

BIOLOGICAL SCREENING OF OILS FROM *ZIZYPHUS OXYPHYLLA* EDGEW

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Abstract

The oil obtained by solid phase extraction of the *n*-hexane fraction from stem (WO-1 and WO-2) and leaves (WO-5) of *Zizyphus oxyphylla* Edgew was evaluated *In vitro* for their antimicrobial, insecticidal, cytotoxic and phytotoxic potential. Bacterial strains used for the experiment were *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Shigella flexneri*, and *Staphylococcus aureus* while fungal strains used were *Aspergillus flavus*, *Candida albicans*, *Candida glabrata*, *Fusarium solani*, *Microsporium canis* and *Trichophyton longifusus*. The tested samples did not show any activity against any of above mentioned bacterial strains while in case of antifungal activity, fractions WO-1, WO-2 and WO-5 exhibited low activity against *Microsporium canis* and *Aspergillus flavus* respectively. The crude oil sub-fraction WO-2 and WO-5 exhibited significant phytotoxicity at higher concentration (1000 mg/mL) against *Lemna minor* plant. WO-1 and WO-5 revealed moderate and good insecticidal activity against *Rhyzopertha dominica* and *Tribolium castaneum* respectively. In brine shrimp lethality assay, no cytotoxicity was observed at any concentration by any oil fraction.

Introduction

Pakistan exhibits a wide range in topography and climate, resulting in vegetation and floristic diversity making it varietal emporium of medicinal plants. Herbal medicines which were considered just a concrete blend of dynamic medical known-how and ancestral experience are now being rationalized and pharmacologically validated. It is estimated that trade in medicinal plants is several millions per year. To satisfy emerging market needs, surveys are being carried out to unearth new plant sources of herbal remedies and medicines. The chief lacuna in gaining prominence of herbal medicines is that many of the concepts elude scientific explanation. So screening of crude extracts is necessary to validate the claimed curative properties of medicinal plants (Nisar *et al.*, 2011; Zia-ul-Haq *et al.*, 2012 a, b, c).

Zizyphus oxyphylla Edgew (*Rhamnaceae*), locally known as elanai and badrai is used by indigenous people to cure different ailments. Its roots are sun dried boiled in water and filtered. The filtered water is used in curing jaundice (Jan *et al.*, 2009). To the best of our knowledge, oil of various parts of this plant has not been screened for *In vitro* biological activities. As our efforts to explore the flora of Pakistan (Nisar *et al.*, 2010, 2011; Zia-ul-Haq *et al.*, 2009, 2010, 2011 a-f; 2012 a-f) for various activities, *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.

Material and Methods

Plant material: The plant material (stem and leaves) was collected from Swat Valley (KPK, Pakistan). Plant was identified by Dr. Hassan Sher, Jehanzaib College of Swat and voucher specimen has been placed in the national herbarium Islamabad with voucher no NH-012 (2004).

Preparation of crude extract and fractionation: The shade dried and powdered plant material was grounded and

extracted with methanol. This extract was filtered and evaporated under vacuum to obtain a thick greenish black gummy mass which was fractionated into *n*-hexane. The *n*-hexane fraction was subjected to column chromatography on silica gel. The oil sub-fractions WO-1 to WO-4 (for stem oil) and WO-5 (for oil from leaves) were obtained using *n*-hexane as eluent, and then the polarity increased gradually using chloroform gradient as shown in Figs. 1 and 2.

