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Determination of Antibacterial, Antioxidant and Cytotoxicity Effect of *Indigofera tinctoria* on Lung Cancer Cell Line NCI-h69

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Abstract: The present study has been under taken with an objective to determine the antibacterial, antioxidant and cytotoxic activity of the leaf extract *Indigofera tinctoria.* The selected medicinal plant was collected from near by region of Coimbatore. Antibacterial, activity was carried against gram positive and gram negative bacteria. Antioxidant property was determined both quantitatively and qualitatively. Determination of cytotoxic activity of leaf extract was carried out on lung cancer cell line. The compound present in the extract were identified by GC-MS analysis. The extract screened for photochemical analysis was found to contain bioactive compounds like flavonoids, saponins, tannins, steroidal terpens, phenols and anthroquinone. The leaf extract had shown the ability to inhibit the growth of gram positive bacteria namely *Bacillus pumilus*, *Staphylococcus aureus* and *Streptococcus pyogenes* and zone of inhibition of 16 and 17 mm, respectively but not shown growth of inhibition on gram negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*. Strong antioxidant activity was observed both qualitatively and quantitatively. The strong antioxidant was observed at 250 μg mL^{-1} with an IC_{50} value of 51.66 which is higher than that of standard ascorbic acid. The cytotoxic effect of leaf extract on lung cancer cell line NCI-H69 was studied. The percentage cell viability of cells was found to decrease at increasing concentration. GC-MS analysis of the leaf extract shows 6 compounds. This study suggests that ethanol extract of *Indigofera tinctoria* have profound antibacterial, antioxidant and cytotoxic effect.

Key words: *Indigofera tinctoria*, antibacterial, antioxidant, cytotoxic activity, phytochemical analysis, GC-MS

INTRODUCTION

Medicinal herbs have a long history in improving human health and curing various diseases. A wide interest has been made for researchers using herbal material in identification of the active components and verification of their efficiency. All modern clinical drugs over 50% are of natural product origin India has an extensive rich heritage of herbal medicine since from the time of Ayurveda with medicinal properties. The Indian people have a tremendous passion for medicinal plants and use them for a wide range of health related applications from a common cold to memory improvement and treatment of poisonous snake bite (Kumari *et al*., 2009).

Various medicinal properties have been attributed to natural herbs. Madicinal plant constitute the main source of new pharmaceuticals and healthcare products (Ivanova *et al*., 2005). The history of plants being used for medicinal purpose is probably old as the history of mankind. The use of medicinal in industrialized societies has been traced to the extraction and development of several drugs from these plants as well as from traditionally used folk medicine (Shrikumar and Ravi, 2007). Extraction and characterization of several active phytocompounds from these green factories have given birth to some high activity profile drugs (Mandal *et al*., 2007). The use of traditional medicine is widespread in India (Jeyachandran and Mahesh, 2007).

Indigofera tinctoria (Fabaceae) is distributed in South and South East Asia, tropical Africa and is introduced in tropical America. In India, it is found almost throughout and cultivated in many parts. The plant *Indigofera tinctoria* Linn. (Fabaceae) popularly known as true indigo is a common remedy for various ailments. It has been cultivated from worldwide centuries. The Indigo dye is shrub one to two meter height. It may be annual, biennial or perennial. Roots and leaves are used epilepsy and hydrophobia. The phytoconstituents are responsible for the pharmacological screening in the presence of phytochemical constituents. Dry powder is used in the treatment of asthma (Savithramma and Rao, 2007). Nili is a reputed drug produced from this plant which is used in Ayurveda for the promotion of hair growth (Joy *et al*., 1998). Decoction of the leaves used in bites and strings of venomous insects and reptiles, to relieve the pain and also burns and scalds (Stepp, 2004). DeFeudis *et al.* (2003) reported that whole plant of *Indigofera tinctoria* Linn.

