EFFICACY OF CLITORIA TERNATEA LEAF EXTRACT AGAINST EXPERIMENTAL ASPERGILLOSIS

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ABSTRACT

Objective: To evaluate the efficacy of methanolic extract of Clitoria ternatea leaf in experimental murine systemic aspergillosis.

Methods: The investigation of methanolic extract of C. ternatea leaf against experimental murine systemic aspergillosis was assessed by the survival rates, quantitative determination of fungal burden in spleen, kidney and lung organs, histopathological evaluation and serum galactomannan index.

Results: Methanolic extract of C. ternatea and amphotericin B demonstrated survival benefits over control. All untreated animals died by day 14. Similarly, both treated groups had significant reductions (P<0.05) in the fungal burden of lungs, kidneys and spleen. By day 28, there was a complete clearance of the fungi from these organs. A decline was also observed in serum galactomannan level of treated animals.

Conclusion: The methanolic extract of C. ternatea was effective against aspergillosis in immunocompromised mice comparable to that of amphotericin. This study merits further clinical investigations of this extract as well as isolation and characterization of their bioactive antifungal chemical constituent(s).

Keywords: Aspergillosis, Immunocompromised mice, Clitoria ternatea extract, Galactomannan, Fungal burden.

Aspergillus spp are ubiquitous in nature and are the most frequent opportunistic organisms producing a wide range of saprophytic and invasive syndromes in the lungs [1]. Infections caused by Aspergillus spp are increasing in importance, especially among immunocompromised hosts. The most commonly affected patients are with hematological malignancies undergoing intensive chemotherapy, bone marrow and stem cells transplant recipients who developed graft-versus-host disease [2,3]. Additionally several predisposing factors for development of invasive aspergillosis include more widespread use of solid organ transplantation, the use of adrenal corticosteroids, the acquired immunodeficiency syndrome (AIDS) [4].

Aspergillus niger has been infrequently described as an invasive pathogen in the past [5,6]. It is an agent of chronic pneumonia, bronchial asthma, invasive aspergillosis (IA) and mycetoma [7]. Amphotericin B (AmpB) is a polyene macrolide with broad-spectrum and potent antifungal activity [8]. Even though AmpB is still a major antifungal drug used for invasive aspergillosis treatment, the clinical use is sometimes limited because of adverse reactions such as renal toxicity, hypokalemia and anemia [8,9]. Also, the appearance of resistant or multiresistant strains to exist agents requires the need for increased research and development of new drugs [10].

Botanically, Clitoria ternatea (butterfly pea) belongs to the family Fabaceae and subfamily Papilionaceae. It is a very well known Ayurvedic medicine used for different ailments. Its extracts possess a wide range of pharmacological activities including antimicrobial, antipyretic, anti-inflammatory, analgesic, diuretic, local anesthetic, anti-diabetic, insecticidal, blood platelet aggregation-inhibiting and for use as a vascular smooth muscle relaxing properties [11]. The leaf extract was found to exhibit considerable antifungal activity against A. niger in a dose-dependent manner with IC50 value 0.4 mg/mL. The scanning electron microscopy (SEM) study demonstrated irreversible deleterious morphological alterations on hyphae and conidiophore of A. niger when treated with leaf extracts [12]. The toxicity of methanolic extracts of C. ternatea leaf has been evaluated and found to be safe even at the dose of 2 g/kg [13]. Therefore, in the present study we investigated the therapy of leaf extract against murine aspergillosis induced in transiently immunocompromised mice.

The raw materials of C. ternatea plant samples were collected at Seberang Jaya, Penang, Malaysia. A voucher specimen (No. 11006) has been deposited at the herbarium of School of Biological Sciences, USM. The leaves were separated and washed with water to remove dirt prior to drying at 40 °C for 3 days. Powdered (200 g) leaves were macerated in methanol (2 L) for 72 h under constant stirring conditions. The extract was then filtered through No. 1 Whatman filter paper and dried under partial vacuum using rotary evaporator (BUCH R-110, Switzerland).

The cyclophosphamide, amphotericin B, saline pellets were purchased from Merck (Darmstadt, Germany), Patelia Aspergillus 62796, immunoenzymatic sandwich microplate assay for the detection of Aspergillus galactomannan antigen in serum were purchased from Bio-Rad (Marnes-la-Coquette, France). The A. niger ATCC 16508 strain were used in this study. The conidial suspension was adjusted to 0.8 x 10^6 conidia/mice by using hemacytometer and verified by plating on potato dextrose agar plates.

