

Phytochemical Screening and Antimicrobial Studies in Leaf Extracts of *Indigofera aspalathoides* (Vahl.)

Tamilarasan Tamil Kumar¹, Seeni Mohamed Salique¹, Mohamed Hussain Muhammad Ilyas¹, Nooruddin Thajuddin², Annamalai Panneerselvam³, Mohamed Khan Syed Ali Padusha⁴, Hussain Syed Jahangir^{1,*}

Tamilarasan Tamil Kumar¹,
Seeni Mohamed Salique¹,
Mohamed Hussain Muhammad Ilyas¹,
Nooruddin Thajuddin²,
Annamalai Panneerselvam³,
Mohamed Khan Syed Ali Padusha⁴,
Hussain Syed Jahangir^{1,*}

¹Post Graduate and Research Department of Botany, Jamal Mohamed College (Autonomous), Tiruchirappalli-620020, Tamil Nadu, INDIA.

²Department of Microbiology, School of Life Sciences, Bharathidasan University, Tiruchirappalli-620024, Tamil Nadu, INDIA.

³Post Graduate and Research Department of Botany and Microbiology, A. Veeriyar Vandayar Memorial Sri Pushpam College (Autonomous), Poondi-613503, Thanjavur District, Tamil Nadu, INDIA.

⁴Post Graduate and Research Department of Chemistry, Jamal Mohamed College (Autonomous), Tiruchirappalli-620020, Tamil Nadu, INDIA.

Correspondence

Dr. H. Syed Jahangir, M.Sc., M.Phil., Ph.D.

Associate Professor, Post Graduate and Research Department of Botany, Jamal Mohamed College (Autonomous), Tiruchirappalli-620020, Tamil Nadu, INDIA.

Phone no : (+91) 9443817623

E-mail: syedbot2000@gmail.com

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ABSTRACT

Introduction: In traditional Indian herbal medicine, the plant *Indigofera aspalathoides* (Vahl.) has been used to treat various human ailments. **Methods and Results:** Various phytochemical compounds (Rf value) such as Tannin (1.14), Flavonoid (1.14), Saponin (0.87), Sterol (0.88) and Phenol (0.86) were found in the leaf extract. The antimicrobial effects of the phytoconstituents were examined for three bacterial and fungal species. The highest anti-bacterial and anti-fungal activities were found in flavonoid compound, where the maximum zone of inhibition was recorded in *Staphylococcus aureus* 18mm compared to positive control chloramphenicol (24mm). 13mm observed in *A. flavus* and *A. ochraceous* where positive control streptomycin exhibits 18mm. GC-MS analysis revealed the presence of three major compounds in 8 α (2H)-Phenanthrenol, 7-ethenyldecahydro-1,1,4 α , 7-tetramethyl-, acetate, [4 α s(4 α α , 4b α , 7 α , 8 α α , 10 α α)], Benzoic acid, 4-methyl-, 2-oxo-2- phenylethyl ester and Cyclohexanol, 5-methyl-2-(1-methylethyl)-, [1S-(1 α ,2 α ,5 α)] in the crude extracts. The preparative HPLC analysis proved the presence of single peak from the crude flavonoid compounds, where the absorption maximum was between 207-290nm exhibited by the UV spectrum analysis. FTIR spectrum confirmed the presence of amide group, phenol group, carboxylic acid, alkynyl, alkene and aromatic ring. NMR studies proved the presence of phenyl group, methyl group and H₂C-CH group and their molecular weight recorded as 353 through the mass spectrum analysis. **Conclusion:** The identified compound considered as the vital compound to design the "green antimicrobial drugs".

Key words: *Indigofera aspalathoides*, Antimicrobial activities, HPLC, GC-MS, NMR, MS.

INTRODUCTION

Plants have been an important source of medicine for thousands of years. Various plant parts like leaves, flowers, fruits, stem and root have their own bioactive compounds used to treat many diseases dated back to prehistory and people of all continents have this old tradition.¹⁻² Today, scientists and the general public recognize their value as a source of new or alternative medicinal products. Recently, wide array of research highlight the potential health beneficial principles from phytal sources. Herbal medicine is based on the natural plant products and that can promote health and alleviate illness. Modern drugs or conventional medicines are often viewed as impersonal, emphasizing crisis intervention. The World Health Organization (WHO) also considers phytotherapy in its health programs and suggests basic procedures for validation of drugs from plant origin³ and estimates that up to 80% of people still rely mainly on traditional remedies such as herbs for their medicine.⁴ Approximately 25-50% of current pharmaceuticals are derived from plants, the surge to produce 'Green Medicine' from natural sources are gradually increased.

Herbal drugs play an important role in health care programmes in developing countries like India⁵ and often been referred to as the Medicinal Garden of the world. The clinical use of plants described in Indian Vedas for curing different diseases. At this stage, India has a unique position in the world where a number of recognized Traditional systems of medicine i.e. Ayurveda, Siddha, Unani, Homeopathy, Yoga and Naturopathy⁶ exists. Indigenous plants are reservoirs of various metabolites and provide unlimited source of important chemicals that have diverse biological properties.⁷ Plant and its derivatives have long history to clinical relevance as a source of potential general chemotherapeutic agents.⁸

Phytochemical tests are considered as the one of the standard pharmacognostical parameter.⁹ Phytochemicals are actually organic compounds which possess medicinal properties.¹⁰ The steadily increasing microbial resistance to existing drugs was a serious problem in antimicrobial therapy and necessitates continuing research into new classes of antimicrobials.¹¹⁻¹² One way to prevent antibiotic

