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To cite this article: Muhammad Nisar, Shafiq Ahmad Tariq, Inamullah Khan Marwat, Muhammad Raza Shah & Ihsan Ali Khan (2009) Antibacterial, antifungal, insecticidal, cytotoxicity and phytotoxicity studies on *Indigofera gerardiana*, Journal of Enzyme Inhibition and Medicinal Chemistry, 24:1, 224-229, DOI: [10.1080/14756360802051313](https://doi.org/10.1080/14756360802051313)

To link to this article: <https://doi.org/10.1080/14756360802051313>



Published online: 01 Feb 2009.



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Antibacterial, antifungal, insecticidal, cytotoxicity and phytotoxicity studies on *Indigofera gerardiana*

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(Received 3 October 2007; in final form 12 February 2008)

Abstract

The antibacterial, antifungal, acute cytotoxicity, phytotoxicity and insecticidal profile of the crude extract and various fractions of *Indigofera gerardiana* have been studied. Six bacterial and fungal strains were used, of which *Salmonella typhi* and *Microsporum canis* were the most susceptible strains with MICs 0.37 mg/mL and 0.09 mg/mL, respectively. The crude extract and the fractions showed low insecticidal activity against *Sitophilus oryzae*, *Rhyzopertha dominica* and *Callosobruchus analis* but no activity against *Tribolium castaneum*. The brine shrimp lethality assay showed absence of any measurable cytotoxicity of the crude extract and fractions, showing a good safety profile at a preliminary level. All the fractions except crude extract revealed profound and highly significant herbicidal activity against *Lemna minor* at the concentration of 1000 µg/mL. *Indigofera gerardiana* was shown by in-vitro assays to be a potential source for natural antifungal, antibacterial and herbicidal agents.

Keywords: *Indigofera gerardiana*, antibacterial, antifungal, insecticidal, herbicidal

Introduction

The genus *indigofera*, comprises of 700 species. All of these herbs or shrubs, distributed throughout the tropical regions of the globe. In Pakistan, it is represented 24 species (Nisar and Ali, 1997). The plant *Indigofera gerardiana* commonly known as Ghorega belongs to leguminosae (Fabeaceae) [1]. It is widely distributed in northern parts of Pakistan and finds various medicinal uses in the indigenous system of medicine. In northern areas of Pakistan, this plant is traditionally used for relieving abdominal and spastic pains and the infectious diseases especially the skin infections involving microorganisms. In literature considerable work has been done on various species of the genus *Indigofera*. *Indigofera daleoides* Benth is used traditionally used for the treatment of diarrhea, was found to be active against various pathogenic bacterial

strains [3]. Similarly *Indigofera oblongifolia* has showed its antimicrobial [4], hepatoprotective [5] and lipoxigenase inhibitory activity [6]. Abubakar et al. has reported the snake-venom neutralizing activity of *Indigofera pulchra* [7]. Antioxidant and free radical scavenging and anti-dyslipidemic activities of *Indigofera tinctoria* has been reported [8,9]. *Indigofera emarginella*. has shown in-vitro antimalarial activity against *Plasmodium falciparum*. Chakrabarti et al. have reported the antidiabetic activity of *Indigofera mysorens* [11].

This study was aimed at investigating the antibacterial and antifungal activities of *Indigofera gerardiana* to explore the scientific basis for its folk use in infectious diseases caused by the microbial pathogens. Brine shrimp lethality assay was also performed to assess its safety. Additionally, we have attempted

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to explore the insight of *Indigofera gerardiana* for its insecticidal and phytotoxicity potential to be developed as natural insecticides and herbicides.

Experimental

Plant material

Indigofera gerardiana was collected as a whole plant from upper Dir, NWFP (Pakistan) during the month of March–April 2005. The plant was identified by Prof Dr Jahandar Shah, plant taxonomist and Vice Chancellor, University of Malakand, Chakdara.

