Research Article

Phytochemical Screening, Antioxidant and Antimicrobial Activity of the Ethanolic leaf Extract of *Tinospora crispa* (L.) Miers [Menispermaceae]

J Akila Shree*, C Krishnaveni,

Department of Botany, Bharathiar University, Coimbatore, Tamil Nadu, INDIA.

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ABSTRACT

The qualitative phytochemical analysis showed the presence of alkaloids, glycosides, flavonoids, tannins and phenols in the ethanol leaf extracts. The ethanol leaf extract showed highest tannin, phenol and flavonoid content that the other extracts such as hexane and water in the phytochemical analysis. *In vitro* antioxidant analysis showed that the ethanol leaf extract shows moderately good free radical scavenging activity when compared with standard Ascorbic acid. The *in vitro* antimicrobial analysis showed good zone of inhibition against most of the tested microorganisms in the concentration dependent manner. The present study showed that the ethanol extract of *Tinospora crispa* leaves has significant number of phytochemicals, antioxidant and antimicrobial activity. Many biologically active secondary metabolites were present in the leaf extract which could be responsible for its therapeutic potential.

Keywords: *Tinospora crispa*, Ethanolic leaf extract, Antioxidant, Anti-microbial, Ethnomedicinal plant.

Correspondence:
Miss. J AkilaShree,
Bharathiar University,
Coimbatore-641046,
Tamil Nadu, INDIA.

Email id: akilashree-jagadeesh@gmail.com

INTRODUCTION

Drugs which are obtained from the plants are easily available, less expensive, safe, and efficient and rarely have side effects. The plants that have been selected for medicinal purposes over thousands of years constitute the most obvious choice of examining the current search for therapeutically effective new drugs such as anticancer drugs,[1] antimicrobial drugs,[2] and antihepatotoxic compounds. According to the World Health Organization (WHO), medicinal plants will be the best source to obtain various drugs. About 80% of individuals from developed countries uses traditional medicines, which has chemical compounds derived from medicinal plants. However, such plants should be investigated for better understanding of their properties, safety, and efficiency.[3]

Medicinal plants contain some organic compounds which provide definite physiological action on the human body and these bioactive substances include tannins, carbohydrates, terpenoids, steroids, alkaloids, and flavonoids.[4,5] These compounds are synthesized by primary or secondary metabolism of living organisms. Secondary metabolites are chemically and taxonomically extremely diverse compounds with obscure function. They are widely used in the human therapy, veterinary, agriculture, scientific research and other fields.[6] A large number of phytochemicals belonging to several chemical classes have been shown to have inhibitory effects on all types of micro-organisms *in vitro*.[7]

Plant products have been part of phytomedicines since time immemorial. This can be derived from barks, roots, flowers, leaves, fruits, seeds.[8] Knowledge of the chemical constituents of plants is desirable because such information will be value for synthesis of complex chemical substances.[9-11]

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Tinospora crispa, a herbaceous climber, has been widely used in traditional medicine for treating various ailments such as contusion, fever, fracture, septicaemia, scabies and other tropical ulcers. A wide range of secondary metabolites such as alkaloids, phenolics, diterpenes, flavones, and triterpenes have been isolated, some of which have also shown corresponding biological activities.[12] It is also known as "akar patawali" or "akar seruntum" in Malaysia and "Borapet" in Thai and "Da ye ruan jin teng" in Chinese, is a herbaceous climbing plant that is widely distributed in South East Asia, particularly in Vietnam, Thailand, Malaysia, Indonesia and India.[13-17] This medicinal herb has been used in the Thai traditional medicine due to its anti-pyretic, anti-inflammatory, antidiabetic, anti-malarial and health maintaining properties.[18] It is also used in Chinese traditional medicine for the treatment of contusion, septicaemia, fracture, scabies, fever, and other tropical ulcer related disorders.[17,19] Oral administration of the decoction of the stem of the plant is claimed to possess anti-malarial and anti-anthelmintic properties whereas decoction of the whole plant has been used as a traditional remedy for diabetes among the Malay community.[15,20-24] A poultice of fresh leaves is reported for treatment of wounds and itches.[25,26] Brotowali is widely known as traditional medicine as antidiabetic agent and antioxidant that accelerate wound healing process.[27]

