphal growth against concentration graph.

Sample Preparation for Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (FESEM LEO Supra 50 VP, Carl Zeiss, Germany) was performed on *A. niger* which was treated with and without *C. surattensis* flower extract. The treated sample was taken at various incubation periods [0 (control), 3, and 7 days] for SEM examinations. Small plugs of 5-10 mm samples were cut and placed on a planchette. The samples were than fixed with 2% osmium tetraoxide for 1 h and followed by freeze drying process for 10 h. Finally the specimens were coated with gold before viewing the sample at different magnifications[5].

Phytochemical Screening

Standard procedures for phytochemical screening described by Evans[6], Parekh and Chanda[7] and Mishra et al[8] were adopted to screen the methanolic extract of *C. surattensis* flower.

Determinations of Total Phenolic Contents

Total phenolic content of *C. surattensis* flower extract was determined using the Folin-Ciocalteau assay according to the previously described method[9]. Total phenolic content was calculated

from the calibration curve of a gallic acid standard solution. Results were expressed as gallic acid equivalents, in mg/g dry extract.

Determination of Total Flavonoid Content

The amount of total flavonoid in the *C. surattensis* flower extract was measured spectrophotometrically following method described by Djeridane et al[10]. Catechin was used as standard to construct a calibration curve. Various concentrations of *C. surattensis* flower extract (1.5 mL) were mixed with of 2% methanolic AlC_{13} solution (1.5 mL). After incubation at room temperature for 15 min, the absorbance of the reaction mixture was measured at 430 nm (UV-9100; Ruili Co., Beijing, China). The flavonoids content was expressed as mg catechin equivalents (CE) in 1 g extract (mg CE/g).

Tannin Estimation

Tannins were estimated spectrophotometrically[11], with minimal modifications. 0.5 ml of suitably diluted extract was taken in a test tube and volume was made up to 2.5 ml with distilled water. 0.25 ml of 1:19 diluted Folin Ciocalteau reagent and 0.5 ml of 20% sodium carbonate solution were added. The solution was kept for 30 minutes at room temperature. Subsequently, absorbance was measured at 775 nm (UV-9100; Ruili Co., Beijing, China) and concentration was estimated with respect to tannic acid as standard. Total tannin in the extract was expressed as equivalent to tannic acid (mg TE/g extract).

Results

Antimicrobial Activity

Methanol extract of *C. surattensis* flower showed significant inhibition zone against *A. niger*. The diameter of the inhibition zone measured for *A. niger* was 15 mm. The extract showed MIC value of 6.25 mg/ml against *A. niger* (Table I). The fungicidal activity against *A. niger* was determined by the agar dilution method. Percentage of hyphal growth inhibition of this fungus was determined at concentrations 0.195, 0.39, 0.78, 1.562, 3.125, 6.25, 12.5, 25, 50 and 100 mg/ml. 100% inhibitions were observed at 12.5 mg/mL. The percentage reduced from 62% at 6.25 mg/ml to 0 at 0.195 mg/ml (Table I). IC_{50} value as 2.49 was obtained from the graph plotted.

SEM Observations of Aspergillus niger Morphology

The efficiency of *C. surattensis* flower crude extract on the ultrastructure of *A. niger* (myceli-

Table I. Antimicrobial activity of *Cassia surattensis* flower.

Mircoorganisms	Zone of inhibition (mm)[a,b]			MIC[c] (mg/ml)	MFC[d] (mg/ml)	Concentration tested for hyphal growth inhibition assay (mg/ml)	Hyphal growth inhibition atselected concentration (%)[b]	
	Cassia surattensis flower extract	M	ME				Cassia surattensis flower extract	M
Aspergillus niger	15	22	0	6.25	12.5	0.195	0	ND[e]
						0.39	18	ND
						0.78	24.7	ND
						1.562	42	ND
						3.125	55.3	ND
						6.25	62	100
						12.5	100	100
						25	100	100
						50	100	100
						100	100	100
						IC^{50}	2.49	< 0.5

