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BIOLOGICAL SCREENING OF DIFFERENT ROOT EXTRACTS OF *EUPHORBIA WALLICHII*

IRSHAD ALI[1*], RUBINA NAZ[1], WAHIB NOOR KHAN[1], RUKHSANA GUL[1], AND M. IQBAL CHOUDHARY[2]

[1]Department of Chemistry, Gomal University, Dera Ismail Khan
[2]International Center for Chemical Sciences, H.E.J. Research Institute of Chemistry, University of Karachi, Karachi

Abstract

Root extracts of *Euphorbia wallichii* obtained from n-hexane (E1), chloroform (E2), ethyl acetate (E3) and n-butanol (E4) were tested *In vitro* for their phytotoxicity, cytotoxicity, antibacterial and antifungal activities. All the extracts at high concentration of 1000 µg ml-1 showed 60-100% phytotoxicity and 50-100% cytotoxicity while at low concentration of 10 µg ml-1 they showed 30-80% phytotoxicity and 25-70% cytotoxicity. Antibacterial study performed against six bacteria viz., *Escherichia coli*, *Bacillus subtilis*, *Shigella flexenari, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhi* showed a trend of inhibition zone in E2 and E3 extracts was found to be the most active while E1 and E4 extracts exhibited moderate to low activity in killing the tested bacteria. No significant antifungal activity for 6 tested fungi was observed.

Introduction

The genus *Euphorbia* belongs to the family Euphorbiaceae (spurge family) consisting of about 2,000 species; most of them are found in China, Western and Central Himalaya, Mediterranean, Middle East, South Africa and southern USA. Most are herbs but some are shrubs or trees. Plants of the genus *Euphorbia* are known to possess considerable medicinal and economical importance. They have been used to cure bronchitis and rheumatism (Satyanarayana *et al*., 1991), skin irritant (Gundidza *et al*., 1993), anti-cancer (Pan *et al*., 1991), anti-tumor and warts (Shi *et al*., 1995), inflammatory disorders (Satti *et al*., 1988), asthma (Ferreira *et al*., 1993) and as analgesic (Vamsidhar *et al*., 2000). Many biologically active diterpenes were found from this genus (Ng *et al*., 1990). A few compounds have been isolated form this plant (Wang *et al*., 2004), however biological screening was totally ignored. Keeping in view the importance of this plant, the current study was designed to screen for biological activities before taking up the main task of isolation, and structural elucidation of pure compounds.

Materials and Methods

Preparation of the extracts: The roots of *E.wallichii* were collected from Donga Gali, Pakistan in August 2003, and identified by Prof. Iftekhar H. Shah, Faculty of Pharmacy, Gomal University, Dera Ismail Khan. A voucher specimen # 3730 is deposited in the herbarium of faculty of Pharmacy, Gomal University Dera Ismail Khan, Pakistan.

Shade-dried roots of *E.wallichii* (25 kg) were grounded and extracted with MeOH at room temperature (3x23L). The combined methanolic extract was filtered and evaporated under vacuum to obtain a thick gummy mass. It was suspended in water and successively extracted with n-hexane (3L, E_1), chloroform (4L, E_2), ethyl acetate (4L, E_3) and n-butanol (4L, E_4). All these extracts (E_1 - E_4) were tested for phytotoxicity, brine shrimp lethality, antibacterial and antifungal activities.

*Corresponding author E-mail: irshad_gomal@yahoo.com

Bioactivity studies: Phytotoxic activity was determined by using the modified protocol of Lemna minor (Atta-ur-Rahman, 1991). The medium was prepared by mixing various constituents in 100 ml distilled water and the pH was adjusted (5.5-6.5) by adding KOH solution. The medium was then autoclaved at 121^{o}C for 15 minutes. The extracts dissolved in ethanol (20mg/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 500, 50 and 5 ppm 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. One other flask supplemented with solvent and reference growth inhibitor (paraquat), served as positive control. 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. The results were interpreted by analyzing the growth regulation in percentage calculated with reference to the negative control by the following formula:

$$\text{Growth regulation (\%)} = \frac{100 - \text{No. of fronds in test sample}}{\text{No of fronds in negative control}} \times 100$$

Brine shrimp lethality bioassay: It is an excellent and simple preliminary method to determine the cytotoxicity of crude plant extract and pure natural compounds. In this method, artificial "sea water" was prepared by dissolving 3.8 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 (50µl) 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. The data were analyzed with a Finney computer program to determine the LD_{50} values.

Antibacterial bioassay: 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 10^4-10^6 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 (E_1-E_4) in the concentration of 1 mg/ml were prepared in dimethyle sulfoxide (DMSO) and 100 µl dilutions were added in their respective wells. The antibacterial activity of extracts (E_1-E_4) was compared with standard drug; the std. drug imepinem was served as positive control.

