Original Article

Fungicidal Effect and Oral Acute Toxicity of Cassia spectabilis Leaf Extract

S. Sangetha 1, Z. Zuraini 1, S. Sasidharan 2, 3, S. Suryani 1

1 School of Distance Education, University Sains Malaysia, Minden 11800, Penang, Malaysia
2 Department of Biotechnology, Faculty of Applied Sciences, AIMST University, Jalan Bedong-Semeling, Batu 3 1/2, Bukit Air Nasi, Bedong 08100, Kedah, Malaysia
3 Department of Biotechnology, AIMST University, Jalan Bedong-Semeling, Batu 3 1/2, Bukit Air Nasi, 08100 Bedong, Kedah, Malaysia

(Received: 11, January 2008. Accepted: 2, June 2008)

Abstract

The fungicidal activity of Cassia spectabilis leaf extracts was investigated using the disk diffusion technique and the broth dilution method. The extract showed a favorable antimicrobial activity against Candida albicans with a minimum inhibition concentration (MIC) value of 6.25 mg/ml. Apart from the fungicidal effects, imaging using scanning electron microscopy (SEM) was done to determine the major alterations in the microstructure of the C. albicans. The main abnormalities noted in the SEM studies were the alterations in morphology and complete collapse of the yeast cells after 36 h of exposure to the extract. The in vitro time-kill study performed using the leaf extract at 1/2, 1 or 2 times of the MIC significantly inhibited the yeast growth with a noticeable drop in optical density (OD) of yeast culture, thus confirming the fungicidal effect of the extract on C. albicans. In addition, in vivo antifungal activity studies on candidiasis in mice showed a 5-fold decrease in Candida in kidneys and blood samples in the groups of animals treated with the extract (2.5 g/kg body weight). In an acute toxicity study using mice, the acute minimum fatal dose of the extract was greater than 2000 mg/kg, and we found no histopathological changes in macroscopic examination by necropsy of mice treated with extract. We conclude that the extract may be safely used as an anticandidal agent.

Key words: Candida albicans, Cassia spectabilis leaf, fungicidal activity, scanning electron microscopy, toxicity

Introduction

Infectious diseases caused by bacteria, fungi, viruses and parasites are still a major threat to public health, despite the tremendous progress in human medicine 6. The past three decades have seen a dramatic increase in microbial resistance to antimicrobial agents 2, 3. Therefore, new antimicrobial agents are needed to deal with this situation. This encouraged us to evaluate the natural resources of our country to identify an antimicrobial agent, particularly against Candida albi-

cans.

The genus Cassia, comprising about 600 species widely distributed worldwide is well known for its diverse biological and pharmacological properties 6. Cassia spectabilis (sin Senna spectabilis) (DC) Irwin et Barn (Leguminosae) is widely grown as an ornamental plant in tropical and subtropical areas, and has been commonly used in traditional medicine for many years. It has also been used in traditional Brazilian medicine for the treatment of flu and cold, as a laxative and purgative 6.

There is still much that is not known about Cassia spectabilis, and research must be conducted on many areas as available data are deficient in the status, extent and utilization of Cassia spectabilis as an antimicrobial agent. The present study was carried out to
determine the fungicidal activity of the extract of *Cassia spectabilis* leaf against *Candida albicans* (Berkhout) and also to report the oral acute toxicity of the extract in mice.

**Material and Methods**

**Microorganism**

*Candida albicans* was used as the test organism and was obtained from our laboratory’s stock culture. The yeast was cultured on Sabouraud dextrose agar at 30°C for 24 h and the stock culture was maintained on Sabouraud dextrose agar slants at 4°C.

**Plant collection and preparation of extracts**

Fresh *Cassia spectabilis* leaf was collected from Penang, Malaysia, in June 2007 and authenticated by the botanist of the School of Biological Sciences at Universiti Sains Malaysia, where the herbarium was deposited. The sun-dried leaf was cut into small pieces and 100 g was boiled in a Soxhlet with 300 ml of methanol for 4 h. The leaf extract was evaporated to dryness in a rotary evaporator. The dried extract was then redissolved in 10% DMSO (v/v) to yield solution containing 100 mg of extract per ml.

**Fungicidal activity**

The fungicidal activity of the extract was determined following the method described by NCCLS. With slight modifications.