[a]Disk diffusion technique; [b]The values (average of triplicate) are diameter of zone of inhibition at 100 mg/ml crude extract, 30 µg/ml Chloramphenicol and 30 µg/ml Micanozole nitrate; [c]Broth dilution method; [d]Agar dilution method; [e]ND, not detected. The MIC values for Escherichia coli, Bacillus subtilis, Micrococcus sp, Proteus mirabilis, Staphylococcus aureus, Candida albicans and Rhizopus sp also determined and the values were 12.5 mg/ml, 6.25 mg/ml, 3.125 mg/ml, 3.125 mg/ml, 1.562 mg/ml, 6.25 mg/ml and 3.125 mg/ml respectively. M = Micanozole nitrate; ME = methanol.

um and conidiophores) treated with and without the extract is shown in Figure 1a-f at the same magnification. Untreated (control) *A. niger* appeared as thick tube like hyphae and with spherical conidiophores attached together as a network in a chain form (Figure 1a and b). When *A. niger* treated with *C. surattensis* flower extract a major alteration on the morphology of hyphae was observed as the incubation period increased. There is obvious changes observed on the hyphae on Day 3 (Figure 1c) where the smooth hyphae cell wall was flattened and shrunken compared to the control cells. Folding, empty and collapsed hyphae were visible on Day 7 (Figure 1e). Treated conidiophores on Day 3 (Figure 1d) did not showed many changes on the microscopic observations. The activity of the extract was observed on the spores on Day 7 where the spore arrangement was altered, outer layer were distorted and shrunken spores were observed at the end of the treatment period (Figure 1f).

Phytochemical Analysis

Phytochemical screening on the methanolic extract revealed the presence of active compounds such as tannins, saponins, phenols, carbohydrate and reducing sugar.

Total Phenolic and Flavonoid Content

The total phenolic content of *C. surattensis* flower extract was determined to be 349.87 ± 5.41 mg/g gallic acid equivalent. In addition, the total flavonoid content of *C. surattensis* flower extract was 89.64 ± 3.21 mg/g catechin equivalent.

Tannin Estimation

Estimation of total tannin is based on oxidation of molecules which contain phenolic hydroxyl groups. For *C. surattensis* flower extract, total tannin was found to be 55.14 ± 3.11 mg/g of samples.

Discussion

Out of the 350,000 plant species identified so far, about 35,000 (some estimate up to 70,000) are used worldwide for therapeutic reasons and less than about 0.5% of these have been studied for their phytochemical and pharmacological potential[12]. This medicinal plants and herbs, thus, embody a huge pool of putative lead bioactive compounds to be exposed for the development of various therapeutic agents for human diseases. *C. surattensis* alleged to have many medicinal properties. Voon et al[13] reported that methanol extract of *C. surattensis* flower exhibited good antibacterial and antifungal activity against *Proteus mirabilis, Staphylococcus aureus, Escherichia coli*, *Salmonella typhi, Micrococcus* spp., *Enterobacter aerogenes, Bacillus subtilis, S. sonnei, A. lipoferum, Klebsiella pneumoniae, Pseudomona aeruginosa*, *Candida albicans* and *Aspergillus niger*. Moreover, Sangetha et al[14] reported that *C. surattensis* flower exhibited good antioxidant activity. They reported that the *C. surattensis* flowers extract revealed the best antioxidant property, presenting much lower IC_{50} values (423.32 µg/ml for DPPH assay and 11.1 µg/ml for xanthine oxidase assay). Furthermore, the highest antioxidant contents (polyphenols) were found for these extracts (657.2392 ± 2.0321 mg GAEs/g extract). Therefore, in this research *C. surattensis* flowers were used to evaluate the antimicrobial activity against *A. niger*. Furthermore, we mainly focus on *A. niger* because it may cause allergic bronchopulmonary disease, invasive aspergillosis, or may be a colonizer of natural or preformed cavities of the human[15,16].

