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PRELIMINARY PHYTOCHEMICAL ANALYSIS OF METHANOLIC EXTRACT OF *Tinospora cordifolia* AND ITS ANTI-BACTERIAL ACTION ON *E.coli* CELL DIVISION

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Key words: E.coli, Bacterial cell division, FtsZ, Berberine, Tinospora cordifolia.

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ABSTRACT

Plan: *The study involves the antibacterial screening the methanolic extract of Tinospora cordifolia and its preliminary phytochemical analysis.*

Preface: *Bacterial infections have become increasingly difficult to treat because of multi drug resistance. Although bacteria have over 200 specific protein targets for therapy, only three are most successfully exploited now. Bacterial cell division proteins are attractive targets for drug development. The "Filamenting temperature-sensitive mutant Z (FtsZ) protein is a major protein of the bacterial cell division machinery and is a potential target for therapy. Inhibition of FtsZ protein results in the inability of bacteria to divide and hence they die. Plant secondary metabolites have been shown to have antibacterial activities. However their mechanism of action is not thoroughly investigated.*

Methodology: *In this study we have used the methanolic extract of Tinospora cordifolia plant and tested its anti-microbial action by zone inhibition method.*

Outcome: *Although the T.cordifolia extract did not show high anti bacterial activity by disc diffusion assay, the E.coli showed elongated structures in the presence of T.cordifolia extract suggesting that the extract is interfering with the bacterial cell division. HPLC analysis of the extract showed the presence of Berberin, which may be the active molecule in inhibiting FtsZ protein and subsequent cell division.*

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1. INTRODUCTION

There has been an unprecedented emergence of new infections and re-emergence of old infections in the last two decades (Morens *et.al,* 2004)[17]. Many infections have become increasingly difficult to treat because of multi drug resistance (Giamarellou 2006)[6]. It is likely that in the future unless new and more effective drugs are discovered, even small infections will result in serious health complications. Conventionally bacterial infections are treated with bacteriostatic or bacteriocidal agents. Bacteriostatic agents do not kill the bacteria but prevent their multiplication. Drugs like sulphonamides and tetracyclines are bacteriostatic agents. Bacteriocidal agents, on the other hand, kill bacteria by acting on some vital target in the bacteria. For example, Beta lactams act on cell wall synthesis, Daptomycin acts on the plasma membrane (Steenbergen *et.al,* 2005; Baltz, 2009)[2] and fluoroquinolones act on DNA. However, bacteria have developed drug resistance due to indiscriminate use of all these antibiotics (Skold, 2000)[20]. Also, the problem of antibiotic resistance has been made worse by the use of new drugs that are merely variants of older overused antibiotics. Hence drug discovery programs are focused on finding new targets for therapy.

Cell division is essential for viability in bacteria. The cell division is governed by the divisome, a multiprotein complex that ensures proper positioning and construction of the septum. Septum formation is followed by a cytokinesis event that separates the two daughter cells (Weiss, 2004; Vicente *et.al,* 2006)[26]. This process ensures that cell division does not occur under unfavorable conditions. For example, damage to genomic DNA or the cell wall can lead to the arrest of cell division, allowing repair mechanisms to be activated (Jani *et.al,* 2015)[10]. Attention is focused on the major bacterial cell division protein FtsZ as the new target for antibacterial therapy. While synthetic molecules which target FtsZ protein are being investigated, natural products from plants can also provide biologically active molecules and template molecules for the development of modified derivatives with enhanced activity and reduced toxicity. Among the plants with anti microbial activity *Tinospora cordifolia* has shown great promise on account of the diverse chemicals found in the plant. *T.cordifola* belongs to the genus Menispermaceae. It is a large, deciduous extensively spreading climbing shrub with several elongated twining branches. Leaves are simple, alternate, estipulate, with long petioles. The plant has several secondary metabolites such as alkaloids, glycosides, lactones, aliphatic and other compounds (Mishra *et.al,* 2014)[15]. Although its anti bacterial action has been reported, the antibacterial target has not been investigated. Hence, in this study, the anti microbial activity of *T.cordifolia* extract and its possible action on FtsZ protein is described.

2. MATERIALS AND METHODS

Fresh plant was collected from Agumbe, Karnataka. The whole plant was shade dried Chopped into fine pieces and pulverized. The pulverized powder was stored at 4°C in an airtight container until use. The plant powder was sequentially extracted with Hexane, Ethyl acetate and Methanol. The fractions were concentrated using Rotary Flash Evaporator at temperatures not exceeding 40^0C. The dried samples were redissolved in methanol and stored at -20°C. Phytochemical analysis of the three extracts was carried out according to the standard phytochemical analytical procedures (Harborne, 1984)[8]. The methods are briefly described as follows:

2.1. Test for Alkaloids

3 ml aqueous extract was stirred with 3 ml of 1% HCl on steam bath. Dragendorffs reagent was then added to mixture. Brown colored precipitate of the resulting precipitate was taken as an evidence for the presence of alkaloid.

