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Phytochemical and Anti-bacterial Activity of *Toddalia asiatica*: A Wild Nutraceutical

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Abstract

Toddalia asiatica (Rutaceae) a woody liana is used traditionally in the treatment of malaria, sprains, cough, fever, neuralgia, epilepsy, dyspepsia, and other disease conditions. A wide range of chemical constituents are found in leaf extracts such as tannin, alkaloids, flavonoids, terpenoids and phenolic compounds and in fruit extract, phytoconstituents such as tannin, saponin, phenolic compounds, and reducing sugar are reportedly present. In recent years, drug resistance to human pathogenic bacteria has been commonly reported from all over the world. In the present scenario of emergence of multiple drug resistance to human pathogenic organisms, this has necessitated a search for new antimicrobial substances from other sources including plants. It was investigated that the leaf and fruit extract of *T. asiatica* showed potential antibacterial activity against human pathogens such as *Streptococcus mutans*, *Streptococcus pyogenes*, *Vibrio cholera,* and *Shigella flexneri*. This study could be helpful to develop antibacterial agent against the tested strains which will help to formulate a new drug effective against these pathogens.

Keywords: Anti-bacterial activity, Future drug, Phytochemistry, *Toddalia asiatica*

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Introduction

Toddalia asiatica (L) Lam. belongs to the family Rutaceae (Syn: *Paullinia asiatica* L., *Toddalia aculeata* Pers.) is a medicinal plant commonly known as Orange climber, distributed in many parts of India.[1,2] This plant is recognized by many local names in different languages such as Chikafusi (Shona), Tunda poda (Odia), Dahan (Marathi), Gato (Shona), Rukato (Shona), Kanj (Hindi) and Jangli mirch (Bengali) etc.[2,3] It has a variable rambling, prickely, sarmentose shrub, ascending to an altitude of 2,500 m. In south India, the plant is very common in the Nilgiri and Palani hills and also in the scrubby jungles of Odisha. In the plains, particularly in dry situations, the plant assumes the form of a low shrub with smaller and narrower leaflets.[4]

T. asiatica is a rambling or scandent very prickly shrub, stem up to 15 m high and 10 cm in diameter, bark of the plant is pale brown, leaves are alternate, 3-foliolate, petiole 2–3 cm long, leaflet sessile, 5–10 cm × 1.8–3.8 cm, obovate-oblong or oblong, crenulate, shortly blunt-acuminate, base acute, glabrous, and dark shining green above with many slender parallel nerves. Inflorescences with male flowers corymbose panicles, with female flowers cymose panicles, bract scale like. Flowers white or yellowish, small, 5 mm diameter, stamen 4 or 5, ovary 4 or 5 locular, berry orange, 7.5–12 mm diameter, 3–5 grooved or hardly lobed, seeds dark brown.[2,5]

It is known as "Milagarani" in Siddha system of medicine and also known as "Kanchana" in Ayurveda.[6] The fruit is traditionally used to treat malaria and coughs; its roots has been used to treat indigestion and influenza and the leaves for lung diseases and rheumatism.[7-9] Tribal people used this plant for multiple applications such as stomach problems, fever, cough, and cold. It is also used in the treatment of various ailments such as rheumatic arthritis, sprains, bronchitis, nausea, diarrhea, and chest pain.[1] It is also used traditionally in Kenya by many communities for the treatment of malaria, toothaches, as well as nasal and bronchial pains.[10] Leaves has been utilized traditionally for the cure of diabetes.[11] Unripe fruits and roots are rubbed with oil to prepare a stimulant liniment for treatment of rheumatism. The flower juice is applied to the stings of wasp for having immediate relief.[3] The plant has also been reported that the root and duramen of *T. asiatica* are mainly rich in coumarins, triterpenoids and alkaloids.[12,13]

The general objective of our study was to determine the pharmacological feature of *T. asiatica* and systematic attempt to analyse anti-bacterial activity of leaves and fruits of *T. asiatica* on selected bacterial strains.

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Materials and Methods

Collection of Plant for Experimental Work

The sample were collected and kept in poly bags tagged with the botanical name and sorted out as per standard sampling procedure and passport description.[14]

Preparation of Extracts

Soxhlet method and percolation were adopted to obtain different extracts;[15,16] The collected experimental plant materials were dried at room temperature under shade and were powdered after drying using mechanical devices. The powdered material of the experimental plant was kept in thimble and extraction was carried out using the Soxhlet apparatus. The residues were collected and left for air drying and dried crude extracts were stored in refrigerator for further phytochemical analysis and antibacterial activities.