From the antimicrobial screening favorable zone of inhibition was obtained for *A. niger*. For MFC the flower extract showed only moderate reduction (42%) at 1.562 mg/ml of hyphal growth, while inhibition was substantial at 12.5 mg/ml with 100%. At 6.25 mg/ml of extract in broth medium fungal growth was completely inhibited but for MFC at 12.5 mg/ml. Earlier antifungal investigations on the aqueous and methanolic extracts of Roman chamomile (*Anthemis nobilis*) dried leaves and *Matricaria (M.) chamomilla* flower heads was done by Magro et al[17] and Rauha et al[18]. Magro et al[17] reported that the aqueous extract of Roman chamomile dried leaves was able to inhibit *A. niger* growth at a concentration of 920 mg/ml. Meanwhile, Rauha et al[18] also shown that the methanolic extract of *M. chamomilla* flower heads also possessed inhibitory effect against the fungus. The flower extract significantly reduced the growth of *A. niger* in a dosage response manner. Antifungal activity by the MIC and MFC methods were further verified by SEM observations. Flower extract completely squashed and severely collapsed hyphae were observed from SEM study, which results in flattening and ultimately death of hyphae. The hyphae showed lack of cytoplasm, damage and loss of integrity and rigidity of the cell wall.

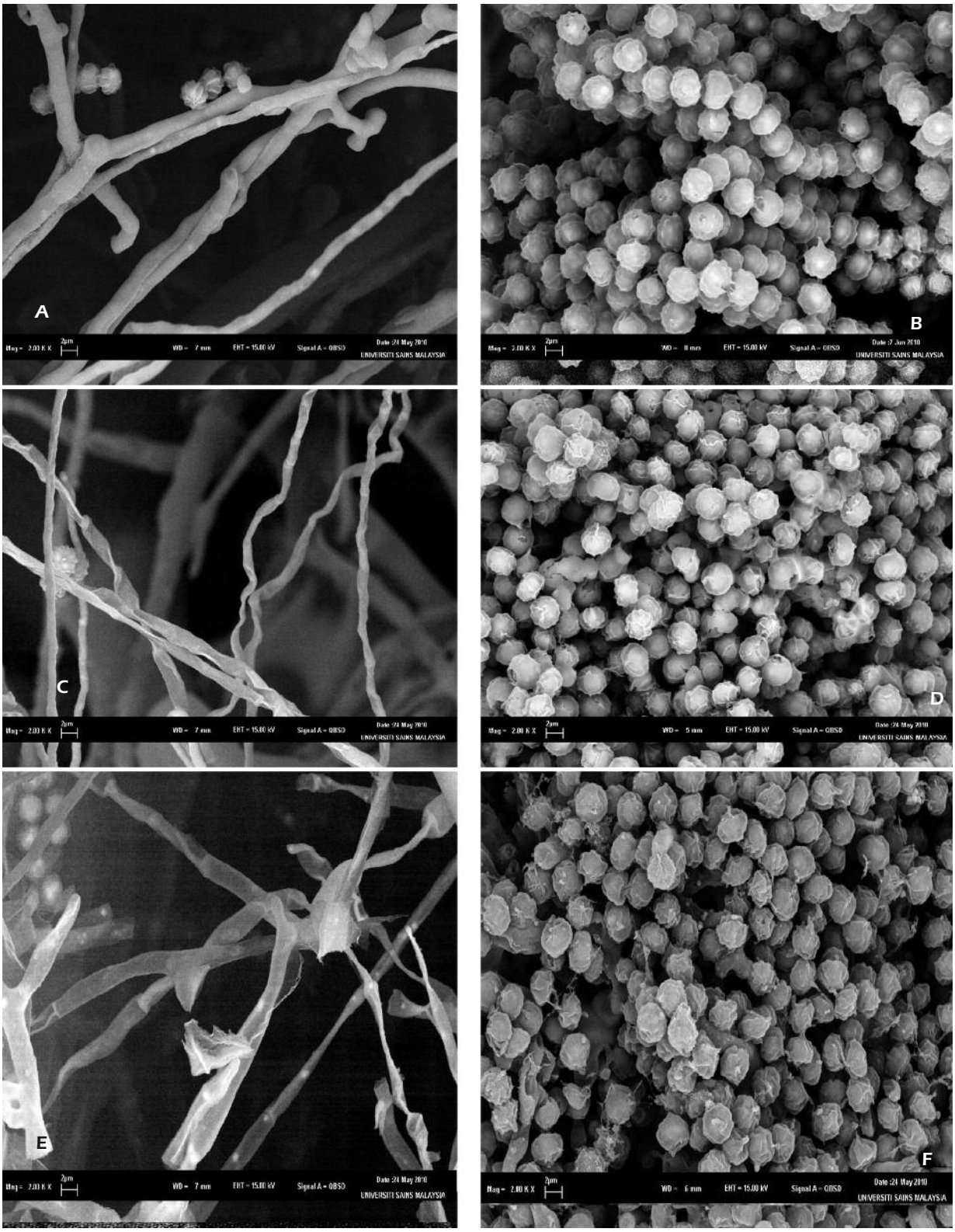

Figure 1. Scanning electron micrographs of *A. niger* conidiophores grown on PDA with and without *C. surattensis* flower extract during 7 days of incubation at 28°C. ***A*** and ***B***, control mycelium and conidiophores. ***C*** and ***E***, flattened and squashed mycelium; and (***D*** and ***F***) shrunken conidiophores treated with 6.25 mg/ml of extract.

Treated conidiophores did not demonstrate many alterations on the SEM microscopic observations. These findings indicate that the mode of antifungal activity of flower extract of *C. surattensis* is an outcome of attack of extract on the plasma membrane and retraction of cytoplasm in the hyphae and ultimately death of the *A. niger* mycelium. Alternatively, Tolouee et al[19] reported such alteration induced by extract may be related to the interference of bioactive components with enzymatic reactions of wall synthesis, which affects morphogenesis and growth of *A. niger*. To confirm this, we will investigate through transmission electron microscopy (TEM) in our future studies. Similar results of ultrastructural changes of fungal morphology have been reported for *A. niger* hyphae treated with *Cympobogon nardus*[20], *Citrus sinensis*[21], *Thymus eriocalyx* and *Thymus x-prolock*[22] and *Cymbopogon citratus*[23].