Female Swiss albino mice of 4 - 5 weeks old, weighing initially about 23 - 28 g were used. The animals were maintained on a 12-h light/dark cycle, at room temperature and were allowed free access to commercial food pellets (Gold Coin, Penang, Malaysia) and tap water. The animals were acclimatized for at least 1 week prior to experimentation. The handling and use of animals was in accordance with institutional guidelines and approval was obtained from the Animals Ethics Committee, Universiti Sains Malaysia, Penang, Malaysia (USM/PPS/50/063) Jld. 2. An immunocompromised state of the mice was confirmed with an examination of their blood profile [14]. Experimental aspergillosis was induced by an intravenous (iv) inoculation of restrained immunocompromised mice with 0.8 x 10^6 conidia/mice of A. niger via the lateral tail on day 3 post-cyclophosphamide immunosuppression [9].
The experiment was carried out in two parts with three groups of mice each. In the first part, the mortality due to aspergillosis was evaluated whereby infected animals (8 mice/group) were followed-up for 28 days and their survival rates and time were recorded. In the second part of the experiment, the mortality due to aspergillosis was evaluated whereby the infected mice (14 mice/group) were sacrificed on days 1, 3, 5, 7, 14, 21 and 28 in groups of 2 each for determination of fungal colonization of organs (lungs, kidneys, spleen) by quantitative determination of fungal burden, histopathological evaluation and serum galactomannan index. Infected mice received either one of the following treatments; methanolic extract of C. ternatea (100 mg/kg, iv) and Amp B (1 mg/kg, iv) daily for 5 consecutive days while control group received vehicle, 2% aqueous Tween 80 (5 ml/kg, iv). Treatments were initiated 2h after fungal colonization and continued for 7 days.

The quantitative measurement of fungal burden was performed by removing the kidneys, spleen and lungs aseptically and homogenized in 1 ml of sterile saline. The tissue homogenates were diluted and plated on PDA, and the plates were incubated at 28°C for 48 h. The fungal burden was expressed as CFU/organ. Triplicate determinations were performed for each organ/group. Organ samples (kidneys and lungs) were fixed in 4% buffered formalin, processed and sectioned by routine histologic techniques after paraffin embedding. Sections were then stained with either hematoxylin and eosin or Grocott’s methamine silver stain (GMS).

The galactomannan assay was performed according to the procedures described by [9]. Blood samples were collected for each sample at days 1, 3, 5, 7, 14, 21 and 28 for the determination of serum galactomannan concentrations (14 mice/group). Serum galactomannan concentrations were determined by the Platelia enzyme immunoassay data were expressed as a serum galactomannan index (GMI). The GMI for each test serum is equal to the OD of a sample divided by the OD of a threshold serum provided by the producer (Bio-Rad). Enzyme immunoassay data were expressed as a serum galactomannan index (GMI). The GMI for each test serum is equal to the OD of a sample divided by the OD of a threshold serum provided in the test kit. Sera with GMIs of less than 0.5 was considered negative. Sera with GMIs of greater than 0.5 were considered positive.

Survival data were analyzed by Kaplan-Meier test and comparisons between groups were performed by the one-way analysis of variance (ANOVA) followed by Tukey-HSD post-hoc test. P values of <0.05 were considered significant in these analyses.

The survival of mice infected systemically with A. niger was monitored up to 28 days following inoculation. All the untreated infected animals died by day 13. Treatment with methanolic extract of C. ternatea leaf significantly improved the survival rate of the infected animals and exhibited zero mortality throughout the 28 days of experimentation, similar to those AmpB treated animals (fig. 1).

In the current murine aspergillosis model used, fungal colonization in organs was assessed quantitatively by enumeration of Aspergillus CFU and qualitatively by histology. Table 1 shows the fungal burden in spleen, kidney and lung homogenates of the infected animals killed at different time intervals post-infection.

