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Pharmacological Screening of *Arceuthobium oxycedri* (Dwarf Mistletoe) of Juniper Forest of Pakistan

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Abstract: The local population of Balochistan, Pakistan considers the *Juniperus excelsa* an important medicinal plant. The species is facing a grave threat by a parasitic and epiphytic angiosperm, dwarf mistletoe, *Arceuthobium oxycedri* (DC) M. Bieb (Viscaceae). The methanolic extract of *A. oxycedri* was studied for its chemical composition and biologically active compounds for the first time. The extract was assayed for antibacterial, antifungal, phytotoxic, cytotoxic and insecticidal activities. The antibacterial and antifungal activities of the extract were determined against ten bacterial and ten fungal strains by agar well diffusion and disc diffusion assay. The extract was highly effective against three bacteria *Pseudomonas aeruginosa, Escherchia coli, Bacillus subtillis* and a fungus *Candida albicans*.. The phytotoxic effects showed that it was extremely toxic for *Lemna acquinoctialis*. It showed high cytoxicity for brine shrimps at all concentrations and was found to be significantly cytotoxic against *Candida albicans* when checked by flow cytometer. However, the extract was not effective against the pests tested.

Key words: Antifungal, antibacterial, phytotoxic, cytotoxic, insecticidal

INTRODUCTION

Ziarat is a summer resort in Balochistan, Pakistan. It lies at an altitude of almost 8000 feet above sea level at $30^{o}.3'$ latitude and $67^{o}.8'$ longitudes. The climate is extremely cold in winters while summers are pleasant. It is rich in biodiversity with a large variety of fauna and indigenous medicinally important flora. The evergreen *Juniper excelsa* forest here is the second largest forest of the world. These are the oldest living species on the planet and are also known as living fossils, their age is up to 2000 years. They have a slow growth rate and a very low potential for regeneration. Its berries have long been in use in folk medicine for kidney disorders and other diseases. The species is facing a great threat due to dwarf mistletoe, an epiphytic angiosperm and a destructive parasite, *Arceuthobium oxycedri* (DC) M. Bieb (Viscaceae). This destroys the host tree within a very short time and has resulted in a substantially increased mortality rate. This disease is infecting approximately a quarter of the forest and a large number of trees have fallen victim to it. It has caused great concern as no effective remedial measures have been found so far to combat this disease except to cut and burn the tree. The ecology and taxonomy of *A. oxycedri* has been briefly studied[1-3], but no work on its chemistry and biological activities was carried out earlier. Therefore in this paper its biological activities and chemical composition is given.

MATERIALS AND METHODS

Plant materials and extraction: The *Arceuthobium oxycedri* was collected from *Juniper excelsa* forest at Ziarat, Balochistan, Pakistan in July 2002. This was authenticated by Dr. Rasool Bakhsh voucher specimen no. MAZ 1287, was deposited in the herbarium of University of Balochistan, Quetta, Pakistan. Two kg whole plants of *A. oxycedri* were dried in shade, ground and soaked in methanol for 10 days. The brown syrupy oily extract (28g) was obtained after evaporating the solvent. The extract was checked for antibacterial, antifungal, phytotoxicity, cytotoxicity and insecticidal activities, The experiments were repeated five times and results are compared with control and expressed in mean, the data was subjected to analysis through MINITAB and a two-way analysis of variance was used to compare the difference among means at 0.05 level of significance ($P<0.05$).

Antifungal and antibacterial activities: The antifungal and antibacterial activities of these crude extracts were determined against 10 fungal and 10 bacterial isolates by agar well diffusion[4] and disk diffusion assays[5]. Sabourad Dextrose Agar and Mycological agar was used as culture media for antifungal activities while Muller Hinton agar and Trypto Soya agar for bacteriological cultures these were obtained from Difco Laboratories, USA. 24h old cultures containing $1x10^{6}$ CFU mL^{-1} were spread on

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media. For agar well diffusion assay 6mm diameter wells were dug in the middle of the media by the help of sterile metallic borer. For agar disk diffusion assay 6mm sterile filter paper disks were placed one in the middle of the agar surface of each petri plate. The extract was dissolved in 20mg mL^{-1} distilled water, from this 10, 15, 20µL was pipette out and added to the respective wells or disks. Zones of inhibition were measured in mm after incubating petri plates at 30°C for 24-48h for bacterial and *Candida albicans* cultures and at 27°C for 7 days for fungal cultures. The results were compared with their respective standard azoles and antibacterial antibiotics i.e. Micanazole, Ketoconazole and Tetracycline, Tobramycin. Distilled water or DMSO was used as negative control for all samples.