The aerial parts are widely used in Thailand, Indonesia and Malaysia as a bitter tonic for the treatment of jaundice, rheumatism, gout, leprosy, urinary disorders, and fever.[28,29] *Tinospora crispa* contains flavonoids and terpenoids which can help to control blood glucose level and accelerate wound healing.[30] This study focus mainly on the phytochemicals present in the ethanol leaf extract and to know their antioxidant and antimicrobial activity.

MATERIALS AND METHODS

Collection and Processing of plant materials

The fresh leaves and stem of *Tinospora crispa* were collected from Ettumanoor, Kerala. The Plant material was identified and authenticated by the Botanical Survey of India, Coimbatore.

The leaves and stem were cleaned to remove unwanted debris, washed under running tap water, air-dried in shade, and powdered using a mixer grinder. The powder was further passed through a 2 mm sieve to obtain finer particles. The powdered samples were further stored in a clean glassware container for future use.

Preparation of plant extracts

20 g of powdered stem material of *Tinospora crispa*., was dispersed in 150 ml of solvents such as hexane, ethanol, and water and subjected to soxhlet extraction for 6-8 hr. The mixture was evaporated to dryness in a rotary flash evaporator and stored in a refrigerator. The condensed extracts were made to 1mg/ml dilution and were used for further phytochemical and pharmacological studies.

Qualitative phytochemical assay

Qualitative phytochemical screening of the stem was carried out with three different solvents as Hexane, Ethanol, and water in order to analyze the class of organic compounds. The extracts of the *Tinospora crispa* stem were analyzed by standard chemical tests (Patil *et al.* 2013) to determine steroids and triterpenoids, alkaloids, flavonoids, tannins, carbohydrates, glycosides, amino acids and proteins.[31]

In vitro Antioxidant Activity

Oxidation is one of the most important processes, that produce free radicals in food, chemicals and even in living systems. Free radicals have a vital role in processes such as food spoilage, chemical materials degradation and also contribute various disorders in humans.

Antioxidants are defined as substances that even at low concentration significantly prevent oxidation of easily oxidizable substrates. The applications of antioxidants are industrially widespread in order to prevent polymers oxidative degradation, auto oxidation of fats, synthetic and natural pigments discoloration, etc. There is an increased interest in using antioxidants for medicinal purposes in the recent years.

DPPH free radical scavenging activity

Preparation of standard solution

Required quantity of ascorbic acid was dissolved in petroleum ether, chloroform, ethyl acetate, ethanol and water to give the concentration of 10, 20, 30, 40, and 50 µg/ml.[32]

Preparation of test sample

Stock solution of samples were prepared by dissolving 10 mg of dried leaf and stem bark extract in 10 ml of petroleum ether, ethyl acetate, ethanol, chloroform, and water to give concentration of 1mg/ml.

Preparation of DPPH solution

4.3 mg of DPPH was dissolved in 3.3 ml petroleum ether, ethyl acetate, chloroform, ethanol and water and it was protected from light by covering the test tubes with aluminium foil.

Protocol for estimation of DPPH scavenging activity

- 150 µl DPPH solution was added to 3 ml of petroleum ether, chloroform, ethyl acetate, ethanol and water extracts and absorbance was taken immediately at 516 nm for control reading.
- Different volume levels of test sample (20, 40, 60, 80 and 100 µl) were screened and made 200 µl of each dose level by dilution with 3 ml petroleum ether, chloroform, ethyl acetate, ethanol and water
- 150 µl DPPH solution was added to each test tube.
- Absorbance was taken at 516 nm in UV-visible spectrophotometer (Shimadzu, UV1700, Japan) after 15 min using methanol as a blank.