2.2. Test for Tannins

$FeCl_3$ Test: About 2 ml of the aqueous extract was stirred with 2 ml of distilled water and few drops of FeCl3 Solution were added. Formation of green precipitate was indication of presence of tannins.

Gelatin test: 1% gelatin solution containing 10% sodium chloride was added to each extract. Formation of precipitate indicated the presence of tannins and phenolic compounds.

2.3. Test for Saponins

5 ml of aqueous extract was shaken vigorously with 5 ml of distilled water in a test tube and warmed. The formation of stable foam was taken as an indication of the presence of saponins.

2.4. Test for Flavonoids

Alkaline reagent test: To 1 ml of aqueous extract, 1 ml of 10% lead acetate solution was added. The formation of a yellow precipitate was taken as a positive test for flavonoids.

Shinoda test (Magnesium Hydrochloride reduction test):-To leaf and bark (mixture) extracts, 5ml. 95% ethanol was added separately. Each mixture was treated with 0.5g magnesium turnings and few drops of conc. HCl. Pink color, if produced, may confirm the presence of flavonoids.

2.5. Test for Terpenoids

Salkowski's test - The extract is treated with chloroform with few drops of concentrated sulphuric acid, shaken well and allowed to stand for some time, formation of yellow coloured lower layer indicated the presence of terpenoids.

2.6. Tests for glycosides

Liebermann's test: 2 ml of the organic extract was dissolved in 2 ml of chloroform and then 2 ml of acetic acid was added in it. The solution was cooled well in ice. Sulphuric acid was then added carefully. A colour change from violet to blue to green indicates the presence of a steroidal nucleus (aglycone portion of the glycoside).

2.7. Tests for steroids

A red colour produced in the upper layer when 2 ml of organic extract was dissolved in 2 ml of chloroform and 2 ml concentrated sulphuric acid was added in it, indicates the presence of steroids.

2.8. HPLC analysis of plant extract

HPLC analysis of the extracts was carried out using Schimadzu – LC-10A, having Eximius PURITAS C18 column (4.6 mm × 250 mm, 5 µm). The mobile phase was Acetonitrile: 0.1% Formic acid in water (45:55), and the separation was under Isocratic condition with a flow rate of 1 ml / min. Sample (10 µL) was injected and the run time was 10 Min. The detector was set at UV-346 nm.

2.9. Anti-bacterial assay

Anti-bacterial assay was carried out by the disc diffusion essentially by the method described by Heatly (1944)[10] as follows: Standard *E.coli* strain was used for the antimicrobial assays. Bacteria were first grown in LB (Luria-Bertani) broth. A 10 µl aliquot of the bacteria was mixed with LB broth and then spread over a 90 mm Petri dish containing 25 ml of 1.5% agar in LB broth. A 20 µl aliquot extract was dropped onto the surface of a 6mm diameter disc such that the disc contained 1000 µg of the materialand the solvent was removed by blowing cold air. The discs were placed on the agar plate along with a disc containing 5µg of ciprofloxacin as reference standard after the agar plate was dried and incubated overnight at 37ºC in the dark. If the sample examined had antimicrobial activity, a clear zone would be formed on the surface of the agar representing inhibition of bacterial growth. The diameter of an inhibition zone around the discs was measured. The values were recorded with the average (mm) of two diameter measurements per disc taken in two directions, roughly perpendicular to each other.

2.10. Inhibition of bacterial cell division

E.coli cells were cultured in a containing LB broth as described above. The LB broth contained 0.25 µg/ml upto 100 µg/ml methanolic extract of *T.cordifolia.* The *E.coli* cells were then observed under 100 X 10 magnification.

3. RESULTS

Table 1. The qualitative phytochemical analysis of the extracts prepared using three solvents

	Ethyl acetate extract	Hexane Extract	Methanol Extract
Alkaloid	+	-	+
Tannins	+	-	+
Terpenoids	+	+	+
Glycosides	+	-	+
Steroids	+	+	+
Saponins	-	-	+
Flavonoids	+	-	+

The methanolic extract had all the classes of phytochemicals tested whereas the hexane extract had only terpenoids and steroids. The ethyl acetate extract had all the components except saponins.

