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Research Paper

Evaluation of Antimicrobial and Antioxidant Properties of Stem Extracts of *Tinospora Cordifolia* (Amrutha balli)

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Abstract
Plants produce a diverse range of bioactive molecules, making them a rich source of different types of medicines and healing properties. *Tinospora cordifolia*, a well-known ayurvedic herb commonly known as giloy, has demonstrated multifaceted benefits, such as anti-inflammatory, analgesic, antibacterial and antioxidant properties in animal, as well as *in-vitro* studies. Hence, in the present study was aimed to evaluate the antioxidant and antimicrobial properties of methanolic stem extracts of *T. cordifolia*. Stem parts of *T. cordifolia* was subjected to successive solvent extraction by continuous hot extraction (Soxhlet) with methanol. Antioxidant assays were carried out by in vitro model using DPPH free radical scavenging activity. *In-vitro* antibacterial activity of methanolic stem extracts of *T. cordifolia* was assessed using agar well diffusion method with Chloramphenicol as positive control and Dimethyl sulfoxide (DMSO) as negative control, the zones of inhibition after 48 hours of was measured in millimeters (mm). The antimicrobial activity of methanolic extract of stem of *T. cordifolia* were also evaluated against some pathogenic microorganisms viz. *P. aeruginosa, S. aureus, K. pneumonia*, and methicillin resistant *Staphylococcus aureus* (MRSA). Results revealed that methanolic stem extract of *T. cordifolia* showed the presence of phytoactives like phenols, tannins, flavonoids, saponins, terpenoids, alkaloids, proteins, and carbohydrates; while glycosides were absent in the methanolic extract of *T. Cordifolia* stem. Stem extracts of *T. cordifolia* possess potential antibacterial activity against *P. aeruginosa, S. aureus, K. pneumonia*, and MRSA. In conclusion, this preliminary study supplies as evidence-based study for methanolic stem extracts of *T. cordifolia* could be used alternative to synthetic antioxidants and antimicrobials. However, it needs to be confirmed further with *in vivo* studies.

Keywords: *T. cordifolia*, Phytochemical screening, Antimicrobial, Antioxidant,

Introduction

Plants are one of the most important sources of medicines. They can synthesize different bioactive molecules such as phenols, flavonoids, vitamins, alkaloids, terpenoids, tannins, glycosides, quinones and many others. Most of the plants used for medicinal purposes have been identified and their uses are well documented and described by various research investigators.[1-3] The use of plants for medicinal purposes is as old as human civilization itself. The evidence of medicinal value of some plants has been observed since ancient times. These plants are still widely used as ethnomedicine around the world. Medicinal plants have been used for curing diseases in different traditional systems of medicine such as Ayurveda, Siddha, European, Tibetan, and Unani.[4]

Herbal medicine is still the mainstay of treatment in about 75%–80% of people in many developing countries for their primary health care because of better cultural acceptability and compatibility with the human body and fewer side effects.[5] Currently, one-half of the pharmaceuticals dispensed having plant origins, very few are intended for use as antimicrobials, since we have relied on bacterial and fungal sources of these activities.[6] From time immemorial, man depends on plants for medicine. There are different ways in which plants have been found useful in medicines. The parts of medicinal plants that may be used are different types of seeds, root, leaf, fruit, flowers or even the whole plant.[7]

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Tinospora cordifolia (Amrutha balli) is one of the most important plants used in indigenous system of medicine. *T. cordifolia* a medicinal plant found in the Asian subcontinent including India, Nepal, Bangladesh Malaysia etc. with great medicinal properties including antioxidants, antimicrobial, anti-diabetic, anti-ageing (Figure 1).[8] It belongs to the *Menispermaceae* family, commonly called as Guduchi in Sanskrit.[4] It is a deciduous climbing shrub with small greenish flowers, having enormous medicinal value in all its parts such as leaves, stem, and also the root.[9] It is a Rasayana (rejuvenator) and anti-aging medicine in Ayurveda, used to improve the immune system and the body resistance against infections.[10] It has also been found that *Tinospora* has antispasmodic,[11] antipyretic,[12] anti-inflammatory,[13] anticomplementary,[14] and immunomodulatory activities.[15] In addition to it, *Tinospora* has been found to exhibit antidiabetic,[16] hepatoprotective,[17] anticancer,[18] and antioxidant properties[19] as well. It has been listed as an insecticide, an antifungal agent, and an antibacterial agent.[20,21]

