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In vitro anti-bacterial activity of *Tinospora cordifolia* leaf extract and its phytochemical screening

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ABSTRACT

Background

Antimicrobial resistance is the main concern worldwide to combat infectious. Over the years studies on leaf extracts *Tinospora cordifolia* have demonstrated the potent role its antibacterial property. The current study is an attempt to test its antibacterial property against Escherichia coli cell division.

Material and methods

Phytochemical screening assay of *T. cordifolia* leaf extract was done using standard procedure and the results showed the presence of alkaloid, carbohydrate, terpenoid, steroid, tannin, amino acid, flavonoid and glycoside components.

Results

HPLC analysis revealed the presence of berberine in *T. cordifolia* leaf extract. Further *E. coli* cells were treated with berberine to study its efficacy in inhibiting cell division. Antibacterial assay was performed by using disc diffusion method.

Conclusion

Among aqueous, methanolic, ethanolic, chloroform, hexane and acetone extract only methanolic extract showed zone of inhibition.

Keywords

T. cordifolia, Berberine, HPLC, *E. coli*

Introduction

Microorganisms, like any other life form, tend to evolve over time. Survival is the key for existence and it must adapt to its surrounding. Any interference to their survival must be overcome. The use of antimicrobials, even when used under appropriate conditions, can lead to antimicrobial resistance let alone its indiscriminate usage resulting in medication ineffectiveness and allowing the infections persist in the body for a longer duration. Resistant strains of microbes continue to proliferate and poses major risk factor for severe infections.

Plant and plant based antimicrobial agents were used against the microbial infection for ages all over the countries. Plant-derived medicine is the major antimicrobial agents for 60 to 80% of populations in the developing countries [1]. Traditionally, crude plant extracts are used as herbal medicine for the treatment of human infectious diseases. Natural compounds present in medicinal plants enhancethe immune status and maintain disease free state [2]. Because of this medicinal products research gains important role in clinical research.

T. cordifolia is one of the most widely used herb in Ayurveda, one of the ancient systems of medicine practiced in India. It belongs to Menispermaceae family and commonly called heart leaved moonseed plant in English, Guduchi in Sanskrit, Giloe in Hindi, Teppatige in Telugu [3]. It has large, deciduous shrub with twinning branches, leaves are simple, alternate and long petiole. Flowers are unisexual and small; fruits are drupes, turning red when ripe. Fruits aggregate of *T. cordifolia* belongs to different classes of alkaloids, steroids, glycosides, diterpenoid lactones, sesquiterpenoids, phenolics, aliphatic compound [4-5]. These compounds are present in all parts of *T. cordifolia* but its concentration is more in root, stem and leaves. Berberine and furanolactone is the main compound of the plant [6]. The plant is of great interest to researchers because of its medicinal properties like anti-bacterial, anti-fungal, anti-cancer, anti-periodic, anti-spasmodic, anti-stress, anti-inflammatory, anti-arthritis, anti-neoplastic, anti-diabetic activity [7]. All parts of the plant are reported to be useful in ethnobotanical survey conducted by ethnobotanist [8]. Medicinal properties of *T. cordifolia* is presently applied in modern medicine and used to overcome the adverse effect of chemotherapy [9]. The plant is also used in the treatment of jaundice, arthritis, urinary tract infections (UTI) etc. and is rich in micronutrients such as calcium, phosphorous, copper, manganese, zinc and iron [3]. Roots, stem and leaves of *T. cordifolia* has no toxic effect on human body when orally consumed [2]. In our present study preliminary phytochemical screening and anti-microbial activity of different extract of leaves of *T. cordifolia* were evaluated using standard procedure.

Material and methods

Collection of plant material

Fresh plant leaves were collected from Nursery Horticulture Department, Mysore, authenticated by Department of Botany, Maharani's College, and Mysore. Leaves were washed in running tap water, shade dried for 15 days.

