In vitro and in vivo antifungal activity of Cassia surattensis flower against Aspergillus niger

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A B S T R A C T
Invasive aspergillosis (IA) in immunocompromised host is a major infectious disease leading to reduce the survival rate of world population. Aspergillus niger is a causative agent causing IA. Cassia surattensis plant is commonly used in rural areas to treat various types of disease. C. surattensis flower extract was evaluated against the systemic aspergillosis model in this study. Qualitative measurement of fungal burden suggested a reduction pattern in the colony forming unit (CFU) of lung, liver, spleen and kidney for the extract treated group. Galactomannan assay showed a decrease of fungal load in the treatment and positive control group with galactomannan index (GMI) value of 1.27 and 0.25 on day 28 but the negative control group showed high level of galactomannan in the serum with GMI value of 3.58. Histopathology examinations of the tissues featured major architecture modifications in the tissues of negative control group. Tissue reparation and recovery from infection were detected in extract treated and positive control group. Time killing fungicidal study of A. niger revealed dependence of the concentration of C. surattensis flower extract.

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1. Introduction
Invasive aspergillosis cause by various Aspergillus sp can be life-threatening in an immunocompromised host [1]. Spores from Aspergillus sp are the responsible agents for the development of aspergillosis. Invasive aspergillosis (IA) has become a leading cause of fatality in the human population especially in the countries that is rising with globalization effects [2]. Patients with prolonged neutropenia, hematological malignancies, receiving high dose corticosteroids therapy, underwent stem cell and solid organ transplantation or with advanced HIV are becoming common victim of IA [3–5]. Aspergillus niger, a filamentous fungi was identified as one of the primary causative agent to IA from Aspergillus genus [6].

A. niger is ubiquitous in the environment. The ecological niche of these fungi is in soil, compost, litter and on decaying plant material. It can grow in wide temperature ranging from 6 to 47 °C. Aspergilli produce small hydrophobic conidia that disperse easily into the air and survive broad range of environmental conditions [7]. Although there is other mode of aspergillosis infection commonly develops as a result of inhalation of Aspergillus spores. Spores from the atmosphere enter the lung and forms tangled mass of fungus fibers. The fungal mass enlarge gradually and destroys the lung tissue in the process, but usually does not spread to other areas unless under certain circumstances. Using the synthetic chemicals to inhibit the fungal growth leads to negative consumer reaction due to different ecological and medical problems - residual toxicity, carcinogenicity, teratogenicity, hormonal imbalance, spermatotoxicity, etc. [8,9]. This problem has become a challenge for scientific investigators to search for alternative antifungal agents from natural resources.

Plant extracts of many higher plants have been reported to exhibit antifungal activity. Based on this challenge, the present study was undertaken to evaluate the efficacy of Cassia surattensis Burm. f., flower extract against experimental aspergillosis. Recent studies also reported the in vitro antifungal activity C. surattensis by Sumathy et al. [10]. The results of this study confirmed that the

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methanol extracts of *C. surattensis* exerted significant antifungal activity against *A. niger* with an IC$_{50}$ of 2.49 mg/ml on the hyphal growth.

*C. surattensis* 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 antioxidiant assessment on *C. surattensis* flowers proved the flowers have good antioxidiant property [11]. Bark and leaves of this shrub is believed to be antihemorrhagic. It is also believed that Balinese rub flowers proved the flowers have good antioxidiant property [11]. Bark and leaves of this shrub is believed to be antihemorrhagic. It is also believed that Balinese rub flowers were collected in Universiti Sains Malaysia, Pulau Pinang, Malaysia. The flowers were separated and cut into small pieces, which were first washed with tap water and then with distilled water. The flowers were then dried in an oven at 60 °C for 7 days, after which the dried flowers were ground into a fine powder using a grinder and stored in clean, labeled airtight bottles. The flower sample was sequentially extracted with methanol by adding approximately 100 g of the dried sample into 400 ml methanol. The extraction was carried out at room temperature by soaking for 7 days with intermittent stirring during the first day. The extracts were filtered through clean muslin cloth and the extraction process was repeated again for a second time by adding another 400 ml of methanol to the sample residue. The filtrate from each extraction was combined and concentrated under vacuum on a rotary evaporator (Buchi, Switzerland) at 40 °C–50 °C in order to evaporate the excess methanol solvent and until a dark yellow methanol extract was produced. The concentrated extract was poured into Petri dishes and brought to dryness at 60 °C in the oven until a paste-like mass was obtained. Then, a paste form extract was sealed in Petri plates and stored at room temperature (RT).

