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Phytochemical and Antimycobacterial Screening of Leaf Extracts of *Indigofera trifoliata*

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ABSTRACT

The present research work mainly focuses on phytochemical and antimycobaceterial screening of leaf extract of *Indigofera trifoliate.* The phytochemical screening is done by preparing different extracts of the following leaves of the plant with the help of different solvents namely chloroform, aqueous, methanol and ethyl acetate. Antitubercular activity was screened by *in vitro* assay method using MABA (Micro plate alamar blue assay) technique. Among the four extracts screened methanolic extract showed significant activity against *Mycobacterium tuberculosis* with minimum inhibitory concentration of 25 µg/ml. whereas aqueous extract did not show any activity at 100 µg/ml. Among the four extracts intermediate activity is shown by Chloroform and Ethyl acetate extract with MIC value of 50 µg/ml. The secondary metabolites like phenols, glycosides, tannins, reducing sugars, terpenoids, flavonoids present in the extract may be responsible for the attributed activity. The following secondary metabolites are identified by general chemical tests. From the above research work carried out, it can be implicated that the flora should be explored still further for different secondary metabolites and other potential compounds as an alternative remedy for multidrug resistant tuberculosis. The natural system of medicine is gaining passion due to their fewer side effects than that of traditional medicines. Different species of *Indigofera* should be explored for conceivable for antitubercular activity.

INTRODUCTION

Tuberculosis is a cannibalistic communicable disease which is affected by various dreadful strains of *Mycobacterium tuberculosis* in human race [(1)]. Tuberculosis accounts for about three million deaths each year throughout the world. It is an alarm as a global health hazard. The TB infected people propel the *Mycobacterium* into the air by cough, sneeze or spits. Development of multidrug resistant strains of *Mycobacterium* towards the traditional drugs it has become necessary to explore the flora and identify the lead molecules to fight against the virulent strains [(2) (3) (4)]. Medicinal plants based therapeutic systems play an important role in meeting the health care needs of around 80% of the world population [(5)]. Out of 2,48,000 species 12,000 plants are known to have medicinal properties. However, less than 10% of all plants have been investigated for phytochemical. **"Herbal drugs**" are gaining increasing importance over chemotherapeutic agents because of their histocompatability, less toxicity, fewer side effects and their cost effectiveness over allopathic formulations. 50% of all drugs in clinical use are of natural product of origin [(6)]. World's 25 best selling pharmaceutical agents, 12 are either natural products or their derivatives [(7)].

These are some of the plants from which compounds are isolated and found to have pharmacological activity [(8,9,10,11)] are well illustrated in Table I.

Table I. Showing some of the important natural plants and their pharmacological activity

S.No.	Plant	Pharmacological Activity
1	*Digitalis purpurea*	Cardiotonic activity
2	*Rauwolfia serpentina*	Antihypertensive agent
3	C*inchona sp*	Antimalarial agent
4	*Papaver somniferum*	Analgesics
5	*podophyllum sp*	Anticancer agent
6	*Atropa belladona*	Anticholinergic
7	*Catharanthus roseus*	Antileukemic agents

The drug discovery from the natural source is an interesting point in which it involves various steps, finally the lead molecule is identified and it is used as a starting material for the synthesis of many synthetic drugs of biological interest, A schematic diagram showing the process of typical medicinal plant drug discovery process is shown in detail in Fig I.