The % reduction and IC_{50} were calculated as follows

- The free radical scavenging activity (FRSA) (% antiradical activity) was calculated using the following equation:

$$\% \text{ Antiradical activity} = \text{Control absorbance} - \text{Sample absorbance} \times 100$$

Control absorbance

Antimicrobial activity

In the recent years, there has been an increasing awareness about the importance of medicinal plants. Drugs from these plants are easily available, efficient, inexpensive, safe and rarely accompanied by side effects. Plants that have been selected for medical use over thousands of years constitute the most obvious starting point for new therapeutically effective drugs such as anticancer drugs and antimicrobial drugs. Recently, medicinal plants usage has been increased than the field of chemotherapy. The medicinal plants have been used as materials for the extraction of active pharmacological agents.

Screening of Antimicrobial Activity

Microbes tested

Antimicrobial activity of extracts was tested against two gram-positive bacteria such as *Staphylococcus aureus* (MT126466), *Bacillus cereus* (MF671985) two-gram negative bacteria such as *Escharichia coli* (MN700632), *Salmonella typhi* (KY787190). Antifungal activity was determined against two fungal pathogens *Aspergillus niger* (MK720633), *Aspergillus flavus* (MH174075). These isolates were obtained from the Centre for Bioscience and Nanoscience Research, Coimbatore, Tamil Nadu, India. The stock cultures of bacteria were maintained on Nutrient Agar slants and fungi on Potato Dextrose Agar slants and stored at 4°C.

Preparation of inoculum

Stock cultures were maintained at 4°C on slopes of Nutrient agar. Composition of Nutrient agar medium for bacterial culture are Peptone – 5 g, beef extract - 3 g, Sodium chloride - 5 g, Agar- 15 g, Distilled water- 1000 ml, pH (at 25°C) at 7.4± 0.2. Active cultures of experiments were prepared by transferring a loopful of cells from the stock cultures to test tube of Nutrient Agar medium for bacteria that were incubated without agitation for 24 hr at 37°C. Potato Dextrose Agar medium was used for fungal cultures. Composition of PDA medium for fungal culture are Potato- 200 g, Dextrose 20 g, Agar- 20 g and Distilled water- 1000 ml, pH 5.6. Active cultures of experiments were prepared by transferring a loopful of cells from the stock cultures to test tube of PDA medium for fungal that were incubated. Chloramphenicol acts as a selective agent to inhibit bacterial overgrowth of competing microorganisms from mixed specimens, while permitting the selective isolation of fungi.

Agar well diffusion method (Olurinola, 1996)

Agar well diffusion method was used to screen the antibacterial and antifungal activities of leaves and stem of *Tinospora crispa* using different solvent extracts such as Hexane, ethanol and water. One ml of fresh bacterial or fungi culture was pipetted in the center of sterile petri dish. NA for bacteria and PDA for fungi poured into the petri dish containing the inoculum and mixed well. Upon solidification, well were made using a sterile cork borer into agar plates containing inoculums.

Then, 10, 20, 30µl[33,34] of each extract (20% w/v) was added to respective wells. The concentration of extracts (20% w/v) has been selected based on our previous literature. Then, the plates were incubated at 37°C for 18-24 hr for bacterial pathogens and 27°C for 48 hr for fungal pathogens. The diameter (mm) clear zone of inhibition was recorded and the experiment was repeated thrice for each replicates the reading was taken in three different fixed direction and average values were observed. Standard fluconazole 5 µg was used as positive control for fungi. Amoxyclav 5 µg was used as positive control for bacteria. DMSO at a concentration of 10% was employed as a negative control.

RESULTS

Qualitative phytochemical analysis

The qualitative phytochemical analysis showed the presence of glycosides, terpenoids, resins and carbohydrates in the hexane extract. The ethanol extract showed the presence of most of the primary

Table 1: Details of Phytoconstituents present in the leaf extract of *Tinospora crispa*.