**Disk diffusion technique**

The test microbe was removed aseptically with an inoculating loop and transferred to a test tube containing 5 ml of sterile distilled water. Sufficient inoculum was added until the turbidity equalled 0.5 McFarland (10^4 colony forming units ml^{-1}) standard (bio-Merieux, Marcy Peteole, France). One ml of the test tube suspension was added to 15-20 ml of Sabouraud dextrose agar before setting aside the seeded agar plate (9 cm in diameter) to solidify for 15 min. Three Whatman filter paper No. 1 disks of 6 mm diameter were used to screen the fungicidal activity. Each sterile disk was impregnated with 20 μl of extract (corresponding to 10.0 mg/ml of crude extract); miconazole nitrate (30 μg/ml, as positive control) and 10% DMSO (v/v) (as negative control) before being placed on the surface of the seeded plates. The plates were incubated at 37°C overnight and examined for zones of growth inhibition.

**Determination of minimum inhibitory concentrations (MIC)**

A 16 h culture was diluted with a sterile physiological saline solution [PS; 0.85% (w/v) sodium chloride] with reference to the 0.5 McFarland standards to achieve inocula of approximately 10^5 colony forming units ml^{-1}. A serial dilution was carried out to give final concentrations between 0.10-50.0 mg crude extract per ml. The tubes were inoculated with 20 μl of the yeast suspension per ml of Mueller-Hinton broth, homogenized and incubated at 37°C. After incubation, 50 μl was withdrawn from each tube, inoculated on agar plates and incubated at 37°C for 24 h. The MIC value was determined as the lowest concentration of crude extract in the broth medium that inhibited visible growth of the test microorganism.

**Scanning electron microscope observations**

Scanning electron microscope (SEM) observations were carried out on *C. albicans* cells. One milliliter of the *C. albicans* cell suspension at the concentration of 1 x 10^6 cells per ml was inoculated on a Sabouraud dextrose agar plate and incubated at 37°C for 12 h. The extract (2 ml), at the concentration of 10.0 mg per ml, was then dropped onto the inoculated agar and further incubated for another 36 h at the same temperature. A 10% DMSO treated culture was used as a control. A small block of yeast containing agar was withdrawn from the inoculated plate at 0, 12, 24 and 36 h and fixed for scanning².

**Time-kill study**

In order to assess the fungicidal effect with 1/2, 1 or 2 fold MIC concentration over time, growth profile curves were plotted⁶. A 16 h culture was harvested by centrifugation, washed twice with phosphate saline and resuspended in phosphate saline. The suspension was adjusted using the McFarland standard and was then further diluted in phosphate saline to achieve approximately 10^5 colony forming units ml^{-1}. Leaf extract was added to aliquots of 25 ml Mueller-Hinton broth (MHB) in a 50 ml Erlenmeyer flask in a water bath at 37°C, in an amount which would achieve concentration of 1/2, 1 and 2 fold MIC (3.13 mg/ml) after addition of the inocula. Extract free medium was used as control. Thereafter 1 ml of inoculum was added to each Erlenmeyer flask. After addition of the inoculum a 1 ml portion was removed the flask and the growth of *C. albicans* was monitored using this portion by measuring the optical density at 540 nm (UV-9100, Ruili Co., China). The growth of *C. albicans* was measured every 4 h for 48 h by the above method.

**In vivo antifungal activity**

**Laboratory animals**

Swiss albino mice male weighing between 25 and 35 g were used. The cages with the mice were placed in a room (temperature 26 ± 2°C) with controlled cycles of 12 h of light and 12 h of darkness; light went on at 7 am and relative humidity was 45-55%. Water and food
were provided to animals ad libitum. The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of the School of Biological Sciences, Universiti Sains Malaysia. Experiments were conducted in accordance with the internationally accepted principles for laboratory animal use and care (EEC Directive of 1986; 86/609/ EEC).

**Antifungal assay**

The standard intravenous (i.v.) inoculation of *C. albicans* used in this study was $1 \times 10^7$ viable cells/ml PBS, of which 0.1 ml was injected into the lateral tail vein of mice\(^1\). Animals (male) were divided into two groups of 20 mice each and received treatment as described in Table 1. All mice were killed by cervical dislocation on day 5 after i.v. *C. albicans* inoculation.

The kidneys of each animal were removed aseptically, and 0.1 ml of blood was withdrawn from the renal artery and 0.1 ml of heparin (25U/ml), as an anticoagulant was added into the blood sample. The kidneys were then, placed in sterile centrifuge tubes and homogenized in 5 ml of sterile PBS. Aliquots from each homogenate and blood samples were serially diluted, plated on Sabouraud dextrose agar plates, and incubated at 37°C for 24 h. All cultures were done in triplicate. The colonies were then enumerated and the colony forming units (CFU) were calculated per gram of organ and per ml of blood sample, respectively.

