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BIOLOGICAL SCREENING OF *IMPATIENS BICOLOR* ROYLE

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Abstract

Crude methanolic extracts of *Impatiens bicolor* Royle as well as its different fraction namely n-hexane (A), dichloromethane (B), ethyl acetate (C), n-butanol (D) and aqueous (E) were tested *In vitro* for their insecticidal, cytotoxic and phytotoxic activities. Only n-hexane (A) fraction showed moderate insecticidal activity while ethyl acetate (C), n-butanol (D) and aqueous (E) fractions indicated low insecticidal activity. All fractions except n-butanol (D) indicated significant phytotoxicity. Cytotoxic results observed were also very low as compared to standard used and only dichloromethane (B) fraction showed cytotoxicity at higher dose while all other fractions as well as crude extract exhibited moderate to low activity in killing the tested brine shrimps.

Introduction

Pakistan has a very rich botanical wealth and a large diversity of plants resources due to its varied climatic and edaphic factors only a small percentage of which have been biochemically investigated (Ahmad *et al*., 2007). The green pharmaceuticals are receiving extraordinary importance and popularity (Ahmad & Husain, 2008). The synthetic drugs approved as safe and efficacious a decade ago had to be recalled and relabeled because of unanticipated side effects. On the other hand, herbal medicines do not have any such effects but have benefits due to the combinations of medicinal ingredients coupled with vitamins and minerals (Hussain *et al*., 2003). In recent years, there has been a consistent growth in the demand for plant-based drugs and several plant products from a variety of species. The increasing use of traditional therapies demands more scientifically sound evidence for the principles behind therapies and for effectiveness of medicines (Patwardhan *et al*., 2005). All plants may not be as useful as claimed, or may have more therapeutic properties than are known traditionally. The therapies are often criticized due to dearth of research, critical evaluation, *In vivo* studies and validations (Fong, 2002; Houghton, 1995) support the safety of uses. Therefore, proper scientific investigations are required to explore the exact medicinal potential of plants.

Impatiens bicolor Royle is an important medicinal plant distributed in northern areas of Pakistan. The plant is used locally as diuretic, tonic and has cooling effect (Gilani *et al*., 2001). Although some work has been done on this plant (Hasan & Tahir, 2005) however its biological screening was totally ignored. In this context as part of our continuous studies on exploring the hidden potential of indigenous flora of Pakistan (Nisar *et al*., 2007, 2008, Zia-ul-Haq *et al*., 2007 a, b; 2008 a, b; 2009) we have screened the extract of *I. bicolor* Royle and its different fractions for various *In vitro* biological activities to evaluate its phytomedicinal potential. The present investigation will provide a broad base for the possibility of further detailed biological studies on *I. bicolor* Royle.

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Material and Methods

Plant material, preparation of crude extract and fractionation: Whole plant of *Impatiens bicolor* Royle was collected from Khwazakhela, Swat, N.W.F.P. Pakistan, during September 2008. A taxonomist, Dr. Hassan Sher, Jahan Zeb Post Graduate College Saidu Sharif, Swat (Pakistan), identified the plant. A voucher, specimen No.18-NH-4-008 was deposited in the National Herbarium, Islamabad, Pakistan.

Shade-dried *I. bicolor* Royle (10 kg) was grounded and extracted with MeOH and water at room temperature. The combined methanolic extract was filtered and evaporated under vacuum to obtain a thick greenish black gummy mass. It was fractionated into n-hexane (A); dichloromethane (B), ethyl acetate (C), n-butanol (D), aqueous (E) as well as crude (F) fractions. All these extracts (A - F) were tested for various biological activities.

Insecticidal activity: The extracts A, B, C, D, E and F, obtained from the extraction of *I. bicolor* Royle, 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 (Ali *et al*., 2009).

The percentage mortality was calculated by the formula:

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

Phytotoxic activity: Phytotoxic activity was determined by using the modified protocol of *Lemna minor* (Ali *et al*., 2009). 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., 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:

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

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.

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 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 (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.

