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The Antifungal Activity of *Sarcococca saligna* Ethanol Extract and its Combination Effect with Fluconazole against Different Resistant Aspergillus Species

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Abstract Microbial resistance is a major drawback in chemotherapy of microbial or fungal infection disease. In this study, the antifungal activity of ethanol extract of a selected plant (*Sarcococca saligna*) has been investigated against clinical isolates of *Aspergillus niger*, *Aspergillus treus*, *Aspergillus flavus*, and *Aspergillus fumigatus*. Also, the enhancement of the antifungal activity of fluconazole by this extract was further evaluated against mentioned test strains. Conventional disk diffusion method was used to assay the antifungal activity of *S. saligna* ethanol extract in the absence and presence of fluconazole. The highest antifungal activity was observed against *A. treus*. The ethanol extract of *S. saligna* enhanced the antifungal activity of fluconazole against *A. niger* and *A. treus* and *A. flavus*. At the highest tested contents (4 mg/disk), 1.15-, 0.64-, and 2.47-fold increases in inhibition zone surface area were observed for *A. niger*, *A. treus*, and *A. flavus*, respectively. However, no enhancing effect was observed for this plant extract against *Aspergillus fumigates* at tested contents (0.5, 1, 2, 3, and 4 mg/disk). In a separate experiment, the general cytotoxicity of the ethanol extract of *S. saligna* was examined with brine shrimp assay. This plant extract showed low cytotoxicity against *Artemia salina* (LC_{50}=186 μg/ml).

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Introduction

The natural response of microorganisms to antibiotic stress is called the antimicrobial resistance, which is developed by prevention of interaction of the drug with the target, efflux of the antibiotic from the cell, and direct destruction or modification of the compound. Concerned about the widespread emergence and dangers of this decreased sensitivity to antibiotics, extensive investigations have been reported to combat this problem [1, 2]. Using resistance modulators with the antibiotics is one of the important alternatives, which deactivates the resistance mechanism of microorganisms and also extends the usefulness of antibiotics with known clinical, pharmacological, and toxicological properties [3, 4]. Besides developing new chemical resistance inhibitors [5–7], plant-based natural products are being searched to work as resistance modulators [8–13].

Aspergillus genus is a famous taxonomic group of molds; some of its species are very important animal and human pathogens. These microorganisms broadly cause localized or systemic aspergillosis diseases [14]. *Aspergillus* species seem susceptible to itraconazole and new triazoles, but they are intrinsically resistant to fluconazole [15, 16]. In our screening program for finding a plant extract to enhance the antifungal activity of fluconazole against different *Aspergillus* species, we selected a plant extract prepared from *Sarcococca saligna* (Buxaceae). In the traditional medicine of Pakistan, the leaves of *S. saligna* are used as laxatives, blood purifiers, and muscular pain killer agents [17]. Several pharmacological activities have been reported for this common wild shrub, including those of cardio-suppressants, vasodilators, tracheal relaxants, cholinesterase inhibitors, antispasmodics, antidiarrheals, antisecretorys, calcium antagonists, and acetylcholinesterase inhibitors [18–20]. Antibacterial activity of *S. saligna* against several human pathogenic bacteria has also been reported [21], but to the best of our knowledge, based on a literature survey, the antifungal activity of ethanol extract of *S. saligna* and its combination with fluconazole against different *Aspergillus* species has not yet been investigated. We report herein that antifungal activity of *S. saligna* plant extract itself and in combination with fluconazole was investigated using the disk diffusion method against the following clinical isolated *Aspergillus* species: *Aspergillus niger*, *Aspergillus treus*, *Aspergillus flavus*, and *Aspergillus fumigates*. The cytotoxicity assessment of the plant extract performed by brine shrimp assay has also been presented.

Materials and Methods

Plant Material and Extraction

The aerial parts of *S. saligna* were collected from Malam Jaba (7,400 ft above sea level), Swat (Pakistan), on May 15, 2005. Taxonomic identification of the plant was done by Prof. Dr. Habib Ahmad, Department of Botany, Hazara University. A voucher specimen (No. 235 HUH) was deposited in the herbarium at the Department of Botany, Hazara University. The plant material was dried in the shade under controlled conditions. The dried plant was ground into powder with a heavy-duty grinding machine. The powdered plant material

(5 kg) was exhaustively extracted at room temperature with 95% ethanol using the percolation method. The extract obtained was evaporated by a vacuum rotary evaporator, keeping the temperature below 40 °C. A gummy residue (320 g, 6.4%) was obtained as a crude extractive. A standard ethanol solution (10 mg/ml) was prepared using the dried plant extract and used for antifungal experiments.