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contains glycoside, indican, indigotine, indirubin and galactomannan composed of galactose, mannose and 2.5% of alkaloids, rotenoids and flavonoids. The plant also has pharmacological activities like hepatoprotective, antidyslipidemic, antiproliferative, antileukaemia and act as anticancer agents.

Medicinal plants represent a rich source of antimicrobial agents. In last few years, a number of studies have been conducted on medicinal herbs in different countries to prove the efficiency of antimicrobial activity. Medicinal plants possess immunomodulatory and antioxidant properties. Plants contain several phytochemicals which possess strong antioxidant activities (Senthilkumar and Venkatesalu, 2009). The antioxidant play an important role in the prevention of chronic ailments such as heart diseases, cancer, diabetes, hypertension, stroke and Alzheimer's disease by protecting the cells from damage caused by free radical the highly reactive oxygen compound (Bakasso *et al*., 2008). Natural products discovered from medicinal plants have played an important role in the treatment of cancer. Cancer is a major public health burden in both developed and developing countries. Cancer is such a segment where researchers are expecting new molecules from herbs that can provide us with tools for fighting against this dreaded disease (Shoeb, 2006). With this background, the present study was undertaken with an aim of evaluating the antibacterial, antioxidant and anticancer activity of the methanolic leaf extract of *Indigofera tinctoria.*

MATERIALS AND METHODS

Collection of medicinal plant: *Indigofera tinctoria* plant was collected from the region of Pollachi, near Coimbatore district, Tamilnadu. The study was carried on 6th October 2009 to 30th September 2010.

Preparation of leaf extract: Fresh leaf part of the plant material was washed under running tap water, air dried and then homogenized to fine powder and stored in airtight bottle. The air dried and powdered leaf of plant material (20 g) was extracted with 200 mL of methanol using Soxhlet apparatus for 24 h. The extract was filtered and evaporated until dryness. The extract was stored at 4°C until further use.

Phytochemical study of *Indigofera tinctoria*: Phytochemical screening for alkaloids, flavonoid, quinone, glycosides, steroid, terpenoids and saponins were analyzed using the powdered leaf extract of *Indigofera tinctoria*. The presence of alkaloids was tested according to Siddiqui and Ali (1997). Flavonoids was identified by using sodium hydroxide and ammonia as reported by Bot *et al.* (2007). Presence of saponin was carried out as Wall *et al.* (1952). Steroidal ring test was done according to Sofowora (1982). The presence of steroidal terpenes was performed as Yen (1971). Tannin was determined by using the method of Trease and Evans (1989). Phenol was confirmed according to Sermakkani and Thangapandian (2010). Borntrager's test for anthroquinone was determined by Trease and Evans (1989).

Thin layer chromatography: The crude leaf extract of Indigofera tinctoria was used for the separation of secondary metabolites by thin layer chromatography. The extract is dissolved in methanol and used to separate the phytochemical compounds are as follows.

Flavonoids: Flavonoids were separated as the method followed by Gracia *et al.* (1993).

Saponins: Saponins were identified using Chloroform: Glacial acetic acid: Methanol: Water (64:34:12:8) as solvent mixture (Mallikharjunah *et al*., 2007).

Tannins: Tannins were identified using n-Butanol: Acetic acid: Water (4:1:5) as solvent mixture (Mamyrbekova-Bekro *et al*., 2008).

Sterols and terpenoids: Sterols and Terpenoids were separated according to Mamyrbekova-Bekro *et al.* (2008).

Phenols: The phenols were separated as determined by Mallikharjunah *et al.* (2007).

Antibacterial activity of indigofera tinctoria

Microorganism: Five bacterial species were collected from, KMCH hospital, Coimbatore namely *Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus pumilus* (Gram positive) and *Escherichia coli*, *Pseudomonas aeruginoas* (Gram negative). The culture were maintained in nutrient agar medium.

Determination of antibacterial activity by agar well diffusion method: The assay was performed using agar well diffusion method as determined by Nair *et al.* (2005).

