Pak. J. Bot., 44(1): 355-359, 2012.

BIOLOGICAL SCREENING OF OILS FROM *IMPATIENS BICOLOR* ROYLE

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Abstract

The oil sub-fractions obtained by solid phase extraction of the n-hexane fraction from aerial parts of the *Impatiens bicolor* Royle, were tested *In vitro* for their antibacterial, antifungal, insecticidal, cytotoxic and phytotoxic activities. Bacterial strains used for the experiment were *Shigella flexeneri*, *Escherchia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa, Salmonella typhi* and *Staphylococcus aureus* while fungal strains included *Candida albicans*, *Aspergillus flavus*, *Fusarium solani*, *Microspoum canis*, *Candida glaberata*n and *Trichophyton longifusis*. The tested samples did not display any activity against above mentioned bacterial strains while in case of antifungal activity, fractions MQ-6 and MQ-7 exhibited low activity against *Microsporum canis.* The crude oil sub-fraction MQ-4 (81.25%) exhibited highly significant phytotoxicity in the tested concentration (1000 mg/mL) against *Lemna* plant. The tested samples revealed low to moderated insecticidal activity against *Tribolium castaneum* while in case of brine shrimp lethality, MQ-5, MQ-6, MQ-7 showed significant cytotoxicity at high dose 1000 µg/mL with LD_{50} value 170.748, 170.79, 161.54 µg/ mL respectively.

Introduction

Medicinal plants are natural and basic source of modern pharmaceutical industry. Both cultivated and wildly grown medicinal plants possess great potentialities. This potential has led to their use since centuries in folklore medicines by indigenous community and is now authenticated by isolation of active principles and biodynamic compounds from them by using ultra modern screening techniques. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found *In vitro* to have antimicrobial properties (Cowan *et al.,* 1999). Historically, plants have provided a good source of anti-infective agents; emetine, quinine, and berberine remain highly effective against microbial infections.

Globally, the quick spread of the infectious diseases is due to many dynamic factors such as poor health care systems, irrational use of available antimicrobial agents, population growth, increase in international travelling, intensive farming practices, and degradation of the environmental factors and failure of available drug therapies by microbial resistance. The pharmacological treatment of diseases by medicines began by using the medicinal herbs (Tyler, 1997). It is evident that search for antimicrobial agents from plants is a wider resurgence of scientific interest to produce new chemotherapeutics. *Impatiens bicolor* Royle is a less explored herb despite its diverse medicinal properties and use by indigenous people. Although some work has been done on this plant (Nisar *et al.,* 2010 a, b) however no study exists on biological screening of plant (aerial parts) oil obtained from n-hexane fraction by column chromatography. In this context as part of our continuous studies on exploring the hidden potential of indigenous flora of Pakistan (Zia-ul-Haq *et al.,* 2007 a, b; 2008 a, b; 2009, 2010 a, b; 2011; Nasir *et al*., 2011) we have screened the oil extracted from *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.

Materials and Methods

Plant material, extraction and fractionation: *Impatiens bicolor* Royle (aerial parts) was collected from Khwazakhela, Swat, N.W.F.P. Pakistan, during September 2008. A voucher, specimen No.18NH-4-008 was deposited in the National Herbarium, Islamabad. Plant material (10 kg) was grounded and extracted with methanol and water (85:15) at room temperature. The combined methanolic 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 MQ-1 to MQ-8 were obtained using *n*-hexane as eluent, and then the polarity increased gradually using dichloromethane gradient as shown in Fig. 1.

Antibacterial activity: The antibacterial activity was checked by the agar–well diffusion method (Kavanagh *et al*., 1963; Carron *et al*., 1987). 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 std. drug imepinem and DMSO were used as positive and negative control. Zone of inhibition (in mm) was recorded visually. The amount of growth in each well was determined visually by comparing with the growth in the control wells (Carron & Maran, 1987; Jorgensen & Turnidge, 1999; Kivack *et al*., 2002; Stepanovic & Anetic, 2003; Bektas & Donmez, 2004; Rashid *et al*., 2009).