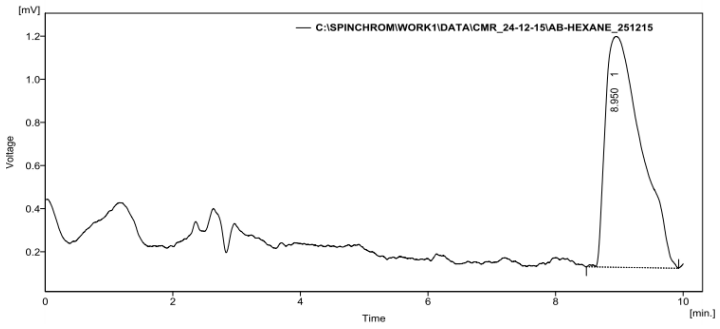

Fig 1. HPLC analysis of Hexane extract of *T.cordifolia*

The hexane extract showed the presence of one peak which was unresolved. The HPLC analysis of Ethyl acetate extract is shown in Fig 2. The HPLC profile of the methanol extract is shown in Fig 3.

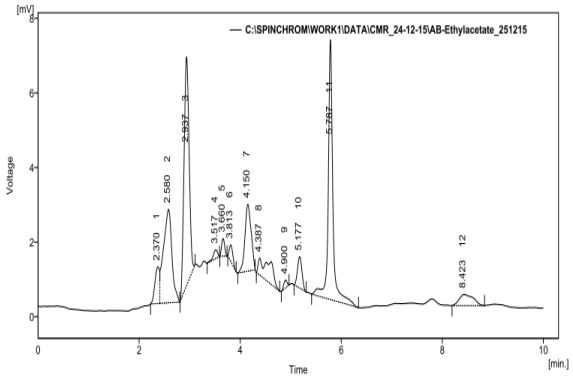

Fig 2. HPLC analysis of Ethyl acetate extract of *T.cordifolia*. The ethyl acetate extract showed the presence of five well- resolved peaks.

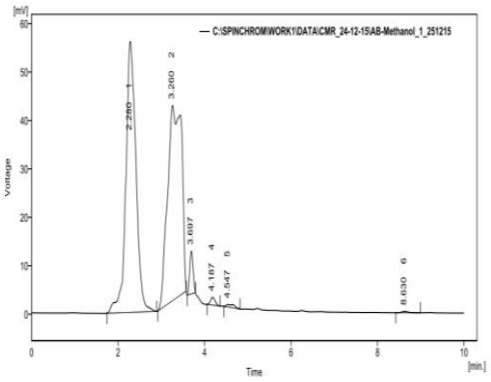

Fig 3. HPLC analysis of methanolic extract of *T.cordifolia*

There were three well-resolved peaks and one unresolved peak in the methanol extract. The peak at retention time 3.6 min corresponded to berberin.

Table 2. The amount of berberin in the three extracts

Sample	*Retention Time*	*AUC*	*Concentration of berberine (µg/g)*
Hexane Extract	-	-	Nil
Ethyl Acetate Ext	3.660	2.005	11.7
Methanol Extract	3.697	48.162	282.90

The amount of Berberine in the methanol extract was about 24 fold higher than that in the ethyl acetate extract.

Table 2. The effect of the extracts on inhibition of growth of *E.coli*

Test Organisms	*Test Compound*	*Concentration (µg/Disk)*	*Zone of Inhibition (in mm)*
Escherichia Coli	Methanolic Extract	1000	7.25 ± 0.25
	Ethyl acetate Extract	1000	-
	Hexane Extract	1000	-
	Ciprofloxacin	5	25.00 ± 0.25

The ethyl acetate and hexane extracts had no inhibitory effect on *E.coli* where the methanolic extract showed inhibitory effect in comparison with ciprofloxacin. The effect of the methanol extract on the cell division of *E.coli* is shown in Fig4.

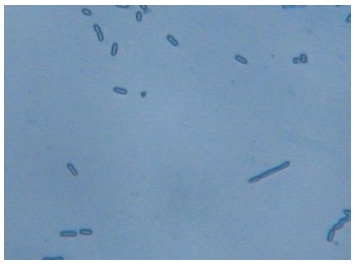

Fig 4. *E.coli* grown in the presence of 25 µg/ml T. cordifolia extract. The *E.coli* cells were elongated showing inhibition of cell division.

4. DISCUSSION

Bioactive compounds are substances that have a beneficial or harmful effect on the cellular function of an organism, living tissue or cells. Plants, microbes, fungi and animals have been a rich source of diverse bioactive molecules. Natural products provide unlimited opportunities for new drug discoveries, because of the diversity of chemical structures (Cosa *et.al,* 2006)[5]. Most common bioactive natural products include antibiotics, mycotoxins, alkaloids, flavonoids, terpenoids, phenolic compounds, pigments, phytosterols growth factors, stanols, steroids, glucosinolates and anthraquinones.

