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An invtro study on anti-obesity and antiradical activities of siddha medicine seenthil chooranam

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Abstract
Traditional systems of medicine have been in trend for treating numerous ailments in many develop and development countries such as India, China, Japan and Korea, Tavian since age-old time. Siddha system of medicine is one of the ancient traditional system of India and practiced mostly in its southern part of India for treating different diseases including even chronic conditions. Seen thil chooranam composed of purified *Tinospora cardifolia*. Pancreatic lipase inhibitors from plant sources may prove as promising side effects lacking anti-obesity therapeutics, present study was conceived with the objective of anti-lipase, antiradical activities of seenthil chooranam decoction. Phytochemical constituents were screened quantifications of total phenolics, tannins, flavonoids, were done by taking tannic acid, quercetin, as reference molecules. Antiradical activities were evaluated by using different free radicals (ABTS, lipid peroxidation, metal chelating, superoxide and nitric oxide scavenging activities). The antiradical activity of seenthil chooranam decoction was proved to be better than the standard ascorbic acid. Antilipolytic function of seenthil chooranamdecoction was assessed using porcine pancreatic lipase (PPL; triacylglycerol lipase, EC 3.1.1.3) in an *in vitro* assay system with *p*-nitrophenyl palmitate (*p*-NPP) as a substrate. From the data of the results obtained, maximum percentage of lipase inhibition was shown by seenthil chooranam decoction (82.3%) than standard drug. In all cases, the coefficients of determination of the regression (R2) were greater than 0.95. IC_{50} are the means ± standard deviations of three determinations.

Keywords: seenthil chooranam, anti-obesity, antiradical, *T. cardifolia*

Introduction

Herbal medicines are used for the treatment of various ailments from ancient times and it is not an exaggeration to say that the use of the herbal drugs is as old as mankind. Herbal medicines are synthesized from the therapeutic experience of generation of practicing physicians of ancient system of medicine for more than hundreds of years (Abbas *et al*., 2014) [1]. Nowadays, researcher shows a great interest in those medicinal agents that are derived from plants because the currently available drugs are either have certain side effects or are highly expensive. Nature has blessed us with enormous wealth of herbal plants which are widely distributed all over the world as a source of therapeutic agents for the prevention and cure of various diseases (Kim *et al*, 2013) [4]. According to WHO, world's 80% population uses herbal medicines for their primary health care needs. Herbal medicines will act as parcels of human society to combat disease from the dawn of civilization. The medicinally important parts of these herbal plants are chemical constituents that produce a desired physiological action on the body (Biren *et al*, 2006) [2].

Since ancient time India uses herbal medicines in the officially alternative systems of health such as Ayurveda, Unani, Sidha, Homeopathy, and Naturopathy. In India, there are more than 2500 plants species which are currently used as herbal medicaments. For than 3000 years, the herbal medicines are used either directly as folk medication or indirectly in the preparation of recent pharmaceuticals. Thus, from the knowledge of traditional plants, one might be able to discover new effective and cheaper drugs. The antioxidant ability of flavonoids depends on the molecular structure and position of hydroxyl groups. It is reported that flavonoids may alter peroxidation kinetics by chelating the lipid packing order and thus stabilize the membranes, prevent the diffusion of free radicals and stop peroxidation. Flavonoids not only bind with protein but can interact with membrane phospholipids by hydrogen bond. *In vitro* study reveals that flavonoids can directly scavenge H_2O_2, ·OH, singlet oxygen or peroxyl radical.

The development of obesity is characterized by a chronic imbalance between energy intake and energy expenditure, and it is often ascribed to changing lifestyles and inadequate dietary habits. Also, decreased energy expenditure is often associated with an inherited low basal metabolic rate, physical in activity, and low capacity for fat oxidation. To reduce body weight and adiposity, a change in lifestyle habits is still the crucial cornerstone. Physical activity might be helpful in the prevention of obesity by elevating the average daily metabolic rate and increasing energy expenditure. Unfortunately, this clinical approach is not long-term lasting, and weight regain is often seen. Following a high-fat diet, the diet-induced thermogenesis is lower than following high-protein and carbohydrate diets, and also fat is more effectively absorbed from the gastrointestinal tract than carbohydrates, which translates into lower energy expenditure when following a high-fat diet. So, high-fat diets produce a metabolically more efficient state, at least in part because of the lower postprandial thermogenic effect of lipids in comparison with carbohydrates (Prachayasittikul *et al*, 2008) [10].

