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Research Article

PRELIMINARY ANTIBACTERIAL AND CYTOTOXICITY EVALUATION OF *INDIGOFERA TINCTORIA* L GRADIENT AND SINGLE EXTRACT

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ABSTRACT

This study examines antibacterial and Cytotoxicity of *Indigofera tinctoria* L. The study was carried out using petroleum ether, acetone, ethanol and water as extracting solvents in the gradation of increasing polarity. The extraction was also done using single extract in 80% ethanol. Antibacterial activity was observed at its maximum in single extract. Maximum antibacterial activity was shown against *Pseudomonas aeruginosa* and *Serratia marcescens*. MIC value of 62.5 mg/ml was observed towards *Pseudomonas aeruginosa* but MBC value was >250 mg/ml and MIC value of 125 mg/ml was observed towards *Serratia marcescens* and MBC value was >250 mg/ml. Preliminary Phytochemical evaluation revealed the presence of alkaloids, flavonoids and phenolics. Cytotoxic study conducted using water extract in *Allium cepa* root meristem. All the tested concentrations of leaf extracts caused significant inhibition in the growth of roots compared with the control. Various chromosomal abnormalities like bridge formation, stickiness of chromosomes at prophase and metaphase stages, disturbance in the orientation of the spindle resulting in wrong directions of chromosome movement, vagrant chromosomes, were the common abnormalities found in this case. There was a considerable increase in the total number of aberrant cells with increasing concentration of the leaf extract.

KEYWORDS: *Indigofera tinctoria,* antibacterial, disc diffusion, Cytotoxicity, gradient extraction

INTRODUCTION

Indigofera tinctoria L. belongs to the family Fabaceae, its synonym is *Indigofera sumatrana* Gaertn. Leaves of the plant are medicinally relevant. The plant is a shrub, flowering occurs in August to December. The plant is observed in degraded forest areas and scrub jungles, also in the plains. The plant is distributed in Paleotropics, widely cultivated in all districts of Kerala. Local names are Amari, Neelayamari. It is a subshrub, found in moist deciduous forests and also in plains, the plant is widely cultivated[1]. *Indigofera tinctoria* is used in constipation, liver disease, heart palpitation and gout[2]. The roots, stems and leaves are used for treating chronic bronchitis, asthama, ulcers, skin diseases and is useful for promoting growth of hair. The juice extracted from the leaves is useful in the treatment of hydrophobia. An extract of the plant is good for epilepsy and neuropathy. The plant possesses anti-toxic property[3]. The flavonoid fraction of *Indigofera tinctoria* had chemopreventive effect against benzeo pyrene induced lung cancer[4]. The peripheral analgesic property of *Indigofera tinctoria* was reported[5]. The methanolic extract of the whole plant possessed antihelmintic activity against *Pheretima posthuma*[6]. The ethanolic leaf extract *Indigofera tinctoria* have the ability to inhibit the growth of gram positive bacteria namely *Staphylococcus aureus*, *Bacillus pumilus* and *Streptococcus pyrogens*. Strong antioxidant activity was observed both qualitatively and quantitatively. The cytotoxic effect of *Indigofera tinctoria* leaf extract on lung cancer cell line NCI-H69 was studied. The percentage cell viability of cells was found to decrease at increasing concentration[7]. The present attempt is to compare antibacterial activity in gradient and 80% ethanolic extract and to observe cytotoxicity in roots of *Allium cepa.*

MATERIALS AND METHODS

Preparation of plant extract

Fresh specimens were collected in the month of December from Kottayam district of Kerala State, India. A voucher specimen (MB 1110) was deposited at the herbarium of St. Thomas College Palai. Leaves and roots were washed properly, air-dried and powdered well and were used for preparing extracts. About 50 gm of powdered material were successively extracted using petroleum ether, acetone, ethanol and water in the gradation of increasing polarity. The extraction was also done using single extract (80% ethanol).

Bacterial strains used

Test organisms were collected from the culture collection of the Institute of Microbial Technology (IMTECH) Chandigarh. These include *Staphylococcus aueus, Vibrio cholerae, Salmonella paratyphi, Escherichia coli, Serratia marcescens, Pseudomonas aeruginosa, Bacillus cereus, Vibrio parahaemolyticus, Streptococcus haemolyticus, and Klebsiella pneumoniae***.** The bacteria were sub cultured on nutrient agar slants, incubated at 37^{o}C for 24 hours and stored at 4^{o}C in the refrigerator to maintain the stock culture.