Solvent extraction

Dried leaves were crushed and finely powdered using pestle and mortar. Leaf powder was extracted with aqueous, methanol, ethanol, chloroform, hexane and acetone by Maceration method [10]. 20gms of well grinded leaf powder was dissolved in 200ml of solvent. The obtained solution was filtered using filter paper and the final filtrate was evaporated to dryness at room temperature. Scraped out the content and kept it at 4º C in order to minimize the possibility of contamination and to maintain its bioactivity for longer period.

I. Phytochemical screening

Various tests were conducted to confirm the presence of phytochemical in *T. cordifolia* leaf extract such as alkaloid, carbohydrate, terpenoid, steroid, saponin, tannin, amino acid, flavonoid, glycoside and protein. Phytochemical analysis was carried out according to following standard procedures.

i. Amino acid:

Ninhydrin Test: 2 drops of ninhydrin solution with 2 ml of extract, boil, and appearance of blue color indicate the presence of amino acid [9].

ii. Alkaloid:

Wagner's test: For 1 ml sample solution adds few drops of Wagner's reagent; reddish brown precipitate will form.

iii. Carbohydrate Test:

Fehling's test: For 1 ml Fehling A and 1 ml Fehling B reagents add 1 ml of extract boiled (10min). Formation of brick red color precipitate indicates the presence of carbohydrate [11].

iv. Terpenoid test:

Salkowski's test: 1 ml of crude extract with 1ml of chloroform, evaporated to dryness, adds 2ml of concentrated H_2SO_4 and heated for about 2 minutes. Grayish color will appear.

v. Saponin test:

Froth test: 2 ml of crude extract with 2ml of distilled water in a test tube, shaken vigorously (warm); formation of stable foam indicates the presence of saponin.

vi. Glycoside test:

Liebermann's test: For 2 ml of the extract add 2 ml of chloroform and 2 ml of acetic acid, solution cooled well in ice, add Sulphuric acid carefully color changes into green.

Table 1: Qualitative phytochemical analysis of *Tinospora cordifolia* leaf extract

Phytochemicals Sample	Alkaloid	carbohydrate	Terpenoid	steroid	Saponin	Tannin	Amino acid	Flavonoid	Glycoside	Protein
Aqueous	+	_	_	+	_	_	+	_	_	+
Methanol	+	_	+	+	_	+	_	_	+	+
Ethanol	+	_	_	+	_	+	_	_	+	+
Chloroform	+	+	_	+	_	_	_	_	_	+
Hexane	+	_	+	_	_	+	_	_	+	+
Acetone	+	_	+	_	_	+	_	+	+	+

+ve Present, -ve Absent

vii. Protein test:
Millon's Test: 3 ml extract and 5 ml of Millon's reagent, heated. Color changes from white precipitate to brick red on heating [12].

viii. Steroid test:
Salkowski's test: 2ml of crude extract dissolved in 2ml of chloroform add 2ml of con. H_2SO_4 sidewise, red color ring is produced [13].

ix. Tannin test:
$FeCl_3$ test: 2ml of extract dissolved in 2ml of distilled water (stirred), add few drops of $FeCl_3$, green precipitate will form [14].

x. Flavonoid test:
FeCl3 test: 1ml of extract dissolved with few drops of ferric chloride, green color will form [15-18].

II. Qualitative analysis of HPLC
Qualitative analysis of HPLC was carried out to check presence of berberine in aqueous, methanol, ethanol, chloroform, hexane and acetone extract. Analysis was carried out using Schimadzu HPLC, C-18 column with a mobile phase of orthophosphoric acid at flow rate of 1ml/min with UV detector at 345nm.

III. Quantitative analysis of HPLC
Berberine standard was purchased from Sigma-Aldrich, Bangalore and it was run against aqueous, methanol, ethanol and acetone extract. Qualitative analysis of HPLC was carried out using C-18 column with a mobile phase of acetonitrile at flow rate of 1ml/min with UV detector at 345nm.