### 2.2. *Aspergillus niger* strain

*A. niger* was obtained from the School of Biological Sciences, Universiti Sains Malaysia, Pulau Pinang, Malaysia. This fungal species was used throughout this study. *A. niger* was grown on a Potato Dextrose Agar (PDA) medium for 4 days at 28 °C. Conidia were harvested by flooding sterile saline on the surface of grown *A. niger* and gently rubbed with a sterile quad loop. The harvested conidia were counted using a hemocytometer to obtain the required concentration (0.8 × 10$^6$ conidia CFU/mice) for infection.

### 2.3. Mice

Female Balb/C pathogen-free mice weighing between 23 and 28 g obtained from the Animal House, Universiti Sains Malaysia, Pulau Pinang were used for this study. The animals were housed in controlled environment with 12 h light–dark cycle at 22 ± 2 °C and allowed for free access to standard pellet diet and water ad libitum. Mice were grouped into 3 groups that serve as negative control, treatment and positive control groups. The experimental procedure was approved by Animal Ethical Committee (USM/Animal Ethics Approval/2010/(59) (261)).

### 2.4. Experimental aspergillosis

As an initial step all the mice were immunosuppressed with a dose of 200 mg/kg cyclophosphamide (CY) by intraperitoneal injection (i.p.), 3 days prior to infection. After that the mice were inoculated with 0.8 × 10$^6$ conidia CFU/mice, 3 day post immunosuppression by intravenous injection (i.v) via lateral tail vein. Treatment began 2 h after fungal inoculation and continued for 5 consecutive days. Infected mice in negative control group received 2% of Tween 20 (i.v), treatment group received 100 mg/kg (i.v) of *C. surattensis* flower extract and positive control received 1 mg/kg (i.v) of Amphotericin B. Four mice from each group were euthanized at days 1, 7 and 28 for quantitative measurement of infection, galactomannan assay determination and histopathology examination. At the selected times after infection and treatment, the mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). Fifteen minutes after the intraperitoneal injection of ketamine and xylazine, the mice were tested for righting, toe-pinch, and palpebral reflexes. In addition, cervical dislocation was performed on the anesthetized mice and blood samples were taken. The blood (0.5 ml) obtained using cardiac puncture with a 25 gauge needle was placed into heparinized anticoagulant tubes. Immediately after cervical dislocation and blood collection, the organs of the mice – lung, liver, spleen and kidney – were excised for further analysis and immersed in Bouin’s solution for at least 1 day or further used for other assays.

### 2.5. *Aspergillus niger* burden assessment

Fungal burden assessment was performed on the lung, liver, spleen and kidney samples of the animals. The organs were removed aseptically and homogenized in 1 ml of sterile saline. Serial dilution was performed on the tissue homogenates and plated on PDA medium. Plates were incubated at 35 °C for 48 h and number of CFU per organ was calculated. Experiments were performed in triplicates [13].

### 2.6. Galactomannan assay

Serum samples from the collected blood were subjected for galactomannan determination. The release of fungal galactomannan in the serum was measured using the Platelia Aspergillus EIA kit (Platelia Aspergillus 62797, Immunoenzymatic detection of galactomannan antigen of Aspergillus in serum, Biomer) in accordance with the manufacturer’s instructions. About, 300 μl of test serum was added to 100 μl of serum treatment solution and heated for 3 min at 100 °C, followed by centrifugation process at 10,000 g for 10 min. Initially, 50 μl of conjugate was added into the monoclonal antibody coated microtiter plate before the addition of 50 μl of supernatant and incubated for 90 min at 37 °C. After 5 washes with washing solutions 200 μl of substrate buffer was added and incubated in dark room for 30 min. The reaction was stopped with addition of 100 μl of stopping solution and optical density (OD) of the sample was measured at 450 nm (reference filter of 620 nm). Serum galactomannan index (GMI) less than 0.40 was considered negative and more than 2.00 as positive. GMI for each test serum is equal to the OD of a sample divided by the OD of a threshold serum provided by the kit.

### 2.7. Histopathological examinations

All the vital organs (lungs, liver and kidneys) isolated from each animal group were fixed in 10% buffered formalin for 48 h in separate vials, routinely processed, and embedded in paraffin wax. Paraffin sections (5 μm) were cut on glass slides and stained with...
Gomori’s methenamine silver (GMS) stain. The slides were examined under a light microscope and the magnified images of the tissues’ structure were captured for further study.