Determination of the Antifungal Activity

A disk diffusion method was used to assay antifungal activity of ethanol extract of *S. saligna* and its combination effect with fluconazole against clinical test strains on Sabouraud dextrose agar (Difco, Germany) plates [22]. Blank paper disks and standard fluconazole paper disks (25 μg) were purchased from Mast (UK) and supplemented with different concentrations of *S. saligna* ethanol extract (0.5, 1, 2, 3, and 4 mg/disk). Standard fluconazole paper disk was also used as control. All the test strains from our collection (*A. niger*, *A. treus*, *A. flavus*, and *A. fumigates*) were previously isolated from Shariati University Hospital (Tehran, Iran) and identified by conventional methods by our colleague (Dr. Sassan Rezaee). A single colony of test strains was streaked on Sabouraud dextrose broth liquid medium (Difco, Germany) and incubated for 3 days at 25 °C. The inocula were prepared by diluting the cultures with 0.9% NaCl to a 0.5 McFarland standard and were applied to the plates along with the standard and prepared disks containing different concentrations of plant extract. After incubation at 25 °C for 3 days, the zones of inhibition were measured. The assays were performed in triplicate.

Brine Shrimp Lethality Assay (BSA)

The cytotoxicity of the *S. saligna* ethanol extract was determined by adaptation of a previously described method [23]. Water life brand brine shrimp (*Artemia salina*) eggs were purchased from the Shilat Center (Tehran, Iran). The eggs were hatched in a flask containing 300 ml artificial seawater made from distilled water. The flask was well aerated with the aid of an air pump and kept in a water bath at 29–30 °C and illuminated by a bright light. The nauplii hatched within 48 h. The extract was dissolved in normal saline. Different concentrations were obtained by serial dilution (10, 100, 500, and 1,000 μg/ml). Solution of each concentration (500 μl) was transferred into clean 24-well plates via a pipette, and to them was added aerated seawater having 10–20 nauplii (500 μl). A check count was performed, and the numbers of alives were noted after 24 h. The mortality end point of the bioassay was determined as the absence of controlled forward motion during 30 s of observation. The controls used were seawater and a well-known cytotoxic alkaloid, berberine hydrochloride. Lethality percentage was determined and LC_{50} calculated based on probit analysis with 95% confidence interval.

Results and Discussion

In this study, for the first time, the antifungal activity of the ethanol extract of *S. saligna* and its combination effect on the fluconazole were investigated against different clinical isolates of *Aspergillus* species (Table 1). As shown in Table 1, no clear zones of inhibition were observed for all test strains around standard fluconazole paper disks, and this confirmed that these test strains were resistant to fluconazole. The ethanol extract prepared from *S. saligna*

Table 1 Zone of inhibition (mm) of ethanol extract of *S. saligna* against *Aspergillus niger*, *Aspergillus treus*, *Aspergillus flavus* and *Aspergillus fumigates* (in absence and in presence of fluconazol at content of 25 μg/disk.

Plant extract (mg/disk)	Mean diameter of inhibition zones (mm)		Increase in fold area
	Without fluconazole (A)	With fluconazole (25 μg) (B)	
Aspergillus niger			
0.0	–	–	0
0.5	9	11	0.49
1.0	11	12	0.19
2.0	12	13	0.17
3.0	13	14	0.16
4.0	15	22	1.15
Aspergillus treus			
0.0	–	–	0
0.5	8	14	2.10
1.0	17	22	0.67
2.0	20	25	0.56
3.0	25	27	0.17
4.0	24	31	0.67
Aspergillus flavus			
0.0	–	–	0
0.5	–	–	0
1.0	–	–	0
2.0	–	9	1.65
3.0	–	10	2.04
4.0	–	11	2.47
Aspergillus fumigates			
0.0	–	–	0
0.5	–	–	0
1.0	–	–	0
2.0	–	–	0
3.0	8	8	0
4.0	9	9	0