Extraction

The plant sample with an appropriate quantity obtained after the preliminary necessary preparations such as drying under shadow for three weeks then chopped into small pieces and pulverized into a fine powder. The powdered plant material (20 kg) was soaked in distilled methanol with occasional stirring at room temperature. After 15 days, the methanol soluble materials were filtered off. The process was repeated 3 times and the filtrate was concentrated *in vacuo* at 40°C to afford dark brown. The crude methanolic extract (40 g) was suspended in distilled water and successively extracted with hexane (11% w/w of crude extract), chloroform (31.9% w/w of crude extract), ethylacetate (21.6% w/w of crude extract), n-butanol (17.2% w/w of crude extract), and finally the aqueous (18.2% w/w of crude extract) fraction was obtained. Each organic extract was then evaporated to dryness. Stock extracts solutions were prepared at 200 mg/mL in distilled water and stored at ambient temperature for further use.

Fungal and bacterial strains

Tests were performed on six fungi and six bacteria reference strains. Bacterial strains were *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6633, *Shigella flexneri* (clinical isolate), *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853 and *Salmonella typhi* ATCC 19430. Fungal strains include *Trichophyton longifusus* (clinical isolate), *Candida albicans* ATCC 2091, *Aspergillus flavus* ATCC 32611, *Microsporum canis* ATCC 11622, *Fusarium solani* 11712 and *Candida glabrata* ATCC 90030. They were maintained on agar slant at 4°C. The strains were activated at 37°C for 24 h on nutrient agar (NA) or Sabouraud glucose agar (SGA) respectively for bacteria and fungi, prior to any screening.

Hole diffusion method. The antimicrobial tests were carried out by the hole diffusion method as described by Berghe and Vlietinck (1991) [12] using a cell suspension of about 1.5×10^{-6} CFU/mL obtained following Mac farland turbidity standard No. 0.5.

The concentration of the suspension was standardised by adjusting the optical density to 0.1 at 600 nm wavelength (SHIMADZU UV-vis spectrophotometer) [13]. Holes of 6 mm diameter were then made on the MHA plate (8 mm thick) and filled with 150 µl of methanolic extract, fractions or standard drug(s) in DMSO. The inoculated plates were incubated at 37°C for 24 h. Antimicrobial activity was evaluated by measuring the diameter of the zone of growth inhibition around the hole. The assay was repeated three times and the mean diameter was recorded. Imepenem, miconazole and amphotericin B were used as standard antibiotics for comparison with extracts and fractions.

MIC determination by macrodilution method. Extracts (10 mg/mL) were dissolved in DMSO and serially diluted with sterile water in microplates in a laminar flow cabinet [14]. The same volume of an actively growing culture of the test bacteria and fungi was added to the different wells and cultures were grown overnight in 100% relative humidity at 37°C. The next morning tetrazolium violet was added to all the wells. Growth was indicated by a violet color of the culture. The lowest concentration of the test solution that led to an inhibition of growth was taken as the MIC. The negative control DMSO had no influence on the growth at the highest concentration used. Imepenem, amphotericin B and miconazole were used as controls for comparison.

Cytotoxicity assay

Brine shrimp (*Artemia salina* leach) eggs were hatched in a shallow rectangular plastic dish (22 × 32 cm), filled with artificial seawater which was prepared with commercial salt mixture and double distilled water. An unequal partition was made in the plastic dish with the help of a perforated device. Approximately 50 mg of eggs were sprinkled into the large compartment that got darkened while the minor compartment was opened to ordinary light. Two days later, nauplii were collected by a pipette from the lighted side. A sample of the test compound was prepared by dissolving 20 mg of each compound in DMF (2 mL). Three variable portions of this stock solution i.e., 500, 50 and 5 mg/mL were transferred to 9 vials (three for each dilution were used for each test sample and LD₅₀ is the mean of three values) and one vial containing DMF (2 mL) was kept as control. The solvent was allowed to evaporate overnight. After two days, when the shrimp larvae were ready, 1 mL of seawater and 10 shrimps were added to each vial (30 shrimps/dilution) and the volume was adjusted with seawater to 5 mL per vial. After 24 h, the number of survivors was counted by following the standard procedure [15,16]. Data were analyzed by Finney computer program to determine the LD₅₀ values [17].

Insecticidal activity

The insecticidal activity of the test compounds was determined by direct contact application using filter paper [18]. 3 mL of each extract/fraction (1 mg/mL) was applied to filter papers (90 mm diameter). After drying, each filter paper was placed in the separate petri dish along with 10 adults of each *Tribolium castaneum*, *Sitophilus oryzae*, *Trogoderma granarium*, *Callosobruchus analis* and *Rhyzopartha dominica*. Permethrin (235.71 µg/cm²) was used as a reference insecticide. All these insects were kept without food for 24 h after which mortality count was done.

