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Antifungal activity from aerial part extracts of *Leptadeniapyrotechnica (Forsskal.) Decne* from the Cholistan Desert, Pakistan

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Abstract

This study was carried out with an objective to investigate the antifungal potentials of aerial part of *leptadeniapyrotechnica(Forssk) Decne*. The aim of the study is to assess the antifungal activity and to determine the zone of inhibition of extracts on fungal strains. The experiment based on ethno-medicinal survey, in the present study, the antifungal activity of methanol, hexane, ethyl acetate and water extracts of aerial part of *leptadeniapyrotechnica*was evaluated for potential antifungal activity against pathogenic fungal strains. The antifungal activity was determined in the extracts using disc diffusion method. The antifungal activities of extracts (1.8, 2.9, 6.5, 12.6, 25, 50, and 75µg/ml) of *leptadeniapyrotechnica*were tested against five fungal strains *Aspergillusflavus, A.fumigatus*, *A. niger*, *A. ustus*and*Candida albicans*. Zone of inhibition of extracts were compared with that of standards drug, Flucanazole antifungal activity. The results showed that the remarkable zone of inhibition of the fungal growth was shown against the tested fungi. The antifungal activity of the *leptadeniapyrotechnica*was due to the presence of various secondary metabolites. Hence, these plants can be used to discover bioactive natural products that may serve as leads in the development of new pharmaceuticals research activities.

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Introduction

The Cholistan Desert is a part of the world's seventh largest desert, the Great Desert, which is stretched along the south border of the Punjab province, Pakistan [1]. The total area of the Cholistan Desert is about 26,000 km²; it lies between 27° 42' and 29° 45' North and 69° 52' and 75° 24' East at an altitude of about 112 m above sea level [2]. Life sustainability revolves round annual precipitation ranging from 100 to 250 mm per annum [3-5].Medicinal plants have been used as traditional treatments for thousands of years being the essence of nutraceuticals, pharmaceuticals, synthetic drugs and folk medicines. The human pathogenic microbes are becoming drug resistant and this issue has led to the urgent exploration of the medicinal plants having lesser side effects and more potential. The present investigation deals with exploring antifungal potential of some medicinal plants of Cholistan desert of Pakistan. Antifungal infections (Candidal and Aspergillosis) specially Candidal infections are real threat for the mankind by two factors i.e. they cause blood-seated infections and development of resistant candidal species against a number of best-known antifungal drugs like Flucanazole, Amphotericin B etc. *Aspergillus*is a common fungus that can be found indoor and outdoor environments. Most people breathe in *Aspergillus* spores every day without being affected. Aspergillosis is a disease caused by this fungus and usually occurs in people with lung diseases or debilitated immune systems. *C. albicans*is a fungus that is pathogenic to human beings [6]. The plantshave been collected from the Cholistan desert. The antifungal prospective of *L. pyrotechnica* was checked for *Aspergillusflavus, A.fumigatus*, *A. niger*, *A. ustus*and*Candida albicans*.Plant extracts of many higher plants have been reported to exhibit antifungal and insecticidal properties in *vitro* and in *vivo* studies (7-10). Traditional knowledge conforms to the study of different solvent fraction extract against antifungal activity collected from Cholistan Desert, Pakistan. The study conducted explore antifungal activity especially *candida albican* is highly human pathogenic from medicinal plants Cholistan Desert. Synthetic antifungal drugs have many side effects, our study find the natural compound for antifungal have few or no side effects.

Materials and methods

Collection of Plant Materials

The fresh and healthy aerial parts of the plant *Leptadeniapyrotechnica* were collected between June and August, 2013 from Bagdad-ul-jadeedcampus, Pakistan. The plant specimens were identified in department of Cholistan Institute of Desert studies with Voucher no. 3471/CIDS/IUB. Plant parts were collected on the basis of the information provided in the ethnobotanical survey. Each specimen/plant material was labeled, numbered, a noted with the date of collection, locality, and their medicinal uses were recorded.

Preparation of Plant Extract

The extraction of the *Leptadeniapyrotechnica* aerial parts was carried out using known standard procedures. The plant materials were dried in shade and powdered in a mechanical grinder. The powder (12.900Kg) of the plant materials were initially defatted with methanol (40 L). The extracts were filtered using Whatman filter paper (No.1).The solvent was evaporated off under high vacuum on rotary evaporator. The dried methanolic extracts (130 g) were used for further fractionation. Dried methanolic extracts (50 g) were dissolved in 200 mL of distilled water. The extraction with petroleum ether (hexane) was done (600 mL X 3) to get hexane extracts 2.9 g. Successive extractions of aqueous extracts was also done with ethyl acetate (600 mL X 3) to get ethyl acetate extracts 20 g.