Antibacterial assay

The disc diffusion method was employed to evaluate the growth inhibition of bacteria by plant extracts[8]. Sterile liquid Mueller Hinton Agar media (pH 7.4 ±2) was transferred into the sterile petridish and after solidification; the bacteria (1 ml broth of approximately 10^5 CFU) were swabbed with a sterile swab under aseptic conditions. Sterile discs prepared using Whatman

No. 4 Filter Paper, of 5-mm diameter were used in the study. The original solvent in which the extract prepared was used as a control. 10 µL of the extract solution was loaded per disc. The discs (including control) were used after drying them in an incubator at 50^{o}C to remove any trace of solvent. Discs were introduced onto the surface of the medium. The plates were incubated overnight at appropriate incubation temperatures. Microbial growth was determined by measuring the diameter of zone of inhibition. Experiments were conducted in more than three replicates and average inhibitory zone diameter was determined along with standard deviation.

Preliminary Detection of Phytochemicals

The crude samples were analysed for phytochemical screening for the detection of alkaloids, phenolics, flavonoids using various spraying reagents. This was done after their separation in silica gel thin layer chromatographic plates as described by Harborne[9].

Cytotoxic analysis

Preparation of the Aqueous Extracts of *Indigofera tinctoria* leaves

The extracts used in this study are crude extracts of *Indigofera tinctoria*. Studying with crude extracts is appropriate because traditional medicinal herbs are generally used as crude extracts. The *I. tinctoria* leaves were rinsed with water, air dried and subsequently ground into fine powder using a kitchen blender. The extract was made by boiling 150 g powdered plant material mixed with 150 ml distilled water. Thereafter, the extracts were filtered through a whatman filter paper to remove particulate matter. Stock solution was diluted with distilled water to make 20%, 40%, 60%, 80% and 100% concentrations.

Cytotoxicity study in Allium cepa

Small bulbs of the common onion, *Allium cepa,* (2*n* = 16) were taken as the test material. Prior to the test, the outer scales of the bulbs and the dry bottom plate were removed without destroying the root primordia. For each extract sample, a series of six bulbs were placed in tap water for 24 h and then onion roots were treated with the *Indigofera tinctoria* leaf extracts of varying concentrations. A set of bulbs was grown using tap water, as control. The roots were allowed to grow for 48 hours. Newly formed root tips were then cut from each bulb and examined for any visible morphological abnormalities. The bulbs with satisfactory root length were used in the study. After 48 h of exposure, several root tips were removed from the bulbs, fixed Carnoy's fluid and stored. Root tips were hydrolysed in 1:1(v/v) ethanol: hydrochloric acid for 2 minutes. Prior to squash preparations roots were repeatedly rinsed in distilled water. Squash preparations were made in acetocarmine. 5 slides were prepared from each concentration and 10 fields were evaluated. The following parameters were used for determination of cytotoxicity: (i) the mitotic index (MI) was calculated as the ratio between the number of mitotic cells and the total number of cells scored and expressed as percentage and (ii) chromosome abnormalities. Photographs were taken from temporary slides as proof. The most frequent abnormalities are shown in microphotographs. After 48 h of exposure to the leaf extract samples, the root lengths and root number were measured and used as an index of general toxicity.

RESULT

Antibacterial activity and Phytochemical analysis of *Indigofera tinctoria* L. were conducted using petroleum ether, acetone, ethanol and water as extracting solvents in the gradation of increasing polarity. The extraction was also done using a single extract, 80% ethanol. Two different extractions were done in order to compare the antibacterial activity of single extract and various gradient solvent extracts. Homoeopathic extracts are usually prepared as single ethanol extract and known as Tinger[10]. Antibacterial activity was tested towards ten pathogenic bacterial strains, of these, *Staphylococcus aureus* showed resistance against *Indigofera tinctoria* in all the extracts taken. The antibacterial activity was observed at its maximum in single extract taken in 80% ethanol (Table 1). Activity was observed against 6 pathogenic bacterial strains, *Vibrio parahaemolyticus, Salmonella paratyphi, Escherichia coli, Serratia marcescens, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa,* other strains showed resistance (Table 1). Maximum activity was shown against *Pseudomonas aeruginosa* and *Serratia marcescens.*

Table 1: Antibacterial activity of *Indigofera tinctoria*, extract taken in 80% ethanol, towards various bacteria

Bacterial strains	Plant part (L=Leaf) (R=Root)	Inhibition zone diameter(in millimetre) and extract used
		80% Ethanol
Staphylococcus aureus	L	-
	R	-
Vibrio cholerae	L	-
	R	-
Salmonella paratyphi	L	16 ± 0.4
	R	14 ± 1.2
Escherichia coli	L	16 ± 0.5
	R	14 ± 1.2
Serratia marcescens	L	18 ± 0.2
	R	20 ± 0.4
Pseudomonas aeruginosa	L	30 ± 0.6
	R	24 ±0.3
Bacillus cereus	L	-
	R	-
Vibrio parahaemolyticus	L	12.2 ± 0.7
	R	11 ± 0.2
Streptococcus haemolyticus	L	-
	R	-
Klebsiella pneumoniae	L	-
	R	12.1 ±0.3