Phytochemicals	Tests	Leaf		
		Hexane	Ethanol	Water
Alkaloids	Mayer's reagent test	-	+	-
	Wagner's reagent test	-	+	+++
	Dragendorff reagent test	-	+	-
Flavonoids	Alkaline reagent test	-	+	+
	Lead acetate test	-	+	-
	Shinoda's test	-	+	+
Glycosides	Keller-Kiliani test	+	+++	++
	Conc. Sulphuric acid test	+	+	-
	Molisch's reagent test	+	+	-
Phenols	Ferric chloride test	-	+	-
	Gelatin test	-	+	-
Steroids	Chloroform test	-	+	-
Terpenoids	Chloroform test	+	-	-
	Lead acetate test	-	+	+
Saponins	Foam test	-	+	+
Quinones		-	+	+
Anthroquinone	Borntrager's test	-	-	+
Resin		+	+	+
Carbohydrates	Molisch's reagent test	+	+	-
	Fehling's reagent test	-	+	+
	Benedict's reagent test	-	+	+
Proteins	Biuret's test	-	+++	-
	Ninhydrin test	-	-	+
	Sulphur test	-	+	-
Lipids	Solubility test	+	+	-

and secondary metabolites viz. alkaloids, flavonoids, glycosides, phenols, terpenoids, tannins, etc. Flavonoids, glycosides, tannins and phenols were present in aqueous extract (Table 1).

In vitro antioxidant analysis

DPPH free radical scavenging activity

The ethanol plant extract showed a concentration-dependent increase in the DPPH free radical scavenging activity. The highest concentration of the plant extract showed good antioxidant activity when compared with the standard Ascorbic acid. The DPPH scavenging potential of the extract and the std. Ascorbic acid were 52.75±0.19 and 82.01±0.20% at 100µg/ml concentration respectively (Table 2, Figure 1,2,3).

Table 2: Antioxidant capacity of ethanol leaf extract of *Tinospora crispa*.

Sl. no.	Concentration (µg/ml)	% RSA	
		Extract	Ascorbic acid
1	20	30.28±0.25	58.16±0.15
2	40	34.71±0.25	64.82±0.19
3	60	43.44±0.35	69.25±0.11
4	80	49.02±0.20	74.71±0.15
5	100	52.75±0.19	82.01±0.20

In vitro antimicrobial activity

Agar well diffusion assay

The results of antimicrobial analysis were concentration dependent. The ethanol leaf extract showed very high antibacterial activity against gram positive bacteria *Bacillus aureus* with the zone of inhibition of 20.3±0.88mm and lowest activity was observed against gram positive bacteria *Bacillus cereus* with 13±1.15mm. Lowest antifungal activity was observed against *Aspergillus niger* with the zone of inhibition of 14±1.15mm and highest activity against *Aspergillus flavus* with 16±0.57mm (Table 3, Figure 4).

DISCUSSION

The medicinal plants are usedl for healing as well as for curing human diseases as they contain various phytochemical constituents.[35] Phytochemical constituents are naturally present in the medicinal plant parts such as leaves, vegetables and roots which have defense mechanism and protect the plants from various diseases. Phytochemicals are both primary and secondary compounds. Chlorophyll, proteins and common sugars are included in primary constituents and secondary compounds have alkaloids, terpenoid and phenolic compounds.[36] The ethanolic extract of *T. crispa* showed the presence alkaloids, flavonoids, glycosides, phenols, terpenoids, tannins, etc. whereas, aqueous extract shows the presence of flavonoids, glycosides, phenols and tannins.