IV. Anti-bacterial activity
Antibacterial activity of *T. cordifolia* leaf extract was carried out using disc diffusion method against Escherichia coli. Aqueous, methanolic, ethanolic, chloroform, hexane and acetone extracts were used as test compound and ampicillin was used as reference standard. Initially *E. coli* was cultured in nutrient broth for 24 hours at 37ºC. Agar plate was prepared by adding 25ml of nutrient agar media into the sterile petri plate, allowed to solidify. 10µl of bacteria was inoculated and streaked on agar plate using cotton swabs. Disc containing different concentration of *T. cordifolia* leaf extract were placed on agar plates. Plates were incubated overnight at 37ºC. Zone of inhibition was measured after 24 hours using ruler and compared with standard ampicillin [19].

Results
Phytochemicals are chemicals produced by plants through primary or secondary metabolism. Preliminary phytochemical screening assay of *T. cordifolia* leaf extract was conducted to check the presence of phytochemicals. *T. cordifolia* leaf extract showed positive test result which indicates the presence of alkaloid, carbohydrate, terpenoid, steroid, tannin, amino acid, flavonoid, glycoside and protein. The test for alkaloid and protein has given positive result and saponin shown negative result in all the six extract. Carbohydrates were present only in chloroform extract, amino acid in aqueous extract and flavonoid in acetone extract. Methanol and acetone extract showed highest number of phytochemicals.

Table 2: Qualitative HPLC analysis of *Tinospora cordifolia* leaf extract

Sample	Retention time
Aqueous	3.609
Ethanol	3.582
Methanol	3.580
Chloroform	Nil
Hexane	Nil
Acetone	3.577

Qualitative analysis of HPLC was carried out to check the presence of berberine in all the six extract. Results revealed the presence of berberine in aqueous, ethanol, methanol and acetone extract and absence of berberine in chloroform and hexane extract. Based on retention time berberine peak was identified. Retention time 3.5 corresponded to berberine [14].

