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Biological screening of selected flora of Pakistan

M. Zia-Ul-Haq[1*], M. Raza Shah[2], Mughal Qayum[3], Sezai Ercisli[4]

[1] Department of Pharmacognosy, University of Karachi, Karachi-75270, Pakistan
[2] H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan
[3] Department of Pharmacy, Abdul Wali Khan University, Mardan-23200, Pakistan
[4] Department of Horticulture, Ataturk University, Erzurum-25240, Turkey

ABSTRACT

Methanolic extracts of different parts of five medicinal plants, *Ferula assafoetida* L. resin, *Grewia asiatica* L. leaves, *Ipomoea hederacea* Jacq. seeds, *Lepidium sativum* L. seeds and *Terminalia chebula* Retz. fruits were tested *in vitro* for their cytotoxic, phytotoxic, insecticidal, nematicidal and anthelmintic activities. *Ipomoea hederacea* showed very significant phytotoxic and cytotoxic activity, with 100% inhibition of *Lemna minor* growth and 100% death of *Artemia salina* at concentrations of 1000 and 100 μg mL^{-1}. *Grewia asiatica* exhibited very weak activities while *Lepidium sativum* and *Ferula assafoetida* showed moderate to good potential in all three bioassays. The results suggest screening of *Ipomoea hederacea* seeds further for isolation of bioactive compounds that may be responsible for its toxic potential.

INTRODUCTION

Pakistan has varied geographic, climatic and vegetation features which are essential to the formation of one of the most diverse flora, which varies through latitudinal and longitudinal gradients. The country is characterized by a wide range of macrohabitats across elevation and climatic gradients, generating high environmental heterogeneity. Some important thematic areas of indigenous flora have been recently covered (Zia-Ul-Haq et al., 2009; 2010; 2011 a, b, c, d, e; 2012 a, b, c, d, e, f; Nisar et al., 2010 a, b, c; 2011), however most of medicinal flora of Pakistan remains unexplored. All plants may not be as useful as claimed, or may have more therapeutic properties than are known traditionally. Claimed therapeutic efficacies of medicinal plants are often criticized due to dearth of research, critical evaluation *and in vivo* studies, questioning the safety of their use. Therefore, proper scientific investigations are required to explore the exact medicinal potential of plants.

Ferula assafoetida L. (*Umbelliferae*), *Grewia asiatica* L. (*Malvaceae*), *Ipomoea hederacea* Jacq. (*Convolvulaceae*), *Lepidium sativum* L. (*Brassicaceae*), and *Terminalia chebula* Retz. (*Combretaceae*) are multi-purpose medicinal plants used in various parts of Pakistan. *Ferula assafoetida* resin is believed to possess sedative, expectorant, analgesic, carminative, stimulant, antiperiodic, antidiabetic, antispasmodic, emmenagogue, vermifuge, laxative, anti-inflammatory, contraceptive and antiepileptic properties (Zia-Ul-Haq et al., 2012d). *Grewia asiatica* is believed to possess antipyretic, antidiabetic, analgesic, antifertility, antibiotic and antimicrobial properties (Zia-Ul-Haq et al., 2012a). *Ipomoea hederaceae* seeds are diuretic, anthelmintic, a blood purifier, and are used for treatment of constipation, inflammations, abdominal diseases, fevers, headaches, bronchitis and dropsy (Nasir and Ali, 1979; Chopra et al. 1986; Joshi, 2000). *Lepidum sativum* seeds are used in chronic enlargement of liver and spleen. The bruised seeds mixed with lime juice are used as local application for the relief of inflammatory and rheumatic pains. The seeds are bitter, themogenic, depurative, rubefacient, galactagogue, emmenagogue, tonic, aphrodisiac and diuretic (Manohar et al., 2012). *Terminalia chebula* fruit is a mild laxative, stomachic, tonic and antispasmodic. It is useful in ophthalmia, hemorrhoids, dental caries, bleeding gums and oral cavity ulcers. Its paste with water is found to be anti-inflammatory, analgesic and to have purifying and healing capacity for wounds (Nadkarni, 1976). Besides their diverse and broad spectrum ethnopharmacological uses, all these plants have some common medicinal uses, since they all possess antimicrobial, antioxidant and anti-inflammatory potential.

Biological screening including cytotoxic, phytotoxic, insecticidal, nematicidal and anthelmintic activities is an important part of the development of new drugs from medicinal plants. These are low cost, quick, and easy benchtop general bioassays used for screening and monitoring of crude extracts of plants. To the best of our knowledge, no systematic investigation has been made on biological screening of the medicinal plants that we have selected. The current study has been designed to screen the methanolic extracts of selected plants for phytotoxicity, cytotoxicity and nematicidal activities. The present research will provide the needed preliminary observations necessary to select among crude extracts, those with potentially useful properties for further biochemical investigations.