Alkaloids are present in Punica granatum, Psidium gujauva, Morus nigra and Prunus persica. Plants containing alkaloids are used as medicines for reducing

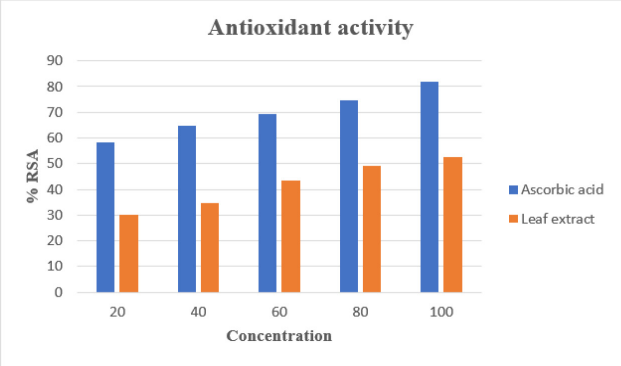

Figure 1: Bar graph showing % RSA of ethanol extract and ascorbic acid against DPPH.

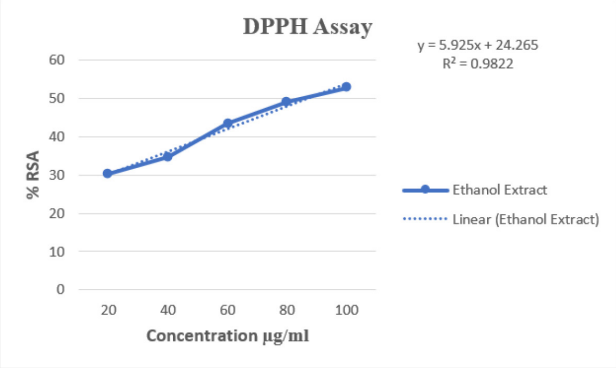

Figure 2: DPPH free radicle scavenging activity of ethanol leaf extract of *Tinospora crispa*.

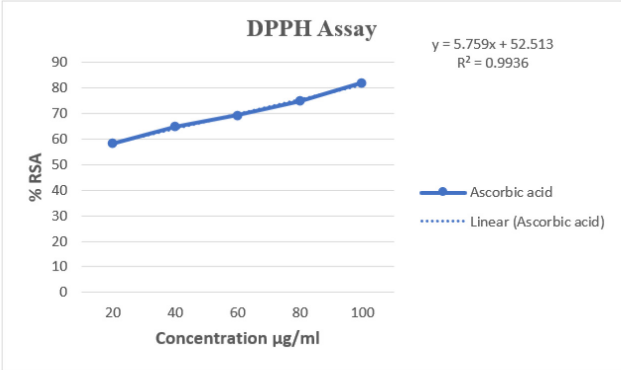

Figure 3: DPPH free radicle scavenging activity of Ascorbic acid.

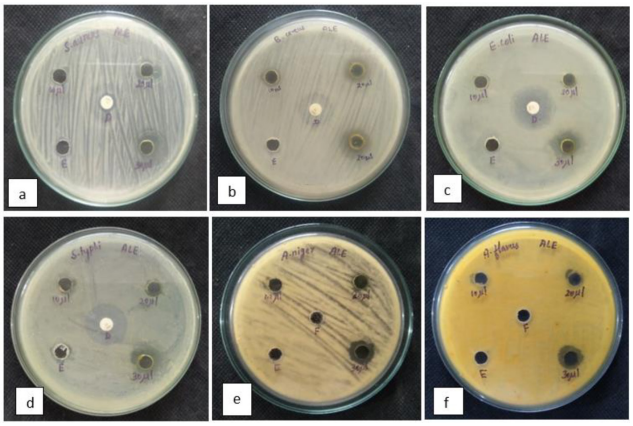

Figure 4: Antimicrobial analysis of ethanol leaf extracts of *Tinospora crispa* against (a) *Staphylococus aureus* (b) *Bacillus cereus* (c) *Escherichia coli* (d) *Salmonella typhii* (e) *Aspergillus niger* and (f) *Aspergillus flavus*.

Table 3: *In vitro* antimicrobial activity of ethanol leaf extracts of *Tinospora crispa* against different bacterial and fungal species.

