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Research Article

TINOSPORA CORDIFOLIA AQUEOUS EXTRACT AMELIORATES THE SYSTEMIC INFECTION OF *ASPERGILLUS FUMIGATUS* IN BALB/C MICE

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ABSTRACT

Objective: The present study was aimed to assess the antifungal activity of *Tinospora cordifolia* aqueous extract (TCAE) against *Aspergillus fumigatus* infection.

Methods: TCAE was tested for *in vitro* antifungal activity against the isolates of *A. fumigatus*, *Aspergillus flavus,* and *Aspergillus niger*. To evaluate *in vivo* activity, various doses (10, 25, and 50 mg/kg) of TCAE were orally administered in *A. fumigatus*-infected mice for 7 days. The combination of prophylactic and therapeutic effect of TCAE was assessed by pre-treating the mice with 10 mg/kg of TCAE for 3 consecutive days before exposing them to *A. fumigatus*. Mice were treated with 10, 25, and 50 mg/kg doses of TCAE for 7 consecutive days' post-*A. fumigatus* infection. The effectiveness of TCAE was evaluated by monitoring the survival rate and assessing the fungal burden in the kidney of the treated mice.

Results: *A. fumigatus*-infected mice treated with TCAE at the doses of 25 and 50 mg/kg exhibited 50% and 20% survival rate, respectively, observed on day 40 post-treatment. Like to the survival data, the fungal burden was also found to be the lowest in the kidney of mice treated with TCAE at a dose of 50 mg/kg. The results showed that pre-treatment with TCAE (10 mg/kg) followed by post-infection treatment with 10, 25, and 50 mg/kg of TCAE for 7 days resulted in 40%, 50%, and 70% survival rate, respectively.

Conclusions: These results suggest that TCAE may potentially be considered for its possible use in the treatment of the systemic infection of *A. fumigatus*.

Keywords: Alternative medicines, *Aspergillus fumigatus*, *Tinospora cordifolia*, Fungal infections.

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INTRODUCTION

The therapeutic use of antibiotics has played a tremendous role in combating various infectious diseases [1]. Although the indiscriminate and extensive use of antifungals has resulted in the emergence of multidrug-resistant isolates of fungal pathogens, which are posing serious challenges to the clinicians [2]. Moreover, some antifungal agents, particularly polyene antibiotics, have been shown to exert serious untoward effects [3,4]. As there are limited numbers of antifungals available in the market, it is important to find suitable replacements for some of the currently used antifungals [5].

Giloy, *Tinospora cordifolia*, has been used for centuries in the Ayurvedic and Unani Systems of the Medicine for the treatment of ailments. It has been demonstrated to have antidiabetic, antioxidant, anti-hepatotoxic, and immunomodulatory properties [6-8]. The aqueous extract of *T. cordifolia* has been shown to protect against *Escherichia coli* and *Staphylococcus aureus* infections [9,10]. The active ingredient, G 1-4A, of *T. cordifolia* stem protected mice against lipopolysaccharide-induced endotoxic shock [11]. It also controlled the drug-resistance *Mycobacterium tuberculosis* by modulating the immune responses [12]. Recently, we showed that aqueous and methanolic extracts of *T. cordifolia* alleviated the *Salmonella typhimurium* infection in a mouse model [13].

Invasive aspergillosis (IA) caused by *Aspergillus fumigatus* is a major cause of mortality in immunocompromised persons, including patients with leukemia, undergoing bone marrow, and solid organ transplantation [14]. Treatment of fungal infections has been a challenge for clinicians due to the limited number of antifungals currently available in the market. Recent upsurge in the frequency of azole-resistant *A. fumigatus* isolates has further limited our antifungal armory. On the other hand, polyene antifungals have been shown to exert an acute renal toxicity in the treated subjects. Thus, it is important to find a plant-derived broad-spectrum antifungal agents that can be used to treat infectious fungal diseases. In the present study, we used *T. cordifolia* aqueous extract (TCAE) against *A. fumigatus* both *in vitro* and in a mouse model. The results of the present study demonstrated that TCAE effectively alleviated the systemic infection of *A. fumigatus* in the mouse model.

MATERIALS AND METHODS

Materials

Sabouraud Dextrose Agar (SDA) was purchased from HiMedia Pvt. Ltd. Mumbai, India. Amphotericin B was purchased from Sigma-Aldrich (St. Louis, Mo, USA).