Antioxidant activity of crude extract of *Indigofera tinctoria*: Antioxidant activity of the crude extract of the leaf of *Indigofera tinctoria* was determined on the basis of its scavenging potential of the stable DPPH (1, 1-diphenyl 1 2-picryl hydrazyl) free radical in both qualitative and quantitative assay.

Qualitative assay: Qualitative assay were performed as the method followed by Rumzhum *et al.* (2008).

Quantitative assay: The crude leaf extract of *Indigofera tinctoria* was mixed with 95% methanol to prepare the stock solution (10 mg 10 mL^{-1}). The test samples were prepared from stock solution by diluting with methanol to attain a concentration of 1000, 500, 250, 100 and 50 μg mL^{-1}, respectively. Diluted test samples (1 mL) were added to 1ml of a 0.004% Methanol solution of DPPH was mixed and was kept at dark for 30 min for reaction to occur. The absorbance was measured at 517 nm using colorimeter. Ascorbic acid was used as a standard. Methanol (1 mL) with DPPH solution (0.004% 1 mL) was used as blank.

The optical density was recorded and (%) inhibition was calculated using the formula:

$$\% \text{ Inhibition of DPPH activity} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

Cytotoxic activity of *Indigofera tinctoria* on lung cancer cell line: The cytotoxic effect of methanolic leaf extract of *Indigofera tinctoria* against NCIH69 (small lung carcinoma) was assayed by MTT assay. Cells were plated at 37°C for 24 h on 24 well plate at density of 10^3-10^4 cells per well, with Minimal Essential Medium (MEM) supplemented with 10% Fetal Calf Serum (FCS). Penicillin (100 units mL^{-1}), Streptomycin (100 μg mL^{-1}) and Amphotericin B (5 μg mL^{-1}) were added to the medium and maintained in a humidified atmosphere (5% CO_2) at 360°C. After 24 h the cells were exposed to different concentration of the extract 10, 50, 100, 250 and 500 μg mL^{-1} were prepared by diluting from stock solution of 5 mg mL^{-1}. The cells were incubated at 360°C in humidified incubator with 5% CO_2 for a period of 72 h. Morphological changes of the cell culture were examined using an inverted microscope.

Gas Chromatography-mass Spectromety (GC-MS) analysis: The methanolic extract of the leaf sample was analyzed by GC-MS (FISONS instrument, Model: GC8000 series and MS-MD800). The GC Column dimension used was 30×0.25×0.5 mm AB-35MS fused silica capillary column. The GC condition maintained were, the injector temperature was about 250°C, Column temperature isothermal at 100°C then programmed to rise up to 250°C at 6°C min^{-1} and be held at this temperature for 10 min. The ion source temperature was 200°C and the interface temperature is 250°C. Helium gas was engaged as a carrier gas at the rate of 1 mL min^{-1}. The spectra were obtained in the EI mode with 70 eV ionization energy. The compounds were identified by comparing with the mass spectrum and matched with the inbuilt library.

RESULTS

Preliminary screening of phytochemical compounds of leaf extract: The Phytochemical screening of leaf extract of *Indigofera tinctoria* was performed which shows the presence of different types of active constituents, namely flavonoids, saponins, sterols, terpenoids, phenolic acids, quinone and tannins which are given in Table 1. The leaf extract did not show the presence of alkaloids, as they did not shown any precipitation on adding few drops of Mayer's reagent to the extract (Tyagi *et al*., 2010).

Qualitative separation of phytochemical compounds by thin layer chromatography: The Rf value was calculated by measuring the distance traveled by the solvent and the solute and corresponding value of each compounds are 0.636, 0.650.0.566, 0.533 and 0.550, respectively. The results were tabulated in the Table 2.