MATERIALS AND METHODS

Preparation of crude extract

The plant materials, *Ferula assafoetida* L. (resin), *Grewia asiatica* L. (leaves), *Ipomoea hederacea* Jacq. (seeds), *Lepidium sativum* L. (seeds), and *Terminalia chebula* Retz. (fruits) were purchased from a local market. These were identified by Dr. Shakeel Ahmad, Bahuddin Zakariya University Multan and a voucher specimen was deposited in the Botany Department of this university. The plant material was air-dried in shade and crushed to a coarse powder separately using mortar and pestle. Plant material (0.5 kg each) was macerated with aqueous methanolic mixture (80:20; v/v), at room temperature for

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*Corresponding author: Sezai Ercisli. Department of Horticulture, Ataturk University, Erzurum-25240, Turkey. E-mail: sercisli@gmail.com

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fifteen days with occasional shaking. The process was repeated three times with the same quantity of solvent mixture. The extracts so obtained were combined, filtered through filter paper under vacuum and concentrated under reduced pressure in a rotary evaporator (model Q-344B – Quimis, Brazil) using a warm water bath (model Q-214M2 - Quimis, Brazil) to obtain a thick gummy mass, which was further dried in a desiccator and stored in an air-tight vial until further use.

Phytotoxic activity

Phytotoxic activity was determined by using the modified protocol of *Lemna minor* (Ali et al., 2009; Nisar et al., 2010 b, c). 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 °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 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., were 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:

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 (Table 1). Paraquat was used as a standard drug, while paraquat and volatile solvent were used as positive and negative controls (Nisar et al., 2010 b, c).

Cytotoxic activity

This activity is an excellent and simple preliminary method to determine the cytotoxicity of crude plant extract and pure natural compounds (Nisar et al., 2010 b, c). 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; brine-shrimp eggs were added (1mg) (*Artemia salina*) and the tank was darkened by covering with aluminum foil. It was allowed to stand for 24 hours at 25 °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. Three replicates were prepared for each concentration, making a total of nine vials. The vials containing material were concentrated, dissolved in DMSO (50 μl) and 5ml "sea water" added to each. Then 10 shrimp were added per vial and allowed to stand for 24 hours; then shrimp were counted and the number of surviving shrimp recorded. Etoposide was used as positive control. The data were analyzed with a Finney computer program to determine the LD_{50} values (Table 2).

Insecticidal activity

Crude extract and all fractions were evaluated against different insects, viz. *Tribolium castaneum*, *Callosbruchus analis*, and *Rhyzopertha dominica*. The test sample was prepared by dissolving 200 mg of crude fractions in 3 ml acetone and loading in Petri dishes covered with filter paper. After 24 hours, 10 test insects were placed in each plate and incubated at 27 °C for 24 hours with 50% relative humidity a in growth chamber. The results were analyzed as percentage mortality, calculated with reference to the positive and negative controls (Table 3). Permethrin was used as a standard drug, while permethrin, acetone and test insects were used as positive and negative controls (Ali et al., 2009; Rashid et al., 2009).

The percentage mortality was calculated by the formula:

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

TABLE 1

Phytotoxic activity of extracts by *Lemna minor* bioassay

Samples	Conc.	% Growth regulation	Conc. of std. drug (μg/ml)
F. assafoetida		60	
G. asiatica		30	
I. hederacea	1000	100	0.015
L. sativum		64	
T. chebula		37	
F. assafoetida		43	
G. asiatica		21	
I. hederacea	100	100	0.015
L. sativum		42	
T. chebula		30	
F. assafoetida		30	
G. asiatica		17	
I. hederacea	10	80	0.015
L. sativum		30	
T. chebula		22	

TABLE 2

Cytotoxic activity of extracts by brine shrimp lethality bioassay

Sample	% deaths at doses			LD_{50}
	1000 μg/ml	100 μg/ml	10 μg/ml	
F. assafoetida	50	40	25	310.3220
G. asiatica	26	25	22	41389. 2
I. hederacea	100	100	70	0.0332
L. sativum	60	48	28	262.4612
T. chebula	26	24	22	346568.9
Etoposide (standard)				7.4625