Mice

BALB/C mice of 10–12 weeks of age were used in this study. Mice were purchased from the animal house facility of King Saud University, Riyadh, Saudi Arabia. The techniques used for bleeding, injection, and sacrifice of animals were approved by an Animal Ethics Committee of the College of Applied Medical Sciences, Qassim University, Buraydah.

Preparation of extracts from the stems of *T. cordifolia*

The dried stems of *T. cordifolia* were procured from the registered attar shop in Buraydah, Saudi Arabia. The plant was identified by Dr. Masihuzzama Khan, Assistant Professor in the Department of Pharmacognosy, Unaizah College of Pharmacy, Unaizah, Qassim University. The stems were powdered and TCAE was prepared as described previously [13].

Determination of the antifungal activity

The antifungal activity of TCAE was determined using the agar well diffusion method [13]. Wells (8-mm diameter) were punched in the

agar. TCAE was dispensed in different wells (20 mg/ml) and incubated at 37°C for 24 h. The antifungal activity of TCAE was assessed by measuring the zone of inhibition. The wells containing amphotericin B (10 mg/ml) and saline were considered as positive and negative controls, respectively.

Preparation of *A. fumigatus* for infection

A. fumigatus was cultured as described in the previous section. Each mouse was infected through the intravenous route with a lethal dose of 7×10^5 viable *A. fumigatus* spores [14].

Treatment of *A. fumigatus*-infected mice with AETC

Mice were treated with various doses (10, 25, and 50 mg/kg) of TCAE orally for 7 days (day 1–7) after *A. fumigatus* infection (day 0). Mice were divided into the following groups: (1) Saline, (2) TCAE - 10 mg/kg, (3) TCAE - 25 mg/kg, and (4) TCAE - 50 mg/kg. Each group was comprised of 10 mice.

Prophylactic use of TCAE against *A. fumigatus*

To determine the prophylactic effect of TCAE against *A. fumigatus*, mice were pre-treated (PT) for 3 consecutive days (−3−−1 days) with a dose of 10 mg/kg of AETC. On day 0, mice were infected with 7×10^5 viable *A. fumigatus* spores through intravenous route. Treatment with 200 µl of 10, 25, and 50 mg/kg of doses of TCAE was given for 7 days to *A. fumigatus*-infected mice that were prophylactically treated or untreated with TCAE (10 mg/kg). Mice were divided into following groups: (1) Saline, (2) PT + TCAE - 10 mg/kg, (3) TCAE - 10 mg/kg, (4) PT + TCAE - 10 mg/kg + TCAE - 10 mg/kg, (5) TCAE - 25 mg/kg, (6) PT + TCAE - 10 mg/kg + TCAE - 25 mg/kg, (7) TCAE - 50 mg/kg, and (8) PT + TCAE - 10 mg/kg + TCAE - 50 mg/kg.

Quantitative analysis of *A. fumigatus* in the kidney

The severity of *A. fumigatus* infection was assessed by determining the fungal load in the kidney of mice untreated or treated with *T. cordifolia* extract. Three mice from each group were sacrificed on day 3 post-*A. fumigatus* infection, and their kidneys were taken out aseptically as described earlier [14]. Briefly, weighed portions of the kidney tissues were homogenized in 5 ml of sterile normal saline, and different dilutions of the suspension were plated on SDA plates. The plates were incubated at 37°C for 48 h. The numbers of viable *A. fumigatus* colonies were counted and the fungal load was determined by multiplying by the dilution factor.

Statistical analyses

Analysis of the survival of mice was performed using Kaplan–Meier curve, and various groups were compared by the log-rank test. Fungal burden (colony-forming units [CFU]) in the kidney was analyzed by one-way ANOVA using GraphPad Prism software version 5.0.

RESULTS

The aqueous extract of *T. cordifolia* shows *in vitro* antifungal activity

The aqueous extract of *T. cordifolia* showed potent activity against *A. fumigatus*, *Aspergillus Flavus,* and *Aspergillus niger* used in the study. A zone of inhibition was calculated as the percentage antifungal activity of amphotericin B (100%). Table 1 shows that *T. cordifolia* extracts showed the antifungal activity against all three strains of *Aspergillus*.

Treatment with TCAE results in increased survival of *A. fumigatus*-infected mice

The therapeutic effect of TCAE was determined by treating *A. fumigatus*-infected mice with the doses of 10, 25, and 50 mg/kg of TCAE for 7 consecutive days. Mice were observed for 40 days for their survival. All *A. fumigatus*-infected mice in the untreated group died by day 10. *A. fumigatus*-infected mice in the groups treated with 25 and 50 mg/kg of TCAE showed 30% and 40% survival, respectively (Fig. 1). The survival rate of mice in the group treated with TCAE at a dose of 50 mg/kg was found to be significantly greater as compared to that of mice in the untreated group ($p<0.01$).

