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Phytochemical and Biological Screening of the Seeds of *Indigofera heterantha* Wall.

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Abstract: Indigofera *heterantha* seeds are used in folk medicine for the treatment of gastrointestinal disorder and abdominal pain. Phytochemical and biological Screening on the seeds extracts were carried out. Alkaloids, steroids, terpenoids, flavonoids, anthraquinones, tannins, phlobatanins, saponins, glycosides and reducing sugars were detected in the extract. The antibacterial activity tested against *Escherichia coli, Bacillus Subtilis, Shigella flexenari, Staphylococcus aureus, Pseudomonas aeruginosa and Salmonella typhi* were negative. The Brine shrimp and insecticidal activity against *Tribolium castaneum, Rhyzopertha dominica* and *Callosbruchuanalis* were found negative at the concentration of 1019.10 µg/cm^2.

Key words: Phytochemical screening · Indigofera heterantha · Antibacterial activity · Insecticidal activity and cytoxicity

INTRODUCTION

Medicinal plants are used by 80% of the world population for health needs. The relationship between man, plants and drugs derived from plants described the history of mankind. Plants are the important source of natural drugs. The plants contain compounds which have potential to be used in modern medicine for the treatment of diseases which are not curable. Pakistan is rich in indigenous floras especially the Khyber Pukhtoonkhwa Province. The country has more than 6000 species of wild plants, out of them about 1500 are considered to be medicinally important. During the past decade, traditional system of medicine has become a burning issue of global importance. Current estimates suggest that in many developing countries, a large population relies heavily on traditional practioners and medicinal plants to meet the primary health care needs, although modern medicine may be available in these countries. Herbal medicines are considered safe and have often maintained popularity for historic and cultural reasons. Currently, many people in the developed countries have begun to turn to complementary and alternative medicine (CAM) therapies, including where conventional health care is provided.

Indigofera heterantha (Fabaceae) wall commonly known an (Indigo Himalayan) is a deciduous shrub 30-60 cm tall widely distributed in the tropical region of the globe [1]. In Pakistan, it is represented by 24 species. The bark of this plant used in folk medicine to treat gastrointestinal disorder and abdominal pain in the Swat Valley of Pakistan [2]. The plant as well as the whole genus is a rich source of bioactive compounds such as monoterpenes [3], tritepenes [3], steroids [3], lignins [3], tannins [4] and alkaloids [5]. Biological activites such as lipogenase [6], dehydrogenase [7], antiulcerogenic [8], antioxidant [9] and antibacterial [10] activities have also been reported. In the light of previous reported data and biological activities the present work was initiated to search for new bioactive constituents in the seeds of this plant.

MATERIALS AND METHODS

Plant Material: The seeds of *Indigofera heterantha* were collected during the month of May 2009 from lower Dir, northern part of Pakistan. Taxonomic identification of the plant was done by Mr. Samin Jan, Associate Professor, Department of Botany, Islamia University, Peshawar,

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

Extraction: The powdered seeds (22 kg) were soaked (cold extraction) in water-methanol (1:19) for seven days. 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 (2.92kg) F1. It was then dissolved in chloroform resulted in to (41g) soluble fraction F2 and remaining insoluble fraction F3.

The chloroform soluble fraction F2 was further fractionated with *n*-hexane and methanol afforded (3g) F2-X and (36g) 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 (1660g) crude fraction F4. The ethyl acetate soluble fraction F4 was further partitioned between diethyl ether and water gave (400g) ethereal crude fraction F4-Z the water fraction (440g) F4-W and insoluble residue (360g) F4-I.

Micro-Organism Collection and Maintenance: The microorganism used in antibacterial activity are: *Escherichia coli*, *Straptodirimus*, *Shigella flexenar*i, *Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella typh*i were collected from stock culture in the PCMD, HEJ research laboratory, university of Karachi, Karachi Pakistan. The organisms were kept on agar in muller hantin agar in the refrigerator at 40°C prior to subculture.

Antimicrobial Activity of the Seed of Indigofera Heteranta Against Selected Bacterial: Antibacterial activity was carried out on the modified agar well diffusion method to study the antimicrobial activity of the seeds of the *Indigofera heterantha*. The medium used was muller-hinton agar.