The methanolic, hexane, ethyl acetate and aqueous extracts were screened against *C. albicans, A. flavus, A. ustus , A. fumigatusand A. niger*in 7 concentrations i.e., 1.8 µg/disc, 2.9 µg/disc, 6.5 µg/disc, 12.6 µg/disc, 25 µg/disc, 50 µg/disc, and 75 µg/ per disc concentration.

Preliminary Phytochemical Screening

The extracts were subjected to preliminary phytochemical testing to detect for the presence of

different chemical groups of compounds. The plant contains triterpenoids, taraxerol, fernenol[7]. *L. pyrotechnica* have chemical constituents led to the isolation of six flavonoids, kaempferol-3-O-α-l-rhamnopyranosyl (1999→699)-O-β-d-glucopyranoside (E-I.1), kaempferol-3-O-β-d-rhamnopyranosyl (1999→699)-O-β-d-glucopyranoside (E-I.2), texasin-7-O-β-d-glucopyranoside E-II.2, kaempferol-3-O-β-d-glucopyranoside (E-III.1), kaempferol (E-IV.1) and kaempferide-3-O-α-l-rhamnopyranosyl (1999→699)-O-β-d-glucopyranoside (E-I.1a). [8]

Test fungi and growth media

The following Fungi

*C. albicans*was 2915; *A. flavus*51*; A. fumigates* 445; *A. ustus*;603 and the voucher number of *A. niger*; 2. were chosen based on their clinical and pharmacological importance. The fungastrains *C. albicans, A. flavus, A. ustus, A. fumigatusand A. niger*were taken from the pathology lab of Quaid-e-Azam Medical College Bahawalpur and First Fungal Culture Bank of Pakistan (FCBP), Institute of Agricultural Sciences (IAGS), P.U., Lahore, Pakistan were used for evaluating antifungal activity. The fungal stock cultures were incubated for 48 hours at 28°C on nutrient for the antifungal screening on solid media Yeast Peptone Dextrose Agar (YPDA) media was prepared.

Antifungal Activity

Determination of zone of inhibition method

*In vitro*antifungal activity was examined for methanol, hexane, ethyl acetate and water extracts. Antifungal activity of plant parts extracts against five pathogenic fungi (*C. albicans, A. flavus, A. ustus, A. fumigatusand A. niger*) by the agar disk diffusion method.Antifungal activity testing by disc diffusion method[9].For the determination of zone of inhibition, pure fungal strains were taken as a standard antifungal for comparison of the results. All the extracts were screened for their antifungal activity against the fungi *C. albicans, A. flavus, A. ustus, A. fumigatusand A. niger*. The sets of eight dilutions (1.8, 2.9, 6.5, 9.8, 12.6, 25, 50, and 75 µg/ml) of *Leptadeniapyrotechnica* extracts and standard drug. Flucanazole used for antifungal activity as standard drugs. The zones of growth inhibition around the disks were measured after 48 hours of in incubation at 28°C. The sensitivities of the microorganism species to the plant extracts were determined by measuring the sizes of inhibitory zones (including the diameter of disk) on the agar surface around the disks and different extracts showing zone of inhibition of 15 mm or more were considered significant and selected for the determination of their MIC by the disc diffusion method [10].

Statistical analysis

All analyses were repeated three times to ensure accuracy. Statistical analysis was carried out using Graph PadInstat.Reported data are expressed as means ± SEM. Student t-test was used in the analysis to determine the level of significance of the various fungus zones of inhibition observed. P-value less than 0.05 were considered significant.

Results

The antifungal activity of the extracts of *Leptadeniapyrotechnica* were studied in different concentrations (1.8, 2.9, 6.5, 9.8, 12.6, 25, 50, and 75µg/ml) against five pathogenic fungal strains. These fungal strains have been selected for the basis of its application purpose of further formulation study.

The antifungal activity of the extracts increased linearly with increase in concentration of extracts (µg/ml). As compared with standard drugs, the results revealed that in the extracts for fungal activity, in the methanolicextracts*A. niger*shows good result as compare with *A. ustus, A.flavus, C. albicans*and*A. fumigatus*. ranged from 1.8 mm to 27.5 mm for*A. Niger* (Table 1).The methanolic aerial parts extracts of *L. pyrotechnica*were also applied against*A. niger*and*A. ustus*which give minimum inhibitory concentration (MIC) 2.9 µg/mL and 12.6 µg/mL, respectively (Table **1**).The methanolic extracts tested on the *A. flavus*which showed minimum inhibitory concentration (MIC) 50 µg/mL (Table 1).

