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Full Length Research Paper

In-vitro pharmacological investigations of aerial parts of *indigofera heterantha*

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***Indigofera heterantha* is locally used for various diseases. In current study we have made an effort to investigate various activities to scientifically validate some of its pharmacological activities. Among *in-vitro* assays no antibacterial activity was found while low antifungal activity was observed. Similarly low insecticidal and low to moderate phytotoxicity was identified in case of fractions of *I. heterantha.* This preliminary study is a starting point for further purification and characterization of the active principles responsible for insecticidal and phytotoxic activities**

Key words: *Indigofera heterantha,* antifungal, insecticidal, cytotoxicity, phytotoxicity.

INTRODUCTION

Genus indigofera, consist of 700 species. All the species are mostly herbs or shrubs distributed thoroughly the tropical region of the globe. In Pakistan it is represented by 24 species. The plant *indigofera heterantha* commonly known as Ghorega belongs to family leguminoseae (Fabeaceae) (Hamayun et al., 2003). It is abundantly available in the northern region of Pakistan with wide varieties of medicinal uses. In northren parts of Pakistan, it is widely used as traditional medicine for treatment against abdominal pain, spastic pain and various skin infections and other infection disease (Nasir and Ali, 1997). Up to our knowledge considerable work is available on genus *indigofera. I. dalecoides* Benth is used for the treatment of diarrhea and various pathogenic bacterial infections (Mathabe et al., 2006). *Indigofera oblogifolia possessed* antimicrobial (Dahot, 1999), hepatoprotective (Shahjahan et al., 2005) and strong lipoxygenase inhibitory activity (Sharif et al., 2005). *Indigofera pulchra* has showed snake-Venom neutralizing activity (Abubakar et al., 2006). *Indigofera tinctoria* showed antioxidant, free radical scavenging activity, and anti-dyslipidemic activity (Prakash et al., 2007). *Indigofera Mysore's* has showed anti diabetic activity. *Indigofera emarginella* showed *in-vitro* antimalarial activity against plasmodium falciparum (Chakrabarti et al., 2006).The aim of this study was to explore the antibacterial and antifungal activity of *indigofera heterantha* to determine the scientific basis for its use as folk medicine to treat microbial pathogen infected and other infectious diseases. Brine shrimps lethality bioassay was also done so as to assess its safety. Besides, we have make an attempt to investigate the importance of *I. heterantha* as an important medicinal plant for its insecticidal and phytotoxicity potential to be used as strong natural insecticide and herbicides.

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Abbreviations: NA, Nutrient agar; **UV,** ultraviolet; **DMSO**, dimethyl sulfoxide; **DMF,** dimethylformamide.

EXPERIMENTAL

Plant material

The aerial parts of *I. heterantha* were collected during the month of May 2009 from Dir Lower, northern parts of Pakistan. The plant was identified and authenticated by Mr. Samin Jan, Associate Professor,

Department of Botany, Islamia University, Peshawar, KPK province, Pakistan. A voucher specimen (No.Sj-36) was deposited in the herbarium of Botany Department, Islamia University, Peshawar, Pakistan.

Extraction

The powdered aeial parts (60 kg) were soaked (cold extraction) in water-methanol (1:19) for seven days as reported earlier (Khan et al., 2011; Nisar et al., 2008; Nisar et al., 2011). The crude water-methanol extract was filtered and concentrated at reduce pressure using rotary evaporator at 50°C, afforded a crude semi solid mass of (kg) F1. It was then dissolved in chloroform resulted in to (g) soluble fraction F2 and remaining insoluble fraction F3. The chloroform soluble fraction F2 was further fractionated with *n*-hexane and methanol afforded (g) F2-X and (g) F2-Y crude extracts respectively using Soxhlet extractor for one day. While the insoluble fraction F3 was further dissolved in ethyl acetate and concentrated afforded (1660 g) crude fraction F4. The ethyl acetate soluble fraction F4 was further partioned between diethyl ether and water gave (g) ethereal crude fraction F4-Z the water fraction (g) F4-W and insoluble residue (g) F4-I.

Fungal and bacterial strain

In this study six fungal and six bacterial strain were used as reported earlier (Jan et al., 2009; Nisar et al., 2009; Shakirullah et al., 2010).The bacterial strain used were *Escherrchia coli ATCC 25922, Bacilus subtilis ATCC 6633, shigella flexeneri* (clinical isolate), *staphylococcus aureus ATCC 25923, Pseudomonas aeruginosa ATCC 27853* and *salmonella typhi ATCC19430.* Fungal strains chosen for this study *Trichophyton longifusus* (clinical Isolate), *Candida albicans* ATCC2091, *Aspergillus flavus* ATCC 32611 *Microspoum canis* ATCC11622, *Fusarium salani* 11712 and *Candida glaberata* ATCC 90030. All these were maintain on agar slant at 4°C the slant was allowed to activate at 37° for 24 h on nutrient agar (NA), for bacteria and fungi, before any screening.