The cultures used in this study were prepared in triplicates and was incubated at a temperature of 37°C for 24 to 72 hours. 0.6 ml of the prepared broth culture of the test organism was taken in a sterile Petri-dish 20 ml of the sterile molten and then MHA was added. Wells were bored in to the medium using 22mg of different fractions of the seeds of *Indigofera heterantha*. *Imipenum* was used as the standard antimicrobial agent at the concentration of 3 mg/ml. The plates were kept in sterilized inoculation chambers for 1 h to facilitate diffusion of the antimicrobial agent into the medium. The plates were then incubated at 37°C for 24 h and the diameters of the zone of inhibition of microbial growth were measured in the plate in millimeters.

Insecticidal Activity of the Seeds of *Indigofera heterantha*: Various fractions were evaluated against different Insects viz, *Tribolium castaneum, Rhyzopertha dominica* and *Callos bruchuanalis*. The test sample was prepared by dissolving 1019.10 μg/cm^2 of crude fractions in 3 ml acetone and loaded in a Petri dish 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.

Cytotoxicity Bioassay: The method used in this study is very simple to determine the cytotoxicity of various exracts. In this method, artificial "sea water" was prepared by dissolving 38 g sea salt per liter of double distilled water and filtered "Sea water" was placed in a small tank; added brine-shrimp eggs (1mg) and was kept covered by covering with aluminum foil to provide darken condition. 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. 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 LD50 values.

Phytochemical Screening: Chemical tests were carried out on the diethyl ether, ethyl acetate, chloroform and water extracts of the seeds *Indigofera heterantha* using standard procedures to identify the constituents as described by Sofowora [11]. Trease [12] and Evans and Harborne [13].

Alkaloids: About 0.2g of the extracts was warmed with 2% H_2SO_4 for two minutes. It was filtered and a few drops of Dragendroffs reagent were added. Orange red precipitate indicated the presence of alkaloids.

Tannins: A small quantity of each xtracts were mixed with water and heated on water bath and filtered. A few drops of ferric chloride was added the filtrate. A dark green solution indicates the presence of tannins.

Anthraquinones: About 0.5 g of the extracts was boiled with 10 % HCl for few minutes in water bath. It was filtered and allowed to cool. Equal volume of $CHCl_3$ was added to the filtrate. Few drops of 10% ammonia was added to the mixture and heated. Formation of rose-pink color indicates the presence of anthraquinones.

Glycosides: The extracts were hydrolyzed with HCl and neutralized with NaOH solution. A few drops of Fehling solution A and B were added. Red precipitate indicates the presence of glycosides.

Reducing Sugars: The extracts were shaken with distilled water and filtered. Then boiled with few drops of Fehling's solution A and B for few minutes. An orange red precipitate indicates the presence of reducing sugars.

Saponins: About 0.2 g of the extract was shaken with 5ml of distilled water and then heated to boil. Frothing (appearance of creamy miss of small bubbles) shows the presence of saponins.

Flavonoids: Extract of about 0.2 g was dissolved in diluted NaOH and HCl was added. A yellow solution that turns colourless indicates the presence of flavonoids.

Phlobatanins: The extract (0.5 g) was dissolved in distilled water and filtered. The filtrate was boiled with 2% HCl solution. Red precipitate shows the presence of phlobatanins.

Steroids: 2 ml of acetic anhydride was added to 0.5 g of the extract of each with 2 ml of H_2SO_4. The colour changed from violet to blue or green in some samples indicate presence of steroids.

Terpenoids (Salkowski Test): 0.2 g of the each extract was mixed with 2 ml of chloroform ($CHCl_3$) and concentrated H_2SO_4 (3ml) was carefully added form a layer. A reddish brown coloration of the interface was formed to indicate positive results for the presence of terpenoids.

RESULTS

The extractive values and the percent yield of ethyl acetate, diethyl ether, water, residue and chloroform extracts of *Indigofera heterantha* seeds are given in the Table 1 while the phytochemical screening of different extracts of seeds of *Indigofera heterantha* are listed in Table 2. The cytotoxicity, antibacterial, Insecticidal and cytotoxic activities are given in Tables 2, 3 and 4. While the phytochemical screening of different extracts of seeds of *Indigofera heterantha* are listed in Table 5.

DISCUSSION

According to the adopted protocol of extraction, the extractive values are given in table showed that the seeds

Table 1: Extractive values of the seeds of Indigofera heterantha in different solvents.