Table 1: The antifungal activity of TCAE

Name of the organism	Percentage inhibition of amphotericin B
Aspergillus fumigatus	52±6.6
Aspergillus flavus	56±8.2
Aspergillus niger	42±6.5

The data represent mean of three different experiments±S.D, TCAE: *Tinospora cordifolia* aqueous extract

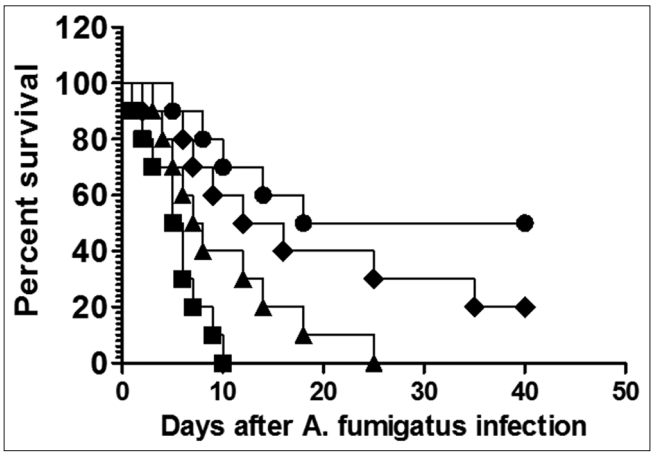

Fig. 1: *Tinospora cordifolia* aqueous extract (TCAE) increases the survival of the *Aspergillus fumigatus*-infected mice. Mice were infected with 7×10^5 colony-forming units of *A. fumigatus* through intravenous route. Treatment with the doses of 10, 25, and 50 mg/kg of TCAE was given for consecutive 7 days' post-infection. Mice were observed for 40 days for their survival; saline (■), TCAE - 10 mg/kg (▲), TCAE - 25 mg/kg (♦), TCAE - 50 mg/kg (●). Untreated control versus TCAE - 50 mg/kg ($p<0.01$)

Mice treated with TCAE showed more resistance against *A. fumigatus* infection

The severity of *A. fumigatus* infection was determined by culturing the tissue homogenates of kidneys of the *A. fumigatus*-infected mice treated or untreated with TCAE. There were higher CFUs of *A. fumigatus* in the kidneys of untreated mice (Fig. 2). Mice treated with 50 mg/kg of TCAE showed least CFUs in their kidney tissue homogenates (Fig. 2).

Prophylactic use of TCAE increases the therapeutic efficacy of the same formulation in combating *A. fumigatus* infection in mice

The combination of prophylactic and therapeutic effect of TCAE was assessed by pre-treating the mice with 10 mg/kg of TCAE for 3 consecutive days before challenging them with *A. fumigatus*. Mice were treated with three doses (10, 25, and 50 mg/kg) of TCAE for 7 consecutive days' post-*A. fumigatus* infection as described in the methods section. The results showed that pre-treatment with TCAE (10 mg/kg) followed by post-infection treatment with 10, 25, and 50 mg/kg of TCAE for 7 days resulted in 40%, 50%, and 70% survival, respectively, on day 40 (Fig. 3). The mice that received only TCAE pre-treatment also showed increased survival compared to untreated mice (Fig. 3).

The assessment of the severity of *Aspergillosis* in the group of immunocompetent mice pre-treated with TCAE showed much reduced fungal loads in their kidneys compared to mice in other groups. Mice that were only PT with TCAE also resisted *A. fumigatus* infection as shown by lower fungal load in their kidneys compared to mice not receiving any TCAE pretreatment (Fig. 4). The group of immunocompetent mice pre-treated with TCAE followed by treatment with 50 mg/kg of TCAE showed the least CFU counts in their kidneys (Fig. 4).

