

Short communication

EFFICACY OF *CLITORIA TERNATEA* LEAF EXTRACT AGAINST EXPERIMENTAL ASPERGILLOSIS

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Received: 14 Jul 2014 Revised and Accepted: 13 Aug 2014

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, bronchiol asthma, invasive aspergillosis (IA) and mycetoma [7]. Amphotericin B (AmpB) is a polyene macrolide with broad-spectrum and potent antifungal activity [8]. Eventhough 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 anaemia [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, antidiabetic, 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 (BUCHI R-110, Switzerland).

The cyclophosphamide, amphotericin B, saline pellets were purchased from Merck (Darmstadt, Germany). Platelia 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 0.8×10^6 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/PPSF/50 (063) Jld. 2). An immunocompromised state in mice was achieved by intraperitoneal injection of the immunosuppressive agent, cyclophosphamide (200 mg/kg). The 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 0.8×10^6 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 morbidity due to aspergillosis was evaluated whereby the infected mice (14 mice/group) were sacrificed on days 1, 3, 5, 7, 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. Infested 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 were 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 methenamine 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 *Aspergillus* EIA a commercial sandwich ELISA kit, and carried out according to the protocol recommended 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 systematically with *A. niger* was monitored up to 28 days following inoculation. All the untreated infected animals died by day 13th. 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).

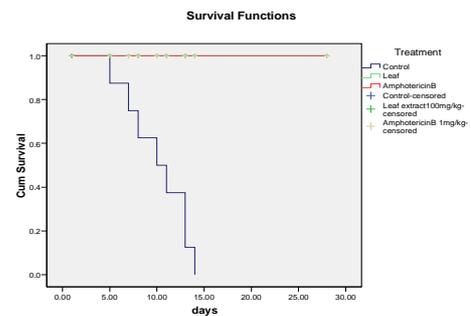


Fig. 1: Survival of *Aspergillus*-infected untreated mice and treated mice with leaf extract and AmpB. The survival of *C. ternatea* leaf and AmpB treated groups are overlapping

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, liver and kidney homogenates of the infected animals killed at days 1, 3, 5, 7, 21 and 28 post-infection.

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

Group	Number of CFU / spleen organ (mean ± SD; n=2)						
	24 h	72 h	5 days	7 days	14 days	21 days	28 days
Control	10000	50000±23571	1472±276	333	n. d.	n. d.	n. d.
Leaf extract	11667±2357	50±24	634±94*	17±23***	0	0	0
AmpB	2217±165*	917±118	117±23**	0***	0	0	0
Group	Number of CFU / kidney organ (mean ± SD; n=2)						
Control	2333±1414	50000±23571	1350±731	50±24	n. d.	n. d.	n. d.
Leaf extract	2333±1414	17±23*	1117±71	0	0	0	0
AmpB	1600±188	1967±377*	67±47*	0	0	0	0
Group	Number of CFU / lung organ (mean ± SD; n=2)						
Control	2667±943	83334±23570	1050±24	500±236	n. d.	n. d.	n. d.
Leaf extract	834±235	333***	1183±212	17±23	0	0	0
AmpB	1834±330	1433±283***	100±47	0	0	0	0

n. d. - Not done, because the animals succumbed, * P < 0.05; ** P < 0.01; *** P < 0.001 when compared to untreated control animals

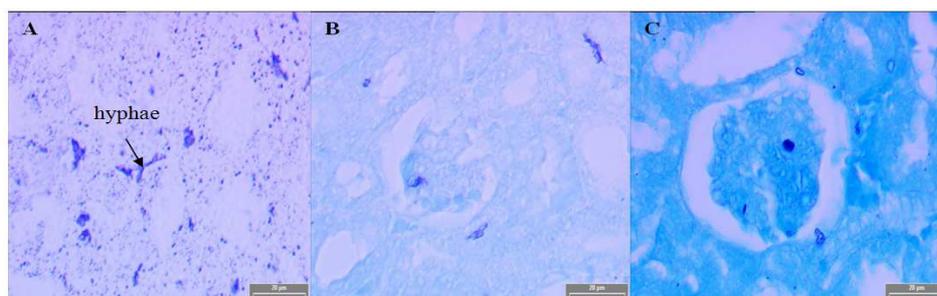


Fig. 2: Histopathology of kidneys. *A. niger* hyphae in the kidney tissue of untreated control (A) shows the developed of hyphae. No evidence of hyphae development was observed in leaf extract (B) and AmpB (C) treated groups

Reduction of fungal spores was noticeable in leaf treated and AmpB treated groups. The effect of methanolic extract of *C. 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 efficacy [15]. In the present study, untreated animals showed high GMI levels till day 7 post-infection (fig. 3).

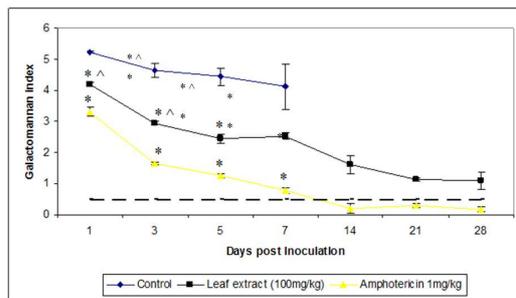


Fig. 3: Galactomannan levels in *Aspergillus*-infected untreated mice and treated mice with leaf extract and AmpB. Galactomannan level was considered negative when GMI < 0.5.

* $P < 0.01$, indicates significant reduction of GMI level when compared with control alone

^ $P < 0.01$, indicates significant difference of GMI level when compared with Amphotericin B alone

The GMI of these animals could not be determined at day 14 onwards because all the animals in this group succumbed. Meanwhile, animals treated with methanolic extract of *C. ternatea* had a gradual and significant ($P < 0.01$) reduction in their GMI levels from day 1 till day 28 of treatment. However, their GMI levels remain positive throughout the study period. In contrast, AmpB treatment significantly ($P < 0.01$) reduced the GMI levels and by day 14 onwards, the GMI levels were found to be negative. These phenomena could be due to occurrence of false positive during detection of serum galactomannan. It was mentioned previously that this false positive could be attributed to cross-reactivity with cyclophosphamide adsorption of dietary GM from damaged gut endothelium and β -lactam antibiotic usage [16].

CONCLUSION

In conclusion, the methanolic extract of *C. ternatea* is effective in experimental aspergillosis comparable to that of amphotericin B, as shown in prolonged survival of the infected mice, significant reduction in the fungal burden and eventually absence of *A. niger* in the spleen, lungs and kidneys. A decline of the galactomannan level in the serum samples was also noticed in *C. ternatea* treated animals though it remains positive throughout the study period.

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.

ACKNOWLEDGEMENT

This research project was supported by the Centre for Drug Research, Universiti Sains Malaysia (USM) under Research University Grant Scheme (1001/CDADAH/813041). The author (L. Kamilla) would also like to acknowledge the scholarship provided by MyPhD, Ministry of Higher Education, Malaysia.

CONFLICT OF INTERESTS

Declared None

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