Results and Discussions

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. So the present study has been carried out to fulfill this gap. Crude methanolic extracts of *Impatiens bicolor* Royle as well as its different fraction namely n-hexane (A); dichloromethane (B), ethyl acetate (C), n-butanol (D), and aqueous (E) were tested *In vitro* for their insecticidal, cytotoxic and phytotoxic activities. These activities proved to be significant in some extracts, while other extracts showed variable response for these bioassays.

The crude methanolic extracts as well as different fraction namely n-hexane (A); dichloromethane (B), ethyl acetate (C), n-butanol (D), and aqueous (E) of *Impatiens bicolor* Royle were evaluated against different insects viz., *Tribolium castaneum*, C*allosbruchus analis* and *Rhyzopertha dominica* (Table 1). Only n-hexane (A) fraction showed moderate insecticidal activity and all other fractions showed very low mortality against tested insect species. Phytotoxicity of all the extracts (A-F) was carried out at three different concentrations i.e., 1000, 100 and 10 µg/ml. The n-hexane (A) fraction was significantly phytotoxic against *Lemna minor* and it was inferred from experiments about its dose dependency (Table 2) i.e., at high concentration it is highly significant and the activity decreased with decrease in concentration. This activity is relatively similar to the standard drug paraquat and indicates the presence of herbicidal compound in n-hexane (A) extract. All other fractions as well as crude extract did not show any potent phytotoxicity even at higher doses i.e., 1000 µg/ml. Phytochemical analysis has indicated presence of a number of polyphenolic compounds and tannins in *I. bicolor* Royle (Hasan & Tahir, 2005). The presence of these compounds, which are toxic, can be another reason for the death of host tissues as these toxins can easily penetrate into the host cells. The phenolic compounds can mediate harmful interactions directly or indirectly by linking autotrophs to each other and to herbivores (Waterman & Mole, 1994). LD_{50} measurements of crude extract as well as its fractions were evaluated against *Artemia salina* brine-shrimp eggs (Table 3). It was evident from the results that dichloromethane (B) fraction followed by ethyl acetate (C) fraction and crude methanolic extract (F) was found to possess markable lethality. This is the first study of this kind on *I. bicolor* Royle and further studies are continued to sort out the natural compounds responsible for theses activities.

Table 1. Insecticidal activity of *Impatiens bicolor* Royle extract and its fractions.

Insect species	% Mortality							
	+ve control	-ve control	A	B	C	D	E	F
C. analis	100	0	0	20	20	20	20	0
T. castaneum	100	0	20	0	20	20	20	0
R. dominica	100	0	40	20	20	20	20	20

n-hexane (A); dichloromethane (B), ethyl acetate (C), n-butanol (D), aqueous (E), crude (F)

Table 2. *In vitro* phytotoxic bioassay of crude extract and different fractions of *Impatiens bicolor* Royle.

Samples	Conc.	No. of fronds samples	Control	% Growth regulation	Conc. of std. drug (µg/ml)
n-hexane (A)		4		80	
Dichloromethane (B)		09		55	
Ethyl acetate (C)	1000	09	20	55	0.015
n-butanol (D)		15		25	
Aqueous (E)		11		45	
Crude (F)		9		55	
n-hexane (A)		18		10	
Dichloromethane (B)		14		30	
Ethyl acetate (C)	100	18	20	10	0.015
n-butanol (D)		17		15	
Aqueous (E)		18		10	
Crude (F)		17		15	
n-hexane (A)		20		0	
Dichloromethane (B)		19		05	
Ethyl acetate (C)	10	19	20	05	0.015
n-butanol (D)		19		5	
Aqueous (E)		19		5	
Crude (F)		18		10	

Table 3. *In vitro* cytotoxic bioassay of different fractions of *Impatiens bicolor* Royle by brine shrimp lethality bioassay.

Extractions	% Deaths at doses			LD_{50}
	10 µg/ml	100 µg/ml	1000 µg/ml	
n-hexane (A)	14	23	28	796.27
Dichloromethane (B)	10	16	28	235.06
Ethyl acetate (C)	11	17	25	251.34
n-butanol (D)	14	22	28	732.35
Aqueous (E)	13	20	26	521.16
Crude (F)	11	19	23	285.79
Etoposide(standard)				7.4625

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