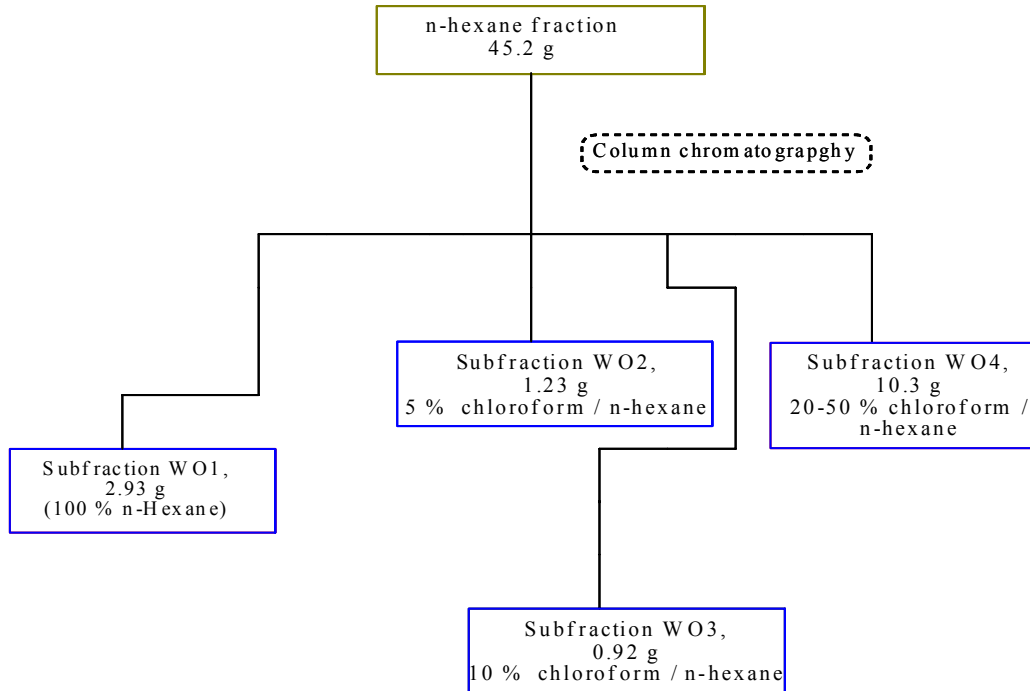
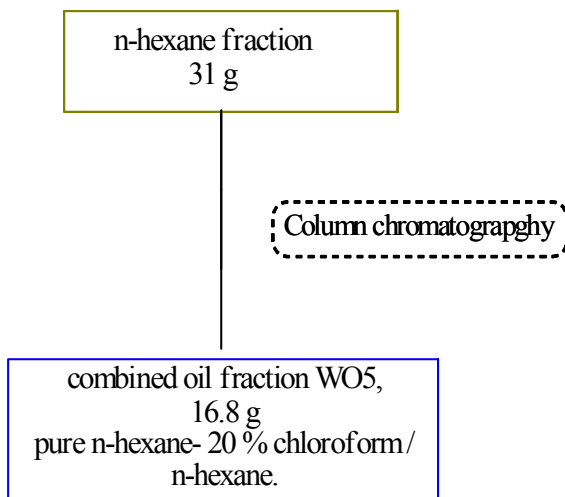
Antibacterial activity: The antibacterial activity was checked by the agar-well diffusion method (Carron *et al.*, 1987; Nisar *et al.*, 2010). One loop full of 24 hours old culture (10⁴-10⁶ CFU) was spread on the surface of Mueller-Hinton Agar plates. Wells were dug in the medium with the help of sterile metallic cork borer. Stock solutions of the test samples in the concentration of 3 mg/ml were prepared in dimethyl sulfoxide (DMSO) and 100 µl dilutions were added in their respective wells. The antibacterial activity of extracts (A-F) was compared with standard drug imipenem; the std. drug imipenem and DMSO were used as positive and negative control. The rate of growth in each well was determined by zone of inhibition (in mm) and was compared with control (Carron & Maran, 1987; Jorgensen & Turnidge, 1999; Kivack *et al.*, 2002; Rashid *et al.*, 2009).

Antifungal activity: The antifungal activity was determined by the Agar Tube Dilution Method (Rashid *et al.*, 2009; Nisar *et al.*, 2010, 2011). Samples were 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) and kept in slanting position at room temperature overnight. The fungal culture was then inoculated on the slant (Table 1). 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:

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

Table 1. Antifungal activity of *Z. oxyphylla* crude oil fractions.

Fungal species	Standard	% Inhibition		
		WO-1	WO-2	WO-5
<i>Trichophyton longifusus</i>	Miconazole 70	-	-	-
<i>Candida albicans</i>	Miconazole 110.8	-	-	-
<i>Aspergillus flavus</i>	Amphotericin 20	-	-	10
<i>Microsporium canis</i>	Miconazole 98.4	35	35	-
<i>Fusarium solani</i>	Miconazole 73	-	-	-
<i>Candida glaberata</i>	Miconazole 110.8	-	-	-

Fig. 1. Schematic diagram for the extraction of oil from stem of *Z. oxyphylla* Edgew.Fig. 2. Schematic diagram for the extraction of oil from leaves of *Z. oxyphylla* Edgew.

Phytotoxic activity: Phytotoxic activity was determined as reported previously (Rashid *et al.*, 2009; Nisar *et al.*,

2010). The medium was prepared in distilled water (100 ml) and pH was adjusted (5.5-6.5) by adding KOH solution. It was then autoclaved for 15 minutes at 121°C. The samples dissolved in ethanol (20 mg/ml) served as stock solution. Sterilized flasks were inoculated with 1000 µl, 100 µl and 10 µl of the stock solution for 500, 50 and 5 ppm respectively. The experiment was run parallel in triplicate. 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. The flasks were plugged with cotton and kept in the growth cabinet for seven days (Table 2). 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:

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

Paraquat was used as a standard drug, while paraquat and volatile solvent were used as positive and negative controls (Rashid *et al.*, 2009).

