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BIOLOGICAL SCREENING OF *ZIZYPHUS OXYPHYLLA* EDGEW LEAVES

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Abstract

Crude methanolic extract and various fractions of *Zizyphus oxyphylla* Edgew leaves were screened for antibacterial, antifungal, phytotoxic, cytotoxic and insecticidal activities. Antibacterial bioassay was performed against six bacteria viz., *Escherichia coli, Bacillus subtilis, Shigella flexenari, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Salmonella typhi* . Ethyl acetate fraction showed good antibacterial activity against *Bacillus subtilus* (16mm zone of inhibition) and *Staphylococcus aureus* (18 mm zone of inhibition). The antifungal activity of these extract performed against 6 fungal strains viz., *Trichophyton longifusus, Candida albicans, Aspergilus flavus, Microsporum canis, Fusarium solani and Candida glaberata* indicated non significant results. Similarly neither of the fractions showed any cytotoxicity while *n*-hexane and ethyl acetate fraction showed moderate (40 %) insecticidal activity. All fractions and crude extract showed significant (60-90 %) phytotoxicity.

Introduction

The genus *Zizyphus* (*Rhamnaceae*) comprises of 100 species, distributed world over including six species indigenous to Pakistan (Qaiser & Nazimuddin, 1984). Digestive disorders, weakness, liver complaints, obesity, urinary troubles, diabetes, skin infections, fever, diarrhea and insomnia are the examples of some diseases in which the folk use of various species of *Zizyphus* has been reported (Kirtikar and Basu, 1984; Han and Park, 1986). Various species of *Zizyphus* have shown various biological activities both in vivo and in vitro such as anti diabetic (Ahmed *et al.,* 2005) antinoceceptive and antipyretic (Adzu *et al*., 2001; Nisar *et al*., 2007), anti spasmodic (Wahida and Nabil, 2009), antiulcerogenic (Wahida *et al*., 2007), antimicrobial (Naglaa, 2002), antioxidant and antilisterial (Sharif *et al*., 2009) and larvicidal properties (De Omena *et al*., 2007).

Zizyphus oxyphylla Edgew is a small or medium size tree and various parts of this plant are used traditionally as remedy of pain, diabetes, allergy, fever, rheumatic and pain. Leaves methanolic extract has shown antinoceceptive and antipyretic activities (Nisar *et al*., 2007). To the best of our knowledge, this plant has not been screened for *In vitro* biological activities. In our efforts to explore the flora of Pakistan (Nisar *et al*., 2007, 2008, 2009 a, b; 2010; Zia-Ul-Haq *et al*., 2007a, b; 2008a, b; 2009; 2010a, b) *Zizyphus oxyphylla* has been subjected to *In vitro* biological activities to rationalize and biologically validate the folk use of this plant for various human ailments.

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Material and Methods

Plant material, preparation of crude extract and fractionation: The plant material was collected from Swat Valley (N.W.F.P Pakistan). Plant was identified by Dr. Hassan Sher, Jehanzaib College Swat and voucher specimen has been deposited in the national herbarium Islamabad with voucher no NH-012 (2004).

The shade dried and powdered plant material (5 Kg) was ground and extracted with methanol. The methanolic extract was filtered and evaporated under vacuum to obtain crude extract (412 g). It was fractionated into *n*-hexane, chloroform, ethyl acetate (Et-acetate), *n*-butanol and aqueous fractions. All these fractions as well as crude extract was screened for antibacterial, antifungal, phytotoxic, cytotoxic and insecticidal activities.

Antibacterial activity: The antibacterial activity was checked by the agar–well diffusion method (Kavanagh *et al*., 1963; Carron *et al*., 1983). In this method one loop full of 24 hours old culture containing approximately 104-106 CFU was spread on the surface of Mueller-Hinton Agar plates. Wells were dug in the medium with the help of sterile metallic cork borer. Stock solutions of the test samples in the concentration of 3 mg/ml were prepared in dimethyle sulfoxide (DMSO) and 100 µl dilutions were added in their respective wells. The antibacterial activity of extracts (A-F) was compared with standard drug imepinem; the std. drug imepinem and DMSO were used as positive and negative control. The antibacterial activity was determined by measuring zones of inhibition usually of each sample wells (Carron & Maran, 1987; Jorgensen & Turnidge, 1999; Kivack *et al*., 2002; Stepanovic & Anetic, 2003; Bektas & Donmez, 2004; Rashid *et al*., 2009).