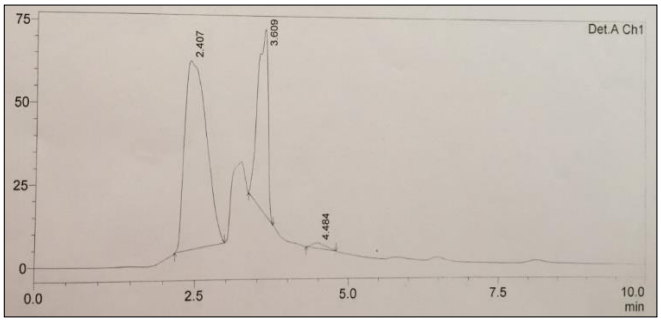

Figure 1: HPLC analysis of aqueous extract of *T. cordifolia*

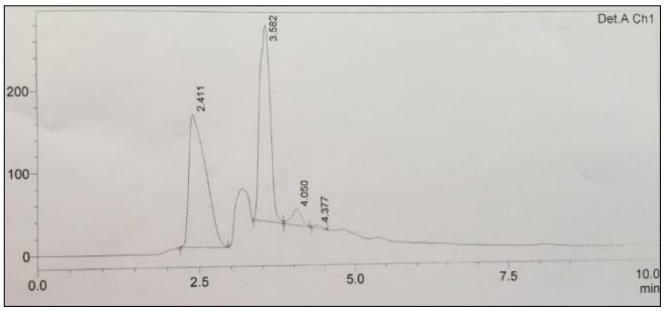

Figure 2: HPLC analysis of methanolic extract of *T. cordifolia*

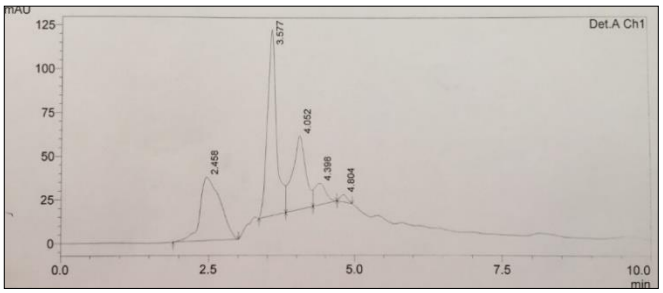

Figure 3: HPLC analysis of ethanolic extract of *T. cordifolia*

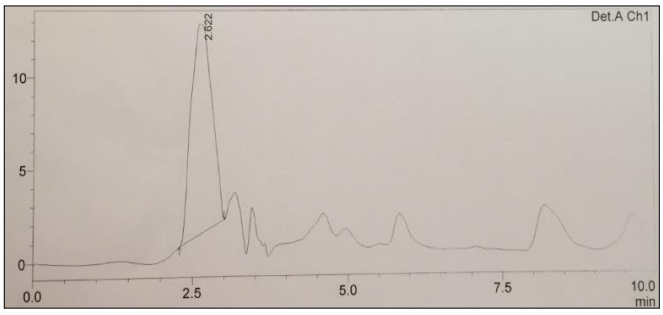

Figure 4: HPLC analysis of chloroform extract of *T. cordifolia*

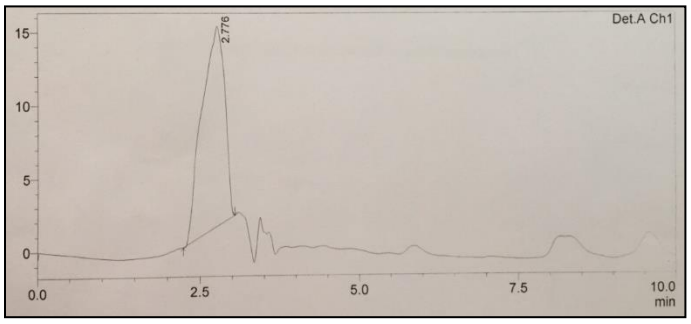

Figure 5: HPLC analysis of hexane extract of T. cordifolia

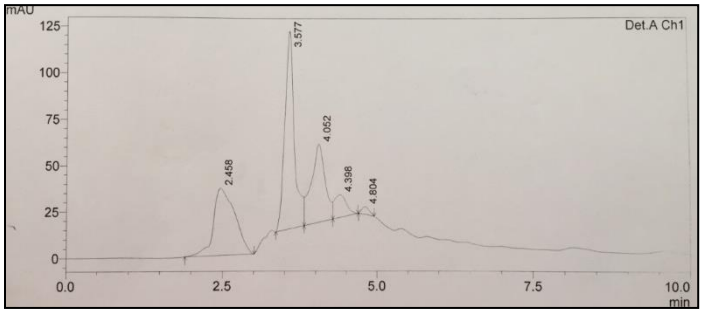

Figure 6: HPLC analysis of acetone extract of *T. cordifolia*

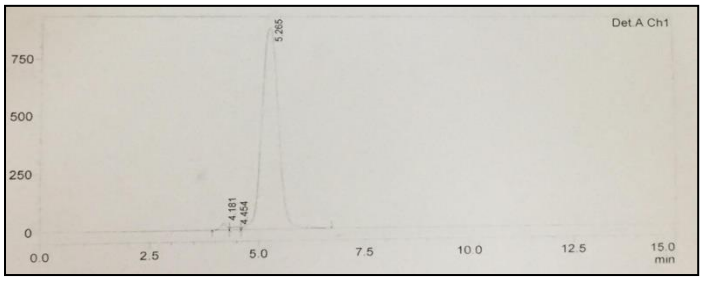

Figure 7: HPLC analysis of Berberine standard (The peak at retention time 5.26 corresponding to Berberine)

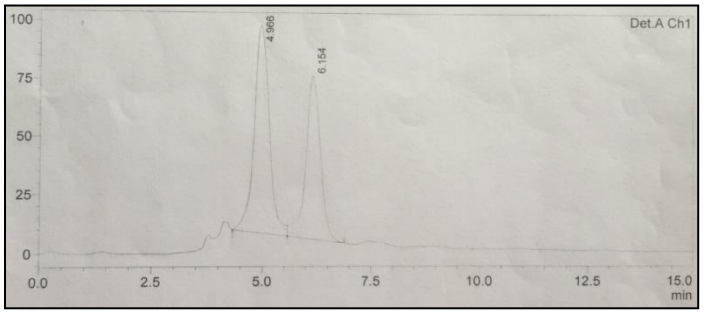

Figure 8: HPLC analysis of methanolic extract of *T. cordifolia* (Analysis showed well separated berberine peak at retention time 4.906)