2.8. Time killing studies

Time killing studies was carried out against A. niger. A. niger inoculum was prepared by growing on PDA slopes and was incubated for 7 days at 30 °C. Slope was flooded with 0.85% saline containing 0.05% Tween 80. Fungal growth was gently probed and resulting suspension was removed and mixed throughout with the use of a vortex mixer. Suspension was adjusted by dilution as necessary to correspond to desired final concentration. A series of doubling dilution of C. surattensis flower extract ranging from 100, 50, 25, 12.5, 6.25, 3.125, 1.562, 0.78, 0.39 and 0.195 mg/ml was prepared in a 96 well microdilution tray. After the addition of inoculum tray was incubated for 48 h at 35 °C. Minimum fungicidal concentration (MFC) was determined by subculturing 10 μl from wells not visibly turbid and spot inoculating on to Sabouraud dextrose agar (SDA) plates and the value was 12.5 mg/ml. MFC was determined as the lowest concentration resulting in no growth on SDA plates and the value was 12.5 mg/ml. MFC was determined by subculturing 10 μl from wells not visibly turbid and spot inoculating on to Sabouraud dextrose agar for 48 h at 30 °C. Minimum fungicidal concentration (MFC) was determined by subculturing 10 μl from wells not visibly turbid and spot inoculating on to Sabouraud dextrose agar (SDA) plates and the value was 12.5 mg/ml. MFC was determined as the lowest concentration resulting in no growth on subculture. C. surattensis flower extract concentration was prepared that corresponded 1/2MFC, MFC and 2MFC. Control contained PBS with the relevant concentration of Tween 20. Fifty microliter of each C. surattensis flower extract at the different concentration determined by 1/2MFC, MFC and 2MFC were added to 5 ml of suspension containing 10⁷ spores/ml and was then incubated at 30 °C in an incubator shaker. Samples were taken after 5, 10, 15, 20, 25, 30, 45, 60, 75, 90, 105 and 120 min and were cultured on potato dextrose agar for 48 h at 30 °C. Fungal colonies were counted after incubation period and the total number of viable spores per ml was calculated.

2.9. Statistical analysis

The organ fungal burden, live spore percentage and galactomannan index were analyzed using one-way ANOVA with Duncan’s variance (SPSS 15) to compare between the groups. All values are mean ± SD. P values of <0.05 were considered significant (p < 0.05).

3. Results

3.1. Aspergillus niger burden assessment

Determination of fungal burden in the visceral organs revealed high levels of conidia in all the organs (lung, liver, spleen and kidney) for negative control on day 1 compared to the treatment and positive control group. Liver and spleen was detected to have high levels of fungal burden in the negative group (Fig. 1). Organ quantified on day 7 revealed a significant reduction pattern of CFUs in both treatment and positive group. But reduction of fungal load in negative group was not observed (Fig. 2). By day 28 the organs from negative group had severe colonization from the A. niger. Significant reduction was observed in the lung, liver, spleen and kidney of the flower extract treated group. The positive control that received Amphotericin B showed clearance in the lung and kidney by the last day (Fig. 3).

3.2. Galactomannan assay

All the serum was tested for the presence of galactomannan to determine the level of A. niger infection on the animals (Fig. 4). Mice from the negative control showed high galactomannan levels throughout the experiment. The galactomannan index (GMI) on day 1 was 2.23 which increased to 2.77 on day 7 and 3.58 by day 28. Serum samples from the shoot treated group had a decline in GMI after day 7 with GMI value of 2.32 to 1.27 on day 28. Progressive decline in galactomannan levels were observed in positive control group that was treated with Amphotericin B. GMI value of 2.25 on day 1 reduced to 0.48 on day 7 and to 0.25 by day 28.

3.3. Histolopathology examination

Histological sections of lung, liver and kidney from all the groups had different degree of pathological changes. Lung tissue from the negative control group presented reduced space of the alveolar sac. Dilated bronchiolo airways and infiltration was observed on lung tissue examined on day 7 (Fig. 5). Massive bronchopneumonia and smooth muscle cell hyperplasia were visually more apparent for the tissue analyzed from this group on the last day (Fig. 5). Mild bronchopneumonia was detected in lung tissue for the treatment group on day 7 but recovered by day 28 (Fig. 5). Small dilated blood cells were obvious in the positive control group but the lung tissue was preserved (Fig. 5). In the liver sections from all the groups conidia were detected around the central vein (Fig. 5). Kidney section from the negative control presented large number of conidia in the glomerulus suggesting acute glomerulonephritis on day 28 compared to those observed on day 1 and day 7 (Fig. 5). In the treatment group reduced number of conidia was observed (Fig. 5). Tissues from the positive control kidney were protected with mild infection (Fig. 5).
initial stage. But, steady decrease in the number of CFUs in the kidney of mice treated with flower extract was observed on day 7 and 28 compared to those in day 1. Complete conidial elimination in the lung and kidney from the positive control animals treated with antifungal drug suggest Amphotericin B is effective in reducing fungal burden. Similar observation was reported where clean kidney was obtained by day 14 in mice administered with antifungal drug [13].