Antifungal bioassay: The antifungal activity was determined by the Agar tube dilution Method (Atta-ur-Rahman *et al*., 1991). The crude extract was dissolved in DMSO (24 mg/1ml). Sterile Sabouraud's dextrose agar medium (5ml) was placed in a test tube and inoculated with the sample solution (400 µg /ml) kept in slanting position at room temperature overnight. The fungal culture was then inoculated on the slant. The samples were incubated for 7 days at 29^{o}C and growth inhibition was observed and percentage growth inhibition was calculated with reference to the negative control by applying the formula:

$$\%\text{inhibition of fungal growth} = 100 - \frac{\text{linear growth and test (mm)}}{\text{linear growth in control (mm)}} \text{ X } 100$$

Miconazole and amphotericin B were used as standard drugs, while miconazole, amphotericin B and DMSO were used as positive and negative controls (Berhge & Vlientinck, 1991; Choudhary & Dur-e-Shawar, 1995; Peters & Gills, 1995; Janaki & Vijayasekaran, 1998; Rashid *et al.,* 2009).

Phytotoxic activity: Phytotoxic activity was determined by using the modified protocol of *Lemna minor* (Atta-ur-Rehman, 1991; Ali *et al.,* 2009; Rashid *et al.,* 2009). The medium was prepared by mixing various constituents in 1000 ml distilled water and the pH was adjusted (5.5-6.5) by adding KOH solution. The medium was then autoclaved at 121°C for 15 minutes. The extracts dissolved in ethanol (20 mg/ml) served as stock solution. Nine sterilized flasks, three for each concentration, were inoculated with 1000 µl, 100 µl and 10 µl of the stock solution for 1000, 100 and 10 µg/ml respectively. The

solvent was allowed to evaporate overnight under sterile conditions. To each flask, medium (20 ml) and plants (10), each containing a rosette of three fronds of *Lemna minor* L., was added. All flasks were plugged with cotton and kept in the growth cabinet for 7 days. The number of fronds per flask were counted and recorded on day seven and their growth regulation in percentage was calculated by the following formula:

$$\text{Growth regulation (\%)} = \text{Mortality (\%)} = \left(\frac{100 - \textit{Number of fronds in test sample}}{\textit{Number of fronds in negative control}} \right) \times 100$$

The result was calculated with reference to the positive and negative control.Paraquat was used as a standard drug, while paraquat and volatile solvent were used as positive and negative controls (Finny, 1971; Hideji & Oshida, 1982; Lewis, 1995; Rashid *et al.,* 2009).

Insecticidal activity: Crude extract and all fractions were evaluated against different insects viz., *Tribolium castaneum*, C*allosbruchus analis,* and *Rhyzopertha dominica*. The test sample was prepared by dissolving 200 mg of crude fractions in 3 ml acetone and loaded in a Petri dishe covered with the filter papers. After 24 hours, 10 test insects were placed in each plate and incubated at 27 °C for 24 hours with 50% relative humidity in growth chamber. The results were analyzed as percentage mortality, calculated with reference to the positive and negative controls. Permethrin was used as a standard drug, while Permethrin, acetone and test insects were used as positive and negative controls (Abbott, 1925; Atta-ur-Rehman, 2001; Tabassum & Naqvi, 1997; Collins, 1998; Atta-ur-Rahman & M. I. Choudhary, 2001; Ali *et al.,* 2009; Rashid *et al.,* 2009).

The percentage mortality was calculated by the formula:

$$\text{Growth regulation (\%)} = \left(\frac{\textit{Number of insects alive in test}}{\textit{Number of insects alive in control}} \right) \times 100$$

Brine shrimp lethality bioassay: It is an excellent and simple preliminary method to determine the cytotoxicity of crude plant extract and pure natural compounds (Ali *et al.,* 2009). In this method, artificial "sea water" was prepared by dissolving 38 g sea salt per liter of double distilled water and filtered (Meyer *et al*., 1982). "Sea water" was placed in a small tank; added brine-shrimp eggs (1mg) (*Artemia salina*) and was darkened by covering with aluminum foil. It was allowed to stand for 24 hours at 25^{o}C which provided a large number of larvae. Twenty milligrams of the concentrated sample was dissolved in 2 ml $CHCl_3$ (20 mg/2 ml) and transferred to 500, 50 and 5 µl vials corresponding to 1000, 100 and 10µg per ml, respectively. Then three replicates were prepared for each concentration making a total of nine vials. The vials containing material was concentrated, dissolved in DMSO (50 µl) and 5ml "sea water" added to each. Then 10 shrimps were added per vial, allowed to stand for 24 hours, shrimps were counted and recorded the number of surviving shrimps. Etoposide was used as positive control. The data were analyzed with a Finney computer program to determine the LD_{50} values (Alves *et al.,* 2000).

Results and Discussion

In recent years, there has been a resurgence of scientific interest in the use of medicinal plants for the development of new pharmacotherapeutic agents. Medicinal plants play an important role for the management of different microbial infections because overmedication and long-term side effects of synthetic drugs have assumed alarming range. Effective, safe and cheap medicinal agents from plants may appear as potential alternatives for controlling microbial infections particularly the resistant cases (Nisar *et al.,* 2010).