### Table 1: Fungal burden in spleen, kidney and lung homogenates of the infected animals killed at different time intervals post-infection.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of CFU / spleen organ (mean ± SD; n=2)</th>
<th>24 h</th>
<th>72 h</th>
<th>5 days</th>
<th>7 days</th>
<th>14 days</th>
<th>21 days</th>
<th>28 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>10000</td>
<td>50000±2357</td>
<td>1472±276</td>
<td>33.3</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Leaf extract</td>
<td></td>
<td>1166±2357</td>
<td>50±24</td>
<td>634±94</td>
<td>17±23**</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AmpB</td>
<td></td>
<td>2217±1655</td>
<td>91±118</td>
<td>117±23***</td>
<td>0***</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of CFU / kidney organ (mean ± SD; n=2)</th>
<th>24 h</th>
<th>72 h</th>
<th>5 days</th>
<th>7 days</th>
<th>14 days</th>
<th>21 days</th>
<th>28 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>2333±1414</td>
<td>50000±2357</td>
<td>1350±731</td>
<td>50±24</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Leaf extract</td>
<td></td>
<td>2333±1414</td>
<td>17±23*</td>
<td>1117±71</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AmpB</td>
<td></td>
<td>1600±189</td>
<td>1967±77*</td>
<td>67±47*</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of CFU / lung organ (mean ± SD; n=2)</th>
<th>24 h</th>
<th>72 h</th>
<th>5 days</th>
<th>7 days</th>
<th>14 days</th>
<th>21 days</th>
<th>28 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>2667±943</td>
<td>8333±23570</td>
<td>1050±24</td>
<td>500±236</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Leaf extract</td>
<td></td>
<td>834±235</td>
<td>33±***</td>
<td>1183±212</td>
<td>17±23</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AmpB</td>
<td></td>
<td>1834±330</td>
<td>1433±283***</td>
<td>100±47</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

n. d – Not done, because the animals succumbed. * P < 0.05; ** P < 0.01; *** P < 0.001 when compared to untreated control animals.
Reduction of fungal spores was noticeable in leaf treated and AmpB treated groups. The effect of methanolic extract of *Clitoria ternatea* on organ fungal colonization in these organs was comparable to that of AmpB. The fungal burden in spleen, kidneys and lungs of *C. ternatea* treated animals showed a reduction during the first week after initiation of treatment and complete clearance by day 14 till the end of study period. The complete clearance of fungal burden after *C. ternatea* treatment confirmed the irreversible deleterious morphological alterations on *A. niger* [12]. Histopathology was performed on lungs and kidneys of mice. Kidneys from untreated controls demonstrated development of fungal hyphae from day 3 onwards (fig. 2). On contrary, there was no evidence of hyphae development observed in both *C. ternatea* leaf and AmpB treated groups. A marked reduction of Aspergilli spores was observed in both lung and kidney histopathology of *C. ternatea* leaf extracts and AmpB treated groups.

Galactomannan (GM) is a polysaccharide antigen isolated from *Aspergillus* species and was the first antigen detected in experimentally infected animals and in patients with invasive aspergillosis [15,16]. The sandwich ELISA system is currently the most sensitive method which detects up to 1 ng of GM/ ml of serum. The sensitivity of ELISA was higher than that of the polymerase chain reaction (PCR) and quantitative galactomannan detection is superior to PCR in diagnosing and monitoring invasive pulmonary aspergillosis in experimental mice model [17]. The detection of serum galactomannan is also an important indication of therapeutic response of a particular antifungal agent. A decrease in the concentration of GM in serum is indicative of treatment indication of therapeutic response of a particular antifungal agent. A model [17]. The detection of serum galactomannan is also an important monitoring invasive pulmonary aspergillosis in experimental mice infected animals and in patients with invasive aspergillosis [15,16]. The sandwich ELISA system is currently the most sensitive method which detects up to 1 ng of GM/ ml of serum. The sensitivity of ELISA was higher than that of the polymerase chain reaction (PCR) and quantitative galactomannan detection is superior to PCR in diagnosing and monitoring invasive pulmonary aspergillosis in experimental mice model [17]. The detection of serum galactomannan is also an important indication of therapeutic response of a particular antifungal agent. A decrease in the concentration of GM in serum is indicative of treatment efficacy [15]. In the present study, untreated animals showed high GMI levels till day 7 post-infection (fig. 3).

The present *in vivo* activity of the *C. ternatea* methanolic leaf extract against *A. niger* further established the *in vitro* activity of *C. ternatea* as already been described in literature [12]. Based on the current study, the *C. ternatea* methanolic leaf extract confirmed the irreversible deleterious morphological alterations to *A. niger*. Further clinical investigations of this extract as well as isolation and characterization of their bioactive antifungal chemicals constituent(s) are merited as natural plant-derived fungicides may be a source of new alternative active compounds, in particular with antifungal activity.

**AKNOWLEDGEMENT**

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**CONFLICT OF INTERESTS**

Declared None

**REFERENCES**


![Fig. 3: Galactomannan levels in *Aspergillus*-infected untreated mice and treated mice with leaf extract and AmpB.](image-url)