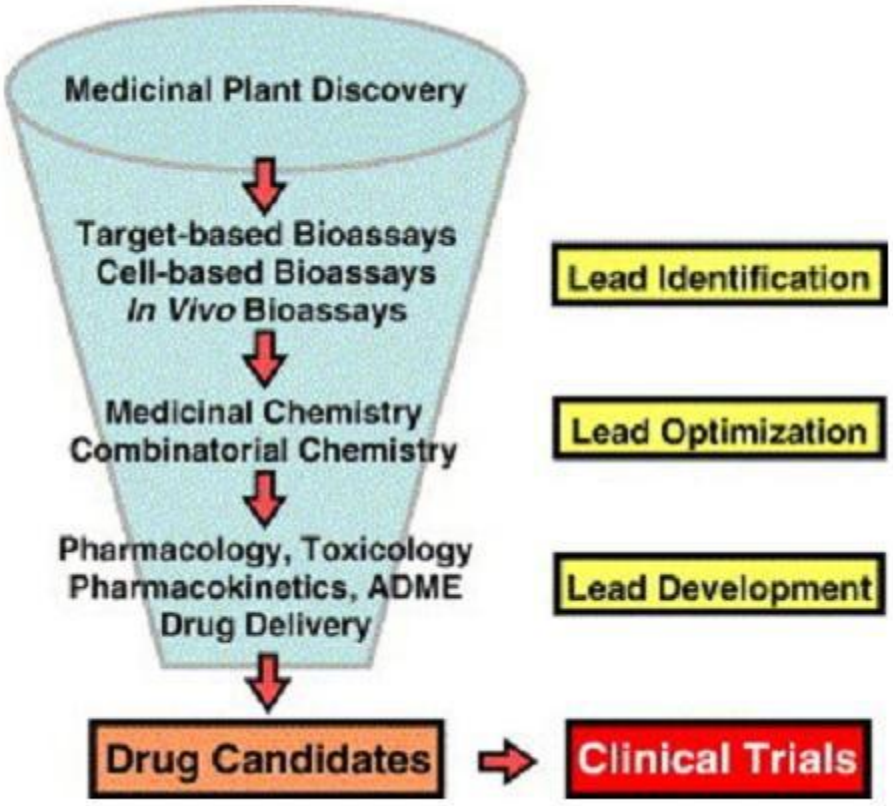

Fig I: Schematic representation of a typical medicinal plant drug discovery process.

Indigofera is a large genus of about 700 species of flowering plants belonging to the family Fabaceae [12, 13]. The image of the plant is shown in Fig 2. The species are mostly shrubs, though some are herbaceous, and a few can become small trees up to 5–6 m (16–20 ft) tall. Most are dry-season or winter deciduous [14]. It is most commonly distributed in China, Australia, Malaysia, East Asia, Indonasia internationally. In India it is mostly distributed in Andhra Pradesh, Arunachal Pradesh, Assam, Bihar, Daman and Diu, Delhi, Goa, Gujarat, Haryana, Himachal Pradesh and other parts [15]. The existing literature reviews of the plant which drives us to explore and appease anti-tuberculosis activity against H37RV of different extracts of *Indigofera trifoliate*

Fig II: Plant Image

PLAN AND OBJECTIVE

The design and objective of the present research work concentrates on the identification of the phytochemical constituents of different extracts of *Indigofera trifoliate* and to screen for antitubercular activity of the following extracts and to focus on the most potent extract among them.

MATERIALS AND METHODS

Collection and authentication of plant material

The leaves of the plant, *Indigofera trifoliate* growing in the local areas of Visakhapatnam of Andhra Pradesh state were collected during the month of September-October. It was identified and authenticated by Dr. S.B.Padal, Dept. of Botany, Andhra University and sample specimen was kept in our laboratory for future reference. Plant material was garbled at first to remove all the dust particles and unwanted material then it was washed thoroughly, initially with tap water and then with distilled water and then allowed to dry in shade. The dried plant material was pulverized to fine powder and stored at room temperature in air tight container until used further.

Preparation of Plant extracts

To 1 Kg of *Indigofera trifoliate* leaf powder, 2 litres of each solvent, viz. Chloroform, Ethyl acetate, Methanol and Distilled water was added consequently for preparing the extracts in increasing solvent polarity (Flow Chart-1). Extraction with the solvent was done for one day at 27^0C, after maceration the supernatant of each solvent was recovered by filtering through Whatmann filter paper. This process was repeated thrice and the respective solvent from the supernatant was evaporated in a Rota vapor to obtain crude extracts which are to be stored at 4^0C until used for evaluation.

The codes are as follows:

CE – Chloroform extract of *Indigofera trifoliate*,

EE – Ethyl acetate extract of *Indigofera trifoliate*,

ME – Methanol extract of *Indigofera trifoliate*,

AE – Aqueous extract of *Indigofera trifoliate.*

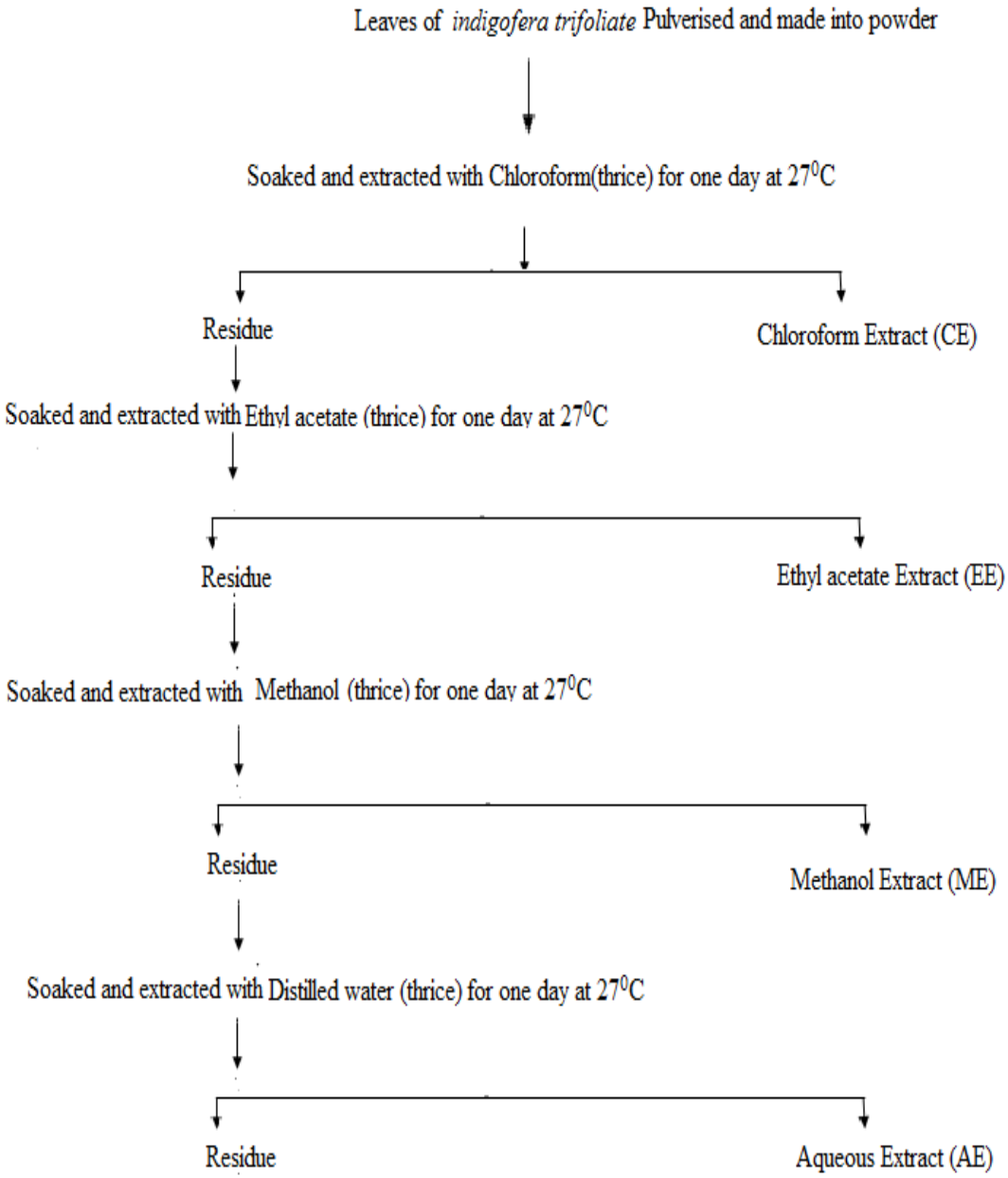

Fig III. Schematic representation of showing extraction procedure from leaves of *Indigofera trifoliate*

Table II. Tests carried out for Preliminary Phytochemical Screening of the extracts

