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BIOLOGICAL SCREENING OF ZIZYPHUS *OXYPHYLLA* EDGEW STEM

MUHAMMAD NISAR[1*], WAQAR AHMAD KALEEM[2], MUGHAL QAYUM[2], INAMULLAH KHAN MARWAT[2], M. ZIA-UL-HAQ[4], IHSAN ALI[1] AND M. I. CHOUDHARY[3]

[1]*Institute of Chemical Sciences, University of Peshawar, Peshawar-25120, NWFP, Pakistan*
[2]*Department of Pharmacy, University of Peshawar, Peshawar, 25120, Pakistan*
[3]*H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan*
[4]*Department of Pharmacognosy, University of Karachi, Karachi-75270, Pakistan*
[*]*Corresponding author: akhundin@yahoo.com*

Abstract

Crude methanolic extract and various fractions of *Zizyphus oxyphylla* Edgew stem were screened for antibacterial, antifungal, phytotoxic, cytotoxic and insecticidal activities. Antibacterial bioassay performed against six bacteria viz., *Escherichia coli, Bacillus subtilis, Shigella flexenari, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Salmonella typhi* indicated that neither crude extract nor any of subsequent fraction exhibited antibacterial potential. The antifungal activity of the extract and fractions screened against five fungal strains viz., *Candida albicans, Aspergilus flavus, Microsporum canis, Fusarium solani and Candida glaberata* indicated non significant results. Similarly neither of the crude or subsequent fractions showed any cytotoxicty or insecticidal activity. However crude extract and fractions showed significant phytotoxicity at higher doses.

Introduction

Zizyphus species (*Rhamnaceae*) are widespread in the Mediterranean region, Africa, Australia, and tropical America (Pawlowska *et al*., 2009). In Pakistan six species of *Zizyphus* has been reported (Qaiser & Nazimuddin, 1984). *Zizyphus* are commonly used in folklore medicine for the treatment of various diseases such as digestive disorders, weakness, liver complaints, obesity, urinary troubles, diabetes, skin infections, loss of appetite, fever, pharyngitis, bronchitis, anaemia, diarrhoea, and insomnia (Han & Park, 1986; Kirtikar & Basu, 1984). *Zizyphus oxyphyla* Edgew (locally known as elanai) is used by indigenous people to cure different ailments. Its roots are sun dried and boiled in water and filtered. The filtered water is used in curing jaundice (Jan *et al.,* 2009).

Pakistan is an exquisite example of biodiversity having a rich tradition of herbal remedies and the majority of its population relies mainly on medicinal plants for health-related matters. Despite widespread use of plant resources in traditional medicines, bioassay analysis of very few plant species have been conducted to investigate their medicinal properties, and to ascertain safety and efficacy of traditional remedies (Nisar *et al.,* 2010b). To the best of our knowledge, this plant has not been screened for *In vitro* biological activities. As our efforts to explore the flora of Pakistan (Nisar *et al*., 2010 a, b, c; Zia-ul-Haq *et al.,* 2007 a, b; 2008 a, b; 2009; 2010 a, b) *Zizyphus oxyphylla* has been subjected to *In vitro* biological activities to biologically validate the folk use of this plant against various human ailments.

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 placed in the national herbarium Islamabad with voucher no NH-012.

The shade dried and powdered plant material (8 Kg) was ground and extracted with methanol. The methanolic extract was filtered and evaporated under vacuum to obtain crude extract (375 g). It was fractionated and 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). 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 standard drug imepinem and DMSO were used as positive and negative control. The antibacterial activity was determined by measuring the zone of inhibition visually of each sample (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 Agar tube dilution Method (Atta-ur-Rahman *et al*., 1991). The crude extract was dissolved in DMSO (24 mg / ml). 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°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 *et al*., 1995; Peters & Gills, 1995; Janaki & Vijayasekaran, 1998; Rashid *et al.,* 2009) (Table 1).

Phytotoxic activity: Phytotoxic activity was determined by using the modified protocol of *Lemna minor* (Atta-ur-Rehman, 1991; 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 and10 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:

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

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 *et al.*, 1982; Lewis, 1995; Rashid *et al.*, 2009) (Table 3).

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 Petri dishes covered with the filter papers. After 24 hours, 10 test insects were placed in each Petri 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 *et al.*, 2001; Rashid *et al.*, 2009).