Table 1. Antifungal activity of Mathanolic extract of *Leptadeniapyrotecnica* showing zones of inhibition (mm).

Fungus	Mean zone of inhibition of methanolic extract (mm)								
	+ve control	1.8 µg/ml	2.9 µg/ml	6.5 µg/ml	12.6 µg/ml	25 µg/ml	50 µg/ml	75 µg/ml	MIC
Candida albicans	33.5±0.500	N.I	N.I	N.I	N.I	N.I	N.I	N.I	N.I
Aspergillusflavus	37.0±1.000	N.I	N.I	N.I	N.I	N.I	3.4 ±0.33**	8.6±0.318**	50 µg/ml
Aspergillusfumigatus	39.7±0.882	N.I	N.I	N.I	N.I	N.I	N.I	N.I	N.I
Aspergillususstus	39.0±1.000	N.I	N.I	N.I	2.6 ±0.306**	7.8±0.296**	14.9±0.581**	19.2±0.441**	12.6µg/ml
Aspergillusniger	41.3±0.250	N.I	1.8 ±0.115**	5.9 ±0.145**	9.4 ±0.306**	11.3±0.371**	21.3±0.651**	27.5±0.289**	2.9 µg/ml

*indicate a significant difference at p<0.05**indicates a significant difference at p<0.01

+ve control = Flucanazole 100 µg/ml, N.I = No inhibition, MIC = minimum inhibitory concentration.

The hexane extracts of *L. pyrotechnica*aerial parts were used to test the antifungal activity against *A. flavus, A. ustus and A. niger*. The hexane extracts when tested against *A. flavus*it gave MIC 2.9 µg/mL (Table 2). The hexane extracts were also found to inhibit *A. niger*and*A. ustus*, the observed MIC were 6.5 µg/mL and 12.6 µg/mL, respectively (Table 2).

Table 2. Antifungal activity of hexane extracts of *Leptadiniapyrotechnica*showing zones of inhibition (mm).

Fungus	Mean zone of inhibition of Hexane extract (mm)						
	+ve control	1.8 µg/ml	2.9 µg/ml	6.5 µg/ml	9.8 µg/ml	12.6 µg/ml	MIC
Candida albicans	12.6 ±0.250	N.I	N.I	N.I	N.I	N.I	N.I
Aspergillusflavus	9.3 ±1.250	N.I	1.8±0.145**	3.3 ±0.176**	4.1±0.088**	6.8 ±0.145	2.9 µg/ml
Aspergillusfumigatus	10.1 ±0.900	N.I	N.I	N.I	N.I	N.I	N.I
Aspergillususstus	11.1 ±0.100	N.I	N.I	N.I	N.I	2.8 ±0.145**	12.6 µg/ml
Aspergillusniger	8.9 ±0.100	N.I	N.I	1.5 ±0.173**	3.2±0.153**	5.4 ±0.260**	6.5 µg/ml

*indicate a significant difference at p<0.05**indicates a significant difference at p<0.01

+ve control = Flucanazole 13 µg/ml, N.I = No inhibition, MIC = minimum inhibitory concentration.

The ethyl acetate extracts of *L. pyrotechnica* were also tested against *A. flavus*. The observed minimum inhibitory concentration was 9.8 µg/mL (Table 3). The ethyl acetate extracts of *L. pyrotechnica* also applied on *A. ustus* that showed MIC 6.5 µg/mL (Table 3).

Table 3. Antifungal activity of Ethyl acetate extracts of *Leptadeniapyrotechnica*showing zones of inhibition (mm).

Fungus	Mean zone of inhibition of ethyl acetate extract (mm)						
	+ve control	1.8 µg/ml	2.9 µg/ml	6.5 µg/ml	9.8 µg/ml	12.6 µg/ml	MIC
Candida albicans	12.6 ±0.250	N.I	N.I	N.I	N.I	N.I	N.I
Aspergillusflavus	9.3 ±1.250	N.I	N.I	N.I	2.0±0.088**	3.9 ±0.115**	9.8 µg/ml
Aspergillusfumigatus	10.1 ±0.900	N.I	N.I	N.I	N.I	N.I	N.I
Aspergillususstus	11.1 ±0.100	N.I	N.I	1.4±0.233**	2.9±0.186**	6.3 ±0.115**	6.5 µg/ml
Aspergillusniger	8.9 ±0.100	N.I	N.I	N.I	N.I	N.I	N.I

*indicate a significant difference at p<0.05**indicates a significant difference at p<0.01

+ve control = Flucanazole 13 µg/ml, N.I = No inhibition, MIC = minimum inhibitory concentration.

