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Phytochemicals, Antimicrobial and Cytotoxic Potential Study of *Tinospora cordifolia*

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Authors' contributions

This work was carried out in collaboration among all authors. Authors AB and MRH designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author LB managed the literature searches. Author ASMS review and correct the manuscript. Authors MZA and RH supervised the entire work. All authors read and approved the final manuscript.

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ABSTRACT

Aims: *Tinospora cordifolia* is a herb used in the traditional folk medicine due to their health benefits. Studies reported the biochemical composition and some biological properties of the plant stem. The aim of the current study was to investigate the antimicrobial and cytotoxic properties from the extract of *Tinospora cordifolia* stem.
Place and Duration of Study: The study was accomplished by eleven months in the Department of Pharmacy, University of Science and Technology Chittagong (USTC), Bangladesh.
Methodology: Presence of phytochemical constituents such as alkaloids, flavonoids and tannins,

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saponins were evaluated. Antimicrobial activity of the stem extract was assayed separately using an agar diffusion method against some gram negative and gram positive bacteria. The cytotoxic potential of the stem extract was evaluated by the Brine-Shrimp Lethality Assay mathod.

Results: The zone of inhibition formed by the methanolic extract in the antimicrobial assays was significant. In addition, cytotoxicity of the extract was found to be significant as compared to the standard gallic acid in Brine-Shrimp lethality assay.

Conclusion: The methanolic extract of *Tinospora cordifolia* may possess significant antimicrobial property which requires more studies to isolate the specific bioactive compound for developing a new antimicrobial agent from this plant.

Keywords: Tinospora cordifolia; phytochemicals screening; antimicrobial activity; cytotoxicity; LC_{50}.

1. INTRODUCTION

Nature has been a source of medicinal principles for many years and an impressive number of modern drugs have been isolated from natural sources. Many of them were isolated and purified by considering their use in the traditional medicine. The original source of many important pharmaceuticals is the medicinal plants used by indigenous people [1]. It has been reported that about 64% of the total global population remains dependent on traditional medicine and medicinal plants for provision of their healthcare purpose [2]. Some phytochemicals produced by various plants have antimicrobial activity allowing these plants to be studied and used for the development of new antimicrobial drugs [3,4]. In the past, plant metabolites were generally considered as sources of anti-nutritional factors. Recent bans and restrictions on the use of animal antibiotic growth promoters stimulate the interest in bioactive secondary metabolites of plant source as alternative performance enhancers [5]. The herb *Tinospora cordifolia* (*T. cordifolia)* belongs to the Menispermaceae family and is commonly known as Gu-lancha or *Tinospora* in English and Giloya or Ambervel in Hindi. It has a long history of use in Ayurvedic medicine (the traditional medicine of India). Evidence hints that *Tinospora* may have anti-cancer [6], immune stimulating [7], anti-diabetic [8], cholesterol-lowering [9] and liver-protective [10] actions. *T. cordifolia* has also shown some promising and rapid action in healing the diabetic foot ulcers [11]. Particularly, it has been reported that ethanolic extracts of *Tinospora cordifolia* show antimicrobial activity towards clinical isolates of methicillin-resistant and carbapenemase-producing bacteria [12]. Antimicrobial activity of different concentrations of *Tinospora cordifolia* against *Streptococcus mutans* has been found to be significant [13]. In addition, the extract of its stem stimulates bile secretion, causes constipation, enriches the blood and cures jaundice [14]. It is useful in skin diseases [15]. The root and stem of *T. cordifolia* are prescribed in combination with other drugs as an anti-dote to snake bite and scorpion sting. Dry barks of *T. cordifolia* have anti-inflammatory [16], anti-allergic [17], anti-spasmodic, antipyretic [18] and anti-leprotic [19] properties. Extract of *T. cordifolia* has also exhibited *in vitro* inactivating property against Hepatitis B and E surface antigen in 48-72 h [20]. *T. cordifolia* was found to be more effective than acetylsalicylic acid in acute inflammation [21]. However, all these findings are far too preliminary to be relied upon. The aim of the present work was to evaluate the phytochemicals, cytotoxic and antimicrobial activities of *T. cordifolia* to assess the scientific basis of the traditional uses of these plants.