Tinospora cordifolia Miers (Menispermaceae) is widely used in theIndian system of medicine in the treatment of various ailments. Categorized as a "Rasayana" in Ayurveda, it is used for its general adaptogenic and pro host

immunomodulatory activity in fighting infections. A great deal of chemical investigation on this plant is already on record and a number of protoberberine and aporphine alkaloids have been reportedto occur on it. *Tinospora Cordifolia* is a large, glabrous, deciduous, climbing shrub. The stem structure is fibrous and the transverse section exhibits a yellowish wood with radially arranged wedge shaped wood bundles, containing large vessels, separated by narrow medullary rays. The barkis creamy white to grey, deeply left spirally and stem contains rosette like lenticels. The leaves are membranous and cordate in shape. Flowers are in axillary position, 2-9 cm long raceme on leaflet branches, unisexual, small and yellow in color. Male flowers are clustered and female are usually solitary.

Materials and methods

Plant collection

Tinospora cardifolia stem was obtained from Herbal garden of Government Siddha Medical College, Arumbakkam, Chennai, Tamil Nadu, India. A plant taxonomist authenticated the plant and samples were kept in the Medicinal Botany herbarium with voucher specimen numbers MB/GSMC-283/2021.

Extraction

Tinospora cardifolia preparation, 100 g of the dried plant was crushed using blender to a paste-like state for 1 min. The homogenised sample was firstly freeze dried in order to reduce moisture content of the sample for a more efficient extraction process. The powder was then soaked in n-Hexane to defat for 24 h. It was then soaked in methanol for 72 h to obtain methanol crude extract, which was concentrated using a rotatory evaporator at 40 °C. The sticky residues were partitioned with chloroform to give chloroform soluble fractions. This was evaporated under reduced pressure and dried using an oven to obtain an anthocynin rich fraction by ethyl acetate (Dasgupta *et al.*, 2014).

Phytochemical Screening

The aqueous extract of from the stem of *Tinospora cardifolia* were subjected to phytochemical screening to determine the presence of secondary metabolites such as alkaloids, flavonoids, terpenoids, tannins, glycosides, saponins and polyphenols using standard procedures (Trease and Evans 1983).

Lipase Inhibition

The ability of the ethyl acetate extract from the stem of *Tinospora cardifolia* to inhibit lipase was measured by Bustanji *et al*. (2011). The lipase activity was determined by measuring the hydrolysis of p-nitrophenol butyrate (pNPB) to p-nitrophenol at 410 nm using UV-VIS spectro photo meter. Lipase assays were performed by incubating 200 µl of plant extract (5mg/ml ethanol) with 100 µl of PPL solution for 5 min at 37°C; then 10µL of the pNPB substrate (100 mM in acetonitrile) was added. The volume was completed to 1 mL using the buffer. The release of pNPB is estimated as the increment increase in absorbance against blank. The percentage of residual activity of PL was determined by comparing the lipase activity in the presence and the absence of the tested inhibitors. Orlistat (100 µg/ml) was used as a positive standard inhibitor control, whereas plant extract was replaced by ethanol to be used as negative control. All experiments were repeated twice.

DPPH (*A, A*-Diphenyl-*B*-Picrylhydrazyl) Assay for Scavenging Activity

DPPH radical scavenging activity was determined according to the method described by Choi *et al*. (2000). Briefly, 1 ml of the ethyl acetate extract from the stem of *Tinospora cardifolia* (25-100 µ/ml) or standards (BHT and ascorbic acid) were mixed with 1.5 ml (0.02 %) of DPPH solution in methanol. The mixture was incubated in the dark for 30 min, and the absorbance was measured at 517 nm in the spectrophotometer (6800 UV-VIS spectrophotometer) using methanol as blank. The percentage of scavenging of DPPH radicals was calculated by using the following formula: where *AS* is the absorbance of the sample; *AC* is the absorbance of the negative control (ethyl acetate without the sample).

Reducing Power Assay

The reducing power was determined according to the Oyaizu *et al*. (1986). Aliquot of 0.2 mL of various concentrations of the ethyl acetate extract from the stem of *Tinospora cardifolia* (25-100 µg/mL) were mixed separately with 0.5 mL of phosphate buffer (0.2 M, pH 6.6) and 0.5 mL of 1% potassium ferricyanide. The mixture was incubated in a water bath at 50°C for 20 min. After cooling at room temperature, 0.5 mL of 10% trichloroacetic acid was added to it followed by centrifugation at 3,000 rpm for 10 min. Supernatant (0.5 mL) was collected and mixed with 0.5 mL of distilled water. Ferric chloride (0.1 mL of 0.1%) was added to it and the mixture was left at room temperature for 10 min. The absorbance was measured at 700 nm. Ascorbic acid was used as positive control.