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resistance of pathogenic species was to use new compounds that were not based on existing synthetic antimicrobial agents.¹³⁻¹⁴ GC-MS is performed to identify the compounds in the ethanol extracts of the plant, and their compounds¹⁵ determined by NIST data library. Many studies have been undertaken with the aim of determining the antimicrobial and phytochemical constituents of medicinal plants and using them for the treatment of both topical and systemic microbial infections as possible alternatives to chemical synthetic drugs to which many infectious microorganisms have become resistant.¹⁶⁻¹⁷

Indigofera aspalathoides (Vahl.) belongs to the family Fabaceae and it grows abundantly in Southern India. This plant has been used in traditional Indian medicine for the treatment of oedematous tumors, gastric hyperacidity, ulcers, toothache and abscesses. The stem was traditionally used for various skin disorders and cancer.¹⁸ The present study was aimed to investigate the phytochemical constituents of *I. aspalathoides* and its antimicrobial studies against the selected different pathogenic microorganisms.

MATERIALS AND METHODS

Plant collection and Crude extraction

Indigofera aspalathoides plants were collected from the fields located in Tamil University campus, Thanjavur (Tamil Nadu, India) and authenticated at Department of Botany, Rapinat Herbarium, St. Joseph's college (Autonomous), Tiruchirappalli (Tamil Nadu, India). All the solvents and chemicals used obtained from MERCK India (AR grade). The leaves were washed under running tap water and rinsed with 0.1% HgCl₂ and then with sterilized distilled water. Surface sterilized leaves were shade dried for 48 h and powdered. Plant powder (1gm) was thoroughly mixed with ethanol (10ml) and kept at 32±2°C for 24 h and filtered by using muslin cloth. The filtrate was centrifuged at 5000 rpm for 10min (REMI, Elektrotechnik Limited, VASI, INDIA) and the supernatant filtered by Whatman filter No.1 under strict aseptic conditions.

Phytochemical screening

The ethanolic leaf extracts of *I. aspalathoides* were qualitatively tested for the presence of phytochemical constituents according to Vogel's technique.¹⁹ The secondary metabolites such as Saponin and sterols,²⁰ phenols,²¹ flavonoids²² and tannins²³ were quantitatively analysed by using thin layer chromatographic methods.

Test Microorganisms

Two Gram negative (*Escherichia coli* and *Salmonella typhi*), one Gram positive bacteria (*Staphylococcus aureus*) and three fungal strains (*Aspergillus niger*, *Aspergillus flavus* and *Aspergillus ochraceous*) procured from Microbial Germ Plasm Culture Collection Unit (Sri Gowri Biotech Research Academy, Thanjavur) and maintained in the laboratory by periodic subculture. The bacteria were maintained on Nutrient agar medium and fungi were maintained on Potato Dextrose agar (PDA) medium. A loopful of bacterial (12 hrs grown) and fungal (36 h grown) culture were sub cultured on Muller Hinton Agar (MHA) and PDA respectively. Colonies of the pure organism were cultured in 10ml broth medium and incubated at 37°C overnight. Cultures were adjusted to suspension density equal to 0.5 Mc Farland turbidity standards²⁴ which has an approximate cell density of 1.5×10⁶ Cfu/ml.²⁵

Determination of Antimicrobial Activity

Sensitivity test for bacterial and fungal strains to ethanolic leaves extract of *I. aspalathoides* was measured by means of zone of inhibition using well diffusion assay. MHA and PDA medium were used for bacterial and fungal species growth. A sterile swab was dipped in broth suspension of 0.5ml MacFarland as standard. The entire surface of the medium was spread uniformly by their respective samples and the wells were made by

sterile well borer and filled with 200 µl of secondary metabolites isolated through TLC method. The inoculated plates were incubated for 24 h at 37°C for bacteria and 72 hrs at 28°C for fungus to find their antibacterial and antifungal efficacy.²⁶ Pure solvent used as negative control and 200µl of a broad spectrum antibiotic chloramphenicol (for bacteria) and streptomycin (for fungi) as standard positive control. Each test was performed in triplicates.

Characterization of the compound

The potent secondary metabolite which showed highest antimicrobial effect against bacterial and fungal strains was further subjected for characterization studies.

Gas Chromatograph-Mass Spectrophotometer (GC-MS) and High Performance Liquid Chromatography (HPLC)

The GC-MS analysis of ethanolic leaf extracts were carried out using a GC Clarus 500 Perkin Elmer (Shelton, USA), equipped and coupled to a mass detector Turbo mass gold – Perkin Elmer Turbomass 5.1 spectrometer with an Elite-1 (100% Dimethyl poly siloxane). Using computer searches on a NIST Ver.2.1 MS data library and comparing the spectrum obtained through GC-MS compounds present were identified.²⁷

The crude compounds isolated from extract were first subjected for analytical HPLC analysis. The compound which showed highest peak was isolated through preparative HPLC analysis with LC-20AT prominence liquid chromatograph pump and SPD-20A prominence UV-Vis detector and Rheodyne type injector (Shimadzu Corporation, Japan).²⁸

UV-Visible spectrophotometer and Fourier Transform Infrared Spectroscopy (FTIR)