2. MATERIALS AND METHODS

2.1 Plant Materials

The plant was collected from Forests of Bandarban, Chittagong Hill Tracts in November2017 and authenticated by Dr. Shaikh Bokhtear Uddin, Associate Professor, Department of Botany, University of Chittagong. A voucher specimen has been deposited at the Department of Chemistry, Chittagong University of Engineering and Technology, Chittagong.

2.2 Preparation of Plant Extract

The plant stems were thoroughly washed with water, cut into small pieces and dried in hot air woven at 55ºC for 3 days followed by at 40 ºC for the next 4 days. After drying the pieces of stems were then coarsely powdered using a suitable grinding mill. About 500g of powdered material was macerated with methanol (1:10) at room temperature for a period of 3 days with occasional shaking and stirring. After that the plant extract was filtered with clean cotton filter followed by Whitman filter paper (No. 1). The

solvent was evaporated by Rotary evaporator (Lab Tech EV311) at 40 ºC under reduced pressure. The extract was then preserved in a refrigerator(2-8ºC) till further use.

2.3 Phytochemicals Screening

2.3.1 Qualitative analysis

The freshly prepared crude extract was qualitatively tested for the presence of chemical constituents. Phytochemicals screening of the extract was performed using the following reagents and chemicals: Alkaloids with Molisch's reagent and concentrated sulfuric acid, flavonoids with the use of Mg and HCl; tannins with ferric chloride solutions and saponin with boiled water and olive oil which has ability to produce stable foam and steroids with Libermann-Burchard reagent. Gum was tested using Molish reagent and concentrated sulfuric acid; reducing sugars with Benedict's reagent; terpenoids with chloroform and conc. sulphuric acid; resin with acetic anhydride and concentrated sulphuric acid, antharaquinone glycosides with chloroform and sodium hydroxide. These were identified by characteristic color changes using standard procedures [22].

2.3.2 Quantitative analysis

2.3.2.1 Alkaloid

Alkaloid was determined using the method [23]. 5 g of powdered stems was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in methanol was added, covered and allowed to stand for 4h. This was filtered and the extract was concentrated on a water bath to 1/4 of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitated was collected and washed with dilute ammonium hydroxide and then filtered. The residue was air dried and weighed. The residue was the alkaloid which was weighed after complete dryness and the percentage was calculated.

2.3.2.2 Flavonoid

Flavonoid was identified by the method followed in the study of Bohm et. al., 1994 [24]. Extraction was performed first. 10 g of the plant sample was extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The whole solution was filtered through Whatman filter paper No. 42 (125 mm). The filtrate was later transferred into a crucible and evaporated to dryness over a water bath and weighed to a constant weight.

2.3.2.3 Saponin

Saponin was determination following the method reported by Obadoni et. al., 2001 [25]. The samples were ground, 20 g of each were put into a conical flask and 100 ml of 20% aqueous ethanol were added. The samples were heated over a hot water bath for 4 h with continuous stirring at about 55°C. The mixture was filtered and the residue was re-extracted again with another 200 ml 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90°C. The concentrate was transferred into a 250 ml separatory funnel and 20ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated and finally collected the aqueous sample. After evaporation the samples were dried in oven and weighed, and saponin content was calculated as percentage.

2.3.3 Physicochemical analysis

2.3.3.1 Moisture

Moisture was determined by the WHO method [26]. 1.0 g each of the respective powdered samples was weighed each on aluminium foil on the automated moisture analyser pan and set at 105°C for 3 h where moisture content percentage of the sample was obtained.