Antibacterial activity of leaf extract: In the present study the methanol leaf extract of *Indigofera tinctoria* had shown the ability to inhibit the growth of microorganisms such as *Bacillus pumilus, Staphylococcus aureus* and *Streptococcus pyogenes* (gram positive bacteria) and no inhibition of growth was observed against gram negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*. The highest zone of inhibition was observed in *Streptococcus pyogenes* and *Staphylococcus aureus* of 17 mm and *Bacillus pumilus* of 16 mm. This result shows

Table 1: Phytochemical analysis of secondary metabolites present in the leaf extract of *Indigofera tinctoria*

Phytochemical compounds	Results
Alkaloids	-
Flavonoids	+
Saponin	+
Tannin	+
Quinon	+
Steroid/Terpenoid	+
Phenols	+

+: Indicate the presence of phytochemical compound, -: Indicate the absence of phytochemical compound

Table 2: Compounds identified from the leaf extract of *Indigofera tinctoria* by TLC

Phytochemical compounds	Color of the spot in the TLC plate	Rf value
Flavonoid	Fluorescence	0.636
Saponin	Yellow	0.650
Tannin	Green	0.566
Sterol/Terpenoid	Greenish black	0.533
Phenol	Dark blue	0.550

Rf: Retention factor (It is the distance traveled by the sample or analyte divided by distance traveled by the solvent front in chromatography)

that the methanolic leaf extract has more potential activity against gram positive bacteria than gram negative bacteria given in Table 3.

Antioxidant activity of leaf extract: The qualitative assay of DPPH showed the color change as yellow with purple background in the TLC plate. In quantitative assay the concentration of 250 μg mL^{-1} showed the IC_{50} value of 51.66 μg mL^{-1} which indicates the presence of strong antioxidant. The result indicates that the antioxidant activity of crude leaf extract is higher than that of standard ascorbic acid. The absorbance of leaf extract and standard ascorbic acid at 517 nm were recorded and tabulated in Table 4.

Cytotoxic activity of methanolic leaf extract on lung cancer cell line NCI-H69: The percentage of cell viability was tabulated in Table 5 which shows the cytotoxity of leaf extract by gradual decrease of cell viability from 50 to 250 μg mL^{-1} concentrations. The lowest cell viability percentage of 43.28 was observed at 250 μg mL^{-1} concentration.

Gas chromatography-mass spectrometry analysis of leaf extract: The peaks were compared to the mass spectra (M800) library and about six compounds were identified namely Methoxy (CAS) anisole (rt.3.417), Octadecane (CAS) N-Octadecance (rt.11.821), Silane, Dimethoxydiphenyl (rt.16.205), Methanone, (2,amino-5-nitro phenyl), Phenyl (rt.17.937), Ethanol, 2-((4, methoxy phenyl) sulphonyl}) para methoxy phenyl 2-hydroxy ethyl (rt.18.428) and (±)7-8 benzyloxy-3,4dihydro-9-methoxy-R-1, T-3-Dimethyl-1H-naphtho (2, 3-C) pyran-5, 10-quin (rt.29.195). The area percentage, chemical formula and the molecular weight of the compounds were shown in Table 6.

Table 3: Antibacterial activity of leaf extract of Indigofera

Test organism	Results	Zone of inhibition (mm)
Gram positive bacteria		
Bacillus pumilus	+	16
Staphylococcus aureus	+	17
Streptococcus pyogenes	+	17
Gram negative bacteria		
Escherichia coli	-	-
Pseudomonas aeruginosa	-	-

Table 4: Antioxidant activity of leaf extract of Indigofera tinctoria Indigofera tinctoria

	Methanol extract of indigofera tinctoria		Standard ascorbic acid	
Concentration (μg mL^{-1})	Absorbance	%Inhibition	Absorbance	%Inhibition
50	0.33	45.00	0.19	68.00
100	0.31	48.33	0.21	65.00
250	0.29	51.66	0.22	63.33
500	0.27	55.00	0.26	56.66
1000	0.25	58.33	0.28	53.33