Bioactivity evaluation is an important part of the development of new drugs from medicinal plants and screening crude extract and various fractions against microorganisms is usually first step during bioactivity evaluation. Both gram positive and gram negative bacteria such as *Staphylococcus aureus***,** *Escherichia coli*, *Pseudomonas aeruginosa, Salmonella typhi, Bacillus subtilus* and *Shigella flexneri* were used to evaluate the antibacterial activity of extract. The result revealed that ethyl acetate fraction showed some good activity (16 mm zone of inhibition) against *Bacillus subtilus* and *(*18 mm zone of inhibition) *Staphylococcus aureusus* while all the other fractions along with the crude did not show any inhibition as shown in Table 1. In the case of antifungal activity six fungi *Trichophyton longifusis, Candida albicans, Candida glabarata, Fusarium solani, Microsporum canis and Aspergillus flavus* were used to see the antifungal activity Results revealed that maximum antifungal activity (35%) was shown against. *Microsporum canis* by crude, *n*-hexane and aqueous fraction each (Table 2) while chloroform and butanolic fraction showed 30% and 20% antifungal activity, respectively. Ethyl acetate and *n*-hexane fraction showed low activity against *Aspergillus flavus* from 10% to 20% respectively. Crude extract showed 10% inhibition against *Fusarium solani.* The knowledge of extent and mode of inhibition of specific compounds which are present in plant extracts, may contribute to the successful application of such natural compounds for treatment of infection disorder like fungal and bacterial diseases (Nisar *et al.,* 2010).

Regarding the phytotoxicity activity result have shown that crude along with all other fraction showed significant activity at highest concentration (1000 µg/ml), maximum activity was shown by crude extract at this concentration that is 90% growth regulation while 60% was the lowest activity as shown by aqueous fraction. The results clearly indicated that phytotoxic activity is dose dependent i.e., high phytotoxcicity at high concentrations and *vice-versa*. Results also showed that neither crude extract nor any of the fractions showed any cytotoxic activity. The result obtained indicated that *n*-hexane and ethyl acetate fraction showed moderate insecticidal activity (40%) against *Callosobruchus analis* while other fractions except crude showed non significant (20%) activity. Crude extract showed non significant activity against *Rhyzopertha dominica.* Crude along with other fraction were inactive against *Tribolium castaneum, Sitophilus oryzea and Trogoderma granarium*.

There does not appear to be any previous report on the biological screening of *Zizyphus oxyphylla* Edgew. Medicinal plants are items of commerce in Pakistan and herbal medicine, are gaining prominence despite the fact that many of the concepts elude scientific explanation. People who were distanced from the traditional systems of medicine are gearing towards green pharmaceuticals as the period of over-romanticizing with allopathic drugs has end and cures and drugs, derived from plants are being integrated in formal health care system. The present study will help the researchers as a basic data for future research in exploiting the hidden potential of this important plant which has not been explored so far.

Table 1. Antibacterial activity of leaves of *Zizyphus oxyphylla* Edgew.

Bacterial species	Zone of inhibition of std. drug* (mm)	Zone of inhibition (mm)					
		Crude	*n*-hexane	Chloroform	Et-acetate	*n*-butanol	Aqueous
Escherichia coli	35	-	-	-	-	-	-
Bacillus subtilis	36	-	-	-	16	-	-
Shigella flexaneri	36	-	-	-	-	-	-
Staphylococcus aureus	43	-	-	-	18	-	-
Pseudomonas aeruginosa	32	-	-	-	-	-	-
Salmonella typhi	40	-	-	-	-	-	-

*Impenum (10 µg disc)

Table 2. Antifungal activity of leaves of *Zizyphus oxyphylla* Edgew.

Fungal species	Standard	% inhibition					
		Crude	*n*-hexane	Chloroform	Et-acetate	*n*-butanol	Aqueous
Trichophyton longifusis	Miconazole 70	-	-	-	-	-	-
Candida albicans	Miconazole 110.8	-	-	-	-		-
Aspergilus flavus	Amphotericin 20	-	20	-	10	-	-
Microsporum canis	Miconazole 98.4	35	35	30	-	20	35
Fusarium solani	Miconazole 73	10	-	-	-	-	-
Candida glaberata	Miconazole 110.8	-	-	-	-	-	-

Table 3. Phytoxicity activity of leaves of *Zizyphus oxyphylla* Edgew.

Conc. of sample (µg/ml)	Conc. of stand drug	% Growth regulation					
		Crude	*n*-hexane	Chloroform	Et-acetate	*n*-butanol	Aqueous
1000		90	85	80	85	75	60
100	0.015	60	50	45	35	35	45
10		40	40	35	25	20	25

Table 4. Insecticidal activity of leaves of *Zizyphus oxyphylla* Edgew.

Name of insect	% Mortality					
	Crude	*n*-hexane	Chloroform	Et-acetate	*n*-butanol	Aqueous
Tribolium castaneum	-	-	-	-	-	-
Sitophilus oryzea	-	-	-	-	-	-
Rhyzopertha dominica	20	-	-	-	-	-
Callosobruchus analis	-	40	20	40	20	20
Trogoderma granarium	-	-	-	-	-	-

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