Cytotoxicity assay with brine shrimp: Cytotoxicity of *A. oxycedri* extract was determined with brine shrimp (*Artemia salina*) following McLaughlin[6]. Larvae were produced from 20mg of eggs when incubated in 4% NaCl in tap water at 27ºC for 48h. The crude extract was dissolved in 20mg $2ml^{-1}$distill water from this solution different concentrations 1000, 100 and 10µL mL^{-1} were made, 3 vials for each concentration and controls were made. 30 shrimp were shifted to each concentration and control vials, after 24h the number of survivors and LC_{50} µg mL^{-1} was calculated.

Cytotoxicity with flow cytometry: Cytotoxicity of *A. oxycedri* extract was also determined through Flow Cytometry (FACScalibur, Becton Dickinson) on 24h old cultures of *Candida albicans.* CA-30 were grown on Sabourad Dextrose Agar and after harvesting and washing twice at 8000 rpm for 10 minutes in saline, were exposed to different concentrations of plant extract. 2g of extract was dissolved in 2ml of water to achieve a concentration of 1g mL^{-1}. Subsequent dilutions of 0.1, 0.1, 0.01, 0.001g mL^{-1} were made using water. 1x 10^6cells mL^{-1} were exposed to different concentrations of extract 1, 0.1, 0.01g mL^{-1}. These were incubated for 1, 2 and 24 h and then stained with Propidium iodide. The fluorescence emitted by the cells was read and the percentage of live and dead was calculated.

Phytotoxicity: Phytotoxicity was checked with Lemna acquinoctialis Welv. These are miniature aquatic monocots used as tools to monitor effects of chemicals as inhibitors or stimulators[7]. Plants were grown in 25 ml flasks containing specifically prepared E-Medium in Fisons Fi-Totron 600H Growth cabinet at 30°C, 50-60 relative humidity, 9'000 lux light intensity and 12 h day 12 h night length for 7 days. Different concentrations 10, 100, 1000 µg mL^{-1} of the extract were made in distilled water and added to the media. Positive and negative controls were made. Paraquat was used as herbicide. 10 plants each with three fronds were placed in each flask and incubated at 30ºC, the number of fronds in each flask was counted after 7 days and their death percentage was calculated.

Insecticidal activity: Five different grain storage pests were reared in wide mouthed bottles. These were then exposed to 1000µg mL^{-1} of the extract dissolved in distilled water and were applied to 90mm diameter filter papers in Petri dishes. Ten adult insects were exposed and they were kept without food for 24h. Mortality rate was determined after 24h. Permethrin was used as positive control and DMSO as negative control.

RESULTS

The methanolic extract of *A. oxycedri* was assayed for its biological activities The antibacterial and antifungal activities revealed that it was significantly active against three bacteria *Pseudomonas aeruginosa, Escherchia coli* (90%) and *Bacillus subtillis* (80%) while it showed no activity against other bacteria tested and the zones of inhibition were comparable to standard antibiotics (Table 2). The extract exhibited significant antifungal activity against human pathogen *C. albicans* (70 %), moderate activity against animal and plant pathogens, *Microsporum canis* (50%) and *Trichophyton longifusus* (40%) respectively. This activity against C. *albicans* was less than the standard azoles (Table 1).

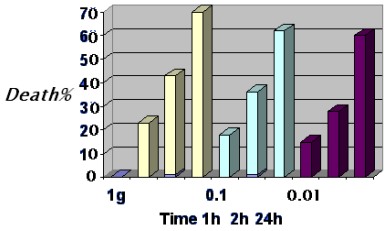

Fig. 1: Cytotoxicity of *A. oxycedri* through flow cytometry

The cytotoxicity was assayed with flow cytometry against *C. albicans*. The extract was significantly toxic at all concentrations. 1g mL^{-1} of the extract killed 70 % cells, 0.1g mL^{-1} killed 62% and 0.01g mL^{-1} killed 48 % of the cells in 24 h (Fig. 1). The *A. oxycedri* extract also exhibited maximum cytotoxicity for brine shrimp at high concentration, 100% of the shrimps were killed at 1000µg mL^{-1} dose level, 96% by 100µg mL^{-1} and 84% by 10µg mL^{-1} (Table 4). It was significantly lethal at all concentrations with LC_{50} of 8.3µg mL^{-1}. This cytotoxicity was comparable to Etoposide used as a standard drug.

Table 1: Antifungal activity

Name of Fungi	growth extract conc. DMSO	growth in -ve control	Inhibition %	Std. Drugs 400µg mL^{-1} +ve control	% Inhibition
Human pathogen					
Aspergillus flavus	100	100	0	AmphotericinB	70
A. niger	100	100		AmphotericinB	
candida albicans	60	100	70	Miconazole	100
C. glaberata	100	100	0	Miconazole	100
Animal Pathogens					
Microsporum canis	50	100	50	Miconazole	98
Plant Pathogen					
Fusarium oxysporum var. lycopersici	100	100	0	Miconazole	
F. moniliformis	100	100	0	Ketoconazole	73
F. solani	100	100	0	Ketoconazole	100
Trichophyton longifusus	60	100	40	Miconazole	70
T. floccosum	0	100	0	Miconazole	80