Galactomannan is a heatstable heteropolysaccharide component in the cell wall of Aspergillus sp. The composition of galactomannan differs between genera and strains [15]. This carbohydrate is released during the growth cycle of Aspergillus [16]. The rise in GMI value in the serum samples from the negative control group indicates the increase in number of conidia of A. niger in the mice as this group of mice was free from antifungal therapy. Mice that received C. surattensis flower extract showed significant reduction in GMI value by end of the study suggesting good antifungal activity exhibited by the flower extract. Amphotericin B is a commercial antifungal drug that used to treat aspergillosis. Administration of Amphotericin B in the positive control group helps to decreased significantly the GMI values. The antibiotics helped to decrease the fungal load in the animals [17].

Histology analysis was performed to identify the infected area in the tissue studied. Generally, the lung becomes the first target to spores from Asperillus sp because spores from the environment gain entry to the human host by inhalation. If the host has poor immune defense than it is gradually lost as the germination and swelling of conidia begins which later mediates aggregation [18,19]. Microscopic examinations on the lungs tissues featured the airwall thickening in all the 3 groups but recovery was observed in both extract treated and positive control groups. Hepatocellular damage was more prominent in the liver from negative control group animals compared to those received C. surattensis flower extract and Amphotericin B. Severe lesions were observed on the kidney tissue in the medulla and cortex area. In this study, the kidneys become the primary target of A. niger infection as the conidia were introduced into the blood by intravenous injection via lateral tail vein [20]. C. surattensis flower extract can be a potential antifungal agent as the flower extract exhibited good antifungal activity towards A. niger in this study from the reduction pattern observed in fungal burden assessment, galactomannan assay and histopathology examinations. Literature revealed that this plant is commonly used to treat fungal infections among traditional healers [12]. However, A. niger infection in the treated and positive control group animal was not only managed with the antifungal activity but also due to the immune system orchestrated by the animals. The role of inflammation as protective response might have lead to tissue reparation and healing of injured tissue due to the fungal infection [8]. The infection was initiated by intravenous injection in this study and not with the natural infection route, which presumably is inhalation. Intranasal inoculation mimics the natural route of infection and would seem to be a more appropriate route than intravenous inoculation. However, intravenous challenge incites a more uniform pattern of disease with different mode of infection [21,22]. Therefore, we used intravenous inoculation and it’s worked well in this study.

Time-kill assay was utilized in this study to verify concentration dependence fungicidal activity and to evaluate the ability of C. surattensis flowers extract to kill the spores of A. niger. The extent of inhibition of fungal spore growth was dependent on the concentration of C. surattensis flowers extract used (Fig. 6). In the case of 1 and 2 times MFC concentrations, the extract inhibited the spore growth remarkably (P < 0.05). The antifungal effect of the C. surattensis flowers extract could be related to several active compounds with biological activities presence in the extract. It should
be noted that there was a gradual increase in inhibition due to the increased concentration of such bioactive compound present in the *C. surattensis* flowers extract. The time-kill assay suggested that *C. surattensis* flowers extract inhibited *A. niger* growth and it also exhibited prolonged antifungal activity against the *A. niger* as determined by time-kill curves.

5. Conclusions

*C. surattensis* flower can be a potential source for new antifungal drug discovery as this flower are commonly used as traditional medicines as they exhibits less harmful effects compared to synthetic compounds. The flower exhibited antifungal activity against *A. niger* infected aspergillosis model. Further studies on the mechanism action of this extract against *A. niger* and the isolation and identification of responsible active compounds for antifungal activity is necessary for new drug discovery.

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References


Fig. 5. Histology of lung, liver and kidney taken from control, *C. surattensis* flower extract treated group and Amphotericin B treated group on Day 28 with 40× magnification. Tissues were stained with GMS (➔: indicates conidia infection). Surattensis flowers extract reduced the live spore percentage remarkably compares with control and show concentration dependence fungicidal activity.

Fig. 6. Effect of *C. surattensis* flower extract on growth of *A. niger* at 0 (PBS), 1/2 MFC, MFC and 2MFC.


