Journal of Medicinal Plants Studies
www.PlantsJournal.com

ISSN 2320-3862
JMPS 2016; 4(6): 18-22

Received: 05-09-2016
Accepted: 06-10-2016

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Phytochemical analysis and antimicrobial activity of *Tiliacora acuminata* (Lam.) F. Thoms. (Menispermaceae)

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Abstract
The present study deals with the phytochemical examination and antimicrobial investigation of *Tiliacora acuminata* (Lam) f. Thoms., an important medicinal plant. The characterizations of compounds present in it were also analyzed using HPLC technique. Qualitative phytochemical analysis of the methanol, ethanol, petroleum ether and aqueous extracts prepared from *T. acuminata* leaf revealed the presence of alkaloids, flavonoids, terpenoids, cardiac glycosides, phenols, saponins and tannins. The High-performance liquid chromatography analysis also confirmed the occurrence of an alkaloid compound, colocynthin in the methanolic leaf extract. The antimicrobial activity was evaluated using methanolic leaf extracts of the plant. The extract was taken in different concentrations. The analysis was done by disc diffusion method, using 3 bacterial and 3 fungal strains. The bacterial strains used for this study were *Serratia spp., Bacillus subtilis* and *Pseudomonas aeruginosa* and the fungal strains were *Actinomycetes spp., Candida albicans* and *Aspergillus niger*. The results showed a maximum inhibitory action against all the bacterial and fungal strains with zones of inhibition ranging from 4mm to 17mm. Thus, the study reflects the potential of *T. acuminata* against the microbes. This observation becomes important in the context of the therapeutical and drug applications of T. *acuminata*.

Keywords: *Tiliacora acuminata*, phytochemical screening, HPLC analysis, colocynthin, methanolic extract, antimicrobial activity

Introduction
Investigation into the components of medicinal plants is a very popular subject of research in chemical and related biological sciences in most developing countries of the world. Some of these plant materials have been known to provide alternative natural products to synthetic and imported drugs. This is the basis for the application of nutrition in therapy and treatment of ailments and diseases as well as provision of raw materials for local pharmaceutical industries [1].

A plant may be considered as a biosynthetic laboratory, not only for the chemical compounds such as carbohydrates, proteins and lipids that are utilized as food by men, but also for a multitude of compounds like glycosides, alkaloids, volatile oils, tannins, etc, that exert a physiological effect [2]. They have enormous potential for research and new drug development. Many secondary metabolites have complex and unique structure and their production can be enhanced by introducing different types of additives into the basal media. About 10,000 plant alkaloids were identified in *Stephania* [3]; many of these pharmacologically active alkaloids are involved in plant defense against pathogens, insects and herbivores. Their potent toxicity makes alkaloids, 'privileged' structures for drug development [4]. The analysis of these alkaloids is important due to their potentially useful pharmacological activities [5] and methods such as high performance liquid chromatography [HPLC] have been used to detect the presence of these [6]. Taking into consideration, the medicinal importance of this plant, the methanol extract of leaf of *Tiliacora acuminata* were analyzed for the first time using HPLC, to identify the phytoconstituents present in it. This work will help to identify the compounds of therapeutic value. A majority of the rich diversity of Indian medicinal plants is yet to be scientifically evaluated for such properties. HPLC is the best technique to identify the bioactive constituents of long chain hydrocarbons, alcohols, acids esters, alkaloids, steroids, amino acid and nitro compounds. This plant has been used as an ingredient in many of the ayurvedic preparations and regarded as an antidote for snake bite [7, 8].

The search for components with antimicrobial activity has gained increasing importance in recent times due to growing worldwide concern about the alarming increase in the rate of infection by antibiotic resistant microorganism [9]. The antimicrobial activity test is an essential technique in many disciplines of science. It is used in pathology, to determine resistance of microbial strains to antimicrobials and in ethnopharmacology research; it is used to determine the efficacy of novel antimicrobials against microorganisms, essentially those of medicinal importance. The test is the first step towards new anti-infective drug development [10].

Tiliacora acuminata is a large woody climber; branches cinereous, striate. Leaves are long, ovate, acuminate, cordate, and truncate or rounded at the base, flowers yellow in elongate, lax, axillary, racemose panicles belonging to the family Menispermaceae. This plant has been used as an ingredient in many of the ayurvedic preparations and regarded as an antidote for snake bite.

Hence, the present study was undertaken to assess the active principles and their medicinal properties of *Tiliacora acuminata* (Lam.) f. Thoms (Menispermaceae) with the following objectives such as, phytochemical analysis, characterization of compound using HPLC and evaluation of antimicrobial activity.