Nematicidal activity

For isolation of *Helicotylenchus indicus* nematodes, 500 mg of soil samples (depth 15-25 cm) were collected from rice paddy (*Oryza sativa* L.) fields. Soil samples were processed by Cobb sieving (Cobb, 1918) and a modified Baermann funnel method (Baermann, 1917). The soil sample was put into a large bucket containing water and the mixture was vigorously stirred into a suspension, which was allowed to settle for about 2 minutes. The heavy soil particles sank to the bottom but nematodes remained suspended in the water. The remaining suspension was slowly poured over a coarse sieve (60 mesh aperture), which was continuously tapped by hand to avoid blocking. The deposit on the sieve was washed with a gentle jet of water into a beaker. This water suspension, containing eel-shaped nematodes, was passed through 200 and 300 mesh sieves. The nematodes thus recovered were mixed and water was decanted after allowing sufficient time for the nematodes to settle down. Then the nematode suspension was poured over a piece of tissue paper attached to a perforated plastic sheet placed in a funnel fitted with a rubber tube and clamped at the lower end. The water contained in the funnel barely touched the bottom of the tissue paper. Care was taken not to allow the debris to float off the edges of the tissue paper. After 24 hours the nematodes wriggled out into the clear water in the funnel and settled at the bottom, and then 100 ml of water containing the nematodes was drawn into a beaker. The nematode suspension was allowed to settle for 2 hours or more, the excess supernatant water was poured off, and the remaining concentrated content was transferred into a cavity block for examination under a stereomicroscope and selecting nematodes (Naqvi et al., 1992). Crude extracts were dissolved in water (passed through Whatman filter paper No.1) to make dilutions of 2%, 1%, 0.5%, and 0.25 %. Experiments were performed under laboratory conditions at 28±2 °C. Glass tubes 15 cm long and 8 cm were used for bioassay. Three ml were taken from all dilutions in each tube. The required amount of nematode suspension (100 freshly hatched second stage juveniles/3ml suspension) was poured into tubes to each of which an equal amount of plant extract had already been poured). Distilled water with nematode larvae was taken as control. The dead nematodes were observed under a stereoscopic binocular microscope after 24 48 and 72 hours and percentage mortality was calculated (Table 4). Nematodes were considered dead if they did not move when probed with a fine needle (Cayrol et al., 1989).

Larvicidal activity

The infective L3 larvae of *Haemonchus contortus* were obtained by culturing fecal samples directly from the rumen of the sheep naturally infected by mixed gastrointestinal nematodes. L3 Larvae were incubated with the plant extracts used at concentrations of 50, 25, 12.5, 6.25 and 3.12 mg/ml of saline phosphate buffer (Ph 7.2). Ivermectin was used as standard. Mortality of the larvae subjected to the above treatments was used as the criterion for anthelmintic activity. Motility was recorded at 0, 1, 2, 3, 4, and 8 hour intervals (Iqbal et al., 2004). LC_{50} was determined using regression analysis and solving the regression equation (Table 5).

RESULTS AND DISCUSSION

With a rich endemic flora, Pakistan has many plant species which are not yet explored; detailed compendia of medicinal species native to Pakistan are being published. Strategies like high-throughput screening, phytochemical profiling, quality controls and standardization of raw materials and finished products, clinical trials and herbal therapeutics will lead to maximum benefits from these plants. The compiled data will be helpful for chemotaxonomy and breeding, as well as authenticity testing of phytopharmaceuticals containing these extracts.

Phytotoxic results of crude extracts of selected plants are shown in Table 1. The criteria used for phytotoxicity were as follows: 0-39% inhibition (low activity), 40-59% inhibition (moderate activity), 60-69% inhibition (good activity),

TABLE 3

Insecticidal activity of of extracts against various insects

Samples	% Mortality		
	C. analis	T.castaneum	R. dominica
F. assafoetida	30	30	25
G. asiatica	20	20	20
I. hederacea	80	70	75
L. sativum	70	65	70
T. chebula	50	50	45

TABLE 4

Nematicidal activity of extracts against *Helicotylenchus indicus*

Samples	**% Mortality observed against concentration**						
	24 hr			**48 hr**			**Control**
	2	**1**	**0.5**	**2**	**1**	**0.5**	
F. assafoetida	49	31	25	57	45	37	1
G. asiatica	22	17	10	39	23	20	1
I. hederacea	90	65	55	96	82	58	2
L. sativum	63	48	28	69	55	33	2
T. chebula	40	29	15	52	39	24	2