Value: Mean±Standard deviation

Table 2: Antibacterial activity of *Indigofera tinctoria* towards various bacterial strains

Bacterial strains	Plant part (L=Leaf) (R=Root)	Inhibition zone diameter(in millimetre) and extract used			
		Petroleum ether	Acetone	Ethanol	Water
Staphylococcus aureus	L	-	-	-	-
	R	-	-	-	-
Vibrio cholerae	L	13±0.43	-	13±0.5	-
	R	5±0.32	-	11±0.2	-
Salmonella paratyphi	L	-	-	-	-
	R	-	-	-	-
Escherichia coli	L	-	12±0.5	-	-
	R	-	14±0.6	-	-
Serratia marcescens	L	-	11±1.2	-	-
	R	-	12±0.7	-	-
Pseudomonas aeruginosa	L	-	9±0.7	-	-
	R	-	10±0.32	-	-
Bacillus cereus	L	-	-	19±0.6	-
	R	-	-	16±0.4	-
Vibrio parahaemolyticus	L	12±0.21	-	-	-
	R	5.2±0.37	-	-	-
Streptococcus haemolyticus	L	-	-	14±0.4	-
	R	-	-	12±0.2	-
Klebsiella pneumoniae	L	11±0.6	14±0.21	-	-
	R	6±0.40	13±0.6	-	-

Value: Mean±Standard Deviation

Table 3: Phytochemical constituents of *Indigofera tinctoria*

Name of plant	Extract used	Phytochemicals			
		Alkaloid	Phenol	Flavonoid	Sugar
Indigofera tinctoria	Petroleum ether	+	+	-	-
	Acetone	+	+	+	+
	Ethanol	+	-	+	-
	Water	-	-	+	+

Values: + present, - absent

Petroleum ether extract exhibited considerable activity against *Vibrio cholerae, Vibrio Parahaemolyticus and Klebsiella pneumoniae.* Acetone extract showed resistance against *Serratia marcescens, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Escherichia coli.* Ethanol extract was active against *Bacillus cereus, Streptococcus haemolyticus* and *Vibrio cholerae*, the other strains showed resistance. Water extract showed no antimicrobial activity (Table 2).

The antibacterial activity was observed at its maximum in single ethanol extract (80% ethanol extract) compared with other extracts obtained after gradient extraction. This difference in activity might be due to the complexity in the number of compounds dissolved in single ethanol extract. The ethanol extract was selected for detailed antibacterial evaluation tests like Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). The MIC and MBC of leaf extract and root extract was tested against *Psedomonas aeruginosa* and *Serratia marcescens* respectively. MIC value of 62.5 mg/ml was observed towards *Pseudomonas aeruginosa* but MBC value was >250 mg/ml and MIC value of 125 mg/ml was observed towards *Serratia marcescens* and MBC value was > 250 mg/ml. Preliminary phytochemical evaluation revealed the presence of alkaloids, flavonoids, phenols, and sugars (Table 3). Alkaloids were detected in petroleum ether, acetone, and ethanol extracts. Phenols were found in petroleum ether and acetone extracts. Flavonoids were detected in acetone, ethanol and water extracts and the presence of sugars were observed in acetone and water extracts.

Cytotoxicity was evaluated using various parameters like (i) the Mitotic Index (MI) was calculated as the ratio between the number of mitotic cells and the total number of cells scored and expressed as percentage and (ii) chromosome abnormalities. Morphological features such as root length and root number were also measured and used as a general index of toxicity.

Table 4: Average root length and root number of *Allium cepa* after treatment with water extract of *Indigofera tinctoria* leaves

Concentration of treatment (in %)	Average root length (in millimeters)	Average root number per bulb
Control	25	12
20	20	9
40	16.2	7
60	14.4	6
80	10	4
100	7.2	3

All the tested concentrations of *Indigofera tinctoria* leaf extracts caused significant inhibition in the growth of roots compared with the control. The inhibition of root number and root length was greater with increasing concentration (Table 4).