S.No.	Name of the test	Procedure
1	Mayer's test (for Alkaloids)	2 ml of plant extract was taken and to it 2 ml of concentrated HCl and Mayer's reagent were added. Green color or white precipitate indicates presence of Alkaloids.
2	Keller-Killiani test (for Cardiac glycosides)	0.5 g of extract was added with 5 ml of water, 2 ml of glacial acetic acid containing one drop of ferric chloride solution was added. This was underlying with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides.
3	Ferric chloride test (for Flavonoids)	About 0.5 g of each extract was boiled with 5 ml of distilled water and then filtered. To 2 ml of this filtrate, a few drops of 10% ferric chloride solution were added. A green-blue or violet coloration indicated the presence of a phenolic hydroxyl group.
4	Xanthoproteic test (for Proteins)	The extract (few mg) was dissolved in 2 ml water and then 0.5 ml of conc. HNO_3 was added in it. Yellow color indicated the presence of proteins.
5	Ferric chloride reagent test (for Tannins)	The test sample of each extract was taken separately in water, warmed and filtered. To a small volume of this filtrate, a few drops of 5% w/v solution of ferric chloride prepared in 90% alcohol were added. Appearance of a dark green or deep blue color indicated the presence of tannins.
6	Salkowaski test (for Sterols and Phenols)	A few milligrams of the plant extract was dissolved in 2 ml chloroform and then 2 ml of conc. H_2SO_4 was added from the sides of the test tube. The test tube was shaken for a few minutes. Red colour development in the chloroform layer indicated the presence of sterols.
7	Foam test (for Saponins)	0.5 gram of each extract was boiled with 5 ml of distilled water and filtered. To the filtrate, about 3 ml of distilled water was

		further added and shaken vigorously for about 5 minutes. Frothing which persisted on warming was taken as an evidence for the presence of saponins.
8	Salkowaski test (for terpenoids)	To 0.5 g of each extract, 2 ml of chloroform was added, followed by a further addition of 3 ml of concentrated H_2SO_4 to form a layer. A reddish brown coloration of the interface indicated the presence of terpenoids
9	Fehling's solution test (for Reducing sugars)	About 0.5 g of each extract was dissolved in distilled water and filtered. The filtrate was heated with 5 ml of equal volumes of Fehling's solution A and B. Formation of a red precipitate of cuprous oxide was an indication of the presence of reducing sugars.
10	Anthraquinone	An aliquot of 0.5 g of the extract was boiled with 10 ml of H_2SO_4 and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipette into another test tube and 1 ml of dilute ammonia was added. The resulting solution was observed for color changes.

Table III: Results of preliminary phytochemical analysis of leaf extract of *Indigofera trifoliate.*

S.No.	Phytochemical constituent	Name of the test	Chloroform	Ethyl acetate	Methanol	Aqueous
1	Alkaloids	Mayer's test	++	+	-	+
2	Cardiac glycosides	Keller-Killiani test	+	++	++	-
3	Flavonoids	Ferric chloride test	-	++	++	+
4	Proteins	Xanthoproteic test	+	+	++	++
5	Tannins	Ferric chloride reagent test	+	++	++	+

6	Terpenoids	Salkowaski test	+	++	++	++
7	Saponins	Foam test	-	-	-	-
8	Sterols	Salkowaski test	++	-	+++	+
9	Sugars	Fehling's solution test	++	++	++	++
10	Anthraquinones		+	++	-	++

+ = slightly Presence.

++ = moderately present.

+++ = Significantly present.

Anti tubercular activity: Micro plate Alamar Blue Assay (MABA)[16]

The anti tubercular activity of crude extracts was determined using the MABA as the analytical method. Briefly, 200 µl of sterile de-ionized water was added to all outer perimeter wells of sterile 96 wells plate to minimize evaporation of medium in the test wells during incubation. The 96 wells plate received 100 µl of the Middle brook 7H9 broth and serial dilutions of compounds were made directly on plate. The final drug concentrations tested were 100 to 0.2 µg/ml. Plates were covered and sealed with Para film and incubated at 37ºC for five days. After this time, 25 µl of freshly prepared 1:1 mixture of Alamar Blue reagent and 10% tween 80 was added to the plate and incubated for 24 hrs. A blue color in the well was interpreted as no bacterial growth, and pink color was scored as growth. The MIC was defined as lowest drug concentration which prevented the color change from pink to blue. The efficacy of plant extracts was compared by repeating the procedure with the standard first line drugs.