Nitric Oxide Radical Scavenging Assay

The nitric oxide radical scavenging assay followed by Panda *et al*. (2009). The extracts were prepared from a 10 mg/mL ethanol crude extract. These were then serially diluted with distilled water to make concentrations from 25-100 µg/mL of ethyl acetate extract from the stem of *Tinospora cardifolia* and standard. These were stored at 4°C for later use. Griess reagent was prepared by mixing equal amounts of 1% sulphanilamide in 2.5% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride in 2.5% phosphoric acid immediately before use. A volume of 0.5 mL of 10 mM sodium nitroprusside in phosphate buffered saline was mixed with 1 mL of the different concentrations of the ethanol extracts (10−100 µg/mL) and incubated at 25°C for 180 mins. The extract was mixed with an equal volume of freshly prepared Griess reagent. Control samples without the extracts but with an equal volume of buffer were prepared in a similar manner as was done for the test samples.

Inhibition of Lipid Peroxidation Activity

Lipid peroxidation induced by Fe^{2+}ascarbate system in egg yolk was assessed as thiobarbituric acid reacting substances (TBARS) by the method of Ohkawa *et al*. (1979). The experimental mixture contained 0.1 ml of egg yolk (25% w/v) in Tris-HCl buffer (20 mM, pH 7.0); KCl (30 mM); $FeSO_4$ $(NH_4)_2SO_4.7H_2O$ (0.06 mM); and different concentrations of ethyl acetate extract from the stem of *Tinospora cardifolia* in a final volume of 0.5 ml. The

experimental mixture was incubated at 37°C for 1 h. After the incubation period, 0.4 ml was collected and treated with 0.2 ml sodium dodecyl sulphate (SDS) (1.1%); 1.5 ml thiobarbituric acid (TBA) (0.8%); and 1.5 ml acetic acid (20%, pH 3.5).

The final volume was made up to 4.0 ml with distilled water and then kept in a water bath at 95 to 100 °C for 1 hour. After cooling, 1.0 ml of distilled water and 5.0 ml of n-butanol and pyridine mixture (15:1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The absorbance of butanol-pyridine layer was recorded at 532 nm in Deep Vision (1371) UV–Vis Spectrophotometer) to quantify TBARS. Inhibition of lipid peroxidation was determined by comparing the optical density (OD) of test sample with control. Ascorbic acid was used as standard. Inhibition of lipid peroxidation (%) by the each extracts was calculated according to 1-(E/C) × 100, where C is the absorbance value of the fully oxidized control and E is absorbance of the test sample.

Statistical Analysis

All results were expressed as mean ± standard deviation (n=3). Significance of differences from the control was determined by Duncan's test and a p value < 0.05 was considered significant.

Result and Discussion

Phytochemical screening

The phytochemical screening of aqueous extract of stem of *T. cardifolia* were studies presently showed the presence of alkaloids, flavonoids, polyphenol, terpenoids, and absence of glycosides and tannin (Table-1).

Table 1: Phytochemical screening of stem of *Tinospora cordifolia*

Sl. No.	Phytochemical Constituents	Observation	Aqueous extract of stem of *T. cardifolia*
1	Alkaloids		
	-Dragendorff's Test	Orange/ red precipitate	+
	-Mayers test	Yellow or white precipitate	+
2.	Flavonoids		
	-Alkalai Reagent	Intense yellow colour	+
	-Lead acetate test	Precipitate formed	+
3.	Glycosides		
	Keller-Killiani test	Reddish brown colour ring formed	-
4.	Tannin-$FeCl_3$ test	Blue black coloration	-
5.	Saponins		
	-Frothing test	Foam	+
6.	Terpenoids		
	-*Salkowski test*	Dark reddish brown color in interface	-
7.	Polyphenols		
	-Ferrozine test	Raddish blue	+
8.	Anthocyanin test		
	Ammonia	Ammonia layer yellow in color	+

The partial characterization of by TLC

The ethyl acetate extract of stem of *Tinospora cardifolia* loaded on Pre-coated TLC plates (60 F_2 54 Merck) and developed with a solvent system of petroleum ether, chloroform and methanol in the ratio of 1:0.5:0.1 were efficient to extract the antioxidant and anti-obesity compound it is used for further studies. The developed plate was viewed under UV 240nm and 360nm (Fig-1).