The spectrum of a single eluted compound with highest peak value from preparative HPLC was further recorded between 190 to 600nm on a UV-Visible spectrophotometer coupled with DAD detector (Agilent Technologies, Cork, Ireland). The pure compound was grinded and dispersed with 95mg of micronized dried IR grade Potassium bromide (KBr). Pellets of samples were prepared using hydraulic press as a salt disc into 1mm pellet and scanned over a wave number range of 4000cm⁻¹ to 400cm⁻¹ using IR Affinity-1 Perkin Elmer 2000 model FTIR spectrometer (Thermo Fisher Scientific Inc., MA, and USA). The wave numbers of different components present in bioactive sample were analyzed using IR solution software and the possible stretches of functional groups were identified.²⁸

Nuclear Magnetic Resonance (NMR) and Mass Spectroscopy (MS) study

¹H NMR spectra of the isolated compound was recorded on a NMR spectrometer (Make: Bruker Biospin, Switzerland, Model 300 MHz AVANCE II) equipped with a 5 mm BBO probe. The experiments were recorded at 298.15 K using the standard pulse sequence library of Top Spin 1.3 followed by processing of the data by using Top Spin 3.2 software. The result graph was compared with the reference chart and possible functional groups present were determined.²⁹

The mass spectrum of the bioactive compounds was recorded on JEOL GC MATE II (USA). The instrument was fitted with HP 5MS capillary column of medium polarity. Helium was used as a carrier gas at a flow rate of 1 mL/min. The sample (1 µL) was injected at the flow rate of 4 mL/min and the purge flow rate was 3 mL/min. The injector and interface temperatures were maintained at 220°C.

RESULTS

Phytochemical screening

Ethanolic leaves extract of *I. aspalathoides* were examined for the presence of phytoconstituents. The results (Table 1) revealed that flavonoids, tannin, phenol, saponin and sterol presence in the ethanolic leaves

Table 1: Qualitative Phytochemical Screening and analysis of *Indigofera aspalathoides* by TLC.

S. No	Compound Tested	Test Applied /Reagent Used	Observation	Result	Rf Value
01.	Tannin	Spot test	Dark green color spot	+	1.14
02.	Flavonoid	Spot test	Yellow color spot	+	1.14
03.	Saponin	Iodine vapours	Yellow color spot	+	0.87
04.	Sterol	Folin-ciocalteu's reagent	Black color spot	+	0.88
05.	Phenol	Folin-ciocalteu's reagent	Blue color spot	+	0.86

+: Presence

Table 2: Antibacterial activity of phytochemicals in *I. aspalathoides* (Vahl.)

S. No.	Bacterial Cultures	Phytochemicals Test (Zone of inhibition mm)				
		Flavonoid	Tannin	Sterol	Phenol	Saponin
1.	<i>Escherichia coli</i>	15	15	10	10	7
2.	<i>Salmonella typhi</i>	16	18	10	5	10
3.	<i>Staphylococcus aureus</i>	18	15	10	-	5

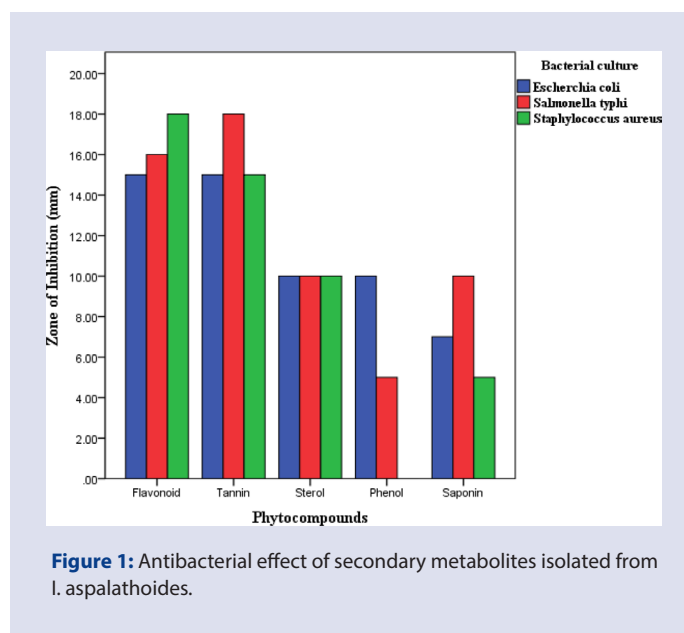
Positive control: Chloramphenicol for *E. coli* - 22mm; *S. typhi* - 20mm; *Staph. aureus* - 24mm.

Negative control: Ethanol - 0mm

extract of *I. aspalathoides*. The identified phytoconstituents were spot identified from TLC and observed under day light condition and they appeared to be in different colors with different Rf values ranging from 0.86 to 1.14.