Hole diffusion method

To carry out anti microbial test hole diffusion method was adopt. Berghe and Vlietinck (1999) by using a cell suspension of 1.5 x 10^{-5}CFU/mL obtained keeping in view farmland turbidity standard No. 0.5. The suspension concentration was standardized by adjusting the optical density to 0.1 at 600 nm (SHIMADZU ultraviolet (UV) visible spectrometer. Holes with 6 mm diameter were made on MHA plate (8 mm thick) and were filled with 150 µL of methanolic extract, fraction or standard drug (s) in Dimethyl sulfoxide (DMSO). The plates were then allowed to incubate at 37°C for 24 h. The extent of anti microbial activity was obtained by measuring the diameter of zone of inhibition around the hole. The bioassay was repeated three times and then the mean diameter was determined. In this study streptomycin, miconazole and amphotericin B were used as standard antibiotics to compare extract and fraction with it.

Cytotoxicity assay

In this bioassay a shallow rectangular plastic dish (22 x 32 cm), filled with artificial sea water was taken. The sea water was prepared with commercial salt mixture mixed with double distilled water. The Brine shrimp (*Artemia salina* leach) eggs were hatched the dish. The dish was made unequally partitioned by using an artificial perforated device. About 50 mg of the eggs were sprinkled into large compartment which becomes darken. The minor compartment was exposed to the ordinary light. After two days, nauplii were collected and removed by a pipette from lighted side. A sample of the compounds to be tested was prepared by dissolving 20 mg of each compound in Dimethylformamide (DMF) (2 ml).Three different stock solution that is, 550, 50, and 5 mg/mL were transferred to 9 vials (three for every dilution were used for each test sample and LD_{50} is the average of the three values) with one vial containing DMF was reserved as a control. The solvent was allowed to evaporate keeping overnight. Two days, later when the shrimp larvae were ready, 1 mL of sea water and 10 shrimp were added to each vial (30 shrimps/ dilution) with a volume adjusted with sea water to 5 mL per vial. After 24 h, the numbers of survivors were counted using standard procedure (McLaughlin et al., 1991; Meyer et al., 1982). The data was analyzed by the use of finny computer program to determine LD_{50} values as reported earlier.

Insecticidal activity

Insecticidal activity was carried out by direct contact application of the test compounds using filter paper (Ahn et al., 1995). In this experiment 3 mL of all extract/fractions (1mg/mL) were applied to the filter papers with (90 mm diameter). After getting drying, each filter paper was allowed to place in a separate Petri dish along 10 adults of each of *Tribolium castaneum, Sitophilus oryzae, Trogoderma granarium, callosobruchus analis* and rhyzopartha dominica. Permethrin (235.71 µ/cm^3) was used as a reference insecticide. These entire insect were allowed to stand without food for 24 h after which the mortality number was counted.

Phytotoxicity assay

In this bioassay the crude extract was tested against *lemna minor* (Ahn et al., 1995).In this study three flask were inoculated with a sufficient stock solution of (20 mg mg/mL) to obtained a final concentration of 500, 50, and 5 µg/mL, respectively. Each flask was then added a 20 mL medium 10 plants each one containing rosette of three fronds. Parquet was used as a standard growth inhibitor. The whole flasks were kept in growth cabinet for incubation up to seven days. After this growth regulation in percentage was determined with reference to the negative control. IC_{50} was obtained by calculating through finny computer program.

Antibacterial activity

Crude extract and all fractions exhibited no antibacterial activity against all bacterial strains used for antibacterial activity.

Antifungal activity

N-hexane fraction (H), Ethyl acetate fraction (E), methanol fraction 1 (M1), methanol fraction (M2), aqueous fraction (A) were studied (Table 1) for their antifungal activity against the *Trichophyton longifusis, Candida albicans, Aspergilus flavus, Microsporum canis, Fusarium solani, Candida glaberata*. All fractions revealed no activity against selected fungal strains except methanol fraction 2 (M2), which showed low activity against *M. canis* as shown in Figure 1.

Insecticidal assay

Different fractions of the aerial parts of *I. heterantha* were evaluated against various Insects viz, *Tribolium castaneum,*

Table 1. Antifungal activities of indigofera *heterantha* aerial parts.

Fungal strain	% Inhibition					
	Std.	H	E	M1	M2	A
Trichophyton longifusis	70[1]					
Candida albicans	110.8[1]					
Aspergilus flavus	20[2]					
Microsporum canis	98.4[1]					15
Fusarium solani	73.10[1]					
Candida glaberata	110.8[1]					

[1]Amphotericin, [2]Miconazole, H=n-hexane fraction, E=ethyl acetate fraction, M1= Methanol fraction1, M2: Methanol fraction 2, A=aqueous fraction.