Materials and Methods

Preparation of plant extract

The mature plant parts of *Tiliacora acuminata* (Lam.) Hook. & Thomson (Menispermaceae) was collected from Paramathi vellore, Tamilnadu. The extraction of the plant was carried out by using known standard procedures. Freshly collected leaf of *T. acuminata* was cut in to small pieces and shade dried. All the dried parts were pulverized by mechanical grinder to get the powder through 100 mesh sieve and stored in an air tight container. Required quantity of powder was weighed and transferred to a conical flask. The powder was treated with various solvents like petroleum ether, ethanol, methanol and aqueous. This process was repeated for a week and the extract was filtered through Whatmann's No.1 filter paper. The filtrate was collected and evaporated up to dryness. The concentrated residue was used for various phytochemical and biological studies.

Preliminary Phytochemical Screening

Qualitative phytochemical tests for the identification of alkaloids, flavonoids, steroids and terpenoids were carried out for all the extracts by the known methods [11, 12]. These tests were carried out in triplicate using various concentrations of sample.

Test for alkaloids

2ml of extract was taken and mixed with few drops of 1%HCl. To 1ml of this mixture, 6 drops of Mayers reagents and Dragendorff reagent were added. Within few minutes yellow creamish precipitate colour in Mayer's reagent and brownish red precipitate and orange precipitate in Dragendorff reagent were appeared.

Test for flavonoids

Shinoda test: 1ml of extract was taken and 3 pieces of magnesium chips were added followed by a few drops of concentrated hydrochloric acid. The appearance of an orange, pink or red to purple colour indicates the presence of flavonoids.

Sulphuric acid test: 1ml of extract was dissolved in few drops of concentrated sulphuric acid and the colour change was observed.

Ferric chloride test: 1ml of extract was added with 2 drops of freshly prepared ferric chloride solution. Appearance of green, blue or violet colours indicates the presence of phenolic hydroxyl group.

Test for terpenoids

Salkowski test: 5ml of extract was mixed with 2ml of chloroform and 3ml of sulphuric acid carefully added to form a layer. A reddish brown colour indicates the presence of terpenoids.

Test for cardiac glycosides

Keller-Killani test: 5ml of extract was treated with 2ml of glacial acetic acid containing 1 drop of ferric chloride solution. This was under laid with 1ml of concentrated sulphuric acid. A brown ring of interface indicates the presence of cardiac glycosides.

Test for phenols

1ml of extract was added with 1ml of alcohol and then few drops of neutral ferric chloride solution were added. The result was observed.

Test for sterols

Salkowski test: 2ml of extract was treated with 2ml of chloroform and 2ml of concentrated sulphuric acid added from the side of the test tube and shaken for few minute. The development of red colour in chloroform layer indicated the presence of steroids.

Liebermann-Burchard test: 1ml of extract was taken in a test tube and 1ml of chloroform concentrated sulphuric acid was added. A blue colour exhibited by chloroform layer and green fluorescence by acid layer suggested the presence of sterols.

Test for saponin

Foam test: 1ml of extract solution was diluted with distilled water and to 20 ml shaken in a graduated cylinder for 15 minutes. Development of stable form suggested the presence of saponins.

Test for Tannins

Braemer's test: 1ml of 5% ferric chloride was added to 5ml of extract. The resultant solution was shaken vigorously and the result was observed.

Test for resin:

5ml of copper sulphate was added to 5ml of extract. The resultant solution was shaken vigorously and allowed to separate. A green coloured precipitate indicated the presence of resin, while the same mixed with ethanol gave a light blue colouration.

HPLC Analysis

Preparative HPLC purification was performed using Knauer LC system equipped with a vacuum degasser, quaternary solvent mixer and K-2600 UV detector. The preparative column Merck Lichrocart 100 RP-18 end-capped (10 × 250 mm, 10µm) was used for colocynthin separation. The analytical HPLC system consisted of an Agilent 1100 series of G1312A binary pump, G1387A WPALS auto-sampler and

G1314A UV detector (Agilent Technologies, Waldbronn, Germany). The Chemstation for LC 3D (Rev. A.10.01 [1635]) was used for data acquisition. The column was a reversed phase (Luna C18 250 × 4.6 mm, 5 µm) column (Phenomenex Inc., Aschaffenburg, Germany) equipped with C18 security guard cartridge (Luna 4 × 3.0 mm). HPLC parameters were as follows: pump mode, isocratic; solvent system, 30:10:60:0.1 (v/v) methanol: isopropanol: water: trifluoroacetic acid; solvent flow rate, 0.5 mL/min; detection wavelength, 237 nm; sample injection volume, 20 µL; run time, 20.0 min. The resulting peak areas were used to calculate the amount of analyte in sample. Each data point was based on the average of three replicate measurements.