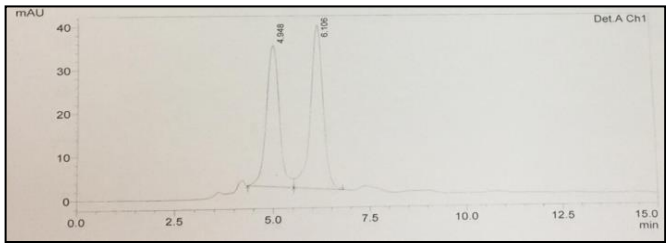

Figure 9: HPLC analysis of ethanolic extract of *T. cordifolia* (HPLC analysis of ethanolic extract showed well separated berberine peak at retention time 4.948)

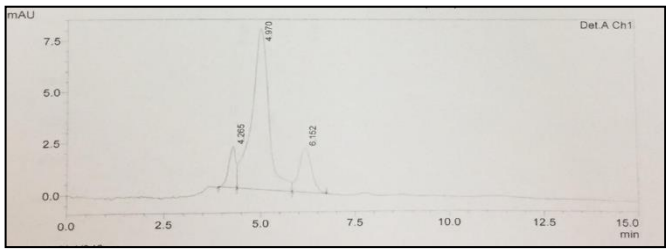

Figure 10: HPLC analysis of aqueous extract of *T. cordifolia* (HPLC analysis of aqueous showed sharp and well resolved berberine peak at retention time of 4.970)

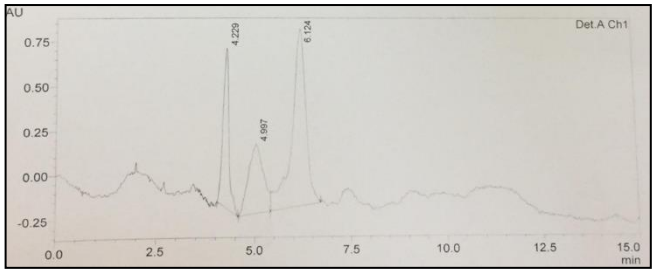

Figure 11: HPLC analysis of acetone extract of *T. cordifolia* (HPLC analysis of acetone extract showed sharp and well separated berberine peak at retention time 6.124)

Table 3: Quantitative analysis of HPLC

Sample	Concentration (%)
Aqueous	0.2
Ethanol	0.5
Methanol	1.1
Acetone	0.1

Quantitative analysis of HPLC was carried out to know the concentration of berberine in aqueous, ethanol, methanol and acetone extract along with berberine standard. In qualitative analysis chloroform and hexane extract showed the absence of berberine, hence chloroform and hexane extract was not taken in quantitative analysis. Based on the retention time of berberine standard, berberine peak was identified in remaining chromatogram. In quantitative analysis methanolic extract showed 1.1% of berberine.

Table 4: Anti-bacterial activity

Test organism	Test compound	Concentration(µl/disc)	Zone of inhibition
Escherichia coli	Methanol	10µl	0.3mm
		20µl	0.5mm
		30µl	0.8mm
	Aqueous	10µl, 20µl, 30µl	Nil
	Ethanol	10µl, 20µl, 30µl	Nil
	Chloroform	10µl, 20µl, 30µl	Nil
	Hexane	10µl, 20µl, 30µl	Nil
	Acetone	10µl, 20µl, 30µl	Nil
	Ampicillin	10µl	1.6mm
		20µl	1.6mm
		30µl	1.6mm

T. cordifolia leaf extract was used as test compound to inhibit the growth of *E. coli* with Ampicillin as reference. Methanolic extract showed zone of inhibition or inhibitory effect on growth of *E. coli.* Compared to the remaining extracts which may be attributed to chemical constituents responsible for antibacterial activity are more soluble in methanolic extract.