The percentage mortality was calculated by the formula:

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

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 25ºC 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 the number of surviving shrimps recorded. 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) (Table 2).

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.,* 2010a). 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 (Nisar *et al.,* 2010a). Bacterial strains both gram positive and gram negative were used to evaluate the antibacterial activity of extract. Interestingly neither crude extract nor any of subsequent fractions showed any activity. Five fungal strains viz., *Candida albicans, Candida glabarata, Fusarium solani, Microsporum canis* and *Aspergillus flavus* were used to assess the antifungal activity. Results revealed that maximum antifungal activity (35%) was shown against *Microsporum canis* by *n*-hexane fraction followed by crude extract and ethyl acetate fraction each of which showed maximum antifungal activity (30%) inhibition followed by n-butanolic fraction that exhibited 20% antifungal activity (Table 1). Crude extract and aqueous fraction showed 10% inhibition against *Aspergillus flavus* and *Fusarium solani* respectively. Crude along with other fraction were inactive against *Tribolium castaneum, Sitophilus oryzea and Trogoderma granarium*.

Regarding to the phytotoxicity activity results have shown that crude along with all other fraction showed significant activity at highest concentration (1000 µg/ml). 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.

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 (Nisar *et al.,* 2010c).

Table 1. Antifungal bioassay.

Test organism	% Inhibition						Standard
	A	B	C	D	E	F	
C. albicans	-	-	-	-	-	-	Miconazole110.8
A. flavus	10	-	-	-	-	-	Amphotericin20
M. canis	30	35	10	30	20	-	Miconazole98.4
F. solani	-	-	-	-	-	10	Miconazole73.25
C. glabarata	-	-	-	-	-	-	Miconazole110.8

Crude (A), n-hexane (B), Chloroform (C), Ethyl acetate (D), n-butanol (E), Aqueous (F)

Table 2. *In vitro* cytotoxic bioassay of *Zizyphus oxyphylla* Edgew stem.

Extractions	% Deaths at doses			LD_{50}
	10 µg/ml	100 µg/ml	1000 µg/ml	
Crude (A)	28	26	22	26756.99
n-hexane (B)	28	26	24	377166.8
Chloroform (C)	26	24	20	20295.30
Ethyl acetate (D)	27	24	22	70820.41
n-butanol (E)	26	24	22	346568.9
Aqueous (F)	26	24	22	346568.9
Etoposide (standard)				7.4625

Table 3. *In vitro* phytotoxic bioassay of *Zizyphus oxyphylla* Edgew stem.

Samples	Conc.	No. of fronds samples	Control	% Growth regulation	Conc. of std. drug (µg/ml)
Crude (A)		5		75	
n-hexane (B)		5		75	
Chloroform(C)	1000	4	20	80	0.015
Ethyl acetate (D)		7		85	
n-butanol (E)		5		75	
Aqueous (F)		3		85	
Crude (A)		14		30	
n-hexane (B)		6		70	
Chloroform(C)	100	5	20	75	0.015
Ethyl acetate (D)		12		40	
n-butanol (E)		8		60	
Aqueous (F)		11		45	
Crude (A)		15		05	
n-hexane (B)		10		50	
Chloroform(C)	10	10	20	50	0.015
Ethyl acetate (D)		15		25	
n-butanol (E)		16		20	
Aqueous (F)		13		35	

References

Abbott, W.S. 1925. A method of computing effectiveness of insecticides. *J. Econ. Ent.*, 18(2): 265-67.

Ahmed, O.A., S.Y. Salim, M.H. Assaf, R.H. Abdel-Hady. 2005. Antidiabetic activity and toxicity of *Zizyphus spina-christi* leaves. *J. Ethnopharm.*, 101: 129-138.

Ali, I, N. Rubina, N.K. Wahib, G. Rukhsana and M.I. Choudhary. 2009. Biological screening of different root extracts of *Euphorbia wallichii*. *Pak. J. Bot*., 41(4): 1737-1741.

Alves, T.M.A., A.F. Silva, M. Brandao, T.S.M. Grandi, E.F.A. Smania, J.A. Smania and C.L. Zani. 2000. Biological screening of Brazilian medicinal plants. *Mem Inst Oswaldo Cruz*., 95: 367-373.