Antimicrobial assay

Tested Organisms

The bacterial strains used for this study include *Serratia spp., Bacillus subtilus and Pseudomonas aeruginosa a*nd the fungal strains were *Actinomycetes spp., Candida albicans* and *Aspergillus niger*. All these cultures were maintained on nutrient and potato dextrose agar medium plates. Two colonies of a 24-hour plate culture of each organism were transferred aseptically into 10 ml nutrient and potato dextrose broth in a test tube and mixed thoroughly using an electric shaker for uniform distribution

Disc Diffusion Assay [13]

Petri dishes were plated with Nutrient agar and Potato Dextrose agar medium were prepared according to the manufacturer's manual and allowed for 30 minutes to solidify. The test organisms were then spread on the surface of the media using a sterile swap stick. The different solvent extracts of plants were (10mg/ml) introduced on the disc (0.7cm) and then allowed to dry. Then the disc was impregnated on the agar plates and Ampicillin was used as reference drug for the bacteria. The various extracts of *T. acuminata* were tested against three fungi and 10µg Ampicillin used as the reference drug for the fungi. The plates were then incubated at 37º C for 24 h. Microbial growth was determined by measuring the diameter of zone of inhibition.

Results and Discussion

Phytochemical screening

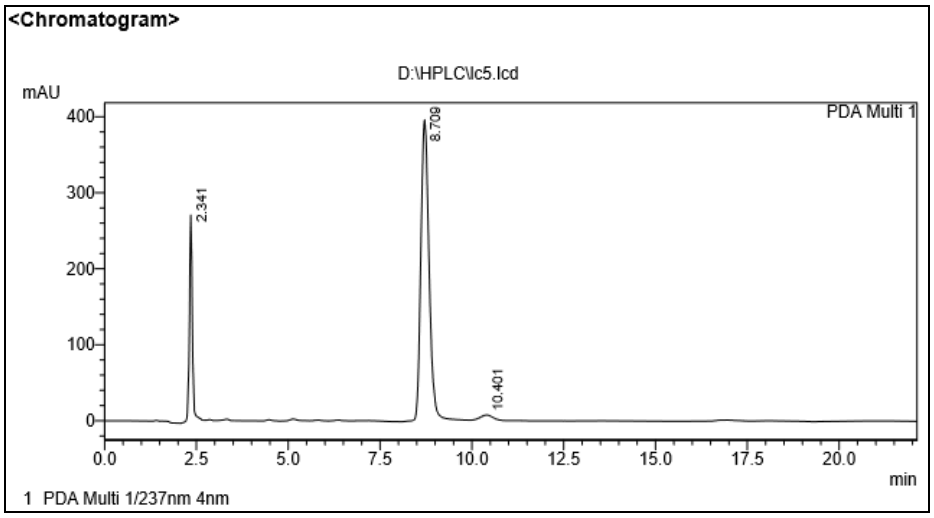
The distribution of different phytochemical constituents in various solvents like petroleum ether, ethanol, methanol and aqueous leaf extracts of *Tiliacora acuminata* was evaluated qualitatively. The results indicated the presence of alkaloids, flavonoids, saponins, tannins, phenols, cardiac glycosides and terpenoids in methanolic and ethanolic extracts (Table-1). Out of the four solvents, methanolic extract showed the maximum result. Most of the Menispermaceae family members possess high amounts of alkaloid than the other phytocompounds. The alkaloids are Liriodenine, dicentrinone, corydine, isocorydine, roemerine, N-methylliriodendronine, 2-O, N-dimethylliriodendronine, aloe-emodin, isocorydine, aporphines, atheroospermidine, tephalagine and dehydrostephalagine reported from *Stephania dinklagei* [14, 15, 16].