DISCUSSION

The presence of alkaloids, phenols, flavonoids, and sugars, might be responsible for the antibacterial activity of the active extracts. The present investigation clarified the antibacterial property of plant towards the clinically important multi-drug resistant strains, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. The infection of this pathogen is common in patients receiving treatment of severe burns or other traumatic skin damage and in people suffering from cystic fibrosis. This pathogen colonises the lungs of patients and increases the mortality rate of the individuals[11]. Another observation is that both polar and non-polar extracts showed considerable activity against various pathogenic bacterial strains. Both leaf and root extracts showed resistance towards gram positive and gram negative bacterial strains. The present results supported the various medicinal uses of the plant in treating skin diseases, respiratory and urinary infections, cough etc.

Cytogenetic analysis was performed on treated *Allium cepa* root meristem. *Indigofera tinctoria* leaf extracts provoked strong inhibition of mitotic index, with increasing concentration of leaf extract. Various chromosomal abnormalities were also observed. Bridge formation during anaphase and telophase, which include di and multi bridges, stickiness of chromosomes at prophase and metaphase stages, disturbance in the orientation of the spindle resulting in wrong directions of chromosome movement, vagrant chromosomes, were the common abnormalities met with. There was a considerable increase in the total number of aberrant cells with increasing concentration of the leaf extract.

In this study, toxic effect of *Indigofera tinctoria* leaf extract was evaluated by analyzing root growth and root morphology. The higher concentrations of *Indigofera tinctoria* leaf extract caused an inhibition of root growth. Cytotoxicity was estimated by observing cytological parameters such as the mitotic index and number of chromosome abnormalities, including chromosome bridges, stickiness, polar deviations, vagrant chromosomes etc. A positive correlation was found between inhibition of root growth and decrease of mitotic index. Mitotic index measures the proportion of cells in the M-phase of the cell cycle and its inhibition could be interpreted as cellular death or a delay in the cell proliferation kinetics[12]. Reduction in the mitotic activity could be due to inhibition of DNA synthesis or a blocking in the G2 phase of the cell cycle, preventing the cell from entering mitosis[13]. The decrease of mitotic index level shows that experimental material had mitodepressive effect resulting in the inhibition of cells access to mitosis[14]. This effect could be formed by decreased ATP level or suppression of the engine of energy production[15].

The observation of sticky metaphase reinforces the toxic effect of *indigofera tinctoria* leaf extracts. Metaphases with sticky chromosome, loses their normal appearance, and they are seen with a sticky "surface", causing chromosome agglomeration[16]. Sticky chromosome result due to the depolymerisation of DNA[17] and partial dissolution of nucleoproteins[18]. Bridges are usually formed due to the presence of dicentric chromosomes. Bridge formation can cause chromosome breakage resulting in the formation of chromosome fragments, which may in turn lead to micronuclei formation, aneuploidy etc. Vagrant chromosome formation may be attributed to the failure of spindle apparatus to organize and function normally resulting in unequal pulling of chromosomes towards the poles. Vagrant chromosomes can also lead to the formation of micronuclei[19].

The results suggest that the tested concentrations of *Indigofera tinctoria* leaf extracts have inhibitory effect on mitosis in *Allium cepa* root meristem. It may prevent DNA synthesis. The leaf-extract also shows cytotoxic activity. Crude extracts of *Indigofera tinctora* leaves were used in the study because; crude extracts are common in traditional system of medicine. Working with crude extracts means working with complex mixtures of biologically active compounds, some of these compounds can be cytotoxic. The results of this study suggest that, *Indigofera tinctoria* have beneficial effects as a medicinal herb but it can cause serious problems and damage on cells when used improperly.

CONCLUSION

The antibacterial screening, phytochemical analysis and Cytotoxic effect of *Indigofera tinctoria* showed the following result. Maximum antibacterial activity was observed in single 80% ethanol extract. *Pseudomonas aeruginosa* and *Serratia marcescens* showed the highest zone of inhibition. Water extracts did not show antimicrobial activity. MIC value of 62.5 mg/ml was observed towards *Pseudomonas aeruginosa* but MBC value was >250 mg/ml and MIC value of 125 mg/ml was observed towards *Serratia marcescens* and MBC value was >250 mg/ml. Preliminary phytochemical evaluation revealed the presence of alkaloids, flavonoids, phenols, and sugars. All the tested concentrations of *Indigofera tinctoria* leaf extracts caused significant inhibition in the growth of roots compared with the control. The inhibition of root number and root length was greater with increasing concentration. Various chromosomal abnormalities were also observed. Bridge formation during anaphase and telophase, which include di and multi bridges, stickiness of chromosomes at prophase and metaphase stages, disturbance in the orientation of the spindle resulting in wrong directions of chromosome movement, vagrant chromosomes, were the common abnormalities met with. The results of this study suggest that, although *Indigofera tinctoria* has beneficial effects as a medicinal herb, it can cause serious problems and damage on cells when used improperly.

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