Atta-ur-Rahman, M.I. Choudhary and J.T. William. 2001. *Bioassay techniques for drug development*. Harward academic Publisher, 67-68.

Atta-ur-Rehman. 1991. *Studies in Natural Product Chemistr*. Elsevier Science Publishers Netherlands, 9: 383-384.

Bektas, T., E. Donmez, M. Unlu, F. Candan, D. Daferera, G. Vardar-Unlu, M. Polissiou and A. Sokmen. 2004. Antimicrobial and antioxidative activities of the essential oils and methanol extracts of *Salvia cryptantha* (Montbret et Aucher ex Benth) and *Salvia multicaulis* (Vahl). *Food Chem.*, 84(4): 519-525.

Berhge, V. and A.J. Vlietinck. 1991. In: *Methods in plants biochemistry*. *Assays for Bioactivity*. (Ed.): K. Hostettmann. Academic Press, London. 6: 47-59 (Chapter 3).

Carron, R.A., J.M. Maran, L. Montero-Fernandozaigo and A.A. Dominguez. 1987. *Plantes Medicinales et Phytotherapic*, 21: 195-202.

Choudhary, M.I., Dur-e-Shahwar, Z. Parveen, A. Jabbar, I. Ali and Atta-ur-Rahman. 1995. Antifungal steroidal lactones from *Withania coagulance*. *Phytochem.*, 40(4): 1243-6.

Collins, P.J. 1998. Resistance to grain protectants and fumigants in insect pests of stored products in Australia. In: (Ed.): H.J. Banks, E.J. Wright and K.A. Dameevski. CSIRO Stored Grain Research Laboratory, Presented in Australian Post Harvest Technical Conference, 55- 57.

Finny, D.J. 1971. *Probit Analysis*. 3rd edition, Cambridge University Press, Cambridge, 333.

Han B.H. and M.H. Park. 1986. *Folk medicine: The art and science*, The American Chemical Society, Washington, p. 206.

Hideji, I., Y. Oshida, A. Ikuta, H. Inatomi and T. Adachi. 1982. Phenolic plant growth inhibitors from the flowers of *Cucurbita pepo*. *Phytochemistry*, 21(8): 1935-1937.

Jan, G., M.A. Khan and F. Gul. 2009.Ethnomedicinal plants used against jaundice in Dir Kohistan Valleys (NWFP), Pakistan. *Ethnobot. Leaflet.*, 13: 1029-41.

Janaki, S. and V. Vijayasekaran. 1998. Antifungal activities of *Agalia roxburghiana* (W&A), MIQ, Var, Beddome.i. *Biomedicine*, 18(2): 86-89.

Jorgensen, J.H., J.D. Turnidge and J.A. Washington. 1999. Antibacterial susceptibility tests; dilution and disk diffusion methods, 1526-1543. In: *Manual of clinical microbiology*. (Ed.): P.R. Murray, Z.J. Baron, Pfaller, F.C. Tenover and R. H. Yolken. 7th edition, *Amer. Society Microbio.*, Washington, D.C.

Kavanagh, F. 1963. *Analytical Microbiology.* Academic Press London, 125-141.

Kirtikar, K.R. and B.D. Basu. 1984. *Indian Medicinal Plants*. Lalit Mohan Publication, Allahabad, India.

Kivack, B., T. Mert and H.T. Ozturk. 2002. Antimicrobial and cytotoxic activities of *Ceratonia siliqua* L., extracts. *Turk. J. Biol.*, 26: 197-200.

Lewis, M.A. 1995. Use of fresh water in phytotoxicity testing: *A Review Environmental Pollution*, 87(3): 319-336.

Meyer, N.B., R.N. Ferrigni, E.J. Putnam, B.L. Jacobsen, E.D. Nichols and I.J. McLaughlin. 1982. Brine shrimp: A convenient general bioassay for active plant constituents. *J. Planta Medica*, 45: 31-35.

Nisar, M., M. Qayum, M.R. Shah, W.A. Kaleem, I. Ali and M. Zia-ul-Haq. 2010a. Antimicrobial screening of *Impatiens bicolor royle. Pak. J. Bot.*, 42(1): 523-526.