Sl. no.	Sample	Type of pathogen	Name of pathogen	Negative control	Positive control	Leaf extract (µl)		
						10	20	30
1	Ethanol leaf extract	Gram positive bacteria	*S. aureus*	NIL	19±0.57	8±0.57	13.3±0.88	20.3±0.88
			B. cereus	NIL	16±0.57	5.33±0.88	9±1.52	13±1.15
		Gram negative bacteria	*E. coli*	NIL	19.3±0.88	8.33±0.88	10.6±1.20	15.6±1.20
			S. typhii	NIL	20±0.57	7.33±0.88	9.66±0.66	15.3±0.88
		Fungi	*A. niger*	NIL	18.3±0.88	7.33±0.88	11±1.52	14±1.15
			A. flavus	NIL	18±0.57	7.33±0.88	11±1.15	16±0.57

headache and fever. These are attributed for antibacterial and analgesic properties.[37] In previous studies it was reported that flavonoids and terpenoids were present in aqueous extract of the Punica granatum while alkaloids and phlobatannins were found to be absent in it.

The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability.[38] Radical scavenging activities are very important to prevent the deleterious role of free radicals in different diseases, including cancer. DPPH free radical scavenging is an accepted mechanism for screening the antioxidant activity of plant extracts. Antioxidant activity was determined in 96-well microtiter plates by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity method. This method is used for quick and reliable measurement of *in vitro* antioxidant activities of plant extracts and pure compounds.[39,40] Antioxidant activity of all extracts tested was generally dose-dependent, with highest concentrations of the extracts showing the highest antioxidant activity. Flueggea virosa contains high amounts of bergenin.[41] The ethanol leaf extract showed the highest antioxidant activity in 100 µg/ml (61.7%), the % RSA increased with increase in concentration of plant extract. Pearling samples of *Avena sativa* had the highest antioxidant activity (73.4-77.6%), followed by flour (52.6-60.8%), aspirations (56.5%), and trichomes (47.4%).[42]

Medicinal plants contain several phytochemicals such as flavonoids, tannins, alkaloids, and terpenoids, which possess antioxidant and antimicrobial properties.[43] The ethanol leaf extract of *Tinospora crispa* at the concentration of 30 µl showed good zone of inhibition against most of the tested bacteria and fungi like *B. cereus, A. niger, S. typhii,* etc. The ethanol extract shows good inhibiton again gram-positive bacteria than gram negative bacteria and fungi. Similar studies on the ethanolic extract of rosemary shows inhibitory effect against pathogenic microbes *E. coli* and *S. aureus*. In the antifungal analysis, the ethanolic extract of thyme and clove had valuable results against CA with inhibition zone (25.2 ± 1.4, and 15.8 ± 1.2), respectively.[44]

CONCLUSION

The qualitative phytochemical screening of the ethanol leaf extract of *Tinospora crispa* plant showed the presence of alkaloids, glycosides, flavonoids, phenols, and tannins. The *in vitro* antioxidant analysis showed that the ethanol leaf extract showed comparatively good antioxidant property with the standard Ascorbic acid. *In vitro* antimicrobial analysis showed good zone of inhibition against the tested microbes when compared with the standards. From the above analysis it has been proved that the ethnomedicinal plant *T. crispa* has medicinal properties which can cure a lot of diseases.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

FRSA: Free radical scavenging activity; **DPPH:** 2,2-Diphenyl-1-picryl-hydrazyl-hydrate; **PDA:** Potato dextrose agar medium; **DMSO:** Dimethyl sulfoxide.

SUMMARY

The present study summarises that the traditional medicinal plant *Tinospora crispa* shows the presence of secondaries metabolites such as Alkaloids, phenols, tannins, glycosides, etc. The antioxidant analysis showed good activity comparatively against the standard ascorbic acid. The antimicrobial activity showed good zone of inhibition against few pathogenic microrganisms and thus this plant possess antioxidant and antimicrobial properties which be studied further in the modern medicinal field.

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