Conc. 400µg m L^{-1}; ($P<0.05$)

Table 2: Antibacterial activity

Extract 200 µg m L^{-1}	*Bacillus subtillis*	*B. cereus*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Corynebacterium diphtheriae*
Zones of inhibition in mm	27±2	15±1	32±2	29±1	12±1
Tobramycin/ Tetracyclin	35±2	30±2	34±1	32±2	32±2

No activity against these bacteria was observed; *Proteus mirabilis, Salmonella typhi, Shigella dysentrin, Klebsilla pneumoniae, Staphylococcus aureus,* showed no activity; n=5; Tetracycline and Tobramycin were used as standard antibiotics. ($P<0.05$).

Table 3: Cytotoxicity through Brine Shrimp assay

Dose µg m L^{-1}	No. of shrimp	Average no. of survivors	% Inhibition
1000	30	0	100
100	30	1±2	3.33
10	30	5±2	16.66

Brine shrimp (*Artemia salina*);LC_{50}; 8.3µg; Std. drug Etoposide LD_{50}; 7.4625

Table 4: Phytotoxicity assay

Name of plant	*A.oxyc-edri* extract gmL^{-1}	Average No. of fronds			% growth inhibition
		Sam-ple	Control (-ve)	Control (+ve)	
L. acqui- notialis	1000	0	32	0	100
	100	19	32	0	41
	10	24	32	0	22

Herbicide; Paraquat 0.902µg m L^{-1} killed all plants; n=5; ($P<0.05$).

Table 5: Insecticidal activity

Insects	1	2	3	4	5	+ve control	-ve control
Dose extract µg cm^{-2} 1571.33	0%	30%	30%	0%	10%	100%	0%

1. *Callosubruchus analis*, 2. *Tribolium castaneum*, 3. *Rhyzopertha dominica*, 4. *Sitophilus oryzae*, 5. *Thigoderma ghanarium*. Positive control; DMSO; Std. Drug; Permeththrin, Conc. 235.71 µg cm^{-2} ($P<0.05$)

The methanolic extract of *A. oxycedri* was significantly phytotoxic for *L. acquinoctialis* and the toxicity was dose dependent at the highest concentration i.e. 1000 µg mL^{-1} of the extract killed 100% of the plants, 100 µg mL^{-1} killed 40% plants while 10 µg mL^{-1} inhibit growth of 22% of the total population (Table 5). This activity was less than that of herbiside paraquat used as positive control.

The extract had non-significant activity against any pests; it killed only 30% of the population of *Tribolium castaneum* and *Rhyzopertha dominica*. It did not affect the growth of other insects tested.

DISCUSSION AND CONCLUSION

The crude methanolic extract of *A. oxycedri* exhibited significant activity against three bacteria *Pseudomonas aeruginosa, Escherchia coli* and *Bacillus subtillis.* These activities were comparable to Tetracycline and Tobramycin. Similar activity has been reported from other medicinal plants[8]. The extract showed good antifungal activity against human pathogen *C. albicans* but this activity was not comparable with standard azoles. It also exhibited maximum cytotoxicity for brine shrimps as 100% of the shrimps were killed at high dose level.

It was significantly lethal at all concentrations with LC_{50} of 8.3µg mL^{-1}. The cytotoxicity assayed with flow cytometry also significantly reduced the growth of *C. albicans* in 24h. As *A. oxycedri* is an epiphyte on *Juniper excelsa*, its cytotoxicity might be responsible for the damage it causes to this fossil species.

The methanolic extract was significantly phytotoxic for *L. acquinoctialis* and was dose dependent i.e. highly significant at highest concentration and activity decreased with the decrease in concentration. This activity is similar to herbicide Paraquat and can be suggested as a cause for the death of the host. This indicates presence of some herbicidal compound in the extract. These phytotoxic effects are also close to allelopathic effects of a weed *Eragostis poaides's*[9]. Large amount of phenols and tannins were present in the extract. The presence of Phenols and tannins which are toxic compounds produced by the parasite can be another reason for the mortality of the host tissue as these toxins can easily penetrate the host cells. The phenolics can mediate harmful interactions directly or indirectly by linking autotrophs to each other and to herbivores[10]. Isolation and purification of natural compounds is in progress.

These phytotoxic and cytotoxic activities of *A. oxycedri* can also be responsible for the death of *J. excelsa*, which is sensitive to this toxicity produced inside its tissues. *A. oxycedri* is a fast growing plant; it gets most of its nutrients from the host's nutrients as a parasite and releases toxic compounds which inhibit the development and growth of J. *excelsa*. As *J. excelsa* is a very slow growing fossil tree it cannot compete with *A. oxycedri* and eventually falls victim to it. To find a cure for this disease and to save the precious *Juniper excelsa* forest bioassay guided isolation and identification of these specific toxic compounds is in progress.

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