Phytotoxicity assay

Phytotoxic activity of the crude extract was tested against the *Lemna minor* L. [19]. Three flasks were inoculated with sufficient stock solution (20 mg/mL) to give a final concentration of 500, 50 and 5 µg/mL respectively. To each flask, 20 mL medium and 10 plants each containing a rosette of three fronds, was added. Paraquat was used as reference growth inhibitor. All flasks were incubated in the growth cabinet for seven days after which the growth regulation in percentage was calculated with reference to the negative control. IC₅₀ was calculated with a Finney computer program.

Results and discussion

Antibacterial activity

Crude extract and the fractions showed zone of inhibition in mm against various bacterial strains (Table I). Crude methanolic extract of *Indigofera gerardiana*. (SI-1), n-hexane fraction (SI-2), butanol fraction (SI-5) and the aqueous fraction (SI-6) showed good MICs and inhibited *S. aureus*, *S. flexeneri*, *P. aeruginosa*, *E. coli* and *S. typhi* (Tables I and II). The fraction SI-2 remained the most active fraction (MIC = 0.37, 0.41 and 0.62 mg/mL for *S. typhi*, *S. aureus* and *E. coli* respectively).

Antifungal activity

The *Indigofera gerardiana* crude extract (SI-1), n-hexane (SI-2), chloroform (SI-3) and aqueous fraction exhibited the most interesting inhibitory activities against *T. longifusus*, *C. albicans*, *A. flavus*, *M. canis* and *F. solani*. Crude extract and its fractions exhibited zone of inhibition (in mm) against these strains (Table III). *M. canis* and *F. solani* were the highly susceptible strains. The species *M. canis* was inhibited remarkably with MIC ranging from 90 µg/mL to 290 µg/mL, followed by the *F. solani* with MIC ranging from 150 µg/mL to 810 µg/mL, *C. albicans* (MIC ranging from 170 µg/mL

Table I. Antibacterial activity of crude extract and the fractions of *Indigofera gerardiana* represented as zones of inhibition of bacterial growth (in mm). 0.2 mL of the test samples was 10 mg/mL, while for imipenem it was 10 µg/disc.

Name of Bacteria	Zones of inhibition of bacterial growth (in mm) by various samples						
	Std. drug	SI-1	SI-2	SI-3	SI-4	SI-5	SI-6
<i>Escherchia coli</i>	24	–	10	9	–	–	11
<i>Bacillus subtilis</i>	23	–	–	–	–	–	–
<i>Shigella flexeneri</i>	28	11	–	–	12	9	–
<i>Staphylococcus aureus</i>	27	11	13	9	–	–	–
<i>Pseudomonas aeruginosa</i>	20	–	–	–	–	11	9
<i>Salmonella typhi</i>	26	8	15	–	–	7	12

Std. drug: imepenem, SI-1 = Crude extract; SI-2 = n-hexane fraction; SI-3 = Chloroform fraction; SI-4 = Ethylacetate fraction; SI-5 = Butanol fraction and SI-6 = Aqueous fraction.

Table II. Antibacterial activity of crude extract and the fractions of *Indigofera gerardiana* represented as Minimum Inhibitory Concentration (MIC, µg/mL).

Name of Bacteria	Minimum Inhibitory Concentration (MIC, mg/mL)						
	Std. drug	SI-1	SI-2	SI-3	SI-4	SI-5	SI-6
<i>Escherchia coli</i>	0.00019	50	0.62	0.83	>100	>100	0.54
<i>Bacillus subtilis</i>	0.00022	>200	50	>100	>100	>100	>200
<i>Shigella flexeneri</i>	0.00013	0.51	>100	>100	0.46	0.79	50
<i>Staphylococcus aureus</i>	0.00016	0.64	0.41	0.81	>100	>200	>200
<i>Pseudomonas aeruginosa</i>	0.00031	100	50	>100	50	0.59	0.78
<i>Salmonella typhi</i>	0.00017	0.89	0.37	50	50	0.96	0.49

Std. drug: imepenem, SI-1 = Crude extract; SI-2 = n-hexane fraction; SI-3 = Chloroform fraction; SI-4 = Ethylacetate fraction; SI-5 = Butanol fraction and SI-6 = Aqueous fraction.