Figure 1: Showing *Tinospora cordifolia*

T. cordifolia is a rich source of alkaloids, furan diterpenoids, clerodane, norditerpenoids, sequiterpenoids, phenolics, lignans, sterols, aliphatic compounds, polysaccharides, essential oil, and fatty acids. In its pharmacological actions, *T. cordifolia* targets body organs, mainly kidney, liver, and spleen.[22] The genus *T. cordifolia* has been widely investigated by several workers and reported to contain several phytochemicals with marked therapeutic activity.[23,24] Free radicals form in our body as a result of biological oxidation. Oxidation is a natural process in organisms to produce energy to fuel biological cycles. Oxidation by-products of normal metabolism cause extensive damage to DNA, protein, and lipids, constituting a major contribution to ageing and to degenerative disease. Oxidative damage is associated with chronic degenerative diseases, including cancer, coronary artery disease, hypertension, and diabetes.[25]

An antioxidant is a chemical that prevents the oxidation of other chemicals. They protect the key cell components by neutralizing the damaging effects of free radicals, which are natural by-products of cell metabolism.[26] Antioxidants occur naturally in many fruits and are able to neutralize free radicals by donating an electron and convert them into harmless molecules.[27] Antimicrobial features have been found in its root, stem, and leaf extracts on pathogenic microorganisms.[28] Ethanolic and aqueous extracts of *T. cordifolia* have been successfully tested against various bacteria such as *Staphylococcus epidermidis, Escherichia coli, Aspergillus niger, Candida albicans*, and *Staphylococcus aureus*.[29] With this background, the present study was undertaken to with the main objective to assess the antioxidant and antimicrobial properties of stem extracts of *T. cordifolia*.

Materials and Methods

Collection of plant material

The stem of *T. cordifolia* were collected in and around Chikkaballapura district, Karnataka, India. The stems were sprayed with ethanol, and then shade dried at room temperature for 10 days. The dried stem were crushed to fine powder with help of electric grinder and stored in airtight containers for further analysis.

Extraction

Approximately 50 g of dried and coarsely powdered stem of *T. cordifolia* were subjected to successive solvent extraction by continuous hot extraction (Soxhlet) with 550 mL of methanol. All the extracts were concentrated by distilling the solvent in a rotary flash evaporator. The extracts were preserved in airtight containers and stored at room temperature until further use.[30.31]

Phytochemical Screening

Phytochemical screening was carried out on the methanol extracts of stem of *T. cordifolia* by using standard procedure to detect constituents as described by sofora,[32] Trease and Evans[33] and Harborne.[34]

Test for Alkaloids

Approximately 0.2g of extract was warmed with 2% H_2SO_4 (2.0 mL) for two minutes. The reaction mixture was filtered and few drops of Dragendrof's reagent was added to the filtrate. Orange red precipitation showed the presence of alkaloids moiety.

Test for Tannins and Phenolic Compounds

The stem extract of *T. cordifolia* in **s**mall quantity was mixed with water and heated on water bath and filtered. To the filtrate, few drops of ferric chloride ($FeCl_3$) was added. A dark green colouration indicate the presence of tannins.

Test for Glycosides

About 0.6g of extract was hydrolyzed with HCl and neutralized with NaOH solution and few drops of Fehling's solution A and B were added. Formation of red precipitate indicates the presence of glycosides.

Test for Reducing Sugars

The extract was shaken with distilled water and filtered. Few drops of Fehling's solution A and B were added and boiled for few minutes. Formation of an orange red precipitate confirms the presence of reducing sugar.

Test for Saponins

About 0.2g of extract was shaken with 5 mL of distilled water and then heated to boil. Frothing (appearance of creamy miss of small bubbles) showed the presence of saponins.

Test for Flavonoids

0.2 g of extract was dissolved in diluted 10%NaOH and few drops of 2M HCl was added. A yellow solution that turns into colorless indicate the presence of flavonoids.

Test for Steroids

2 mL of acetic anhydride was added to 0.5g of extract and then added 2 mL of H_2SO_4. The change of color from violet to blue or green or red showed the presence of steroids.

Test for Terpenoids

0.3g of extract was mixed with 2 mL of chloroform ($CHCl_3$) and 3 mL of concentrated 6M H_2SO_4 was carefully added to form a layer. Reddish brown coloration at the interface was formed which indicate positive results for the presence of terpenoids.

Test for Proteins and Amino acids

To the 0.3g of extract few drops of 0.2% ninhydrin solution was added and heated for 5 minutes. Blue colouration indicate the presence of proteins.