The aqueous extracts of *L. pyrotechnica*did not show any inhibitory effect against all tested fungal strains (Table 4). These results of all the extracts were also compared with the flucanazole, a standard drug (Table 1, 2, 3 & 4).

Discussion

Antimicrobial properties of medicinal plants are

being increasingly reported from different parts of the world. The World Health Organization estimates that plant extract or their active constituents are used as folk medicine in traditional therapies of 80% of the world's population. Methanolic extracts showed maximum activity against *A. niger* (27.5 mm) at higher `dose. At dose 75 µg/mL, 50 µg/mL, 25 µg/mL, 12.6 µg/mL, 6.5 µg/mL and 2.9 µg/mL with inhibition zone was 27.5 mm, 21.3 mm, 11.3 mm, 9.4 mm, 5.9 mm and 1.8 mm, respectively. The dose at 1.8 µg/mL did not give zone of inhibition for the *A. niger*. The methanolic extract showed MIC 2.9 µg/mL against *A. niger* (Table 1). The methanolic extracts showed higher statistical difference at the doses 75 µg/mL, 50 µg/mL, 25 µg/mL, 12.6 µg/mL, 6.5 µg/mL and 2.9 µg/mL ($p < 0.01$) against *A. niger* (Table 1). The methanolic extracts of aerial parts of the plant tested on *A. ustus*at dose 75 µg/mL it gave inhibition 19.2 mm; at 50 µg/mL zone of inhibition appeared as 14.9 mm; at 25 µg/mL it gave inhibition 7.8 mm; at 12.6 µg/mL it imparted inhibition 2.6 mm; while at 6.5 µg/mL, 2.9 µg/mL and 1.8 µg/mL did not give any zone of inhibition.

Table 4. Antifungal activity of aqueous extracts of *Leptadiniapyrotechnica* showing zones of inhibition (mm).

Fungus	Mean zone of inhibition of aqueous extract (mm)						
	+ve control	1.8 µg/ml	2.9 µg/ml	6.5 µg/ml	9.8 µg/ml	12.6 µg/ml	MIC
Candida albicans	12.6 ±0.250	N.I	N.I	N.I	N.I	N.I	N.I
Aspergillusflavus	9.3 ±1.250	N.I	N.I	N.I	N.I	N.I	N.I
Aspergillusfumigatus	10.1 ±0.900	N.I	N.I	N.I	N.I	N.I	N.I
Aspergillusustus	11.1 ±0.100	N.I	N.I	N.I	N.I	N.I	N.I
Aspergillusniger	8.9 ±0.100	N.I	N.I	N.I	N.I	N.I	N.I

+ve control = Flucanazole 13 µg/ml, N.I = No inhibition, MIC = minimum inhibitory concentration.

The minimum inhibitory concentration for the methanolic extract of plant was 12.6 µg/mL (Table 1). The extracts of the plants also showed higher statistical difference at 75 µg/mL, 50 µg/mL, 25 µg/mL and 12.6 µg/mL ($p < 0.01$) when tested on *A. ustus* (Table 1). The methanolic extracts of plant applied on *A. flavus* which showed activity only at two doses i.e 75 µg/mL and 50 µg/mL that gave zone of inhibition 8.6 mm and 3.4 mm. At the doses 25 µg/mL, 12.6 µg/mL, 6.5 µg/mL, 2.9 µg/mL and 1.8 µg/mL it did not showed activity against *A. flavus*. The observed MIC for the methanolic extracts of plant was 50 µg/mL (Table 1). The methanolic extracts applied on the *A. flavus*showed higher statistical difference ($p < 0.01$) at the 75 µg/mL and 50 µg/mL (Table**1**). However, these results of methanolic extracts for the tested fungi were comparable to positive control, flucanazole (Table 1).