Table 2. Phytotoxicity activity of *Z. oxyphylla* crude oil fractions.

Conc. of sample (µg/ml)	Conc. of stand. drug	% Growth regulation		
		WO-1	WO-2	WO-5
1000	0.015	45	80	70.59
100		40	35	29.42
10		25	15	23.53

Insecticidal activity: Insecticidal activity was evaluated against three insects viz., *Rhyzopertha dominica*, *Callosobruchus analis* and *Tribolium castaneum*. Samples were dissolved in acetone (3 ml) and loaded in Petri dishes sheltered with the filter papers. After 24 h, ten insects were placed in each plate and incubated at 27°C for 24 hours with relative humidity (50%) in growth chamber. Permethrin was used as a standard drug, while

permethrin, acetone and test insects were used as positive and negative controls (Rashid *et al.*, 2009; Ali *et al.*, 2009) (Table 3). The percentage mortality was calculated by the formula:

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

Table 3. Insecticidal activity of *Z. oxyphylla* crude oil fractions.

Name of insect	+ve control	-ve control	% Mortality		
			WO-1	WO-2	WO-5
<i>Tribolium castaneum</i>	100	0	-	-	60
<i>Rhyzopertha dominica</i>	100	0	40	20	20
<i>Callosobruchus analis</i>	100	0	20	-	-

Brine shrimp lethality bioassay: Artificial “sea water” was prepared by dissolving sea salt (3.8 g/L) in double distilled water (Meyer *et al.*, 1982). “Sea water” obtained was filtered in a small tank; brine-shrimp eggs (1 mg) were added and tank was darkened by covering with aluminum foil. It was allowed to stand at 25°C for 24 h which provided a large number of larvae. Sample (20 mg) was dissolved in CHCl₃ (2 ml) and transferred to 500, 50 and 5 µl vials corresponding to 1000, 100 and 10µg per

ml, respectively. 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 “sea water” (5 ml) was added to each. Then 10 shrimps were added per vial, allowed to stand for 24 hours, shrimps were counted and number of surviving shrimps was recorded. Etoposide was used as positive control. The data were analyzed with a Finney computer program to determine the LD₅₀ values (Table 4).

Table 4. *In vitro* cytotoxic bioassay of *Z. oxyphylla* crude oil fractions.

Extractions	% Deaths at doses			LD ₅₀
	1000 µg/ml	100 µg/ml	10 µg/ml	
WO-1	24	26	28	377166.8
WO-2	25	26	27	1581233
WO-5	24	26	29	56463.5
Etoposide (standard)				7.4625

Total No. of shrimps used during experiment = 30

Results and Discussions

Documentation and validation of various pharmacological potentials of ethnomedical lore has taken a front seat recently in Pakistan. 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 including *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Bacillus subtilus* and *Shigella flexneri* were used to

evaluate the antibacterial activity of oil. The result revealed that oil sub-fractions did not show any inhibition and showed strong correlation to our previously antimicrobial investigation (Qayum *et al.*, 2012). In the case of antifungal activity six fungi viz., *Trichophyton longifusus*, *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 WO-1 and WO-2. There was no inhibitory effect of oil sub-fractions against remaining fungal strains.

Regarding to the phytotoxicity activity, results have shown that WO-2 and WO-5 fraction showed significant activity at highest concentration (1000 µg/ml), maximum activity was shown by WO-2 and WO-5 at this concentration that is 80% and 70.59% growth regulation while 15% and 23.13% was the lowest activity by same fractions. Similarly WO-1 at this concentration showed 45% growth regulation while 25% was the lowest activity by same fraction. The results clearly indicated that phytotoxic activity is dose dependent i.e., high phytotoxicity at high concentrations and vice-versa. In case of insecticidal activity, WO-2 showed non-significant activity while WO-1 and WO-5 revealed moderate and good insecticidal activity against *Rhyzopertha dominica* and *Tribolium castaneum* respectively. Oil sub-fractions remained inactive against *Callosobruchus analis*. LD₅₀ measurements of crude oil sub-fractions were evaluated against *Artemia salina* brine-shrimp eggs. No cytotoxic effects were observed. There does not appear to be any previous report on the biological screening of *Zizyphus oxyphylla* Edgew oil and further studies are continued to sort out the natural compounds responsible for these activities. Development of industries based on medicinal plants should be included as a priority area for translating lab findings to industrial scale to break confinement from academic pursuits.

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