2.3.3.2 Total ash

Total ash was determined following the WHO method [26]. 2.0 g of the respective powdered samples was ignited in a previously ignited and tarred crucible at 500°C for about 3 h until the sample was white, indicating the absence of carbon, and was cooled in a desiccators and was later weighed.

2.4 Antibacterial Assay

2.4.1 Test organisms

In total, twelve strains of pathogenic bacteria including four Gram-positive bacteria - *Bacillus megaterium, Bacillus subtilis, Staphylococcus*

aureus, Sarcina lutea, and eight strains of Gram negative bacteria - *Salmonella paratyphi, Salmonella tiphi, Vivrio parahemolyticus, Vivrio mimicus, Escherichia coli, Shigella dysenteriae, Pseudomonas aureus, and Shigella boydii* were used to assess the antibacterial activity (Table 1). The strains were collected from the Microbiology Department, University of Dhaka, Bangladesh. All bacterial cultures were maintained in NA slants/ plates; stored at 37°C and periodically sub-cultured.

2.4.2 Antimicrobial assay method

The antimicrobial activity for different extracts was determined by the disc diffusion method [27]. Both gram positive and gram-negative bacterial strains were used for the test. The bacterial strains used for the investigation are listed in Table 1. Solutions of known concentration (mg/ml) of the test samples were made by dissolving measured amount of the samples in calculated volume of solvents. Dried and sterilized filter paper discs (6 mm diameter) were then impregnated with known amounts of the test substances using micropipette. Discs containing the test material were placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic discs (Kanamycin 30µg/disc) and blank discs (impregnated with solvents) were used as a positive and negative control. These plates were then incubated at 37ºC for 24 h to allow maximum growth of the organisms. The test materials having antibacterial activity inhibited the growth of the microorganisms and a clear, distinct zone of inhibition was visualized surrounding the medium. The antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in millimeter. The experiment was performed three times and the mean of the readings was calculated.

2.5 Cytotoxicity Screening

Brine-Shrimp lethality assay (BSLA) is widely used in the bioassay for the bioactive compounds by the method [28]. Here simple zoological organism (*Artemia salina*) was used as a convenient monitor for the screening. The eggs of the brine shrimp were collected and hatched in artificial seawater (3.8% NaCl solution) for 48 hr to mature shrimp called nauplii. The cytotoxicity assay was performed on brine shrimp nauplii using aforementioned Meyer method. The test samples (extract) were prepared by dissolving them in distilled water (20 mg/ml). 2.5, 5, 10, 20, 40, and 80 µl of solutions for each test sample were taken in 6 vials and 4ml of sea water was added to each vial containing 30-35 brine shrimp nauplii. So the concentrations of the test sample in the vials were 12.5, 25, 50, 100, 200, and 400 µg/ml respectively. A vial containing 50 µl dimethyl sulfoxide (DMSO) diluted to 5 ml was used as a control. Standard Gallic acid was used as positive control. Then matured shrimps were applied to each of all experimental vials and control vial. After 24 hours, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial were counted. From this data, the percentage (%) of lethality of the brine shrimp nauplii was calculated for each concentration.

2.6 Statistical Analysis

Data from the experiments were analyzed using the Statistical Package for Social Science (SPSS) software for windows version 17 (SPSS Inc., Chicago, Illinois, USA). All the triplicate data were expressed as Mean ± SD as appropriate. Statistical analysis of the results was performed by using the ANOVA (analysis of variance) followed by Bonferroni post hoc and Dunnett test. The limit of significance was set at $p<0.05$. The LC_{50} values were calculated from linear regression analysis.

3. RESULTS

3.1 Qualitative and Quantitative Phytochemical Screening

Phytochemical screening of the crude extract revealed the presence of tannins, flavonoids, saponins, gums, steroids, alkaloids, reducing sugar and terpenoids. The intensity of the component content were high in alkaloids, resin and antharaquinone glycosides, moderately present in steroids, terpenoids and others present in lower amounts (Table 1).

