Asian Journal of Pharmaceutical Education and Research
Vol -9, Issue-1, January-March 2020
ISSN:2278 7496

RESEARCH ARTICLE Impact Factor: 5.019

PHYTOCHEMICAL ANALYSIS AND *IN VITRO* SCREENING FOR ANTIOXIDANT, ANTIMICROBIAL, ANTIDIABETIC PROPERTIES OF *TINOSPORA CRISPA* LEAVES

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Received 21 Dec. 2019; Revised 28 Dec. 2019; Accepted 02 Jan. 2019, Available online 15 Jan. 2020

ABSTRACT

Medicinal plants have therapeutic potential and are used worldwide to treat various diseases. *Tinospora crispa* (L.) Hook f & Thomson (Menispermaceae) found in the rainforests or mixed deciduous forests in Asia and Africa is used in traditional medicines to treat numerous health conditions. In this study, *Tinospora crispa* have been investigated for their antioxidant, antimicrobial, and antidiabetic activities. Qualitative analysis of various phytochemical constituents and quantitative analysis of total phenol and flavonoids were determined by the well-known test protocol available in the literature. The hydroalcoholic extracts of *Tinospora crispa* leaves exhibited significant and dose-dependent antioxidant activity including electron-donation ability. To analyze the antimicrobial activity, hydroalcoholic extracts of *Tinospora crispa* was tested by a well diffusion method against four selected strain and which shows significant inhibitory action against all the tested strain. In addition, the hydroalcoholic extracts of *Tinospora crispa* leaves exhibited the dose dependent α-amylase inhibitory activity with an IC_{50} value of acarbose and extract was found to 131.75 and 168.79µg/ml respectively indicating that *Tinospora crispa* is a promising source as a herbal medicine.

Keywords: *Tinospora crispa*, Phytochemical Analysis, Antioxidant, Antimicrobial, Antidiabetic Activity.

INTRODUCTION

Natural products, mostly from plants, have begun to gain worldwide interest for promoting healthcare and have been used as conventional or complementary medicines due to toxicity and side effects of synthetic drugs 1. In addition, natural products are known not only as a rich source of structurally diverse substances with a wide range of biological activities, but also as a primary source for synthesized drugs [1,2]. Therefore, the investigation of the pharmaceutical properties of medicinal plants and the analyses of their natural products are an important aspect when developing alternative or adjunctive therapies. The biological and pharmaceutical activities in medicinal plants are mostly mediated by the presence of secondary metabolites including phenolic and flavonoid compounds. These compounds exhibit a wide range of pharmaceutical properties, such as antioxidant, antimicrobial, anti-inflammatory and anticancer properties [3]. Although a variety of plants are known to be good sources of these compounds, their contents are dependent on a number of factors including the climatic conditions, ripeness of the material, their tissues, and genetic factors [4]. *Tinospora crispa* (Menispermaceae) a climber plant found in tropical and subtropical India and parts of the Far East (such as Indonesia, Malaysia, Thailand and Vietnam) and

in primary rainforest or mixed deciduous forest [5,6]. The plant has been recently showing an ethno-pharmaceutical uses for the treatment of fever, diabetes, hypertension, cholera, rheumatism, hyperglycemia, wounds, intestinal worms, and skin infections. Besides that, *Tinospora crispa* is also used to treat tooth and stomachaches, coughs, asthma and pleurisy [7-11]. It was revealed that the chemical constituents isolated from various parts of *Tinospora crispa* contained flavonoid and quaternary alkaloids including flavavone-O-glycosides (apigenin), berberine, picroretoside, palmatine [12,13], borapetol Aand B, borapetoside A and B, tinocrisposide, N-formylanondine, N-formylnornuciferine, N-acetyl nornuciferine, c-sitosterol, picroretine and tinotubride[14]. Two newtriperpenes, cycloeucalenol and cycloeucalenone from *Tinospora crispa* were previously isolated [15]. Hence, the present investigation was performed to find the phytochemical constituents, antimicrobial activity, antidiabetic, and antioxidant activities of the *Tinospora crispa* extract with intention of motivation for usage of plant parts with less adverse implications for the survival of the plant species.