Solvents	Extracts (g) w/w	% yield
Hexane	3.0	0.10
Chloroform	41.0	1.40
Ethyl acetate	1660	56.80
Ether	400	24.00
Methanol-Water	2920	13.20
Water	440	26.50

Table 2: Antibacterial activities of seeds extracts of indigofera heterantha.

Microorganisms	Gram	EA	E	W	R	C
Escherichia coli	-	0	0	0	0	0
Bacillus subtilis	+	0	0	0	0	0
Shigella flexenari	-	0	0	0	0	0
Staphylococcus aureus	+	0	0	0	0	0
Seudomonas aeruginosa	-	0	0	0	0	0
Salmonella typhi	-	0	0	0	0	0

Key word: EA=EtOAc, E=Ether, W=Water, R=Residue, C=Chloroform.

Table 3: Insecticidal activities of the seeds extracts of Indigofera heterantha.

Insect	N0s	A	B	C	D	E
Tribolium castaneum	100	0%	0%	0%	0%	0%
Rhyzopertha dominica	100	0%	0%	0%	0%	0%
Callosbruchuanalis	100	20%	0%	0%	0%	0%

Key Words: A=EtoAc, B=Ether, C=Aqueous, D=Residue, E=Chloroform.

Table 4: Brine shrimp cytotoxic activity of the seeds extracts of Indigofera heterantha

Extract	Dose µg/ml (STD)	No of Shrimps	Mortality %	LD50µg/ml	LD50µg/ml
Chloroform	1000, 100, 10	30(t)	43.3, 23.3, 16.6	3314.79, 42152, 474.86	7.4625
Diethyl Ether	1000, 100, 10	30(t)	46.6, 26.6, 16.6	1775.28, 34458, 349.04	7.4625
Ethyl Acetate	1000, 100 ,10	30(t)	46.6, 33.3, 13.3	1175.80, 32879, 290.74	7.4625
Residue	1000, 100, 10	30(t)	43.3, 23.3, 66.6	1653.13, 79477, 483.77	7.4625

Table 5: Phytochemical Screening of the seeds extracts of Indigofera heterantha.

Chemical Components	Chloroform Extract	Ethyl acetate Extract	Diethyl Extract	Water Extract	Residue Extract
Alkaloids	+	-	-	-	-
Steroids	+	+	+	+	+
Terpenoids	+	+	+	-	+
Flavonides	+	-	-	-	-
Anthraqunones	-	-	-	-	-
Tannins	+	+	+	+	+
Phlobatanins	-	-	-	-	-
Saponins	-	-	+	+	-
Glycosides	+	-	-	-	-
Reducing sugars	-	+	+	+	-

contains little quantity of monoterpenes and fatty acids (0.1% yield) while the chloroform fraction is also low (1.4%). The ethyl acetate fraction (56%) and ether fraction (24%) were high showed the presence of polar components in the seeds extract (13.2%). The water extract was (26.5%).

Phytochemical screening of extracts revealed the presence of steroids, terpenoids, flavonides, glycosides tannins, saponins, glycosides and reducing sugars. The ethyl acetate fraction showed similar results except the absence of flavonides.

The presence of alkaloids in the seeds has not been reported before.

The medicinal properties such as insecticidal and cytotoxicity of ethyl acetate extract may be attributed to the presence of steroid, terpenoids, tannins and reducing sugars.

The crude Di-Ethyl ether extracts obtained showed the presence of steroids, terpenoids and tannins and reducing sugars. The presence of such components in this fraction may have cytotoxicity activity but no work have been reported so far. Similarly the water extracts showed the presence of Steriods, tannins, saponins and reducing sugar only. The cytotoxicity activity of this fraction may be attributed to the chemical constituents present in this fraction. No work has been reported. The residue contains steroids, terpenoids and tannins. These components in this fraction may be ascertained cytotoxicity activity but no work have been done so far.

The chloroform fraction contains Alkaloids, Steroids, Terpenoids, Flavonides, Tannins, Glycoside and Reducing sugars. This fraction shows cytotoxicity activity. The specific activity that may be attributed to the presence of alkaloids need further investigation The use of the species of this genus in gastrointestinal pain and to cure abdominal pain as well the presence of flavonoids and tannins attribute its use in folk medicine

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