Antimicrobial assay

The phytoconstituents in the ethanolic leaves extract of *I. aspalathoides* exhibited varied activity against the tested bacteria and fungi. 200µl of Chloramphenicol was used as positive control whereas the inhibition zones were found as 22 mm, 20 mm and 24 mm for *E. coli*, *S. typhi* and *Staph. aureus* respectively. Among the phytochemical compounds, the highest bacterial inhibition zone was observed against *Staph. aureus* (18mm), followed by *S. typhi* (16 mm), and least inhibition zone was noted in *E. coli* (15mm) on flavonoid treatment. Tannin results showed with highest inhibition observed against *S. typhi* (18 mm), followed by *Staph. aureus* and *E. coli* (15 mm). 10mm of zone of inhibition was observed in *S. typhi*, *Staph. aureus* and *E. coli* on treated with sterol, phenol results showed with highest inhibition against *E. coli* (10mm), followed by *S. typhi* (5 mm) and no results was found in *Staph. aureus*. Saponin result shows highest zone of inhibition against *S. typhi* (10 mm), followed by *E. coli* (7 mm) and *Staph. aureus* (5 mm) (Table 2 and Figure 1). Streptomycin (200 µl) was used as positive control which showed 18mm inhibition zone against the three fungal species. Flavonoids from *I. aspalathoides* possess antifungal activity with the highest diameter zone of inhibition against *Aspergillus flavus* and *Aspergillus ochraceous* (13mm), and least inhibition zone was observed in *Aspergillus niger*

**Figure 1: Antibacterial effect of secondary metabolites isolated from *I. aspalathoides*.****Table 3: Antifungal activity of phytochemicals in *I. aspalathoides* (Vahl.)**

S.No.	Fungal Cultures	Phytochemicals Test (Zone of inhibition mm)				
		Flavonoid	Tannin	Sterol	Phenol	Saponin
1.	<i>Aspergillus niger</i>	12	10	9	13	12
2.	<i>Aspergillus flavus</i>	13	12	13	12	13
3.	<i>Aspergillus ochraceous</i>	13	12	7	10	12

Positive control: Streptomycin - 18mm; Negative control: Ethanol - 0mm

(12mm). Tannin results showed highest inhibition against *A. flavus* and *A. ochraceous* (12mm) and least inhibition zone in *A. niger* (10mm). Sterol results showed with highest inhibition in *A. flavus* (13mm), followed by *A. niger* (9mm) and least inhibition zone in *A. ochraceous* (7mm). Phenol results showed highest inhibition zone in *A. niger* (13mm), followed by *A. flavus* (12mm) and least inhibition zone in *A. ochraceous* (10mm). Saponin results showed highest inhibition against *A. flavus* (13mm), followed by least inhibition zone in *A. ochraceous* and *A. niger* (12mm) (Table 3 and Figure 2). Flavonoid was found to be more potential against bacterial and fungal pathogens. So, therefore flavonoids from *I. aspalathoides* were further elucidated for characterization studies.

GC-MS

The crude ethanolic leaves extract of *I. aspalathoides* was analyzed by GC-MS. A total of 11 different organic compounds were identified and their constituents (Table 4 and Figure 3) were as follows: 3-Methyl-2-(2-oxopropyl)furan (2.50%), Cyclobutanone, 2,2,3-trimethyl-(3.33%), 2,4,6,8-Tetramethyl-1-undecene (0.83%), α -Cubebene (2.92%), (Z,Z)- α -Farnesene (5.00%), 2-Pyridinemethanamine-N-methyl- (0.42%), (+)-Epibicyclosquiphellandrene (5.42%), Hydrazine, 1,2-dimethyl-(0.42%), Cyclohexanol, 5-methyl-2-(1-methylethyl)-, [1S-(1 α ,2 α ,5 α)]- (6.67%), 8 α (2H)-Phenanthrenol, 7-ethenyldodecahydro-1,1,4 α , 7-tetramethyl-, acetate, [4 α s(4 α , 4 β , 7 α , 8 α , 10 α)] (55.42%), Benzoic acid, 4-methyl-, 2-oxo-2- phenylethyl ester (17.08%) respectively.

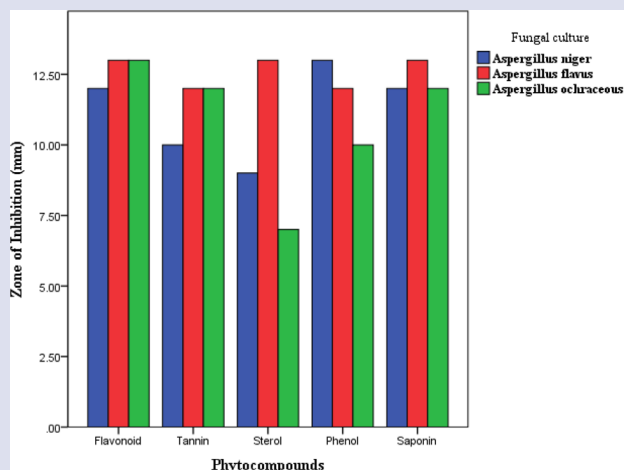


Figure 2: Antifungal effect of secondary metabolites isolated from *I. aspalathoides*.

HPLC

Analytical HPLC chromatogram revealed the presence of five different peaks of the crude flavonoids (Figure 4). The highest peak (62.51%) was chosen for the present study. Through preparative HPLC analysis, a single compound was eluted at the retention time of 5.002 with the peak area and height of 100% is designated as 'A' compound (Figure 5). The eluted compound was further subjected to partial structural elucidation through UV, FTIR, NMR and MS studies.

UV

The UV-visible spectroscopic profile of eluted compound (Figure 6) of the plant extract was studied at a wavelength range of 190 to 1100 nm. Three major bands were recorded at wavelength range of 207 nm, 223 nm and 271nm with absorbance value of 2.1, 2.4 and 1.7 respectively.