Materials and Methods

Plant material

The leaves of *Tinospora crispa* was collected from local area of Bhopal (M.P.) in the month of May, 2019. The leaves were separated and washed with sterile distilled water to remove the adhering dust particles and other unwanted materials. The leaf was air dried under room temperature. The dried plant samples were cut and grinded to make it in powder form. The powdered samples were stored in clean, dry and sterile container for further use.

Chemical reagents

All the chemicals used in this study were obtained from Hi Media Laboratories Pvt. Ltd. Mumbai, India), SD Fine- Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India). All the chemicals used in this study were of analytical grade.

Extraction procedure

The shade dried material was coarsely powdered and subjected to extraction with petroleum ether by maceration. The extraction was continued till the defatting of the material had taken place. 77.5 gm of dried plant material were exhaustively extracted with hydroalcoholic solvent (ethanol: water: 80: 20) using maceration method. The extracts were evaporated above their boiling points and stored in an air tight container free from any contamination until it was used. Finally, the percentage yields were calculated of the dried extracts [16].

Qualitative phytochemical analysis of plant extract

The *Tinospora crispa* extracts obtained was subjected to the preliminary phytochemical analysis following standard methods by Khandelwal and Kokate [17, 18]. The extract was screened to identify the

presence or absence of various active principles like phenolic compounds, carbohydrates, flavonoids, glycosides, saponins, alkaloids, fats or fixed oils, protein and amino acid and tannins.

Quantification of Secondary Metabolites

Quantitative analysis is an important tool for the determination of quantity of phytoconstituents present in plant extracts. For this TPC and TFC are determined. Hydroalcoholic extract obtained from *Tinospora crispa* plant material of subjected to estimate the presence of TPC and TFC by standard procedure.

Total Phenol Determination

The total phenolic content was determined using the modified method of Olufunmiso *et al* [19]. A volume of 2 ml of *Tinospora crispa* hydroalcoholic extracts or standard was mixed with 1 ml of Folin Ciocalteau reagent (previously diluted with distilled water 1:10 v/v) and 1 ml (7.5g/l) of sodium carbonate. The mixture was vortexed for 15s and allowed to stand for 15min at 40°C for colour development. The absorbance was measured at 765 nm using UV/visible spectrophotometer. The total phenolic content was calculated from the standard graph of gallic acid and the results were expressed as gallic acid equivalent (mg/100mg).

Total Flavonoids Determination

The total flavonoid content was determined using the method of Olufunmiso *et al* [19]. 1 ml of 2% $AlCl_3$ methanolic solution was added to 3 ml of extracts or standard and allowed to stand for 60 min at room temperature; the absorbance of the reaction mixture was measured at 420 nm using UV/visible spectrophotometer The content of flavonoids was calculated using standard graph of quercetin and the results were expressed as quercetin equivalent (mg/100mg).

Thin layer chromatography

Thin layer chromatography is based on the adsorption phenomenon. In this type of chromatography mobile phase containing the dissolved solutes passes over the surface of stationary phase. Each solvent extract was subjected to thin layer chromatography (TLC) as per conventional one dimensional ascending method using silica gel 60F254, 7X6 cm (Merck) were cut with ordinary household scissors. Plate markings were made with soft pencil. Glass capillaries were used to spot the sample for TLC applied sample volume 1-micro litre by using capillary at distance of 1 cm at 5 tracks. In the twin trough chamber with different solvent system toluene: ethyl acetate: formic acid solvent system used. After pre-saturation with mobile phase for 20 min for development were used. After the run plates are dried and sprayed freshly prepared iodine reagents were used to detect the bands on the TLC plates. The movement of the active compound was expressed by its retention factor (Rf), values were calculated for different samples.