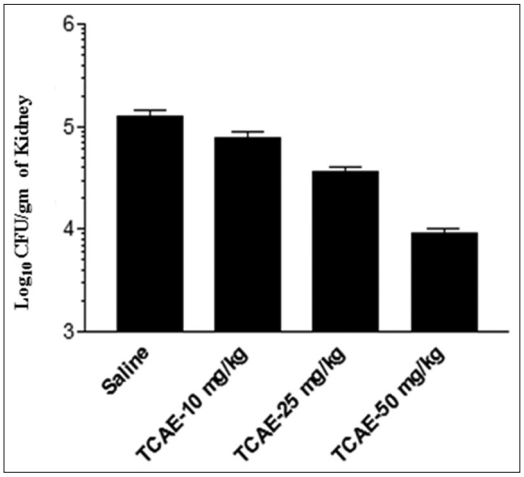

Fig. 2: Treatment with *Tinospora cordifolia* aqueous extract (TCAE) reduces the fungal burden in the kidney of *Aspergillus fumigatus*-infected mice. Mice were infected with 7×10^5 colony-forming units of *A. fumigatus* through intravenous route. Treatment with various doses (10, 25, and 50 mg/kg) of TCAE was given for consecutive 7 days' post-*A. fumigatus* infection. On day 3 post-*A. fumigatus* infection, three mice from each untreated or treated group were sacrificed and the kidneys were taken out for homogenization. The kidney tissue homogenates were cultured to determine the fungal burden. Untreated control versus TCAE-50 mg/kg ($p<0.01$), untreated control versus TCAE-25 mg/kg ($p<0.05$)

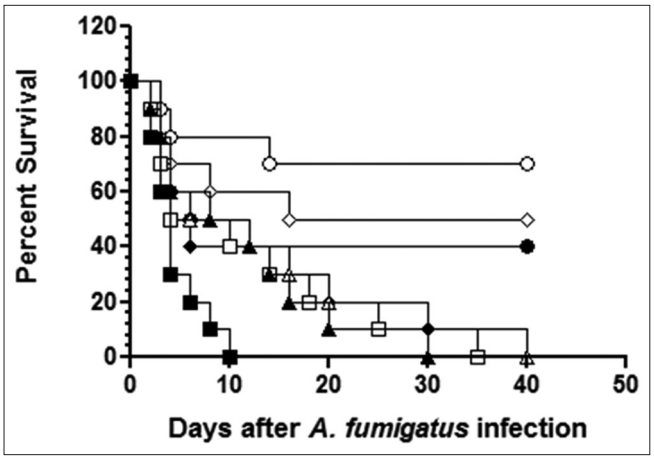

Fig. 3: The combination of prophylactic and therapeutic use of *Tinospora cordifolia* aqueous extract (TCAE) was more effective in the treatment of *Aspergillus fumigatus* infection in mice. Mice were pre-treated with a dose of 10 mg/kg of TCAE for consecutive 3 days. After pre-treatment with TCAE, mice were infected with 7×10^5 colony-forming units of *A. fumigatus* through intravenous route. Treatment with various doses (10, 25, and 50 mg/kg) of TCAE was given for consecutive 7 days' post-infection. Mice were observed for 40 days to monitor their mortality and morbidity. Saline (■), TCAE - 10 mg/kg (▲), PT + TCAE - 10 mg/kg (□), PT + TCAE - 10 mg/kg + TCAE - 10 mg/kg (∆), TCAE - 25 mg/kg (♦), PT + TCAE - 10 mg/kg + TCAE - 25 mg/kg (◊), TCAE - 25 mg/kg (○), PT + TCAE - 10 mg/kg + TCAE - 25 mg/kg (■). Untreated control versus PT-TCAE + TCAE - 25 mg/kg ($P<0.05$), Untreated control versus TCAE-50 mg/kg ($p<0.05$), untreated control versus PT-TCAE + TCAE-50 mg/kg ($p<0.01$)

DISCUSSION

In the recent years, the antibiotic resistance ability of *A. fumigatus* has contributed to increased frequency of *Aspergillosis* in human