**Oral acute toxicity**

In this assay, male and female mice (30-35 g) were used. The animals, housed in cages at 22°C were starved overnight with free access to water. Two groups of 10 animals (control and test group) were formed, each containing an equal number of males and females. A dose limit of 2000 mg/kg body weight of the extract dissolved in water was administered intragastrically to animals from the test group; the animals in the control group received water. In each case the volume administered was 10 ml/kg body weight. Following administration, the animals were closely observed during the first 3 h, and occasionally thereafter for 14 days, for toxic signs and symptoms, and death; the weight was measured daily. During the 14-day period, dead animals were autopsied and at the end of the period, survivors were sacrificed to examine gross changes in the vital organs\(^10\).

Body weight evolution and weight of the organs from the control and the test group were compared using the t-test run on the software SPSS for Windows.

**Results**

**Antimicrobial activity**

The results of fungicidal activity of the extract against *C. albicans* are given in Table 2. The extract exhibited a favorable activity against the yeast tested. The zone of clearance produced by the commercial antibiotic disk was larger than that produced by the extract disk. The agar dilution method recorded the MIC value of 6.25 mg/ml.

**Scanning electron microscope observation**

Fig. 1 shows the SEM photomicrographs of the untreated and extract treated cells of *C. albicans* at various times of exposure to the crude extract of *Cassia spectabilis*. Untreated cells (A) showed many oval cells smooth in appearance and some at a budding stage. After 12 hours of exposure (B), a mild effect of the extract was observed. The 24 hour treated cells (C) had a rough appearance compared to the untreated control cells with the formation of invaginations. After 36 hours of exposure (D), completely collapsed

<table>
<thead>
<tr>
<th>Table 1. Details of experimental groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (control) i.v. Candida 24h gap, follow by treatment with PBS (i.p. once daily for 3 days)</td>
</tr>
<tr>
<td>Group 2 (curative) i.v. Candida 24h gap, follow by treatment with <em>Cassia spectabilis</em> extract, 2.5 g/kg body weight (i.p. once daily for 3 days)</td>
</tr>
</tbody>
</table>

i.v., intravenous; i.p., intraperitoneal; PBS, phosphate buffer solution

<table>
<thead>
<tr>
<th>Table 2. Fungicidal activity (zone of inhibition and MIC(^c)) of <em>Cassia spectabilis</em> leaf extract compared to commercial antibiotic miconazole nitrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microorganism</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
</tr>
</tbody>
</table>

\(^a\) Agar dilution method, mean value N=3.

\(^b\) The values (average of triplicate) are diameter of zone of inhibition at 100 mg/ml crude extract and 30 mg/ml miconazole nitrate.

\(^c\) MIC: minimal inhibitory concentration.
and cavitated cells were seen. It was believed that at this stage, the *C. albicans* cells had lost their metabolic function completely.

**Time-kill study**

The time-kill studies were performed over a period of 48 h with yeast being exposed to 1, 1/2 or 2 × MIC of the *Cassia spectabilis* leaf extract. Fig. 2 shows the results of the time-kill curves for *C. albicans*. At 1/2 × MIC, the *Cassia spectabilis* leaf extract demonstrated a large drop in OD after 20 h, which led to a stationary phase of yeast growth compared with the control. At 1 × MIC, this extract caused complete yeast eradication after only 4 h, and at 2 × MIC, there was complete eradication within 2 h. The time-kill curves described above confirm the potency of *Cassia spectabilis* leaf extract as an antifungal agent against *C. albicans*.

**In vivo antifungal activity**

Table 3 shows the mean of CFU/g organ and CFU/ml of blood from the two groups. In group 2 animals that received a 2.5 g/kg body weight dose of the plant extract followed by inoculation of *C. albicans*, a significant reduction (*P*<0.05) in CFU was observed in kidney and blood samples studied. A 5-fold difference was present in the kidney and blood samples of the treated group compared with those of the control group.

**Oral acute toxicity**

No toxic symptoms or death was observed in any of the animals and all of them lived up to 14 days. An autopsy at the end of the experimental period revealed no apparent changes in any organs; there were also no changes in either the corporal weight or the weight of the principal organs and all animals exhibited a gain in body weight. Table 4 shows the effect of the extract on the principal organ weight relative to body weight. In the daily consumption of food and water, water intake oscillated around 150 and 200 ml per day, respectively, in females and males in both groups. Food intake exhibited the same pattern in each sex and group. Therefore, the acute minimum fatal dose of *Cassia spectabilis* leaf extract for mice is over 2000 mg/kg b.w.