Antioxidant Assay

The modified literature protocol of Blois was used for antioxidant assay.[35,36] Briefly 2, 2-diphenyl-1-picrylhydrazyl (DPPH) solution (1mL;1mM) was prepared in methanol and mixed with sample solution (3mL, containing 20-100ug) in methanol. The control was also run which contains only methanol. The hydrogen atom or electron donation abilities of extract and standards were measured from the bleaching of the purple-colored methanol solution of 2, 2-diphenyl1-picrylhydrazyl (DPPH). The absorbance was measured at 517 nm after 30 min incubation. Decreasing of the DPPH solution absorbance indicates an increase of the DPPH radical-scavenging activity. Scavenging of free radicals by DPPH as percent radical scavenging activities (%RSA) was calculated by using the formula; DPPH% = (Control abs – Extract abs / Control) × 100. The IC_{50} value was determined by using linear regression equation *i.e.*, Y = Mx + C; Here, Y = 50, M and C values were derived from the linear graph trendline.

Evaluation of Antibacterial Activity

Pathogenic Microorganisms

The multiple antibiotic-resistant isolates viz. *Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumonia,* and methicillin resistant *Staphylococcus aureus* (MRSA) were isolated from clinical samples of local hospital in and around Chikkaballapura and confirmed by various microscopic evaluation like Gram's staining.[37] Motility, capsule and spore formation was confirmed as per the procedure prescribed by Collins and Lyne.[38] All the bacterial pathogens were further confirmed by suitable biochemical tests,[39] and used for antimicrobial activity studies.

The direct colony suspension method is the most convenient method for inoculum preparation. The inoculum was prepared by making a direct broth or saline suspension of isolated colonies selected from an agar plate. The suspension was adjusted to achieve a turbidity equivalent to a 0.5 McFarland standard. This results in a suspension containing approximately 1 to 2 × 10^8 colony-forming units (CFU)/mL. To perform this step accurately, used adequate light to visually compare the inoculum tube and the 0.5 McFarland standard against a card with a white background and contrasting black lines. Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, the pathogenic bacteria culture was inoculated into culture plates to screen for antibacterial properties.

Determination of Antibacterial Activities

Antibacterial activities of plant extracts were tested by agar well diffusion method.[40] The culture plates were prepared by pouring 20 ml of sterile Muller Hinton agar (MHA). 1 ml inoculums suspension was spread uniformly over the agar medium using a sterile glass rod to get uniform distribution of bacteria. A sterile cork borer (6 mm) was used to make wells in each plate for extracts. These plates were labeled and each plant extracts (at a concentration of 100, 50, 25, 12.5 mg/ml) were added aseptically into the well. Also, 5% DMSO and chloramphenicol (10 µg) were used as negative and positive control respectively. Plates containing drug were left for one hour in order to diffuse properly in media and to get dry. Then the plates were incubated for 24 h at 37 ºC during which the activity was evidenced by the presence of a zone of inhibition surrounding the well. Each test was repeated three times and the antibacterial activity was expressed as the mean of the diameter of the inhibition zones (mm) produced by the plant extracts when compared to the controls.

Results and Discussion

In the present study the phytochemical screening of *T. cordifolia* stem extract tested revealed that the presence of medically bioactive compounds in the stem. Stem extracts of *T. cordifolia* showed presence of phytoactives like phenols, tannins, flavonoids, saponins, terpenoids, alkaloids, proteins, and carbohydrates; while glycosides were absent in the methanolic extract of *T. Cordifolia* stem (Table 1).

Table 1. Phytochemical screening of methanolic stem extract of *T. cordifolia*

Phytochemical constituent	***T. cordifolia* (Stem)**
Alkaloids	+
Polyphenols	+
Tannins	+
Glycosides	-
Reducing Sugars	+
Saponins	+
Flavonoids	+
Steroids	+
Terpenoids	+
Proteins and amino acids	+

Antioxidant Activity

DPPH is often used to determine free radical scavenging activity of natural compounds due to its stability as a radical.[41] The presence of unpaired electron imparts a strong absorbance at 517 nm, giving the radical a purple color. With the exposure to antioxidants, it undergoes reduction, decreasing absorbance due to the formation of yellow colored anti-radical diphenyl picryl hydrazine (DPPH). The degree of colour change from purple to yellow is a measure of scavenging potential of the antioxidants in the extracts in terms of hydrogen donating ability.[42] The methanolic stem extracts of *T. cordifolia* showed scavenging activity against the free radicals. The methanolic stem extract showed the highest scavenging activity (68.37%) at 10 mg/mL and lowest (41.93%) at 2 mg/mL (Table 2). It was found that when the concentration of the extract increased, the absorbance value gets decreased regularly. Similar results were also observed by Upadhyay et al., in the methanolic stem extract of *T. cordifolia* and found that at a concentration of 2 mg/mL, DPPH free radical scavenging activity was low (44%).[43] Praveen et al. reported that methanolic stem of extract *T. cordifolia* procured from the local market of Mysore showed the highest scavenging activity.[44]

Table 2: Antioxidant activity of methanolic stem extract of *T. cordifolia*

Concentration of extract (mg/mL)	**DPPH Scavenging Activity (%)**
2	41.93
4	47.60
6	56.26
8	62.64
10	68.37