FTIR

The FTIR spectrum was performed to identify the presence of functional groups in the purified compound 'A' of *I. aspalathoides* based on the peak values in the infrared region. The major bands were observed at 3944.39, 3832.76, 3415.38, 2963.75, 2649.50, 2544.83, 2388.65, 2060.32, 1640.07, 1372.78, 1033.79 and 666.69cm⁻¹. The peaks at 3944.39 and 3832.76cm⁻¹ were attributed to amide N-H stretch. 3415.38cm⁻¹ corresponds to phenol O-H stretch. The peak at 2963.75, 2649.50, 2544.83cm⁻¹ represents carboxylic acid O-H stretch. 2388.65, 2060.32cm⁻¹ attributes to alkynyl C≡C. 1640.07cm⁻¹ corresponds to C=C stretch and 1372cm⁻¹ corresponds to C-H bending. 1033.79cm⁻¹ attributed to C-F stretch and 666.69cm⁻¹ represents C-H bending (Figure 7).

NMR

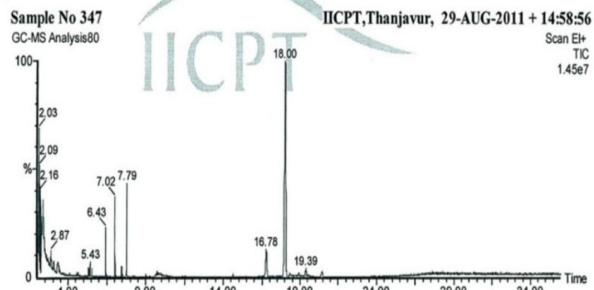
The eluted 'A' compound through NMR analysis revealed the presence of multiplets at 57.6-7.8 ppm that indicated the presence of phenyl group. Multiplets found in the region of 1.2-1.8 ppm showed the presence of methylene group. Presence of triplet and a doublet at 3.9-4.23ppm confirmed the presence of H₂C-CH group (Figure 8).

* Components identified in the Methanol extract of *Indigofera aspalathoides* Sample S.No – 347- [GC MS study]

No.	RT	Name of the compound	Molecular	MW	Peak Area %
1.	2.87	3-Methyl-2-(2-oxopropyl)furan	C ₈ H ₁₀ O ₂	138	2.50
2.	3.30	Cyclobutanone, 2,2,3-trimethyl-	C ₇ H ₁₂ O	112	3.33
3.	5.43	2,4,6,8-Tetramethyl-1-undecene	C ₁₅ H ₃₀	210	0.83
4.	6.43	δ-Cubebene	C ₁₅ H ₂₄	204	2.92
5.	7.02	(Z,Z)-δ-Farnesene	C ₁₅ H ₂₄	204	5.00
6.	7.46	2-Pyridinemethanamine, N-methyl-	C ₇ H ₁₀ N ₂	122	0.42
7.	7.79	(+)-Epi-bicyclosesquiphellandrene	C ₁₅ H ₂₄	204	5.42
8.	9.75	Hydrazine, 1,2-dimethyl-	C ₂ H ₆ N ₂	60	0.42
9.	16.78	Cyclohexanol, 5-methyl-2-(1-methylethyl)-, [1S-(1α,2β,5α)]-	C ₁₀ H ₂₀ O	156	6.67
10.	18.00	8α(2H)-Phenanthrenol, 7-ethenyldodecahydro-1,1,4a,7-tetramethyl-, acetate, [4α-(4α,4b,7b,8a,10a)]-	C ₂₂ H ₃₆ O ₂	332	55.42
11.	19.39	Benzoic acid, 4-methyl-, 2-oxo-2-phenylethyl ester	C ₁₆ H ₁₄ O ₃	254	17.08

Note: *Parameters tested are not covered under the scope of NABL accreditation

GC-MS Chromatogram of Methanol extract of *Indigofera aspalathoides* sample



Thanjavur
Date: 02.09.11
IICPT, Thanjavur, 29-AUG-2011 + 14:58:56
Scan E1+
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Table 4 and Figure 3: GC-MS analysis of the ethanolic leaves extract of *I. aspalathoides*.

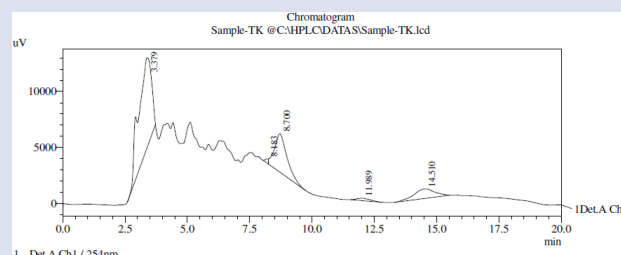


Figure 4: Analytical HPLC analysis of *I. aspalathoides*.