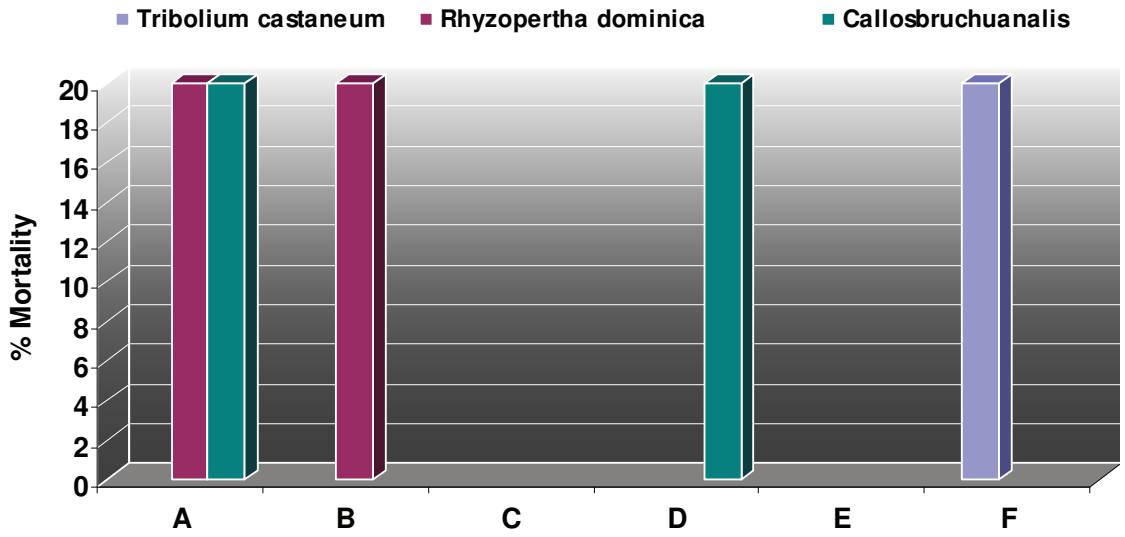

Figure 1. Insecticidal activities of the extracts of *indigofera heterantha* aerial parts.

Table 2. Brine shrimp cytotoxic activity of the extracts of *Indigofera heterantha aerial parts.*

Extract	Dose (µg/ml)	No. of shrimps	Mortality %	LD_{50}µg/ml
n-Hexane	1000, 100, 10	30(t)	43.3, 23.3, 16.6	3314.79, 42152, 474.86
Ethyl acetate	1000, 100, 10	30(t)	46.6, 26.6, 16.6	1775.28, 34458, 349.04
Methanol 1	1000, 100, 10	30(t)	46.6, 33.3, 13.3	1175.80, 32879, 290.74
Methanol 2	1000, 100, 10	30(t)	46.6, 33.3, 13.3	1175.80, 32879, 290.74
Aqueous	1000, 100, 10	30(t)	43.3, 23.3, 66.6	1653.13, 79477, 483.77

Rhyzopertha dominica and *Callosbruchuanalis.* The test sample was prepared by dissolving crude fractions in 3 ml acetone and placed in a Petri dish with the filter papers covered. After duration of 24 h, 10 test insects were added in each plate and incubated at 27°C for 24 h with 50% relative humidity in growth chamber. Results were obtained as percentage mortality, which was calculated with reference to the positive and negative controls. In this bioassay Permethrin was used as a standard drug, while Permethrin, acetone and test insects were used as positive and negative controls. All the fraction tested shows no activity the insects *castaneum, R. dominica* and *Callosbruchuanalis,* except n-hexane fraction (N1), Ethyl acetate fraction (E) which showed 20% activity against *R.* dominica. N-hexane fraction (H) and Methanol fraction 1 (M1) showed (Figure 1) 20% activity against *Callosbruchuanalis*. The residue fraction (A) showed 20% activity against *T. castaneum*.

Cytotoxicity assay

No significant (Table 2), cytotoxicity was found which indicates preliminary safety profile of the fractions of crude extract.

Cytotoxicity data supports ethnopharmacological use of this plant without causing significant cytotoxicty

Phytotoxicity assay

Results of phytotoxicity assay of various fractions of the aerial parts of *I. heterantha* are shown in the Figure. n-hexane fraction (H), Ethyl acetate fraction (E) and methanol fraction 1 (M1) showed good phytotoxicity activity at 1000 µg/ml concentration. Similarly Methanol fraction 2 (M2) also showed moderate phytotoxicity. However aqueous fraction (A) showed low activity at 1000 µg/ml concentration. All fraction showed moderate activity at 100 µg/ml while low activity at 10 µg/ml.

DISCUSSION

The current investigation strongly supported the traditional use of *I. heterantha* for various pathological conditions. Interestingly no antibacterial activity was found while low antifungal activity was found. Similarly low insecticidal and low to moderate phytotoxicity was observed in case of fractions of *I. heterantha.* These activities may be attributed to the presence of alkaloids, phenols, polyphenols, saponins, tannins, anthraquinones, steroids and especially the diterpenes, found in the crude extract and the fractions thereof (Araruna and Carlos, 2010). These phytochemical groups/families of natural products are known to display various activities. Further purification and characterization of the active principles from fractions for insecticidal and phytotoxicity studies will provide a better understanding of the pharmacological mechanisms

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