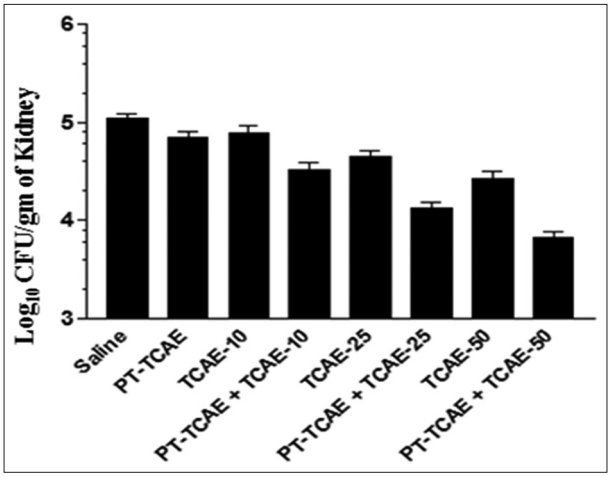

Fig. 4: Prophylactic treatment with *Tinospora cordifolia* aqueous extract (TCAE) increases the therapeutic efficacy of the same formulation against *Aspergillus fumigatus*. Mice were PT with a dose of 10 mg/kg of TCAE for consecutive 3 days. After pre-treatment with TCAE, mice were infected with 7×10^5 CFU of *A. fumigatus* through intravenous route. Treatment with various doses (10, 25, and 50 mg/kg) of TCAE was given for consecutive 7 days' post-infection. On day 3 post-*A. fumigatus* infection, three mice from each untreated or treated group were sacrificed and the kidneys were taken out for homogenization. The kidney tissue homogenates were cultured to determine fungal burden. Untreated control versus PT-TCAE + TCAE - 50 mg/kg ($p<0.05$), untreated control versus PT-TCAE + TCAE - 25 mg/kg ($p<0.01$), untreated control versus TCAE - 50 mg/kg ($p<0.05$), untreated control versus PT - TCAE + TCAE - 50 mg/kg ($p<0.001$)

population [15]. The use of plants and their bioactive constituents has emerged as a promising alternative to traditional drugs in the treatment of fungal infections [16]. We have earlier shown that thymoquinone, a major bioactive constituent of *Nigella sativa* seeds, possesses a strong activity against *Candida albicans* in a mouse model [16]. Due to the problem of antibiotic resistance and prevalence of immune suppression, the treatment of *Aspergillosis* is posing a big threat to human society. Plant-derived antifungal agents can selectively act on various targets with fewer side effects. Since there are less chances of fungal resistance against herbal preparations due to their multiple mechanisms of action, the use of medicinal plants may be proved a better option for the treatment of drug-resistant *Aspergillosis*.

In the present study, the aqueous extract of *T. cordifolia* showed its activity against different *Aspergillus* spp. including *A. fumigatus*, *A. flavus,* and *A. niger*. TCAE was tested against *A. fumigatus*, *A. flavus,* and *A. niger* in *in vitro* studies and was effective in inhibiting the growth of these fungi. This encouraged us to use TCAE against *A. fumigatus* in a murine model. Interestingly, the result of *in vivo* studies confirmed the activity of TCAE against *A. fumigatus*. *A. fumigatus*-infected mice showed increased survival and less fungal load in their kidney tissues after treatment with TCAE, particularly at higher doses.

T. cordifolia and its constituents have been shown to possess immunostimulating activities [13]. An α-D-glucan, a constituent found in *T. cordifolia*, has shown its efficacy in stimulating NK cells, B cells, and T cells with simultaneous production of various immune-stimulatory cytokines. This stimulation of immune cells can play a very crucial role in combating the fungal infectious diseases. We have recently shown that both aqueous and methanolic extracts of *T. cordifolia* stimulate the secretion of important cytokines by macrophages that contribute to the elimination of *S. typhimurium* infection from mice [13]. The immune-stimulating effect of *T. cordifolia* is also evident from the results of

our *in vivo* studies as the combination of prophylactic and therapeutic use of TCAE was most effective against *A. fumigatus*. Thus, the use of *T. cordifolia* extracts for the treatment of *Aspergillosis* seems to have superiority over commonly used antibiotics as earlier possesses both immunostimulatory and antifungal activities.

Extensive use of antibiotics causes systemic toxicity and immune suppression in the treated patients and predisposes them to opportunistic bacterial and fungal infections. The use of herbal medicine minimizes the chances of toxicity, which may support its use for extended periods. Although more extensive studies are needed before considering *T. cordifolia* as an attractive and safe option in the treatment for *Aspergillosis*. Furthermore, this preparation may also be studied for its implications to treat other opportunistic infections in immunocompromised persons due to its immunopotentiating properties.

CONCLUSIONS

The results of the present study suggest that some bioactive constituents present in TCAE may be responsible for its activity against *A. fumigatus*. It is essential that further study should be continued to isolate and purify the bioactive components of *T. cordifolia* that are responsible for antifungal activity.

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AUTHORS' CONTRIBUTIONS

Dr. Masood A. Khan designed and performed the experiments, analyzed the data, and wrote the manuscript.

CONFLICTS OF INTEREST

Authors do not have any conflicts of interest.

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