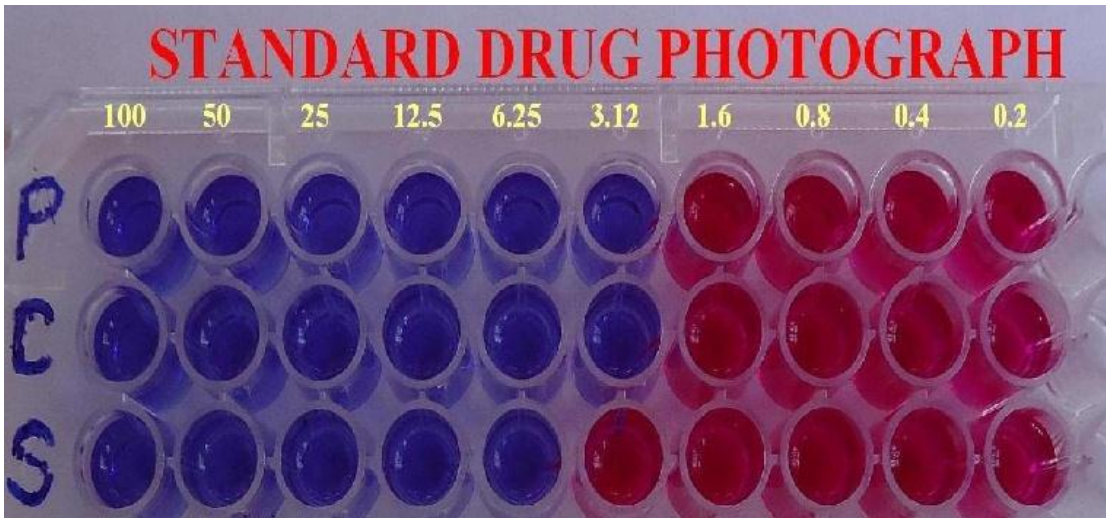

Fig IV: Standard drug Photograph showing Anti-TB activity

Strain used: *M. tuberculosis* (H37 RV strain)

Standard values for the Anti-Tb test which was performed.

Pyrazinamide- 3.125 µg/ml

Streptomycin- 6.25 µg/ml

Ciprofloxacin- 3.125 µg/ml

Fig V: Photograph showing Anti-TB activity of different extracts of *Indigofera trifoliate*

Table IV. MABA results for different extracts along with standard drugs

S.No.	Samples	100 µg/ml	50 µg /ml	25 µg /ml	12.5 µg/ ml	6.25 µg/ ml	3.12 µg/ ml	1.6 µg/ ml	0.8 µg/ ml
1	CE	S	S	R	R	R	R	R	R
2	AE	R	R	R	R	R	R	R	R
3	ME	S	S	S	R	R	R	R	R
4	EE	S	S	R	R	R	R	R	R
*	P	S	S	S	S	S	R	R	R
*	C	S	S	S	S	S	R	R	R
*	S	S	S	S	S	R	R	R	R

S: Sensitive; R: Resistance

CE: Chloroform extract of *Indigofera trifoliate.*

EE: Ethyl acetate extract of *Indigofera trifoliate.*

ME: Methanolic extract of *Indigofera trifoliate.*

AE: Aqueous extract of *Indigofera trifoliate.*

* Standard drugs

P: Pyrazinamide

C: Ciprofloxacin

S: Streptomycin.

RESULTS AND DISCUSSIONS

From the results we can implicate that three extracts namely Chloroform, Ethyl acetate and Methanol are found active against *Mycobacterium tuberculosis* (H37RV) strain. Out of the four extracts screened Methanol was found to be potent among the other three extracts possessing minimum inhibitory concentration (MIC) of 25 µg/ml. Equipotent extracts were found to be Ethyl acetate and Chloroform extracts with MIC value of 50 µg/ml. whereas the aqueous extract did not show potency at 100 µg/ml. The methanolic extract showed potency which is bordering to the potency of streptomycin which is shown in the Table IV.

From the above results one can appraise that the compounds which are having high lipid solubility may be responsible for their potency as antimycobacterial agents. The secondary

metabolites mainly the phenols, sterols, flavonoids, reducing sugars which are identified in the phytochemical investigation may be responsible for the activity against *Mycobacterium*. The potency may be attributed to the penetration coefficient of the secondary metabolites and the possible mechanism may be the cell wall disruption of the virulent strain of *Mycobacterium*.

CONCLUSION

From the present study it is clearly evident that the use of flokflore of this plant exhibit antitubercular activity thus it supports phytochemical investigation of other terrestrial sources which are responsible for pharmacological activity. Further the extract should be processed and different compounds are to be isolated and the secondary metabolite which is responsible for the activity should be found out.

FUTURE SCOPE

Traditional system of medicines causes a lot of side effects like teratogenicity, discoloration of skin, hypersensitive reactions. So it is necessary to find an alternative for lowering of the side effects. Natural products are found to be most promising and viable alternative for conventional medicines in the treatment of tuberculosis. The natural system of medicine is mostly known for their histocompatibility and Bio friendly nature.

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