**Discussion and Conclusions**

The antimicrobial activity of *Cassia spectabilis* leaf extract against *C. albicans* was examined in this study,
and its potency was qualitatively and quantitatively evaluated by the presence or absence of an inhibition zone, zone diameter and MIC value. We mainly focused on *C. albicans* in this study because the increasing prevalence of drug resistant *C. albicans* recovered from hospitalized patients is a major concern worldwide. *C. albicans* is also currently the most abundant and important species of this genus and its known to be responsible for infections in humans, causing vulvovaginitis, oral thrush, nosocomial infection and candidiasis.

The time-kill assays were utilized in this study to confirm MIC findings and to evaluate the ability of *Cassia spectabilis* leaf extract to eliminate *C. albicans* growth in vitro. In the case of 1 and 2 fold MIC concentrations the extract inhibited the yeast growth within 2 to 4 h and subsequent regrowth was not seen. The time-kill assay suggested that the extract significantly

### Table 3. Effect of aqueous extract of *Cassia spectabilis* leaf on *Candida albicans* recovered from kidney and blood of mice

<table>
<thead>
<tr>
<th></th>
<th>Kidney</th>
<th>Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (control) i.v. <em>Candida</em> + i.p. PBS</td>
<td>2.21 × 10^5 ± 1531</td>
<td>2.60 × 10^5 ± 10831</td>
</tr>
<tr>
<td>Group 2 (curative) i.v. <em>Candida</em> + i.p. Extract</td>
<td>3.92 × 10^5 ± 65</td>
<td>4.70 × 10^5 ± 53</td>
</tr>
</tbody>
</table>

All values are colony-forming units (CFU/g organ or CFU/ml of blood) expressed as mean ± SEM of 20 determinations.

^P<0.05 compared with control (Student *t*-test)

### Table 4. Effect of *Cassia spectabilis* leaf extract on organ to body weight index (%) in mice

<table>
<thead>
<tr>
<th>Organ</th>
<th>Control Male</th>
<th>Crude extract Male</th>
<th>Control Female</th>
<th>Crude extract Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>1.43 ± 0.01</td>
<td>1.42 ± 0.01</td>
<td>1.35 ± 0.01</td>
<td>1.36 ± 0.01</td>
</tr>
<tr>
<td>Liver</td>
<td>7.10 ± 0.06</td>
<td>7.11 ± 0.06</td>
<td>6.93 ± 0.01</td>
<td>6.63 ± 0.01</td>
</tr>
<tr>
<td>Lung</td>
<td>1.23 ± 0.01</td>
<td>1.27 ± 0.01</td>
<td>1.14 ± 0.01</td>
<td>1.12 ± 0.01</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.53 ± 0.02</td>
<td>0.51 ± 0.02</td>
<td>0.45 ± 0.01</td>
<td>0.47 ± 0.01</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>32 ± 0.58</td>
<td>31 ± 0.55</td>
<td>30 ± 0.50</td>
<td>29 ± 0.48</td>
</tr>
</tbody>
</table>

Organ body index was calculated as (organ weight × 100)/body weight.

* Crude extract of *Cassia spectabilis* was administered to mice at a dose of 2000 mg/kg.

Values are mean ± S.D. (n = 6).

^P>0.05 (*t* test)

Fig. 2. Growth profile of *Candida albicans* in Mueller-Hinton broth with 0 (Control) and 1/2, 1 or 2 fold MIC concentration (6.25 mg/ml) concentration of *Cassia spectabilis* leaf extract.
inhibited *C. albicans* growth and also prolonged antimicrobial activity against the organism as determined by time-kill curves. In this study the methanolic extract of *Cassia spectabilis* leaf was shown to have significant antifungal activity in a murine model when given by intraperitoneal injection followed by inoculation of *C. albicans*, indicating a strong antifungal activity.

Furthermore, the SEM study showed that the extract could completely collapse the yeast cells and inhibit the growth of *C. albicans*. Hence, *C. albicans* infection could be treated with the extract, as the MIC for this yeast was found to be only 6.25 mg/mL. The acute minimum fatal dose of the crude extract was greater than 2000 mg/kg in rats, which is the single high dose recommended by OECD guidelines for testing oral acute toxicity\(^{16}\). Thus, our test suggested that *Cassia spectabilis* leaf do not cause any apparent acute toxicity. The results of the current study concur with the use of this plant by native people as an herb.

Hence, *Cassia spectabilis* leaf extract could be utilized in developing more effective antimicrobial drugs in the management of nosocomial infections caused by *C. albicans*. In addition, it may find use as an antifungal agent in known dosages especially in rural communities where conventional drugs are unaffordable or unavailable and health facilities inaccessible. The results of the present study may suggest that *Cassia spectabilis* leaf extract possesses compound (s) with anti-yeast properties against this organism. Further purification of the active compound (s) with antimicrobial activity against *C. albicans* of the extract is therefore suggested for further studies.

References