Table III. Antifungal activity of crude extract and the fractions of *Indigofera gerardiana* represented as % inhibition of fungal growth. 0.2 mL of the test samples was 20 mg/mL, while for miconazole and amphotericin B it was 10 µg/disc and 5 µg/disc, respectively.

Name of Fungus	% Inhibition of Fungal Growth By Various Samples						
	Std. drug	SI-1	SI-2	SI-3	SI-4	SI-5	SI-6
<i>Trichophyton longifusus</i>	100 ¹	60	–	–	50	–	30
<i>Candida albicans</i>	100 ¹	50	–	10	80	–	10
<i>Aspergillus flavus</i>	100 ²	–	20	60	–	20	40
<i>Microsporum canis</i>	100 ¹	50	90	50	40	90	70
<i>Fusarium solani</i>	100 ¹	40	70	20	40	20	40
<i>Candida glaberata</i>	100 ¹	–	–	–	–	–	–

¹ Standard Drug = Miconazole; ² Standard Drug = Amphotericin BSI-1 = Crude extract; SI-2 = *n*-hexane fraction; SI-3 = Chloroform fraction; SI-4 = Ethylacetate fraction; SI-5 = Butanol fraction and SI-6 = Aqueous fraction.Table IV. Antifungal activity of crude extract and the fractions of *Indigofera gerardiana* represented as Minimum Inhibitory Concentration (MIC, µg/mL).

Name of Fungus	Minimum Inhibitory Concentration (MIC, µg/mL)						
	Std. drug	SI-1	SI-2	SI-3	SI-4	SI-5	SI-6
<i>Trichophyton longifusus</i>	1.4 ¹	190	–	> 10000	210	–	680
<i>Candida albicans</i>	1.8 ¹	220	50000	50	170	–	940
<i>Aspergillus flavus</i>	2.3 ²	50000	780	180	50000	830	230
<i>Microsporum canis</i>	1.6	260	90	210	290	110	160
<i>Fusarium solani</i>	1.1 ¹	270	150	810	280	790	210
<i>Candida glaberata</i>	0.5 ¹	–	–	–	–	50000	50000

¹ Standard Drug = Miconazole; ² Standard Drug = Amphotericin BSI-1 = Crude extract; SI-2 = *n*-hexane fraction; SI-3 = Chloroform fraction; SI-4 = Ethylacetate fraction; SI-5 = Butanol fraction and SI-6 = Aqueous fraction.

to 5000 µg/mL), *A. flavus* (MIC from 180 µg/mL to 5000 µg/mL) and *T. longifusus* (MIC in the range from 190 µg/mL to > 10000 µg/mL). However, all the fractions as well as crude extract showed negligible activity against *Candida glaberata* (Table IV).

Insecticidal activity

Four insects (*T. castaneum*, *S. oryzae*, *C. analis* and *R. dominica*) were used in the experiment (Figure 1) for contact-mortality model of insecticidal bioassay. Only three insects *S. oryzae*, *R. dominica* and *C. analis* (pulse beetle) were found weakly susceptible to the tested extract and fractions. Concentration used in the experiment for each extract/fractions was 1 mg/mL. Insect pests cause damage to stored grain and processed products by reducing dry weight and nutritional value [20]. Around the world, residual chemical insecticides and fumigation are currently the methods of choice for the control of stored-product insects [21]. The results reveals that crude extract, the subsequent organic (only *n*-hexane, *n*-butanol) and aqueous fractions possess low insecticidal activities.

Cytotoxicity assay

Acute cytotoxicity of the crude extract and its fractions was found to be absent at the tested concentrations

(10, 100 and 1000 µg/mL) in the brine shrimp lethality assay. This study is useful in evaluating the toxicity of the two metal complexes of diclofenac, which declared them safe for use as antifungal and antibacterial agents.