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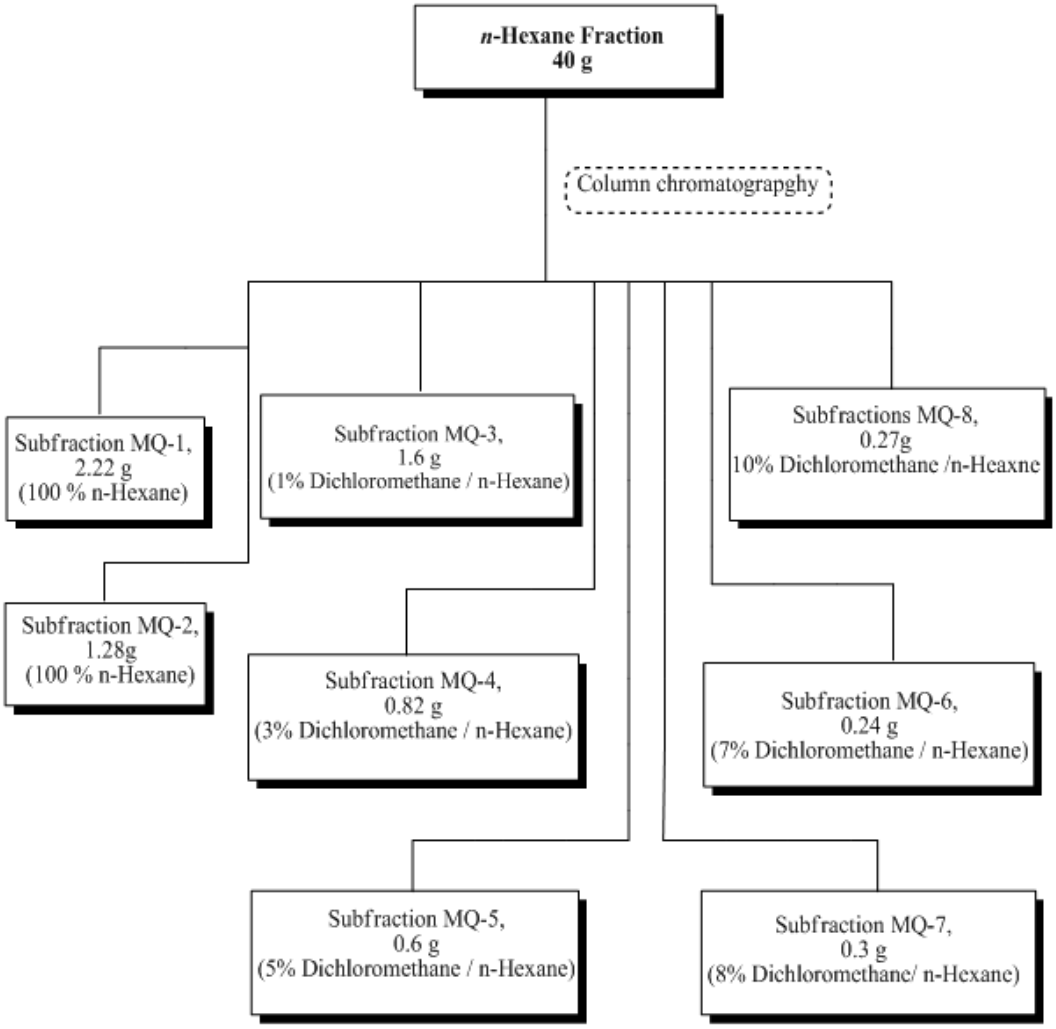

Fig. 1. Extraction scheme of oil fractions of *Impatiens bicolor* Royle.

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:

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

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

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

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 (Rashid *et al.,* 2009).

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 dishes 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 (Rashid *et al.,* 2009; Ali *et al.,* 2009).

The percentage mortality was calculated by the formula:

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

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 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.

Results and Discussion

Plants have been used since ancient times for the treatment of various diseases from flu to cancer. Approximately two-thirds to three-quarters of the world's population rely on medicinal flora as their main source of medicines (Anon., 2000). There has been an increasing interest in studying the biological properties of plant extracts as well as oils obtained from n-hexane fraction through column chromatography. The antibacterial activities of the crude oil sub-fractions obtained from the solid phase extraction of the n-hexane fraction were found inactive against above mentioned bacterial strains and showed strong correlation to our previous antimicrobial investigation on this plant (Nisar *et al*., 2010 a, b, c). For antifungal activities, MQ-6 and MQ-7 exhibited low activity against *Microsporum canis i.e.* 30 % growth inhibition, while remaining oil sub-fractions MQ-1, MQ-2, MQ-3, MQ-4 and MQ-5 were found inactive against *Microsporum canis.* Similarly there was no inhibitory effect of oil sub-fractions against remaining fungal strains (Table 1).