Table 5: Percentage cell viability of leaf extract of Indigofera tinctoria on NCI-H69

Concentration (μg mL^{-1})	Percentage of cell viability (%)
50	92.16
100	81.45
150	69.32
200	57.18
250	43.28

DISCUSSION

Preliminary phytochemical screening revealed the presence of flavonoid, phenols, tannins, saponin, steroidal terpenes and quinones. The leaf extract did not show the presence of alkaloids. These compounds may have remarkable antibacterial, antioxidant and cytotoxic effects. Most plant extracts were found to have major bioactive compounds to be present in different solvent extract. Each bioactive compound has it specific activity for example alkaloids have been used to treat diseases like malaria, pain killers and managing heart diseases (Oomah, 2003). Phytochemical study of the crude powder of 53 plants showed the presence of alkaloids, tannins, cardiac glycosides steroids and saponins. Total phenolic and flavonoid content showed that the methanol extract of *Mangifera indica* showed the highest total phenolic content while acetone extract of *Aristolohia bracteolate* showed highest flavonoid content than other plant extracts (Vaghasiya *et al*., 2011). Antibacterial activity of fresh leaf of *Indigofera tinctoria* showed that the methanolic extract of the plant inhibited the growth of

Table 6: Compounds present in the leaf extract by GC-MS

Retention time	Compound name	Formula	Molecular weight	Area (%)
3.417	Methoxy(CAS) anisole	C_7H_80	108	21.44
11.821	Octadecane (CAS) N-Octadecance	$C_{18}H_{38}$	254	0.40
16.205	Silane, Dimethoxydiphenyl	$C_{14}H_{16}O_2S_1$	244	20.09
17.937	Methanone, (2,amino-5- nitro phenyl),Phenyl	$C_{13}H_{10}O_3N_2$	242	5.01
18.428	Ethanol,2-((4, methoxy phenyl) sulphonyl}) para methoxy phenyl 2-hydroxy ethyl	$C_9H_{12}O_4S$	216	24.64
				3787.00
29.195	(+-) 7-8 benzyloxy-3, 4dihydro-9-methoxy-R-1, T-3-Dimethyl-1H-naphtho (2,3-C) pyran-5, 10-quin	$C_{23}H_{22}O_5$	3787	17.25

Staphylococcus aureus, *Streptococcus pyogenes* and *Bacillus pumilus*. The zone of inhibition measured for *Staphylococcus aureus* and *Streptococcus pyogenes* (gram positive bacteria) using well diffusion method were 17 mm and *Bacillus pumilus* of 16 mm (Table 3). This is similar to the finding of Akroum *et al.* (2009) who reported that methanol to be the best solvent for extraction of most plant active principle such as antibacterial and anticancer. The methanolic extract of 16 Algerian plants had the antimicrobial activity than the ethanolic extract. The antimicrobial activity of the methanolic extract appears to have a broad spectrum of activity on both gram positive and gram negative bacteria. From the above said reference the present study indicates that methanol is the appropriate solvent to extract bioactive compounds. According to Rahman *et al.* (2011), the organic extract of *Argemone mexicana* may act as an alternative to synthetic bactericides which might have significant applications in pharmaceutical or other industries for controlling pathogenic bacteria.