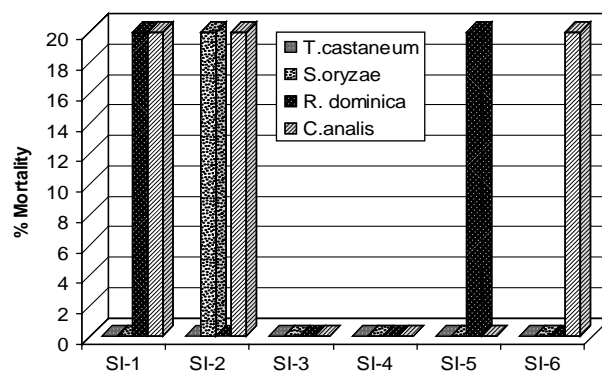


Figure 1. Insecticidal activity of the crude extract and fractions of *Indigofera gerardiana* at the concentration of 1 mg/mL. SI-1 = Crude extract; SI-2 = *n*-hexane fraction; SI-3 = Chloroform fraction; SI-4 = Ethylacetate fraction; SI-5 = Butanol fraction and SI-6 = Aqueous fraction. *T. castaneum* = *Tribolium castaneum*; *S. oryzae* = *Sitophilus oryzae*; *R. dominica* = *Rhyzopertha dominica*; *C. analis* = *Callosobruchus analis*.

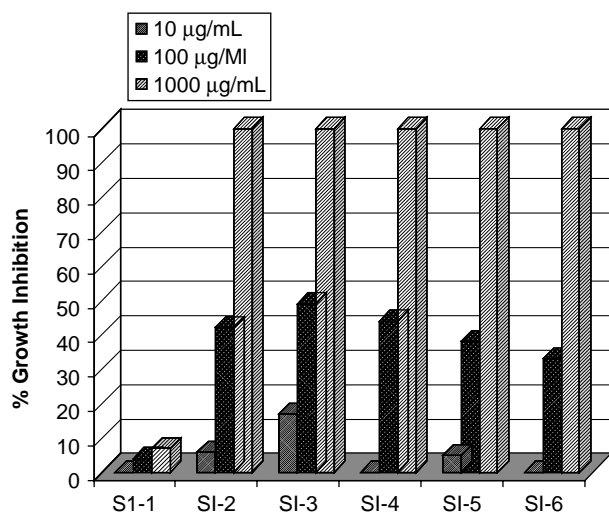


Figure 2. Phytotoxicity (herbicidal) activity of the crude extract and fractions of *Indigofera gerardiana*. SI-1 = Crude extract; SI-2 = *n*-hexane fraction; SI-3 = Chloroform fraction; SI-4 = Ethylacetate fraction; SI-5 = Butanol fraction and SI-6 = Aqueous fraction.

Phytotoxicity assay

The results of phytotoxicity assay of the test compounds are shown in Figure 2. All the fractions except crude extract have exhibited highly significant phytotoxicity in the highest tested concentration (1000 µg/mL) with 100% inhibition of growth of *L. minor*. They also exhibited moderate inhibitory activities at concentration of 100 µg/mL but showed low herbicidal activities at 10 µg/mL concentration. There is a need to discover new herbicides since the number of herbicide-resistant weeds is increasing and conventional synthetic herbicides are becoming less and less effective against the resistant weed biotypes [22] in addition to environmental and health related concerns. Therefore, new herbicides from natural sources are currently receiving more attention which could be appropriate and non-hazardous alternatives to the currently used synthetic agrochemicals as the natural products, generally, are effective, biodegradable and thus posing less threat to the environment. The results obtained from the current study indicated that *Indigofera gerardiana* might be useful as natural herbicides and could be a source of bioactive agrochemicals.

Conclusion

Increasing emergence of resistant bacterial and fungal strains requires timely and prompt measures in terms of developing new and effective antimicrobial agents [23–31]. In this study we have made an effort to investigate new potential applications of *Indigofera gerardiana*. Antifungal and antibacterial activities of *Indigofera gerardiana* identified in the current study shows their potential for the treatment of bacterial and

fungal infections in plants and animals caused by selected pathogenic strains. Insect pests are one of the main problem in storing food grains and other food materials as well as plants. Though insecticidal activities of the extract and fractions were found to be low against insects *Sitophilus oryzae*, *Rhyzopertha dominica* and *Callosobruchus analis* yet this study indicates that further screening and purification of the active compounds followed by structural modifications may lead to enhancement of their potency as well as their efficacy. However herbicidal activity was highly significant that requires the extract to be further screened and developed as a potential weedicide. The in-vitro cytotoxicity assay indicates their safety in terms of acute cytotoxicity though further studies should be conducted to assess their safety in animal models. In short, *Indigofera gerardiana* has a potential to be a source of valuable lead compounds against microbial infections as well as weeds.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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