Table 1. Antifungal activity of crude oil fractions.

Fungal species	Standard	% Inhibition						
		MQ-1	MQ-2	MQ-3	MQ-4	MQ-5	MQ-6	MQ-7
Trichophyton longifusis	Miconazole 70	-	-	-	-	-	-	-
Candida albicans	Miconazole 110.8	-	-	-	-	-	-	-
Aspergilus flavus	Amphotericin 20	5	10	-	5	5	-	-
Microsporum canis	Miconazole 98.4	-	20	20	20	20	30	30
Fusarium solani	Miconazole 73	-	-	10	-	-	10	10
Candida glaberata	Miconazole 110.8	-	-	-	-	-	-	-

Regarding the phytotoxicity activity MQ-4 (81.25%) showed significant growth inhibition at the dose of 1000 µg/mL and indicates that growth inhibition is dose dependent. Similarly, MQ-2, MQ-5, MQ-6, and MQ-7 showed 68.75% growth inhibition at the dose of 1000 µg/mL and considered as good phytotoxic activity (Table 2). In case of insecticidal activity, MQ-2 and MQ-5 showed moderate activity (40% mortality), while MQ-1, MQ-3, MQ-4, and MQ-6 exhibited non-significant activity (20% mortality) against *Tribolium castaneum*. Oil sub-fractions MQ-2 and MQ-3 remained inactive against *Callosobruchus analis* and *Rhyzoperthz dominica*.(Table 3) MQ-4 and MQ-5 exhibited 20% mortality against *Rhyzoperthz dominica*, similarly MQ-1 and MQ-5 also showed 20% killing against *Callosobruchus analis* which was considered non significant. LD50 measurements of crude oil sub-fractions were evaluated against *Artemia salina* brine-shrimp eggs (Table 4). It was evident from the results that MQ-5, MQ-6, MQ-7 showed significant cytotoxicity at high dose 1000 µg/mL with LD_{50} value 170.748, 170.79, 161.54 µg/mL respectively. Different activities observed by different fractions may be due to presence of different chemical constituents present in all these fractions. Actually its chemical constituents, their quantity and composition that makes oil fractions responsible for varying level of responses against various microbial strains as well as against brine shrimp and *Lemna minor*.Previous studies also report various biological activities of oils obtained from n-hexane fraction of plants (Green *et al.,* 2011). 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 these activities.

Table 2. Phytotoxicity activity of crude oil fractions.

Conc. of sample (µg/ml)	Conc. of stand. drug	% Growth regulation						
		MQ-1	MQ-2	MQ-3	MQ-4	MQ-5	MQ-6	MQ-7
1000		75	68.75	75	81.25	68.75	68.75	68.75
100	0.015	56.25	50	50	37.5	56.25	43.75	50
10		37.5	18.75	25	25	37.5	6.25	18.15

Table 3. Insecticidal activity of crude oil fractions.

Name of insect	% Mortality								
	+ive control	-ive control	MQ-1	MQ-2	MQ-3	MQ-4	MQ-5	MQ-6	MQ-7
Tribolium castaneum	100	0	20	40	20	20	40	20	-
Rhyzopertha dominica	100	0	-	-	-	20	20	-	-
Callosobruchus analis	100	0	20	-	-	-	20	-	-

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

Extractions	% Deaths at doses			LD_{50}
	1000 µg/ml	100 µg/ml	10 µg/ml	
MQ-1	6	4	2	3771.67
MQ-2	5	4	2	4733.74
MQ-3	3	2	1	5067.53
MQ-4	10	6	2	5942.34
MQ-5	26	8	4	170.748
MQ-6	26	10	2	170.79
MQ-7	28	6	4	161.54
Etoposide (standard)				7.4625

Total No. of shrimps used during experiment = 30

Acknowledgement

We are grateful to Dr. Hassan Sher of Jahan Zeb Postgraduate College, Saidu Sharif, Swat for identifying the plant.

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(Received for publication 26 February 2010)