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Antibacterial Activity

The enormous heritage of vast natural, time-tested medicinal resources is worth exploring the possibility of developing efficient, economically viable, and clinically acceptable antimicrobials for human application. One among them is *T. cordifolia*, an indispensable medicinal plant, referred to in Ayurveda as "Amruth" or the "Nectar of Immortality" in recognition of its ability to impart youthfulness, vitality, and longevity. Preclinical and clinical pharmacological studies affirm the importance of its therapeutic efficacy and hence have placed it as a novel candidate to be used as the primary drug in the treatment of different ailments.[45] The antimicrobial activity of methanolic extract of *T. cordifolia* is tested against different bacterial strain. All the bacterial strain were treated with different concentration of methanol extract stem part of *T. cordifolia* (12.5 mg/mL, 25 mg/ mL, 50 mg/ mL, and 100 mg/ mL) and 5% DMSO as negative control and 10 microgram chloramphenicol as positive control. Antibacterial activities of all the four different concentrations against selected bacterial strains were recorded in the form of zone of inhibition and measured in millimeter (mm). The highest zone of inhibition (18.5 mm) was observed against MRSA and lowest zone of inhibition (14.9 mm) was observed against *P. aeruginosa* at 100 mg/mL. At 50 mg/mL the highest zone of inhibition (16.5 mm) was observed against *S. aureus* and lowest zone of inhibition (13.5 mm) was observed against *P. aeruginosa* and *K. pneumonia*. Whereas the highest zone of inhibition (11.9 mm) was seen against MRSA and lowest zone of inhibition (9.2 mm) against *K. pneumonia* at 25 mg/mL. At 12.5 mg/mL the highest zone of inhibition (11.5 mm) was observed against *S. aureus* and lowest zone of inhibition (3.8 mm) was observed against *K. pneumoniae.* The reference standard Chloramphenicol showed highest zone of inhibition (24.5 mm) against MRSA and the lowest zone of inhibition (17.9 mm) against at 10 µ/mg *K. pneumonia.* These findings revealed that the tested stem extracts of *T. cordifolia* possess potential antibacterial activity against *P. aeruginosa, S. aureus, K. pneumonia,* and MRSA as shown in Table 3.

Table 3: Antibacterial activities of methanolic stem extract of *T. cordifolia*

Bacterial strain	**Zone of inhibition(mm)**					
			Methanolic stem extract of *T. cordifolia*			
	Negative Control	**Positive Control**	**12.5 mg/mL**	**25 mg/mL**	**50 mg/mL**	**100 mg/mL**
P. aeruginosa	-	19.5	9.5	11.2	13.5	14.9
S. aureus	-	21.2	11.5	13.2	16.5	18.2
K. pneumonia	-	17.9	3.8	9.2	13.5	15.2
MRSA	-	24.5	9.5	11.9	15.5	18.5

The results of antibacterial activities of methanolic extracts of stem part of *T. cordifolia* exhibited in our study were comparable with the previous studies reported in the literature against *E. coli, S. aureus, Pseudomonas aeruginosa,* and *Bacillus subtilis*.[46-48] Some studies found that the maximum antimicrobial property was exhibited against *S. aureus*.[49] The antimicrobial property of *T. cordifolia* against bacteria could be attributed to the secondary

metabolites and the phytochemicals *viz.* quinones, polyphenols, alkaloids, flavonoids, tannins, coumarins, terpenoids, lectins, and polypeptides present in it.[50,51]

Quinones and flavonoids bind to adhesins form complexes with cell wall and inactivate bacterial enzymes. While, terpenoids, polyphenols, and tannins cause membrane disruption and form metal ion complexes, and thus inactivating the bacteria.[52] In the present study, chloramphenicol was found to be more effective and exhibited better antimicrobial properties against all tested bacteria, as compared to *T. cordifolia* stem extract. On the contrary, *Tinospora* is abundantly available, easily accessible, economically feasible, and culturally acceptable and may possess minimal side-effects.

Conclusion

In conclusion results of present study delineated that methanolic stem extracts of *T. cordifolia* exhibited effective antioxidant and antibacterial properties. These biological activities of stem extract of *T. cordifolia* could attributable to phytoactives like phenols, tannins, flavonoids, saponins, terpenoids, alkaloids, proteins, and carbohydrates present in it. Therefore, this preliminary study supplies as evidence-based study for methanolic stem extracts of *T. cordifolia* could be used alternative to synthetic antioxidants and antimicrobials since methanolic stem extracts of *T. cordifolia* mimic the biological activities of synthetic antioxidant and antimicrobials. However, dosage and safety & toxicity studies are recommended to carry out *in-vivo* for successful therapeutic modality.

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