The DPPH assay is based on the measurement of the relative inhibition of the extract, test at various concentrations. Chemicals which are able to change the color of DPPH free radical from purple to yellow can be considered as antioxidants and therefore, a radical scavenger (Hinneburg *et al.*, 2006). The methanol extract of leaves, stem and seed of C.occidentalis have significant antioxidant capacities (Arya and Yadav, 2011). In the present study, the antioxidant activity of methanol leaf extract of *Indigofera tinctoria* was observed at various concentrations from 50-100 μg mL^{-1}. The value of 50% Inhibition Concentration (IC_{50}) was found to 51.66 at 250 μg mL^{-1} concentration. The result showed that the leaf extract has strong antioxidant than the standard ascorbic acid. In earlier study, the leaf extract of *Indigofera* species showed good antioxidant activity. Among the species, *Indigofera tinctoria* showed the good antioxidant activity with an IC_{50} 3.79 at 0.08 μg mL^{-1} (Bakasso *et al.*, 2008). Similarly the aqueous soluble fraction of the methanol extract of *Centella asiatica* revealed potent antioxidant activity with an IC_{50} value at 7 μg mL^{-1} On the other hand, the carbon tetrachloride, n-hexane soluble fraction showed moderate antioxidant activity with the IC_{50} value of 40 and 298 μg mL^{-1}, respectively (Obayed Ullah *et al.*, 2009). According to Anokwuru *et al.* (2011) the methanolic extract of *Hibiscus sabdariffa calyx* gave the highest extraction yield of phenolic contents 29.2 mg GAE/g, ethanol extract yield 27.6 mgGAE/g, acetone extract yield 19.3 mgGAE/g and water extract yielded 20.2 mgGAE/g. The antioxidant activity against DPPH free radicals shows 78±0.25% inhibition, for methanolic extract, ethanol extract inhibited 69±0.46%, acetone extract inhibited 37±0.015 and water extract inhibited 63±9.97%. The results of present study show high antioxidant when compared to the previous study.

The present study on cytotoxic activity of methanol leaf extract of *Indigofera tinctoria* on NCI-H69 showed the morphological changes. The percentage cell viability of each concentration was observed in the Table 5. The lowest cell viability percentage of 46% was showed at 250 μg mL^{-1}. Recent study revealed that the morphological changes were observed and clearly proved that the flavanoidal portion of *Indigofera tinctoria* showed cytotoxic on A-549 cell line (Kameswaran and Ramanibai, 2008). Rahman *et al.* (2008) reported that among the preliminary cytotoxicity screening of some medicinal plants of Bangladesh, *Indigofera tinctoria* showed highly lethal to brine shrimp nauplii. The study indicates that *Indigofera tinctoria* has good antitumor activity as supported by the earlier studies of GC/MS analysis of leaf extract of *Indigofera tinctoria* was performed. The chromatogram showing 10 peaks were compared with that of mass spectra library and among them six compounds were identified.

The leaf extract has showed antibacterial, antioxidant and cytotoxic activity but the compound responsible for the activity is not studied. Similar results were obtained during the study of the GC/MS analysis by Malek *et al.* (2009) in ethyl acetate extract of *Goniothalamus umbrosus* which showed about 42 compounds. Mangunwidjaja *et al.* (2006) reported that GC-MS analysis of Indonesian *Croton tiglium* seed showed 17 peaks with 8 identified peaks in various percentage of fatty acid. The main components were 9, 12, octadecadienoic acid (rt.73.163), Octadec-9enoic acid (rt.73.438) 9, 12-actadecadienoic acid (rt.70.721), respectively. These compounds were found to have potential antioxidant and anticancer activity. The chemical and biological composition *Fagonia indica* Brum. F the antitumour activity of the alcoholic extracts and their fractions (ethyl acetate and butanol) which contain mainly flavonoids constituents were reported by Shehab *et al.* (2011).

CONCLUSION

In the present study methanolic extract of *Indigofera tinctoria* have highest antibacterial, antioxidant and cytotoxity activity. The photochemical analysis shows the presence of flavonoids, saponins, tannins, steroidal terpens, phenols and anthroquinonesence. Antibacterial activity of the leaf extract shows the ability to inhibit the growth of gram positive bacteria. High antioxidant activity

was present. The cytotoxic effect of leaf extract on lung cancer cell line NCI-H69 shows good percentage cell viability. Thus, by increasing the concentration of the extract decreasing of the cancer cells were observed. GC-MS analysis of the leaf extract shows 6 compounds that might have various biological and pharmacological activities. The obtained results could form a good basis for further investigation in the potential discovery of new natural bioactive compounds.

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