Phytochemical Assays

Phytochemical analysis was carried out on different solvent extract of the leaves and fruits (*T. asiatica*) using standard procedure to identify the bioactive compounds.[15,17,18]

Test for Tannin

Five ml of plant extract was added with 5 drops of 10% lead acetate. Formation of a light-yellow precipitate indicates the presence of tannin.

Test for Saponin

One ml of the extract was boiled in 10 ml of distilled water and filtered with Whatman 42 filter paper. 5 ml of filtrate was mixed with 2 ml of normal distilled water and shaken vigorously. Occurrence of stable persistent froth indicates the presence of saponin.

Test for Flavonoids

To 1 ml of the extract, few drops of dilute sodium hydroxide were added. Presence of flavonoids is indicated upon production of an intense yellow colour in the plant extract which became colourless on addition of 2–3 drops of 50% dilute acid.

Test for Terpenoid

0.5 g of plant extract was mixed with 2 ml of chloroform and equal volume of concentrated sulphuric acid was added. Terpenoids presence is confirmed by a reddish-brown colouration of interface.

Test for Phenolic compounds

Two ml of plant extract was added with 5 drops of 1% ferric chloride and 1 ml of potassium ferro cyanide, a bluish-green solution showed the presence of phenolic compound.

Test for Reducing Sugar

0.5 g of plant extract was dissolved with distilled water and filtered. The filtrate was boiled with 2 drops of Fehling's solution A and B for 5 min. An orange-red precipitate obtained indicates the presence of reducing sugar.

Test for Steroid

2 ml of plant extract was dissolved in 5 ml chloroform and then 5 ml of concentrated sulphuric acid was added. Formation of two phases (upper red and lower yellow with green fluorescence) indicates the presence of steroid.

Test for Alkaloids

5 ml of plant extract was mixed with 3 ml of aqueous HCL on water bath and then filtered. 1 ml of Dragendorff's was added in the filtrate. The occurrence of an orange-red precipitate indicates the presence of alkaloids in the sample extract.

Test for Carbonyl

2 ml of plant extract was added with 2 drops of 2, 4-dinitrophenyl hydrazine solution and thoroughly shaken, yellow crystal formation indicates presence of carbonyl.

Antibacterial Activity

The leaves and fruit extracts of experimental plant were screened for antibacterial activity against two Gram-positive bacteria *Streptococcus mutans* (Microbial Type Culture Collection [MTCC] 497) and *Streptococcus pyogenes* (MTCC 1926); three Gram-negative bacteria *Vibrio cholerae* (MTCC 3906), *Shigella flexneri* (MTCC 1457). All used MTCC bacterial strains were collected from the Institute of Microbial Technology, Chandigarh. Antibacterial activity was done using slight modification of standard methods of agar well diffusion assay;[19] disc diffusion method[20] and broth dilution assay.[21]

Agar Well Diffusion Assay

Agar well diffusion method[19] was followed to test the antibacterial activity of extracts of experimental plant parts against the five bacterial strains. Nutrient agar plates were prepared as per manufacturer's instructions. 100 µl of nutrient broth cultures of the test microbes prepared a day before were poured over the plates uniformly and a lawn culture was prepared using a sterile spreader in a laminar hood. Wells (6 mm) were made using sterile borer. Stock solutions of samples were prepared in 100 % DMSO (Sigma) and twofold serial dilutions were made in amount of 100 µl per well at concentration of 0.25 and 0.5 mg/ml. 100 µl of samples were added by sterile syringes into the wells in three above-mentioned concentration and allowed to diffuse at room temperature for 2 h. Plates were incubated at 35 ± 2°C for 18-24 h. Kanamycin served as standard antibiotics control. Triplicates were maintained and the experiment was repeated thrice. For each replicates the readings (diameter of zone of inhibition in cm) were taken and the mean ± SD values (diameter of zone of inhibition) were recorded.

Disc Diffusion Assay

Antibacterial activity using disc diffusion assay was done using the 6 mm of disc prepared from Whatman filter paper.[20] Each extract was dissolved in dimethyl sulfoxide. The sets of dilutions (10 µg/disc and 50 µg/disc) of crude extracts and standard drugs were prepared. 6 mm of discs were kept in the drugs for 12 h before placing to the agar plates. The zones of growth inhibition around the disks were measured after 18–24 h of in incubation at 37°C for bacteria. The sensitivities of the microbial species to the plant extracts were determined by measuring the sizes of inhibition zones (including the diameter of disc) on the agar surface around the discs, and values <8 mm were considered as not active against microorganisms.