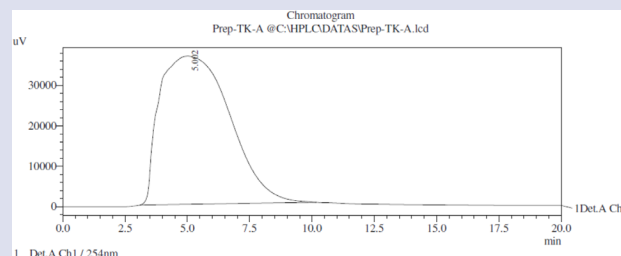


Figure 5: Preparative HPLC analysis of *I. aspalathoides*

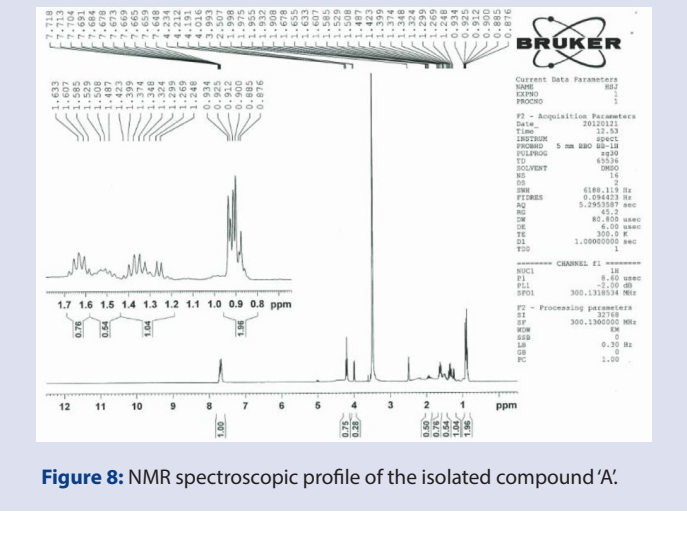
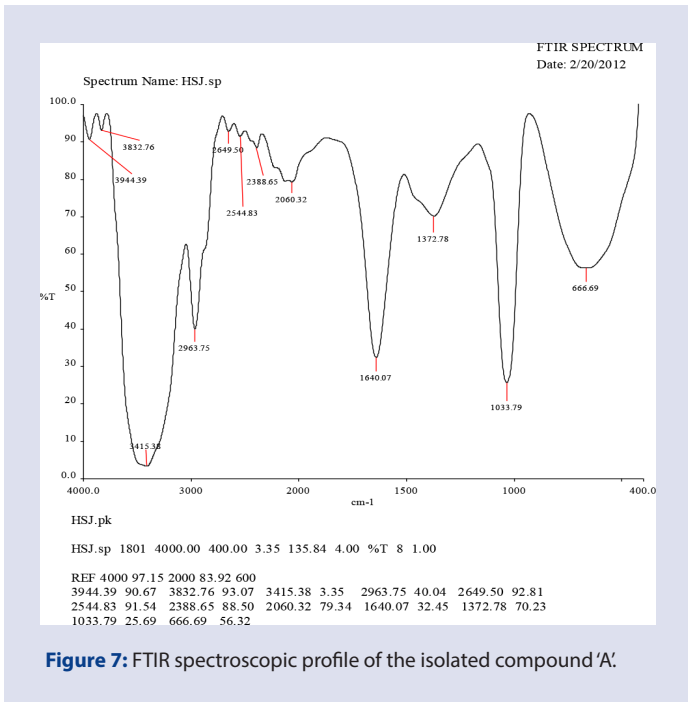
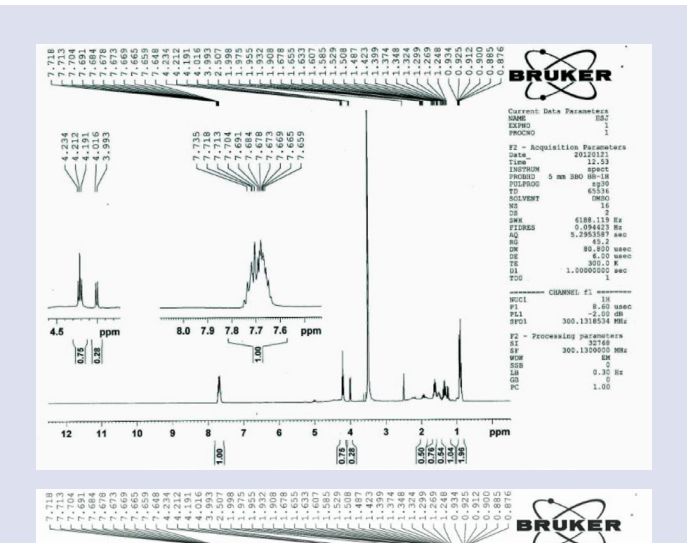
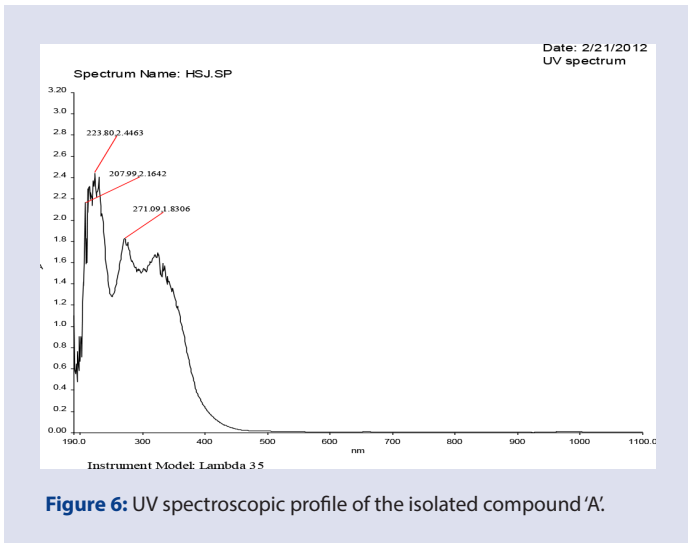


Figure 8: NMR spectroscopic profile of the isolated compound 'A'.

MS study

A Peak observed at m/e 353 is assigned to molecular ion peak and hence the molecular weight of the fragment is 353. A highest peak appearing at m/e 137 assigned to the base peak. This peak is corroborated the maximum amount of the fragment and whose molecular weight is 137. Others peaks were appeared at m/e 148 and 273 accounted for other major fragments (Figure 9).