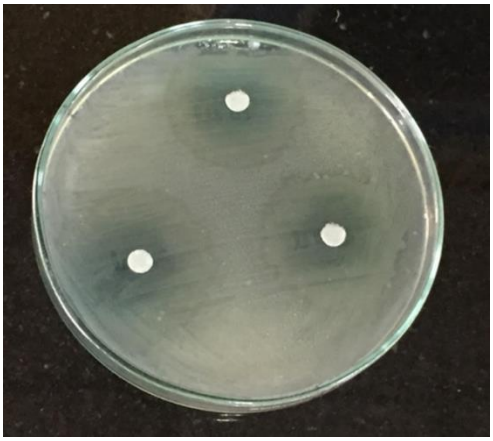

Figure 12: Inhibition of growth of *E. coli* by Ampicillin

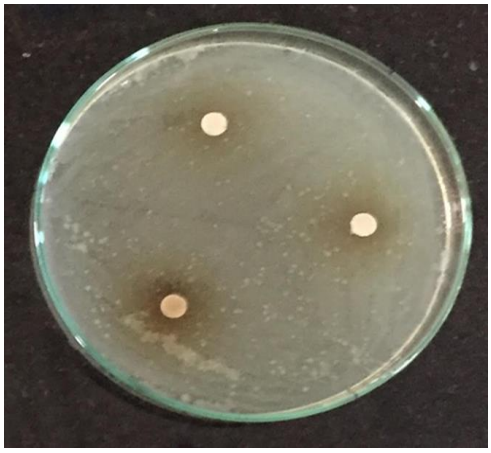

Figure13: Inhibition of growth of *E. coli* by methanolic extract

Discussion

Phytochemicals are biologically active compound found in plants. Naturally they help plants to defend against microbes and these important properties very useful for future drug discovery [20-22]. In this study, preliminary phytochemical screening assay was conducted by using aqueous, methanolic, ethanolic, chloroform, hexane and acetone extract using standard procedure. Result revealed the presence of alkaloid, carbohydrate, terpenoid, steroid, saponin, tannin, amino acid, flavonoid, glycoside and protein. Methanol and acetone extract showed more number of phytochemical. Preliminary phytochemical screening assay proved that *T. cordifolia* leaf extract is rich in primary and secondary constituents. Qualitative analysis of HPLC was carried out using aqueous, methanol, ethanol, chloroform, hexane and acetone extract. Aqueous, methanol, ethanol and acetone extract showed presence of berberine [23]. Chloroform and hexane extract did not show berberine peak. Quantitative analysis of HPLC was carried out with aqueous, methanol, ethanol and acetone extract. Aqueous extract contains 0.2%, methanol extract contains 1.1%, ethanol extract contains 0.5% and acetone extract contains 0.1% of berberine [24]. Methanol extract contains more concentration of berberine compare to other extracts. Anti-bacterial assay was performed with aqueous, methanolic, ethanolic, chloroform, hexane and acetone extract [25-29]. Among that methanol extract showed anti-bacterial activity. Remaining extracts did not show much inhibitory activity. The result obtained from this experiment is just a rough estimate in finding the medicinal property in terms of antibacterial activity of the plant.

Conclusion

The present study indicates *T. cordifolia* leaf extract was rich in primary and secondary constituents. Most of the biologically active phytochemicals were present in methanolic extract. In HPLC analysis berberine was present in aqueous, methanolic, ethanolic and acetone extract its concentration was more in methanolic extract. In anti-bacterial activity methanolic extract was effective against *E. coli*. Since the methanolic extract showed positive result in every assay, the present results can be considered beneficial for further investigation.

Abbreviations

urinary tract infections (UTI)

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Authors' contribution

Conceptualization, data curation was done by PB, PS, RN and BMK., formal analysis, manuscript writing, and editing was done by all authors. Final manuscript was approved by all authors.

Competing interests

The authors declare no conflicts of interest.

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