Nisar, M., M. Qayum, M.R. Shah, W.A. Kaleem, H.L. Siddiqui and M. Zia-ul-Haq. 2010b. Biological screening of *Impatiens bicolor Royle. Pak. J. Bot.*, 43: 1903-1907.

Nisar, M., W.A. Kaleem, M. Qayum, A. Hussain and M. Zia-ul-Haq. 2010c. Biological screening of *Zizyphus oxyphylla* Edgew leaves. *Pak. J. Bot.*, 42(1): 4063-4069.

Nisar, M., S.A. Tariq and Ihsanullah. 2009. Nutritional levels of *Indigofera gerardiana* wall and *Crataegus songrica* k. Koch. *Pak. J. Bot*., 41(3): 1359-1361.

Pawlowska, A.M., F. Camangi, A. Bader and A. Braca. 2009. Flavonoids of *Zizyphus jujuba* L., and *Zizyphus spina-christi* (L.) Willd (Rhamnaceae) fruits. *Food Chem*., 112(4): 858-862.

Peters, W. and H.M. Gilles. 1995. *Colour Atlas of Tropical Medicine and Parasitology*. Mosby-Wolfe.

Qaiser, M. and S. Nazimudin.1984. *Flora of Pakistan.* (Eds.): E. Nasir and S.I. Ali. 140: 9-17.

Rashid, R., M. Farah and M.N. Mirza .2009. Biological screening of *Salvia cabulica. Pak. J. Bot*., 41(3): 1453-1462.

Sharif, M.A., V.K. Bajpai and S.C. Kang. 2009. Antioxidant and antilisterial effect of seed essential oil and organic extracts from *Zizyphus jujube*. *Food Chem. Toxic.*, 47: 2374-2380.

Stepanovic, S., N. Antic, I. Dakic and M. Svabic-Vlahovic. 2003. *In vitro* antimicrobial activity of propolis and synergism between propolis and antimicrobial drugs. *Micro. Res.*, 158(4): 353-357.

Tabassum, R., S.N.H. Naqvi, M.A. Azmi, S.M. Nurulain and M.F. Khan.1997. Residual effect of a neem fraction, nimolicine and an insect growth regulator, dimilin, against stored grain pest *Callosobruchus analis*. *Proc. Pakistan Congr. Zool*., 17: 165-170.

Zia-ul-Haq, M., M. Ahmad and M. Akhter. 2010b. Nematicidal activity of selected flora of Pakistan. *Pak. J. Bot.*, 43: 2119-2123.

Zia-ul-Haq, M., S. Iqbal, S. Ahmad, M. Imran, A. Niaz and M.I. Bhanger. 2007a. Nutritional and compositional study of desi chickpea (*Cicer arietinum* L.) cultivars grown in Punjab, Pakistan. *Food Chem*., 105: 1357-1363.

Zia-ul-Haq, M., M. Ahmad S. Iqbal, S. Ahmad and H. Ali. 2007b. Characterization and compositional studies of oil from seeds of desi chickpea (*Cicer arietinum* L.) cultivars grown in Pakistan. *J. Am. Oil Chem. Soc.*, 84: 1143-1148.

Zia-ul-Haq, M., S. Ahmad, E. Chiavaro, Mehjabeen and S. Ahmed. 2010a. Studies of oil from cowpea (*Vigna unguiculata* (L.) walp.) cultivars commonly grown in Pakistan. *Pak. J. Bot.*, 42(2): 214-220.

Zia-ul-Haq, M., S. Ahmad, M. Ahmad, S. Iqbal and K.M. Khawar. 2009. Effects of cultivar and row spacing on tocopherol and sterol composition of chickpea (*Cicer arietinum* L.) seed oil. *Tari. Bili. Derg.*, 15: 25-30.

Zia-ul-Haq, M., S. Iqbal and M. Ahmad. 2008a. Characteristics of oil from seeds of 4 mungbean (*Vigna radiata* (L.) Wilczek) cultivars grown in Pakistan. *J. Am. Oil Chem. Soc.*, 85: 851-856.

Zia-ul-Haq, M., S. Iqbal, S. Ahmad, M.I. Bhanger and R. Amarowicz. 2008 b. Antioxidant Potential of Desi Chickpea varieties commonly consumed in Pakistan. *J. Food Lipid.*, 15: 26-342.

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