DISCUSSION

Bioactive phytochemicals in plants are of different molecular make-up and they were differently soluble in solvents with different polarity hydrophilic and hydrophobic nature. Various compounds extracted from *Elephantopus scuber* powdered leaf and rhizome samples whereas the methanol solvent elute more compounds such as flavanoids, phenol, steroid, tannin, terpene, xanthoprotien, sugars from leaf extracts and phenol, saponin, steroid, terpene, sugars from rhizome extracts.³⁰ In the present study

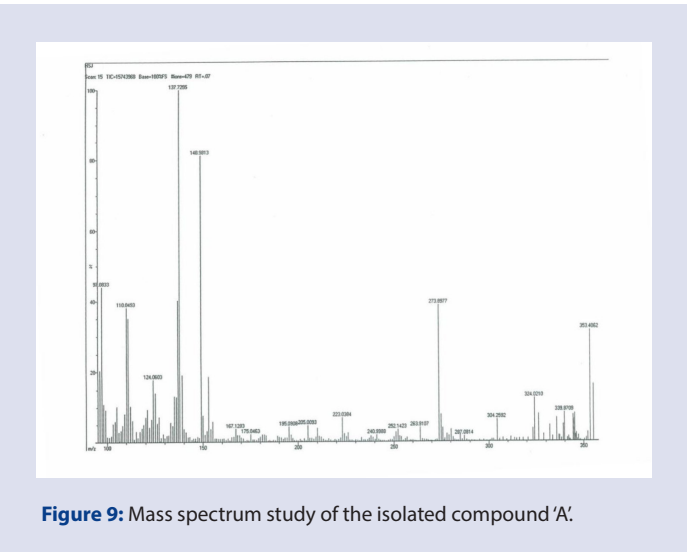


Figure 9: Mass spectrum study of the isolated compound 'A'.

ethanol was used as a solvent source for the extraction of secondary metabolites in a dissolved state. Phytochemical studies were carried out on *I. aspalathoides* that indicated the presence of flavonoids, tannin, phenol, saponin and sterol. Flavonoids were hydroxylated phenolic substances which have been found *in vitro* and act as an effective anti-microbial substances³¹ against a wide array of microorganisms due to

their ability to complex with extracellular and soluble proteins and bacterial cell walls.³² Phenolics and polyphenols were the largest groups of secondary metabolites which exhibit antimicrobial activity.³³ The site(s) and number of phenol groups were thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity.³⁴

The variation in the antibacterial activity of flavonoids was known to be related to their chemical structure, especially in regard to the number and positions of methoxy and phenolic groups within their structures.³⁵⁻³⁶ The antimicrobial effects of tannins have also been widely recognized.³⁷ Saponins have been implicated as bioactive antibacterial agents of plants.³⁸⁻³⁹ Plant steroids were known to be important for their cardio-tonic activities, possess insecticidal and anti-microbial properties. In the present study the highest antibacterial activities observed in flavonoid content of *I. aspalathoides* against *Staph. aureus* (18mm), followed by *S. typhi* (16mm) and *E. coli* (15mm) followed by tannins, sterols and saponins. Similar results were also found against fungal pathogens.

However, the positive control such as chloramphenicol showed the highest zone of inhibition for bacteria such as 22mm for *E. coli*, 20mm for *S. typhi* and 24mm for *Staph. aureus*. Similarly the streptomycin showed the highest zone of inhibition for *A. niger* (18mm), *A. flavus* (18mm) and *A. ochraceous* (18mm). Even though, the pathogenic bacteria as well as fungi developed resistant activity against the selected pure compound Chloramphenicol and streptomycin. However, the 200 μ l of *I. aspalathoides* ethanolic leaf extracts prevents the resistance formation in the *E. coli*, *S. typhi*, and *Staph. aureus* and this may be due to the presence of flavonoids in the leaf extract. Further purification of the ethanolic leaf extracts may be useful to control the resistance formation and also prevent the pathogenesis.

A total of 11 different compounds were identified through GC-MS analysis of *I. aspalathoides*. Most of the major compounds from the extract were biologically active molecules. They were considered to be a part of plants defense systems, and as such have been included in a large group of protective molecules found in plants named “phytoanticipins” or “phyto-protectants”.⁴⁰⁻⁴¹ GC-MS analysis studies revealed the presence of twenty five compounds from the ethanolic leaf extracts of *C. halicacabum* where Cyclohexane-1, 4, 5-triol-3-one-1-carboxylic acid and 1-hydroxytetradecane represented as major compounds.⁴²

3, 7, 11, 15-tetramethyl-2-hexadecen-1-ol, Squalene, 1-lodo-2-methylundecane, Vitamin E and Heptadecane, 2, 6, 10, 14-tetramethyl were also noted in the ethyl acetate extract of *H. arnottiana*.⁴³ 1, 2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester and 9,12-tetradecadiene-1-ol-acetate and 3-chloro-N-(2-phenylethyl) propanamide were identified in stem bark of *Mangifera indica* (L.).⁴⁴ Thus, the identification of a good number of compounds from *I. aspalathoides* by GC-MS might have some ecological significance.