TABLE 5

Larvicidal activity (LC_{50} mg/ml) of extracts against *Haemonchus contortus*

Samples	Larvicidal Activity(LC_{50})
F. assafoetida	9.11
G. asiatica	17.21
I. hederacea	2.07
L. sativum	6.52
T. chebula	4.82
Ivermectin (standard)	3.19

above 70% inhibition (significant activity). *Ipomoea hederacea* appeared to have very significant phytotoxic activity, with 100% inhibition of *Lemna minor* growth at high concentrations (1000 and 100 μg/mL). *Ferula assafoetida* and *Lepidium sativum* showed a good response (60-64%) at a concentration of 1000 μg/mL. *Grewia asiatica* and *Terminalia chebula* showed low weedicidal activity (30-37%) at a concentration of 1000 μg/mL. The data suggests the potential of *Ipomoea hederacea* for further phytochemical analysis as a potent herbicide. Interference of weeds obviously reduces the quality and quantity of agricultural crops and is responsible for huge economic losses all over the world. Synthetic herbicides are extensively used for the control of weeds in agricultural sectors. However, various factors that restrict the use of synthetic herbicides include water and soil pollution, herbicide-resistant weed populations, herbicide residues and detrimental effects on non-target species. In recent times, more emphasis has been placed on natural allelochemicals from plants for weed control in crop production especially to cope with the problem of weed resistance (Li et al., 2003; Saeed et al., 2010).

Cytotoxicity of crude extracts of selected plants is shown in Table 2. *Ipomoea hederacea* exhibited significant cytotoxic effect at high concentrations of 1000 and 100 μg/mL. *G. asiatica* and *T. chebula* exhibited weak lethality, with LD_{50} values of 41389.2 and 346568.9 μg/mL, respectively. *Ferula assafoetida* and *Lepidium sativum* showed a moderate response, with 50-60% deaths at a concentration of 1000 μg/mL. This test is used as litmus test for presence of anticancer compounds. Our results suggest the presence of natural anticancer products in *Ipomoea hederacea* seed extract. Our results are supported by recent findings of Parekh (Parekh et al., 2012) which proved anticancerous activity of *Ipomoea hederacea* seed extract.

Insects control relies heavily on the use of synthetic insecticides; however, their widespread use has led to various problems such as the development of insect strains resistant to insecticides (Zettler and Cuperus, 1990; White, 1995; Ribeiro et al., 2003), toxic residues on stored grain, toxicity to consumers and increasing costs of application. There is an urgent need to develop safe alternatives that are of low cost, convenient to use and environmentally friendly. Insecticidal results of investigated plant extracts are shown in Table 3. The results indicated that *Ipomoea hederacea* and *Lepidium sativum* seed extracts showed the highest insecticidal activity against all three insects, while the remaining extracts had moderate to average activity against all insects.

Nematodes are pathogens which cause serious damage to agricultural crops and plants. These nematodes may be controlled by synthetic nematicides. However, environmental and human health concerns are resulting in increased restrictions on use of synthetic nematicides. Some safe procedures for nematode control have been developed based on biological control agents and organic amendments (Noling and Becker, 1994); one of them is use of plant extracts against these nematodes. This method has drawn great interest from researchers in the prevention of nematodes due to the environmental pollution problems induced by synthetic nematicides. Three different concentrations were used; 2%, 1% and 0.5% with examination after 24 and 48 hours. The results (Table 4) indicated that *Ipomoea hederacea* seed extract exhibited very strong nematicidal capacity against *H. indicus*. *Ferula assafoetida* has shown activity against *Pheretima posthuma* (Naikwadi and Bhalodia, 2009).

The LC_{50} value for methanolic extracts against *Haemonchus contortus* are presented in Table 5. The results indicate that *L. sativum* and *T. chebula* and *I. hederacea* have significant anthelmintic activity. The greatst anthelmintic activity was shown by *I. hederacea*, whose LC_{50} was 2.07 mg/ml and the lowest was observed for *G. asiatica*, 17.21 mg/ml. *Haemonchus contortus* is responsible for severe economic losses in sheep and goat breeding globally. The results confirm the traditional use of *L. sativum* and *T. chebula* for anthelmintic purposes (Abbas et al., 2002; Kamaraj and Rahuman, 2011).

The results clearly indicated that all activities are dose dependent, i.e. high activity at high concentrations and *vice versa*. The possible action of plant extracts is attributed to the bioactive compounds present as secondary metabolites in the form of condensed tannins, saponins, polyphenols, and flavonoids. Chemical analyses and biological and pharmacological assays provide objective evidence to validate traditional uses by indigenous communities. Further, to export herbs it is necessary to put them into value-added products. The current study will open avenues for future in depth research on these plants.

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