Antioxidant activity

DPPH radical scavenging assay

DPPH scavenging activity was measured by modified method of Olufunmiso *et al* [19]. DPPH scavenging activity was measured by the spectrophotometer. Stock solution (6 mg in 100ml methanol) was prepared such that 1.5 ml of it in 1.5 ml of methanol gave an initial absorbance. Decrease in the absorbance in presence of sample extract at different concentration (10-100 µg/ml) was noted after 15 minutes. 1.5 ml of DPPH solution was taken and volume made till 3 ml with methanol, absorbance was taken immediately at 517 nm for control reading. 1.5 ml of DPPH and 1.5 ml of the test sample of different concentration were put in a series of volumetric flasks and final volume was adjusted to 3 ml with methanol. Three test samples were taken and each processed similarly. Finally the mean was taken. Absorbance at zero time was taken for each concentration. Final decrease in absorbance was noted of DPPH with the sample at different concentration after 15 minutes at 517 nm. The percentage inhibition of free radical DPPH was calculated from the following equation: % inhibition = [(absorbance of control - absorbance of sample)/absorbance of control] × 100%. Though the activity is expressed as 50% inhibitory concentration (IC50), IC50 was calculated based on the percentage of DPPH radicals scavenged. The lower the IC50 value, the higher is the antioxidant activity.

Antimicrobial activity

The well diffusion method was used to determine the antibacterial activity of the extract prepared from the *Tinospora crispa* using standard procedure of Bauer *et al* [20]. The drug used in standard preparation was ciprofloxacin of IP grade. The antibacterial activity was performed by using 24hr culture of *Bacillus subtilis, Staphylococcus aureus, Salmonella bongori* and *Klebsiella pneumoniae.* There were 3 concentration used which are 25, 50 and 100 mg/ml for each extracted phytochemicals in antibiogram studies. Its essential feature is the placing of wells with the antibiotics on the surfaces of agar immediately after inoculation with the organism tested. Undiluted overnight broth cultures should never be used as an inoculum. The plates were incubated at 37ºC for 24 hr. and then examined for clear zones of inhibition around the wells impregnated with particular concentration of drug. The diameter of zone of inhibition of each wall was recorded.

Anti diabetic activity

Inhibition of alpha amylase enzyme

A total of 500 µl of test samples and standard drug (10-50µg/ml) were added to 500 µl of 0.20 mM phosphate buffer (pH 6.9) containing α-amylase (0.5mg/ml) solution and were incubated at 25°C for 10 min. After these, 500 µl of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added to each tube. The reaction mixtures were then incubated at 25°C for 10 min. The reaction was stopped with 1.0 ml of 3, 5 dinitrosalicylic acid colour reagent. The test tubes were then incubated in a boiling

water bath for 5 min, cooled to room temperature. The reaction mixture was then diluted after adding 10 ml distilled water and absorbance was measured at 540 nm. Control represent 100% enzyme activity and were conducted in similar way by replacing extract with vehicle [21].

RESULTS AND DISCUSSION

The crude extracts so obtained after the maceration extraction process, extracts was further concentrated on water bath for evaporate the solvents completely to obtain the actual yield of extraction. To obtain the percentage yield of extraction is very important phenomenon in phytochemical extraction to evaluate the standard extraction efficiency for a particular plant, different parts of same plant or different solvents used. The yield of extracts obtained from sample using Pet ether and hydroalcoholic as solvents are depicted in the Table 1.

Table 1 % Yield of plant material

S. No.	Solvents	*Tinospora crispa*
1	Pet ether	1.36%
2.	Hydroalcoholic	4.65%

Phytochemical analysis of hydroalcoholic extract of leaf sample of *Tinospora crispa* showed the presence of flavonoid, phenols, amino acid, protein, saponins and diterpines while, alkaloid and carbohydrate were not detected. From hydroalcoholic extract which exhibited the presence of flavonoid, phenols, amino acid, protein and saponins but alkaloids, glycosides and carbohydrate were reported to be absent (Table 2).