The processing of a crude source material to provide a sample suitable for HPLC analysis as well as the solvent selection for sample reconstitution have a significant bearing on the overall success of natural product isolation.⁴⁵ The results from HPLC crude extract profile for leaves of *I. aspalathoides* shows five peaks at the retention time between 0 to 20mins using methanol and water as solvent. Out of which the highest peak was only selected for further characterization. Similarly, Gallic acid, caffeic acid, rutin, quercetin and ferulic acid in the flavanoids from the methanolic leaf extracts of *I. aspalathoides* were observed.⁴⁶

The result of this study shows that the UV-Vis spectrum scanning of flavonoid prepared with ethanol solvent produced absorbance spectra at wavelength range of 207nm, 223nm and 271nm. Peak locations or the peak wavelengths were specific for different bioactive compound groups and the peak height depends on the concentration of the compound. Flavonoids have an absorption maximum around 240-290nm, which

was affected by the conjugation of ring structure and its substitution pattern.⁴⁷ The spectra for phenolic compounds (tannins) typically lie in the range of 230-290nm. The result of UV-Vis spectroscopic analysis confirms the presence of tannins and flavonoids in the ethanolic extract of *I. aspalathoides*.

FTIR identifies and elucidates the chemical constituents⁴⁸ which providing accurate measurements of whole range of biological specimens.⁴⁹ The ethanolic extract of *I. aspalathoides* exhibited amide group, phenol group, carboxylic acid, alkynyl, alkene and aromatic rings. Identification of phenol and Flavonoid compounds in the ethanolic leaf extracts of *Cardiospermum halicacabum*⁴² which is similar to our results. Flavonoids and phenolic acids have the properties of antibacterial, antifungal, antiviral, hepatoprotective, immunomodulating, and anti-inflammatory.⁵⁰ The presence of amino acids, alkenes, nitrates, nitrites, ethers, esters, aldehydes, alkynes, aromatic compounds, organic halogen compounds and carbohydrates in *Ananus comosus* peel by IR spectrum.⁵¹ Methanolic leaves extract of *Clitoria ternatea* exhibited the presence of phenols, alkanes, primary amines, aromatic amines, carboxylic acids, alkenes, primary and secondary amines.⁵²

NMR spectroscopy provides the detailed structural information about organic compounds in the solution.⁵³ NMR studies revealed that the presence of phenyl group, methyl group and H₂C-CH group in the leaf extracts of *I. aspalathoides*. But in methanol leaf extracts of *E. agallocha* exhibited the presence of aliphatic alicyclic compounds, β -Substituted aliphatic compounds and α -Mono substituted aliphatic compounds.⁵⁴ This kind of analysis showed the presence of phenolic compounds and flavonoids, which can be isolated and further screened for different kinds of biologically active compounds and their activities depending upon the therapeutic uses.

CONCLUSION

It is hoped that this study would lead to the establishment of some biologically active molecules that could be used to formulate potential antimicrobial drugs of natural origin. Further studies will be needed to progress the *I. aspalathoides* ethanolic leaves extract on the same pathogens and as well as different obligate pathogens and also to focus on the isolated bioactive compound for its bio-efficacy and bioavailability in the arena of drug discovery.

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CONFLICT OF INTEREST

No conflict of interest.

ABBREVIATIONS

Rf: Retention factor; **GC-MS**: Gas chromatography mass spectroscopy; **HPLC**: High performance liquid chromatography; **UV spec**: Ultra violet spectroscopy; **FTIR**: Fourier transform infrared spectroscopy; **MS**: Mass spectroscopy; **NMR**: Nuclear magnetic resonance; **nm**: Nanometer; **HgCl₂**: Mercuric chloride; **rpm**: Rotation per minute; **Cfu/ml**: Colonies forming unit per milliliter; **TLC**: Thin layer chromatography; **NIST**:

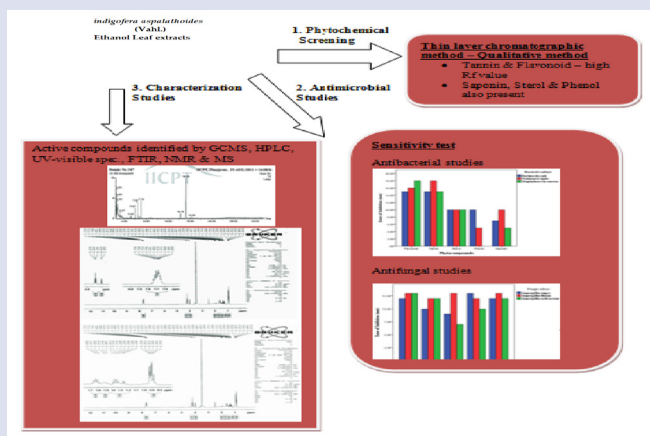
National institute of standards and technology; LC: Liquid chromatography; DAD: Diode array detector; IR: Infrared.

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GRAPHICAL ABSTRACT



SUMMARY

- In this study, various phytochemical compounds were isolated from the ethanolic leaf extract of *Indigofera aspalathoides* (Vahl.)
- Antimicrobial studies were performed for the isolated compounds.
- GC-MS were performed for the identification of the compounds
- HPLC, FTIR and NMR analysis were performed for the isolation and characterization of compounds
- This plant extract contains bioactive compound, effectively worked against obligate pathogens

ABOUT AUTHORS



Tamarasan Tamil Kumar, completed his Post graduate in the year 2009 and Master of Philosophy in 2012 in the Department of Botany, Jamal Mohamed College (Autonomous), Tiruchirappalli, Tamil Nadu (India). He started his research in the year 2012 in the same institution especially as specialization in Environmental Microbiology and Bioremediation.