The objective of performing phytochemical screening is to preliminarily characterize the active principles that are responsible for observed bioactivity by the extract when treated against microorganism[24]. In this study the presence of various secondary metabolites in *C. surattensis* flower extract may contributed to the observed antifungal activity. Previous study on *Terminalia* species reported that the antifungal activity observed in their study could be due to the presence of tannins and saponins[25]. Tannins at high concentrations can act as antifungal agents by coagulating the protoplasm of the microorganism[26]. Saponins is commonly stored in plant cells as inactive precursor but easily can be converted into biologically active antibiotics by enzyme activities immediately after infection. This compound will act by disrupting the membrane integrity of fungal cells. Phenolic and flavonoid compounds that were presence in the methanolic extract of *C. surattensis* flower is also reported to possessed antimicrobial activity[27]. It is possible that these compounds were mainly responsible for the observed antibacterial and antifungal effects in this study. However, further phytochemical studies are needed to isolate the active compound(s) responsible for this pharmacological activity.

Conclusions

At present, there is growing interest in using natural products such as medicinal plant extracts for the development of antifungal agents to reduce the infection of pathogenic microorganism. Therefore, *C. surattensis* flower extract is appears to have promise as a safe alternative natural product. The present study clearly shows the mode of action of extract against the test pathogen *A. niger*. In conclusion, *C. surattensis* flower extract is an excellent candidate for use as the lead compounds for the development of novel antifungal agents.

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Conflict of Interest

None to declare.

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