Antifungal bioassay: The antifungal activity was determined by the Agar Well Diffusion Method (Atta-ur-Rahman *et al*., 1991). In this method Griseofulvin was used as the standard drug. The crude extract was dissolved in DMSO (50 mg / 5ml). 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.

Table 1. Phytotoxic bioassay.

Extractions	Concentration of extractions µg/ml	% Inhibition
n-hexane (E_1)	1000	60
	100	40
	10	30
Chloroform (E_2)	1000	100
	100	70
	10	50
Ethyl acetate (E_3)	1000	100
	100	100
	10	80
n-butanol (E_4)	1000	65
	100	46
	10	32

Results and Discussion

Phytotoxic bioassay: Phytotoxicity of all the extracts (E_1-E_4) was carried out at three different concentrations i.e., 1000, 100 and 10 µg/ml. E_3 was found to be highly active, having 100%, 100%, 80% plant growth inhibition at all the three concentrations while E_2 exhibited second most active, having 100% and 70% plant growth inhibition, at the concentration of 1000, 100 µg/ml and showed weakly active at 10 µg/ml. The E_1 and E_4 exhibited moderate activity at 1000 µg/ml concentration whereas no significant activity of these extracts was shown on other concentrations.

Antifungal bioassay: The fungicidal activity of these extracts was performed against six fungi, viz., *Trichophyton longifusus, Candida albicans, Aspergilus flavus, Microsporum canis, Fusarium solani and Candida glaberata*. The results indicated that these extracts were not very active against the tested fungal strains except in E_2 and E_3 which exhibited moderate activity whereas E_1 and E_4 displayed weak activity in killing *A. flavus* and *M. canis* (Table 1). It was further observed that E_2-E_4 were weakly active and E_1 was devoid of any antifungal activity against the rest of the tested fungi.

Brine shrimp lethality bioassay: LD_{50} measurements of E_1-E_4 were evaluated against *Artemia salina* brine-shrimp eggs (Table 2). It was evident from the results that E_3 was found to be significant lethality with LD_{50} value of 0.0332 µg /ml, E_2 exhibited high lethality with LD_{50} value of 0.7334 µg /ml whereas E_1 and E_4 showed moderate lethality with LD_{50} value of 310.3220 and 262.4612 µg /ml.

Antibacterial bioassay: The antibacterial study was performed against six bacteria viz., *Escherichia coli, Bacillus subtilis, Shigella flexenari, Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella typh.* The data indicated that the E_2 and E_3 were same and found to be most effective, exhibiting high activity whereas E_1 and E_4 showed moderate activity in killing the *B. subtilis, P. aeruginosa* and *S. typhi* (Table 3). The E_2 and E_3 were also found to possess pronounced moderate activity while E_1 and E_4 revealed low activity against *E. coli, S. flexenari*. In case of *S. aureus,* the inhibition was the same and showed low activity in E_2 and E_3 but E_1 and E_4 remained totally ineffective, which showed no activity due to the resistance.

Table 2. Brine shrimp lethality bioassay.

Extractions	% Deaths at doses			LD_{50} µg/ml	Results
	1000 µg/ ml	100 µg/ml	10 µg/ml		
n-hexane (E_1)	50	40	25	310.3220	++
Chloroform (E_2)	100	85	60	0.7334	+++
Ethyl acetate (E_3)	100	100	70	0.0332	++++
n-butanol (E_4)	60	48	28	262.4612	++
Etoposide (positive control)	90	78	56	1.0321	+++

Key: ++++ = significant activity; +++ = high activity; ++ = moderate activity

Table 3. Antibacterial bioassay.

Name of bacteria	Extractions activities zone of inhibition of std. drugs (Imepinem)				
	n-hexane (E_1) 100 µl	$CHCl_3$ (E_2) 100 µl	EtOAc (F_3) 100 µl	n-butanol (F_4) 100 µl	100 µl
E. coli	+	++	++	+	++
B. subtilis	++	+++	++	++	+++
S. flexenari	+	++	++	+	++
S. aureus	_	+	+	_	++
P. aeruginosa	++	++	+++	++	+++
S. typhi	++	+++	+++	++	+++

Key: +++ = high activity; ++ = moderate activity; + = low activity; - = no activity.

Table 4. Antifungal bioassay.

Name of fungus	n-hexane (E_1)	$CHCl_3$ (E_2)	EtOAc (E_3)	n-butanol (E_4)	Std. drugs MIC µ g/ ml
T. langifusus	-	+	+	+	Miconazole 70
C. albicans	-	+	+	+	Miconazole 110.8
A. flavus	+	++	++	+	Amphotericin B20
M. canis	+	++	++	+	Miconazole 98.4
F. solani	-	+	+	+	Miconazole 73.25
C. glaberata	-	+	+	+	Miconazole 110.8

Key: ++ = Moderate activity, + = Low activity, Concentration of Sample. 400µg/ml of DMSO, Incubation Temp: 29°C, Incubation period: 7 days

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