The hexane extracts applied on *A. flavus*at 12.6 µg/mL, 9.8 µg/mL, 6.5 µg/mL and 2.9 µg/mL showed inhibition zones 6.8 mm, 4.1 mm, 3.3 mm and 1.8 mm while at 1.8 µg/mL did not show any activity. The minimum inhibitory concentration 2.9 µg/mL was observed for *A. flavus* (Table 2). The hexane extracts showed higher statistical difference at 9.8 µg/mL, 6.5 µg/mL and 2.9 µg/mL ($p < 0.01$) on the *A. flavus*whereas, the higher dose 12.6 µg/mL ($p > 0.05$) was not statistical good against *A. flavus*(Table 2). The hexane extracts of aerial parts of plant showed MIC 6.5 µg/mL for *A. niger*. At the dose of 12.6 µg/mL it gave 5.4 mm zone of inhibition, at 9.8 µg/mL it resulted 3.2 mm zone of inhibition, and at 6.5 µg/mL it imparted 1.5 mm zone of inhibition while 2.9 µg/mL and 1.8 µg/mL did not show any antifungal activity for *A. niger* (Table **2**). The hexane extracts of the plant also showed a higher statistical difference at the doses 12.6 µg/mL, 9.8 µg/mL and 6.5 µg/mL ($p < 0.01$) on the *A. niger* (Table 2). The hexane extracts of *L. pyrotechnica*were tested on *A. ustus*which showed antifungal activity only at 12.6 µg/mL (2.8 mm zone of inhibition); while other doses 9.8 µg/mL, 6.5µg/mL, 2.9 µg/mL and 1.8 µg/mL did not show antifungal activity against *A. ustus* (Table

2). The hexane extracts showed a good statistical difference only at 12.6 µg/mL ($p < 0.01$) against *A. ustus*(Table **2**). These results of hexane extracts for all the fungi were comparable to standard drug, flucanazole (Table 2).

The ethyl acetate extracts of *L. pyrotechnica* were also checked for its antifungal potential against fungal strains i.e*A. flavus*and*A. ustus*. The ethyl acetate extracts were tested on *A. flavus*at different doses which gave different zone of inhibition. At the dose 12.6µg/mL it gave 3.9 mm zone of inhibition, at 9.8µg/mL it showed 2.0 mm zone of inhibition and 6.5 µg/mL, 2.9µg/mL and 1.8 µg/mL it did not show antifungal activity for *A. flavus*. The observed MIC of the ethyl acetate extracts of the plant was 9.8µg/mL (Table**3**). Thus, ethyl acetate extracts showed higher statistical difference only at two doses 12.6 µg/mL and 9.8 µg/mL ($p < 0.01$) against *A. flavus* (Table**3**). The ethyl acetate extracts of *L. pyrotechnica*were checked on *A. ustus*at the concentration of 12.6µg/mL, 9.8 µg/mL and 6.5 µg/mL that gave zone of inhibitions 6.3 mm, 2.9 mm and 1.4 mm, respectively. At the concentration of 2.9 µg/mL and 1.8 µg/mL it did not show antifungal activity against the *A. ustus*. The minimum inhibitory concentration of the plant was 6.5 µg/mL against *A. ustus* (Table **3**). Ethyl acetate extracts showed higher statistical difference at the doses 12.6 µg/mL, 9.8 µg/mL and 6.5 µg/mL ($p < 0.01$) against *A. ustus*(Table **3**). The antifungal activity of ethyl acetate was comparable to positive control, flucanazole (Table 3). The water extracts of *L. pyrotechnica*did not show antifungal activity against all five tested fungal strains (Table **4**).

The present study has shown that the extracts of *L. pyrotechnica* possess remarkable antifungal activity against many pathogenic fungi. This antifungal activity is due to presence of phytochemicals. Thus, there is a possibility of developing this plant a source of antifungal agent and further investigations are necessary to identify the bioactive principles. In the present work, the extracts obtained from *Leptadeniapyrotechnica* show strong activity against most of the tested fungal strains. The results were compared with standard antibiotic drugs. In this screening work, extracts of *Leptadeniapyrotechnica* were found to be inactive against *Candida albicans* and *Aspergillusfumigatus* whereas, fungal strains (*Aspergillusniger, Aspergillususstus*and *Aspergillusflavus*) were resistant to all the extracts of *Leptadeniapyrotechnica*. The methanolic extract was good against *Aspergillusniger,* the hexane extract showed good antifungal activity against *Aspergillusflavus*and the ethyl acetate extract of *L. pyrotechnicashowed good antifungal activity for Aspergillususstus*.

The above results show that the activity of methanolic extracts of *Leptadeniapyrotechnica* shows significant antifungal activity. This study also shows the presence of different phytochemicals with biological activity that can be of valuable therapeutic index.

Conclusion

The present study justified the claimed uses of aerial parts in the traditional system of medicine to treat various infectious disease caused by the microbes. However, further studies are needed to better evaluate the potential effectiveness of the crude extracts as the antifungal agents. The present results will form the basis for selection of plant species for further investigation in the potential discovery of new natural bioactive compounds. Further studies which aimed at the isolation and structure elucidation of antibacterial active constituents from the plant have been initiated.

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