Table 2 Phytochemical screening of extract *of Tinospora crispa*

S. No.	Constituents	Hydroalcoholic extract
1.	**Alkaloids**	
	Mayer's Test	-ve
	Wagner's Test	-ve
	Dragendroff's test	-ve
	Hager's test	-ve
2.	**Glycosides**	
	Modified Borntrager's Test	-ve
	Legal's test	-ve
3.	**Flavonoids**	
	Lead acetate	+ve
	Alkaline test	+ve
4.	**Phenolics**	
	Ferric Chloride Test	+ve
5.	**Proteins and Amino acids**	
	Xanthoproteic test	+ve

	Ninhydrin Test	-ve
6.	**Carbohydrates**	
	Molisch's Test	-ve
	Benedict's Test	-ve
	Fehling's test	+ve
7.	**Saponins**	
	Froth Test	+ve
	Foam test	-ve
8.	**Diterpins**	
	Copper acetate test	-ve

The total phenolic content (TPC) was expressed as mg/100mg of gallic acid equivalent of dry extract sample using the equation obtained from the calibration curve: **$Y = 0.042X+0.002$, $R^2= 0.999$,** where X is the gallic acid equivalent (GAE) and Y is the absorbance. The total flavonoid content (TFC) was expressed as mg/100mg of quercetin equivalent of dry extract sample using the equation obtained from the calibration curve: **$Y = 0.06X+0.019$, $R^2= 0.999$,** where X is the quercetin equivalent (QE) and Y is the absorbance (Table 3).

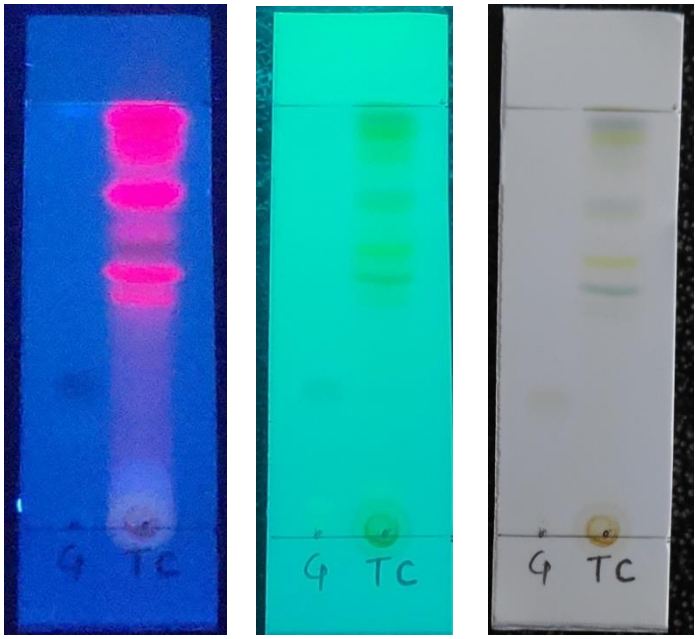
Table 3 Total phenolic and total flavonoid content of *Tinospora crispa (Leaves)*

S. No.	Extract	Total Phenol (mg/100mg)	Total Flavonoid (mg/100mg)
1.	**Hydroalcoholic**	0.698	1.021

The result of thin layer chromatography of *Tinospora crispa* leaves extracts is presented in table 4 and Fig 1. The TLC studies of the hydroalcoholic extract of *Tinospora crispa* with solvent system Toluene: Ethyl acetate: Formic acid in ratio of (7:5:1) and (5:4:1) for gallic acid Rf values was found 0.53.

Table 4 Calculation of R_f. Value

S. No.	Compound	Extract	Rf Value
1.	Gallic acid	Toluene: Ethyl acetate: Formic acid (7:5:1)	0.53



Long UV **Short UV** **Normal Light**

Fig. 1 Spot-1 Gallic acid, Spot-2 Leaves extract of *Tinospora crispa*

DPPH radical scavenging assay measured hydrogen donating nature of extracts. Under DPPH radical scavenging activity, the inhibitory concentration 50% (IC_{50}) value of *Tinospora crispa* hydroalcoholic extract was found to be 60.555µg/ml as compared to that of ascorbic acid (17.681µg/ml). A dose dependent activity with respect to concentration was observed Table 5.