Minimum Inhibitory Concentration (MIC) using Broth Dilution Assay

All the extracts of the experimental plant were screened for their antibacterial activity (modified method of Rai *et al*. 2010). Antibacterial activity was assessed by MIC by serial dilution method. Selected colonies of aforesaid bacteria were picked off to a fresh isolation plate and inoculated in corresponding tubes containing 5 ml of trypticase soya broth. The broth was incubated for 8 ± 1 h at 35 ± 2 °C until there was visible growth. Mc Farland No.5 standard and Phosphate Buffer Saline were used to adjust the turbidity to get 105 cfu/mL.

Data Interpretation

After the incubation, the tubes showing no visible growth after 8 h till 12 h were considered to be inhibition of bacteria which represent MIC values of a respective concentration. Inoculum control showed visible growth due to no antimicrobial agents, whereas the broth control showed no growth due to the absence of bacteria. Triplicates were maintained and the experiment was repeated thrice, for each replicate. The readings were taken as foresaid.

Results

The present study is based on both field and laboratory work. The crude powder of the collected experimental plant (*T. asiatica*) was extracted using Soxhlet method. The extracts were used in the qualitative analysis of secondary metabolites, antimicrobial activity using Agar Well Diffusion, disc diffusion, and MIC assay.

Phytochemical Screening

An attempt has been made to evaluate the presence of bioactive compounds in the experimental plant through qualitative tests. The different extracts of *T. asiatica* were analyzed to study the presence of secondary metabolites. The phytochemical screening revealed the presence of diverse secondary metabolites which are of high pharmacological significance. The common secondary metabolites present in leaf extracts are tannin, alkaloids, flavonoids, terpenoids, and phenolic compounds and in fruit extract, we found tannin, saponin, phenolic compounds, reducing sugar, etc. [Tables 1 and 2].

Evaluation of Antimicrobial Activity

Agar well diffusion

Antimicrobial activity of different extracts of the experimental plant (*T. asiatica*) was determined against two Gram-positive bacteria *S. mutans* (MTCC 497) and *S. pyogenes* (MTCC 1926); two Gram-negative bacteria *V. cholerae* (MTCC 3906), *S. flexneri* (MTCC 1457). The results revealed that the aqueous and methanolic extract of leaf showed highest zone of inhibition against *V. cholerae* and *S. flexneri*. Fruit extracts also showed the highest zone of inhibition against *V. cholerae* and *S. flexneri* [Table 3].

Disc diffusion assay

Disc diffusion assay was carried out and it was examined that there was zone of inhibition for aqueous and methanol extract for all the pathogens. The results revealed that the aqueous and methanolic extract of leaf showed highest zone of inhibition against *V. cholerae* and *S. flexneri*. Fruit extracts also showed highest zone of inhibition against *V. cholerae* and *S. flexneri* [Table 4].

Broth dilution assay

Broth dilution was carried out using different extracts to determine the MIC values of all the extracts against *S. mutans*, *S. pyogenes*, *V. cholerae* and *S. flexneri*. The result showed that the aqueous extract of leaf showed MIC at 250 mg/ml. *V. cholerae*, *S. flexneri, S. mutans* and *S. pyogenes* at 300 mg/ml. Methanolic extract of leaf showed MIC at 400 mg/ml for *V. cholerae* and *S. flexneri* and 450 mg/ml for *S. mutans* and *S. pyogenes.* The aqueous extract of fruit showed MIC at 250 mg/ml against *V. cholerae* and 500 mg/ml for *S. mutans* and *S. pyogenes*. For all tested pathogens MIC of methanolic extract of fruit is 500 mg/ml [Table 5].

Discussion

The pharmacological value of secondary metabolites from the plants is increasing due to the constant discovery of their potential roles in health care and lead chemicals for new drug development. Plant synthesized many compounds with complex molecular structures, as a result of secondary metabolism. Some of the compounds and their derivatives such as alkaloids, flavonoids, tannins, terpenes, and phenolic compounds have antimicrobial properties against many pathogens. In 2008 Rajkumar *et al*. reported that the essential oil of the leaves was studied for its antimicrobial activities by disc diffusion method and was found to be effective against *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella*.[2] Bangajavalli in 2019 reported the presence of carbohydrates, saponins, flavonoids, alkaloids, cardiac glycosides, phenols, coumarins, and steroids have been confirmed in the extracts of the *T. asiatica*. Moreover, phytochemicals such as alkaloids, flavonoids, cardiac glycosides, coumarins, and steroids are also present in the sample. These secondary metabolites contribute significantly