Mean surface area of the inhibition zone (mm^2) was calculated for fluconazol from the mean diameter. Fold increase for fluconazol in each dose was calculated as $(b^2 - a^2)/a^2$, where a and b are the areas of inhibition zones for A and B, respectively. All experiments were done in triplicate and standard deviations were negligible. In the absence of bacterial growth inhibition zones, the disks' diameters (7 mm) were used to calculate the fold increase in columns 5 and 6.

showed antifungal activity at content of ≥0.5 mg/disk against *A. niger* and *A. treus* (Table 1). The antifungal activity of the plant extract against the mentioned test strains was dose-dependent and increased with the increase in the plant extract concentrations. In contrast, the ethanol extract of *S. saligna* did not show antifungal activity against *A. flavus* (Table 1) at contents used for the bioassay (0.5, 1, 2, 3, and 4 mg/disk). Furthermore, another tested strain (*A. fumigates*) was less susceptible to the ethanol extract of *S. saligna* compared to *A. niger* and *A. treus* (Table 1). No inhibition zones were observed for *A.*

fumigates at lowest contents of *S. saligna* ethanol extract (0.5, 1, and 2 mg/disk) (Table 1). The combination effect of this plant extract at the same amounts (0.5, 1, 2, 3, and 4 mg/disk) with fluconazole (25 μg/disk) was also investigated against the mentioned *Aspergillus* species and has been reported in Table 1. The ethanol extract of *S. saligna* enhanced the antifungal activity of fluconazole against *A. niger* and *A. treus* and *A. flavus*. At the highest tested contents of 4 mg/disk, 1.15-, 0.64-, and 2.47-fold increases in inhibition zone surface area were observed for *A. niger*, *A. treus*, and *A. flavus*, respectively. However, no enhancing effect was observed by the plant extract against *A. fumigates* at tested concentrations of the extract.

In a separate experiment, the general cytotoxicity of the ethanol extract of *S. saligna* and a control sample (berberine hydrochloride) were examined with brine shrimp assay. Berberine hydrochloride showed a considerable toxicity (26 μg/ml) against *A. salina* (Table 2). In contrast, the ethanol extract of *S. saligna* demonstrated low cytotoxicity against *A. salina* (LC_{50} >1,000 μg/ml). Minimum lethal concentrations of the ethanol extract of *S. saligna* plant were greater than 1,000 μg/ml; therefore, they were inactive.

Conclusions

Multi-drug resistance is a medical problem faced world-wide and has therefore led researchers in the search for new antimicrobial drugs or resistance modulators, particularly from natural resources [1]. Recently, various natural products or synthetic compounds have been reported to increase the antibacterial activity of the presently used antibiotics against different clinical isolated resistant test strains [5–13]. The essential oil of *Cinnamon zirconium* has been reported to enhance the activity of clindamycin against toxicogenic *Clostridium difficile* [11]. We have also recently reported that the ethanol extract of *Berberis integerrima* and its active component (1-methyl malate) can increase the antibacterial activity of ampicillin against the clinical isolates of *Staphylococcus aureus* [13]. The ethanol extract of *S. saligna* showed antifungal activity against the test strains used (except *A. flavus*). Different clinical *Aspergillus* species that were used during this study were resistant to fluconazole, but the antifungal activity of fluconazole was enhanced in the presence of *S. saligna* ethanol extract against mentioned test strains (except *A. fumigatus*). It also suggested that co-administration of the natural product with fluconazole would enhance its potency in vivo too. Applying pharmacological indications of fluconazole extended its use to that against aspergillosis. Fractionation and identification of the active constituents present responsible for the reported results in the ethanol extract of *S. saligna* are underway through bioassay-guided isolation and structural characterization in our research laboratories.

Table 2 The cytotoxicity potential of *S. saligna* ethanol extract on *Artemia salina* larva.

Sample	Mortality (%)				LC_{50} (μg/ml)	Confidence interval (95%)
	10 (μg/ml)	100 (μg/ml)	500 (μg/ml)	1,000 (μg/ml)		
S. saligna ethanol extract	28.5	30.3	28.3	31.5	>1,000	–
Berberine HCl	33.6	76.2	98.4	102.8	26	16–38

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