Table 5 % Inhibition of ascorbic acid, hydroalcoholic extract of *Tinospora crispa* using DPPH method

S. No.	Concentration (µg/ml)	% Inhibition	
		Ascorbic acid	Hydroalcoholic extract
1	10	44.65±0.21	33.15±0.41
2	20	48.62±0.58	40.63±0.36
3	40	65.34±0.24	44.98±0.54
4	60	69.65±0.45	49.13±0.65
5	80	77.41±0.63	55.74±0.47
6	100	84.13±0.46	61.22±0.36
IC 50		17.681	60.555

***(n=3, mean ± SD)**

It was clear from the experimental data presented in Table 6 & 7 that antimicrobial activity was showed by hydroalcoholic extract against all the strains. The hydroalcoholic extract of *Tinospora crispa* has shown significant effect at 100mg/ml as compared with the standard ciprofloxacin. It is effective against *Bacillus subtilis, Staphylococcus aureus, Salmonella bongori, Klebsiella pneumoniae* in concentration dependent manner.

Table 6 Antimicrobial activity of *standard drug* against selected microbes

S. No.	Name of drug	Microbes	Zone of inhibition		
			10 µg/ml	20 µg/ml	30 µg/ml
1	Ciprofloxacin	*Bacillus Subtilis*	12±0.5	17±0.74	20±0.15
2		*Staphylococcus aureus*	17±1.69	18±2.62	22±2.16
3		*Salmonella Bongori*	17±0.15	23±0.86	25±0.5
4		*Klebsiella pneumoniae*	19±4.71	28±1.24	36±1.699

***(n=3, mean ± SD)**

Table 7 Antimicrobial activity of hydroalcoholic extract of *Tinospora crispa* against selected microbes

S. No.	Name of microbes	Zone of inhibition Hydroalcoholic extract of *Tinospora crispa*		
		25mg/ml	50 mg/ml	100mg/ml
1.	*Bacillus subtilis,*	11±0.47	14±0.94	16±0.47
2.	*Staphylococcus aureus*	6±0	7±0	7±0.23
3.	*Salmonella Bongori*	12±0.47	13±0.47	15±1.24
4.	*Klebsiella pneumoniae*	10±0	11±0.47	15±0.47

***(n=3, mean ± SD)**

Percentage inhibition of α-amylase activity by *Tinospora crispa* leaves extract was estimated with acarbose as the positive control. *Tinospora crispa* extract show dose dependent activity. IC_{50} value of acarbose and extract was found to 35.33 and 224.45 µg/ml respectively Table 8.

Table 8 Results of *in vitro* antidiabetic studies of *Tinospora crispa* extract

S. No	Acarbose		Hydroalcoholic extract	
	Conc.	% Inhibition	Conc.	% Inhibition
1.	100	42.25	100	35.56
2.	200	59.98	200	55.56
3.	300	68.85	300	76.65
4.	400	75.56	400	80.23
5.	500	79.98	500	89.95
	IC_{50} (µg/ml)	131.75	**IC_{50}**	168.79

Conclusion

In this study, we analyzed the antioxidant, antimicrobial, and antidiabetic activities of *Tinospora crispa*. The overall results of the present study suggest that the hydroalcoholic leaf extract of *Tinospora crispa* could be useful as a source of natural antioxidant agents. In addition, the leaf extract of *Tinospora crispa* was shown to possess notable pharmaceutical activities, indicating that *Tinospora crispa* should be considered as a useful source for herbal medicine. The variation in pharmaceutical activities between organic extracts indicates that the comparative analysis of the metabolome in leaf extracts will be required for the isolation and characterization of the active compounds in *Tinospora crispa*.

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