In vivo efficacy of an antifungal fraction from Pallavicinia lyellii, a liverwort

Almost all the antifungal agents, currently in use, have toxic side effects and are relatively expensive. Therefore, there is an urgent need to determine the in vivo efficacy of the active fraction or the isolate from the liverwort. Therefore, in the present study, the efficacy of the active fraction against aspergillosis caused by A. fumigatus in immuno-compromised mice was studied. The active fraction was also subjected to short term general toxicity evaluation in mice. Mice were reared in the animal house facility of the institute and were fed with standard rodent pellets (Lipton and Co. Bangalore) and water, ad libitum. They were maintained under standard laboratory conditions, temperature (25-28°C), humidity (50-80%) and 12 h light/dark cycle.

The animal house and breeding facility has been registered with CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Government of India, and CPCSEA guidelines are followed (IAEC approval obtained).

Pallavicinia lyellii was collected with their rhizoid on sunny days of June, from the forests near Palode, Thrivunnanthapuram District, Kerala State. The plant was identified by a bryophyte taxonomist of TBGRI and a voucher specimen was deposited. The alcohol extract of the powder of P. lyellii was prepared and subjected to fractionation with n-hexane to obtain the active hexane fraction. This fraction was used for in vivo efficacy and short term toxicity evaluation in mice.

To study short term toxicity, 4 groups of mice, each containing 6 male mice (20-25 g, body weight) were used. One group was kept as the control group and Groups 2, 3 and 4 received 100, 200 and 400 mg/kg of the active fraction, respectively. The drug was administered daily for 15 days (p.o.). The control group received 1% Tween 80 in an identical manner.

The behaviour of the animals was observed daily for one hour in the forenoon (10 to 11 a.m.) for 14 days. The behavioural parameters observed were convulsion, grooming, hyperactivity, sedation, loss of the writhing reflex, heart rate and respiratory rate. Initial and final body weights, water and food intake and state of stools were observed. The animals were killed on the 15th day. Hematological and serum biochemical parameters (glutamate pyruvate transaminase [GPT], glutamate oxaloacetate transaminase [GOT], urea, glucose, cholesterol, triglyceride and protein) were determined following standard methods. Hemoglobin was measured using hemoglobinometer with comparison standards. Liver, spleen, kidneys and heart were dissected, weighed and observed for pathological and morphological changes. The peritoneal macrophages and total leucocytes were counted as described elsewhere.

Mice are normally resistant to the oral or intra-peritoneal route of infection with Aspergillus sp and other fungi. This resistance can be reduced by cortisone treatment, which suppresses the immune function. Therefore, the mice were inoculated subcutaneously with a single dose of 5 mg of hydrocortisone (Samarth Pharma (P) Ltd, Mumbai) and intramuscularly with 30,000 units of long-acting penicillin 2 days before intraperitoneal A. fumigatus spore challenge. The animals were challenged with different quantities (0.01, 0.1, 0.5, 1 and 2 million) of viable spores to determine the minimum number of spores required for 100% mortality. 0.1 million spores were found to be sufficient to kill the mice.

To determine anti-Aspergillus fumigatus activity, 54 male mice weighing 20-25 g were divided into 9 groups of 6 mice in each group. One group was kept as normal control, without any treatment. Seven groups were treated with hydrocortisone and penicillin 48 h after hydrocortisone treatment, 6 groups were challenged with 105 spores per mouse and one group was kept as (hydrocortisone and penicillin) control without spore challenge. Three groups of spore-challenged mice received different doses (25, 50 and 100 mg/kg) of the active n-hexane fraction of alcohol extract; daily for 10 days, p.o., and the 4th and 5th spore-challenged groups received 50 and 100 mg/kg (daily for 10 days, p.o. ketoconazole (Nizral, Janssen-Cilag Pharmaceuticals, Mumbai), respectively; and the 6th spore-challenged group received the vehicle (1% Tween 80) and served as control. (1% Tween 80 was used as a vehicle for both the herbal drug and ketoconazole). One group of normal mice also received the active fraction (100 mg/kg). Mortality was observed daily for 30 days.

In short term limited toxicity evaluation, the active fraction administration for 15 days did not influence any of the parameters studied, in any of the doses used. The active fraction also did not result in any change in general behaviour, body weight, stool state, organ weight (liver, kidneys and spleen), food and water intake, hemoglobin, leucocyte count, peritoneal macrophage count, serum protein, urea, GPT, GOT, alkaline phosphatase, total cholesterol, triglyceride and glucose (data not given).

As shown in Table 1, the administration of 105 spores per mouse to hydrocortisone-treated mice resulted in 100% mortality within 6 days. The active fraction at a dose of 100 mg/kg, p.o. daily, starting from the day of fungal challenge, protected all the fungal-challenged mice. The antifungal activity was dose dependent. Lower dose (50 mg/kg) protected 4 out of 6 mice, while the lowest dose tried (25 mg/kg) was ineffective. The antifungal activity of the active fraction was comparable to the standard drug, ketoconazole.

The antifungal fraction from P. lyellii was found to be effective against aspergillosis-induced mortality in immuno-compromised mice. We have already shown the direct antifungal activity of the herbal drug against the fungus under
in vitro conditions.[11] Further, there is no report on the immuno-
modulatory activity of such liverworts. Therefore, the observed
protection from aspergillosis-induced mortality is likely to be
due to the antifungal activity. This herbal drug appears to be
non toxic whereas the antifungal drugs in current use, including
ketoconazole, have reported toxic side effects after pro-
longed use.

P. lyellii is distributed in India and the biomass can be
easily obtained.[14,15] Thus, P. lyellii is likely to be an attractive
material for developing invaluable antifungal drugs.

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Table 1
Protection with the hexane fraction of alcohol extract from P. lyellii against Aspergillus fumigatus challenge in hydrocortisone-
treated male mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>No of surviving mice</th>
<th>Days after the A. fumigatus challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>6</td>
<td>1st</td>
</tr>
<tr>
<td>Cortisone + penicillin</td>
<td>6</td>
<td>2nd</td>
</tr>
<tr>
<td>Cortisone + penicillin + fungal spore (10³)</td>
<td>6</td>
<td>3rd</td>
</tr>
<tr>
<td>Cortisone + penicillin + fungal spore (10³) + active fraction (25 mg/kg)</td>
<td>6</td>
<td>4th</td>
</tr>
<tr>
<td>Cortisone + penicillin + fungal spore (10³) + active fraction (50 mg/kg)</td>
<td>6</td>
<td>5th</td>
</tr>
<tr>
<td>Cortisone + penicillin + fungal spore (10³) + active fraction (100 mg/kg)</td>
<td>6</td>
<td>6th</td>
</tr>
<tr>
<td>Cortisone + penicillin + fungal spore (10³) + ketoconazole (50 mg/kg)</td>
<td>6</td>
<td>7th</td>
</tr>
<tr>
<td>Cortisone + penicillin + fungal spore (10³) + ketoconazole (100 mg/kg)</td>
<td>6</td>
<td>8th</td>
</tr>
<tr>
<td>Active fraction (100 mg/kg) alone</td>
<td>6</td>
<td>9th</td>
</tr>
</tbody>
</table>

n = 6 in each group. Indicated doses of the herbal drug were administered orally, daily, starting from the day of the A. fumigatus challenge (1st day) for 10 days. Hydrocor-
tsone (5 mg, single dose, s.c.) was administered to suppress the immune function 2 days before the challenge. (Long acting penicillin - 30,000 units i.m. was given two
days before spore challenge.) The mortality was observed for 30 days. No mortality occurred after the 9th day in any of the groups.