The results of quantitative analysis of the *T.* cordifolia were investigated and were summarized in Table 2. The results showed that the alkaloids, the flavonoids and the saponin content of methanol extract were 2.13%, 1.65% and 1.5%, correspondingly. The presence of these bases in the investigated plants accounts for their usefulness as medicinal plants.

Table 1. Phytochemical Screening of *Tinospora cordifolia* stem methanolic extract

Phytoconstituents	Observations
Alkaloid	++
Carbohydrate	+
Glycosides	++
Protein and amino acids	+
Fixed oil and fats	+
Tannins	++
Saponins	+
Steroids (phyto sterols)	++
Flavonoids	+
Phenols	++

**Symbol (++) and (+) signifies moderately and slightly present*

3.2 Physicochemical Analysis

Moisture is also essential for most of the physicochemical reactions in the plant tissues and in its absence life does not exist. Moisture and ash content of *T. cordiofolia* stems were found to be 19.6% and about 13.2% respectively.

3.3 Antimicrobial Screening

The methanol extract of the *T.* cordifolia were screened against twelve human pathogenic bacteria to check antibacterial activities by disc diffusion method which showed valuable zone of inhibition. The specific zone of inhibition against various types of pathogenic bacteria was shown in Table 3.

3.4 Brine Shrimp Lethality Assay

The lethality of the extracts of *T.* cordifolia to brine shrimp was determined on *A. salina* after 24 hours of exposure of the extracts and negative control (DMSO), positive control (gallic acid) [28]. The results of the different extracts of *T.* cordifolia (% mortality at different concentrations and LC_{50} values) were shown in Table 4. The methanol extract of *T.* cordifolia and standard showed about 96.67% and 100% mortality respectively to brine shrimp at 800 µg/ml and the percent mortality increased with an increase in concentration.The LC_{50} obtained from the best-fit line slope were found to be 292.37µg/ml and 174.16 µg/ml for methanol extract and standard respectively (Table 4).

4. DISCUSSION

Phytochemical analysis conducted on the plant extracts revealed the presence of constituents which are known to exhibit medicinal as well as physiological activities [29]. Analysis of the plant extracts revealed the presence of phytochemicals such as phenols, tannins,

Table 2. Quantitative phytochemical analysis of *T. cordiofolia* methanol extract

Tests	Result	Remark
Alkaloid	2.13%	Moderately present
Flavonoid	1.65%	Low flavonoid present
Saponin	1.5%	Low saponin present

Table 3. Antimicrobial activity of *Tinospora cordifolia* methanolic extract

Bacterial samples	Zone of Inhibition (mm)	
	(Kanamycin, 30 µg/disc)	Methanol 100% (800 ug/disc)
Gram positive Bacteria		
Bacillus megaterium	32	9
Sarcina lutea	26	10
Bacillus subtilis	36	14
Staphylococcus aureus	36	8
Gram negative Bacteria		
Salmonella paratyphi	35	10
Salmonella typhi	36	11
Vivrio parahemolyticus	32	12
Vivrio mimicus	34	14
E.coli	32	14
Shigella dysenteriae	32	14
Pseudomonas aureus	31	11
Shigella boydii	32	10

Table 4. Results of Brine Shrimp Lethality Assay on crude methanol extracts for *T. cordifolia*

Tests	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml	400 µg/ml	800 µg/ml	LC_{50} µg/ml
Gallic acid	46.67±2.72	60±4.71	73.33±2.72	80±4.71	96.67±2.72	100±0.0	174.16
T. cordifolia Extract	6.67±2.72	10±0.0	33.33±2.72	60±4.71	73.33±5.44	96.67±2.72	292.37µg
Control	0.1%	0.5%	1%	2.5%	5%	10%	0
DMSO	0	0	0	0	0	0	