Table 1: Phytochemical analysis of leaf extracts of *T. acuminata*

S. No.	Phytochemicals	Different solvents used			
		Pet. Eth.	Ethanol	Methanol	Aqueous
1	Alkaloids a) Mayer's test	+	-	++	++
	b) Dragendroff's test	+	+++	+	+
2	Flavonoids a) Shinoda test	-	+	+	-
	b) Sulphuric Acid test	+	+	++	+
	c) Ferric Chloride test	-	+++	+	+
3	Terpenoids Salkowski test	++	-	-	-
4	Cardiac Glycosides	+	+	-	++
5	Phenol Ferric Chloride test	-	+	++	+
6	Sterols a) Salkowaski test	-	-	-	-
	b) Libermann's test	-	-	-	-
7	Saponins Foam test	++	+	+++	+
8	Tannins	-	-	+	++
9	Resins	-	-	-	-

HPLC Analysis

The HPLC analysis revealed the chromatogram of an alkaloid compound namely, Colocynthin (8.7 min) and clearly eluted at different retention times (2.341, 8.709, 10.401) when methanol: isopropanol: water: triflouro acetic acid was used as one mobile phase and eluted at the rate of (30:10:60:0.1) for 0.5 ml/ minutes. The HPLC profile showed three peaks and the retention time, height, % Area and % Height are depicted (Fig. 1). The physicochemical studies of *Tinospora cordifolia* were carried out and the berberine alkaloid was quantified in different fractions of extract by HPLC [17]. The result shows that the methanol extracts have higher concentration of berberine when compared to other solvent fractions. And also revealed the standardization profile and characterization of berberine compound from *Tinospora cordifolia,* which would be of immense value in botanical identification and authentication of plant drug and may help us in preventing its adulteration.



PeakTable

PDA Ch1 237nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.341	1492918	272597	19.049	40.302
2	8.709	6153269	396265	78.515	58.586
3	10.401	190911	7519	2.436	1.112
Total		7837099	676381	100.000	100.000

Fig 1: HPLC Chromatogram and Peak table of methanolic leaf extract of *T. acuminata*

Antimicrobial activity

The methanolic extract of *Tiliacora acuminata* was assessed for antimicrobial activity by using the disc diffusion method with various concentrations of the plant extracts viz. 25, 50, and 75 mg/ml (Table-2). Three bacterial and three fungal strains were used for the present study. The plant extracts exhibited strong antibacterial effects against the tested bacteria with inhibition zones ranging from 3 to 9 mm. The bacterial strains were *Serratia spp, Bacillus subtilus, Pseudomonas aeruginosa* and *Pseudomonas aeruginosa* was found to be more susceptible to the methanolic leaf extract of *Tiliacora acuminata*. The zone of inhibition for *P. aeruginosa* was maximum (9 mm) at 75 mg extract concentration. The zone of inhibition of *Serratia spp.* (7 mm) at 50 mg of the methanolic extract and the results indicated that the methanolic extract illustrated a significant antibacterial activity, when compared to the control, Ampicillin. The fungal strains *Candida albicans, Actinomycetes spp., Aspergillus niger* were susceptible for the plant methanolic extracts (Table-3). For *Actinomycetes spp*., the zone of inhibition was 17 mm at 75 mg concentration of methanolic extract. *Candida albicans* exhibited the zone of inhibition of 15 mm at 75 mg and 11 mm at 75 mg for *Aspergillus niger***.** It revealed that the plant methanolic leaf extract has the maximum antifungal activity when compared with the control Amoxicillin. Results of present investigation highlights the fact that the methanolic extract exhibited greater antimicrobial activity, because the antimicrobial principles were either polar or non-polar and they were extracted only through the organic solvent medium [18].

Table 2: Antibacterial activity of various concentration of methanolic leaf extracts of *T. acuminata*

S. No.	Bacterial strains	Zones of Inhibition (mm)			
		methanolic leaf extracts concentration (mg/ml)			
		Control (Ampicillin)	25	50	75
1.	*Bacillus subtilus*	4	3	8	5
2.	*Pseudomonas aeruginosa*	4	5	8	9
3.	*Serratia spp.*	7	5	7	3

Table 3: Antifungal activity of various concentration of methanolic leaf extracts of *T. acuminata*

S. No.	Fungal strains	Zones of Inhibition (mm)			
		methanolic leaf extracts concentration (mg/ml)			
		Control (Amoxicillin)	25	50	75
1.	*Actinomycetes spp.*	11	9	14	17
2.	*Candida albicans*	10	6	9	15
3.	*Aspergillus niger*	11	5	7	11

Conclusion

The *Tiliacora acuminata* plant is used in the treatment of various ailments in the history of traditional medicine. The result of the present study concluded that *T. acuminata* leaf could be a source for the phytoconstituents and it could be effectively used as medicine for many diseases. The studies on this species also confirmed the antimicrobial activity which will help to identify the potential traditional properties as a valuable source for the discovery of natural product for pharmaceuticals. The results of this study further reaffirm the existence of strong correlation between plant phytochemicals and antimicrobial activity.

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