Table 1: Phytochemical screening of leaf extract of *T. asiatica*

Plant Name	*Bioactive Compounds*	*Solvents*					
		n-hexane	*Petroleum ether*	*Methanol*	*Aqueous*	*Acetone*	*Ethanol*
T. asiatica (Leaf extract)	Tannin	-	++	+++	+++	+++	++
	Saponin	-	-	++	++	-	-
	Flavonoids	-	++	+++	++	++	-
	Terpenoids	-	++	++	+++	-	-
	Phenolic compounds	++	+++	+++	+++	+++	+++
	Reducing sugar	-	-	++	++	-	-
	Steroid	-	-	-	-	-	-
	Alkaloids	-	+	+++	+++	++	+
	Carbonyl	-	-	-	-	-	-

T. asiatica: *Toddalia asiatica*

Table 2: Phytochemical screening of fruit extract of *T. asiatica*

Plant Name	*Bioactive Compounds*	*Solvents*					
		n-hexane	*Petroleum ether*	*Methanol*	*Aqueous*	*Acetone*	*Ethanol*
T. asiatica (Fruit extract)	Tannin	-	++	+++	+++	+++	++
	Saponin	-	+	+++	+++	++	-
	Flavonoids	-	-	++	++	-	-
	Terpenoids	-	++	++	+++	-	-
	Phenolic compounds	++	+++	+++	+++	+++	+++
	Reducing sugar	-	-	+++	+++	++	-
	Steroid	-	-	-	-	-	-
	Alkaloids	-	-	++	++	+	-
	Carbonyl	-	-	-	-	-	-

+++: High concentration, ++: Mild concentration, +: Low concentration. *T. asiatica*: *Toddalia asiatica*.

Table 3: Antibacterial activity of aqueous and methanolic extract of leaf and fruit of *Toddalia asiatica* using Agar Well Diffusion method

Extracts	*Zone of Inhibition (mm)*			
(100 mg/ml)	*Streptococcus mutans*	*Streptococcus pyogenes*	*Vibrio cholera*	*Shigella flexneri*
Aqueous				
Leaf	0.9	0.9	1.1	1.2
Fruit	0.7	0.7	0.9	0.9
Methanol				
Leaf	0.9	1.0	1.2	1.1
Fruit	0.8	0.9	1.0	1.0

Table 4: Antibacterial activity of aqueous and methanolic extract of leaf and fruit of *Toddalia asiatica* using Disc Diffusion method

Extracts	*Zone of Inhibition (mm)*			
(100 mg/ml)	*Streptococcus mutans*	*Streptococcus pyogenes*	*Vibrio cholera*	*Shigella flexneri*
Aqueous				
Leaf	0.8	0.8	0.9	0.9
Fruit	0.7	0.8	1.0	1.0
Methanol				
Leaf	1.0	1.0	1.1	1.0
Fruit	0.9	0.9	1.0	1.0

Table 5: Broth dilution assay aqueous and methanolic extract of leaf and fruit of *Toddalia asiatica*

Extracts	*MIC*			
(50–500 mg/ml)	*Streptococcus mutans*	*Streptococcus pyogenes*	*Vibrio cholera*	*Shigella flexneri*
Aqueous				
Leaf	300	300	250	250
Fruit	500	500	250	500
Methanol				
Leaf	450	450	400	400
Fruit	500	500	500	500
Inoculum	Growth	Growth	Growth	Growth
Broth	No growth	No growth	No growth	No growth

MIC: Minimum inhibitory concentration

towards the biological activities of medicinal plants such as hypoglycemic, antidiabetic, antioxidant, antimicrobial, anti-inflammatory, anticarcinogenic, antimalarial, anticholinergic, antileprosy activities, etc.[4] Alagaraj and Muthukrishnan in 2020 found the presence of alkaloids, flavonoids, saponins, steroids, glycosides, tannins in the methanolic stem extract of *T. asiatica* and the methanolic extract of this plant also shows the antibacterial activity against various organisms as well as the zone of inhibition.[22]

Conclusion

It was investigated that *T. asiatica* showed potential antibacterial activity against human pathogens like *S. mutans*, *S. pyogenes*, *V. cholerae* and *S. flexneri*. Other investigations are necessary to be done on a wide range of bacteria and fungi to assess the spectrum of such plant parts extracts. Moreover, other parts of the examined plants are also needed to be assessed for their antibacterial activity. Further studies on isolation and chemical structure determination of active compounds from these extracts are necessary for their utilization to treat infections caused by pathogenic and often multidrug resistant bacteria.

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