**Values are expressed as % mortality±SEM at different concentrations (n=3)*

flavonoids, saponins, glycosides, steroids, terpenoids, and alkaloids. The results revealed the presence of medicinally important constituents in the plants studied. Many evidences gathered in previous studies [30-31] in which it was confirmed that the identified phytochemicals were bioactive. Several studies confirmed the presence of these phytochemicals contribute medicinal as well as physiological properties to the plants studied in the treatment of different ailments. Water activity of 0.60 is considered as the lower limits for microbial growth. Below this value of 0.55, all metabolic activities are ceased and DNA is denatured. The level of moisture in herbal products can influence the susceptibility of microbial activities on the sample. A literature revealed that less moisture kept the product microbiologically safe by preventing bacterial, fungal and yeast growth. If moisture content is very high, enzymatic activation may occur and result in loss of therapeutically active substance [32]. Ash value gave the rough idea of the minerals content in the samples.

Isolation of pure, pharmacologically active constituents from plants remains a long and tedious process. For this reason, it is necessary to have methods available which eliminate unnecessary separation procedures. Chemical screening is thus performed to allow localization and targeted isolation of new or useful constituents with potential activities. This procedure enables recognition of known metabolites in extracts or at the earliest stages of separation and is thus economically very important.

In the antimicrobial screening, the methanolic extract was found to be sensitive against almost all microorganisms. The methanolic extract of *T.* cordifolia at a concentration of 800 µg/disc showed moderate to mild activity against the entire tested microorganism. Table 1 shows the antimicrobial activity of Tinospora cordifolia of methanolic extract and kanamycin standard. The growth of *E. coli*, *Bacillus subtilis*, *Vibrio mimicus, Shigella dysenteriae* were moderately inhibited with the zone of inhibition 14 mm, while, it showed mild inhibitory activity against *S. aureus* (8 mm), *S. lutea* (10 mm), *B. megaterium* (9 mm), *S. paratyphi* (10 mm), *S. typhi* (11 mm), *S. boydii* (10 mm) and the remaining. Based on the statistical analysis, this inhibition is moderately significant than the standard broad spectrum antibiotic (30 µM of Kanamycin). These findings can be supported by the observation of Cowan et. al., 1999 that reported some antimicrobial activities of this plant [30]. From the result, regarding to the antimicrobial activity, it can be concluded that the methanol extract of stems of *T. cordifolia* possess prospective broad spectrum anti-microbial potency against the given test organisms.

The brine shrimp lethality assay (BSLA) has been used routinely in the primary screening of the extracts as well as isolated compounds to assess the toxicity towards brine shrimp, which could also provide an indication of possible cytotoxic properties of the test materials. Brine shrimp nauplii have been previously utilized in various bioassay systems. Among these applications have been the analyses of pesticidal residues, mycotoxins, stream pollutants, anesthetics, dinoflagelate toxins, morphine-like compounds, carcinogenicity of phorbol esters and toxicants in marine environment. A number of novel antitumor and pesticidal natural products have been isolated using this bioassay [28,33].

In BSLA (Table 4), the mortality rate of *nauplii* was found to increase with the increase in concentration of compound. It was observed that there was a positive correlation between brine shrimp toxicity and cytotoxicity. Moreover, this significant lethality of the crude plant extracts (LC_{50} values less than 1000 µg/mL) to brine shrimp is indicative of the presence of potent cytotoxic and probably insecticidal compounds

which warrants further investigation. BSLA results might be used to guide the researchers on which part of the plant extracts/fractions to prioritize for further fractionation and isolation of these bioactive compounds. Other cytotoxicity tests and specific bioassays may be done on the isolated bioactive compounds to our upcoming work.

5. CONCLUSION

Presence of different phytochemical constituents in the stem of *T. cordifolia* exhibited bioactivities and various medicinal properties. These novel findings would aid us to conduct bioactivity guided isolation and characterization of leading compounds in due course. Our study also indicated that the extracts of *T. cordifolia* has got profound cytotoxic and antimicrobial effects and might have potential use as medicine. Further studies are required to reveal the responsible bioactive constituents for the aforementioned activities.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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