A variety of constituents have been isolated from *Tinospora cordifolia* plant and their structures were elucidated. They belong to different classes such as alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compounds and polysaccharides (Padmapriya *et.al.,* 2009)[19] Methanol extract of leaves is rich in flavanoids, alkaloids and glycosides (Soni HP *et.al.,* 2011)[22]. An alkaloid, *tinosporin* was identified in the plant (Padmapriya *et.al,* 2009)[19]. The methanol extracts of *Tinospora cordifolia* have been reported to have potential against microbial infections (Narayanan *et.al,* 2011)[11]. The anti-bacterial activity of *Tinospora cordifolia* extracts has been assayed against *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Proteus vulgaris,*

Salmonella typhi, Shigella flexneri, Salmonella paratyphi, Salmonella typhimurium, Pseudomonas aeruginosa, Enterobacter aerogene, and *Serratia marcesenses* (Gram-positive bacteria): (Narayanan *et.al,* 2011; Jayachandran *et.al,* 2003; Tambekar *et.al,* 2009)[11].

In mice models, *T.cordifolia* extract has been reported to function in bacterial clearance and improved phagocytic and intracellular bactericidal capacities of neutrophils (Thatte *et.al.,* 1992)[25]. *T.cordifolia* extract has also been reported to have immunostimulant properties on macrophages (Sengupta *et.al,* 2011)[20]. In our study we have found a wide variety of phytochemicals in the methanolic extract of *T.cordifolia*. The purpose of this study was to see whether any of the compounds can inhibit bacterial growth by acting on a new target, namely the bacterial cell division.

The analysis of bioactive compound present in plants involves several steps. Extraction of bioactive molecules is a crucial step in the analysis of the bioactive molecules. Care has to be taken such that the potentially active molecules are not lost during processing and extraction. The selection of solvents for extraction depends on the nature of the bioactive compound to be extracted. Popular methods include heating with a suitable solvent under reflux, and soxhlet extraction.

Phytochemical screening is a qualitative assay for the presence of various phytochemical classes in a phytochemical extract. This is mostly done by qualitative tests. HPLC is a versatile and widely used technique for the isolation and identification of natural products. This techniques is gaining importance for finger printing of herbal extracts (Fan *et.al,* 2006)[2]. HPLC is also used as a method of quantitative separation of natural products especially using preparative HPLC.

One of the important classes of molecules in the plant extract is alkaloids. Alkaloids have diverse structures and many of them exhibit a range of pharmacological activities including antimicrobial activity **(**Hadi and Bremner, 2001)[7]. Berberine is an isoquinoline alkaloid and is present in many plant species. It is widely used in traditional medicine because of its activity against bacteria, fungi, protozoa and viruses (Kim *et.al,* 2002)[12]. It accumulates in cells driven by the membrane potential and is an excellent DNA intercalator (Iwasa *et.al,* 2001)[9]. It is active on several microorganisms with a target on RNA polymerase, gyrase and topoisomerase IV and on nucleic acid (Yi *et.al,* 2007)[27]. In this study we have found it to have an action on bacterial cell division.

There are approximately 200 conserved essential proteins in bacteria, but the number of currently exploited targets is very small. The most successful antibiotics hit only three targets or pathways: the ribosome, cell wall synthesis and DNA gyrase or DNA topoisomerase (Coates *et.al,* 2002)[3]. Bacterial cell division components are novel and attractive targets for new antibacterial drug discovery (Lock and Harry, 2008; Ma and Ma, 2012)[14]. FtsZ is a major protein of the bacterial cell division machinery and is the first protein to be localized to the proposed site of cell division. FtsZ is a GTPase, and the monomers undergo GTP-dependent polymerization to form protofilaments that aggregate into a macromolecular structure, called the Z ring. Other cell division proteins are then recruited to the Z ring, and a new septum is synthesized, which enables the daughter cells to separate (Errington *et.al,* 2003; Adams and Errington, 2009)[1, 5]. FtsZ is an appealing target for new antibacterial drug discovery for several important reasons. First, it is an essential protein for bacterial viability (Dai and Lutkenhaus, 1991)[4]. Second, FtsZ is a protein which is highly conserved and hence is a potentially broad-spectrum antibacterial target. FtsZ proteins have been identified in most bacteria.

Third, FtsZ is not present in higher eukaryotes, which suggests that FtsZ inhibitors would not interfere with human metabolic pathways. Finally, because cell division proteins are not targeted by any known antibiotics in current use, it is anticipated that there would not be cross-resistance within drug-resistant bacterial populations. It is interesting to note that plant products have molecules which target the bacterial cell division machinery, thus providing template molecules for the synthesis of new anti bacterial drugs.

5. CONCLUSION

Even though the *T.cordifolia* has only weak anti-bacterial activity, the present study shows that its compound /compounds are directed to a new target, namely the FtsZ protein of the cell division machinery. Thus identification and characterization of the molecule/molecules can provide novel antibiotics for effective control of drug resistant bacteria.

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