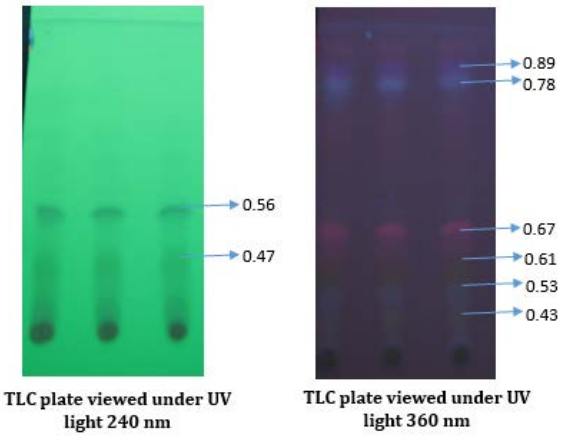

Fig 1: Partial characterization of ethyl acetate extract of stem of *Tinospora cardifolia* by TLC

Anti-obesity activity inhibition of lipase

Result of % lipase inhibition activity of ethyl acetate extract of stem of *T. cardifolia* was presented in Table-2. Ethyl acetate extract of stem of *T. cardifolia* showed significantly ($p < 0.05$) higher % lipase inhibition and EC_{50} as compared to standard orli state, whereas, ethyl acetate extract of stem of *T. cardifolia* showed significantly ($p < 0.05$) lower % lipase inhibition and EC_{50}58.23. Ethyl acetate extract of stem of *T. cardifolia* inhibits the conversion of dietary lipid into fatty acid by hydrolysis. Ethyl acetate extract of stem of *T. cardifolia* it was reported that flavonoid and alkaloids reduced the triglyceride breakdown and work as a bioactive phytoconstituents (Torres-Fuentes *et al*., 2015) [12].

Table 2: Anti-obesity activity inhibition of lipase by ethyl acetate extract of stem of *T. cordifolia*

Different concentration of extract	Ethyl acetate extract of *T. cardifolia*	Standard Orlistate
25 µl/ml	19.32±1.45	16.34±1.73
50 µl/ml	41.35±0.89	38.64±0.89
75 µl/ml	59.35±2.36	56.34±2.35
100 µl/ml	76.32±1.78	72.34±1.78
EC_{50} Value	58.32±1.78	62.34±2.14

Results are expressed as percentage inhibited Lipase formation with respect to control. Each value represents the mean+SD of three experiments

Radical scavenging activity DPPH

The antioxidant activity of ethyl acetate extract of *T. cardifolia* was primarily assessed by 2, 2-diphenyl-1-picrylhydrazyl (DPPH), which is based on the ability of DPPH to react with proton donors such as phenols. However, ethyl acetate extract of *T. cardifolia* free radical scavenging ability remains unknown. The present study showed that ethyl acetate extract of *T. cardifolia* exhibits significant free radical scavenging potential (EC_{50}: 60.23±2.45µg/mL, Table-3). The percentages of free radical scavenging are given in Table-4. This property has been widely used to evaluate the free radical scavenging effect of natural antioxidants (Jao and Ko, 2002).

Table 3: Radical scavenging activity DPPH by ethyl acetate extract of *T. cordifolia*

Different concentration of extract	Ethyl acetate extract of stem of *T. cardifolia*	Standard Vitamin-C
25 µl/ml	18.32±1.54	14.56±0.23
50 µl/ml	34.65±2.36	31.56±1.89
75 µl/ml	47.34±1.78	44.32±1.36
100 µl/ml	73.65±1.46	67.32±2.47
EC_{50} Value	60.23±2.45	64.32±2.47

Results are expressed as percentage radical scavenging activity DPPH formation with respect to control. Each value represents the mean+SD of three experiments.

Inhibition of Lipid Peroxidation

In the present study, egg yolk was used as substrate for free radical mediated lipid peroxidation, which is a non-enzymatic method. Ethyl acetate extract of stem of *T. cardifolia* also inhibited the lipid peroxidation induced by ferrous sulfate in egg yolk homogenates. Maximum inhibition was recorded in Ethyl acetate extract of stem of *T. cardifolia* 74.23% with EC_{50} value 57.32 µl/ml and lowest inhibition percentage ascorbic acid 70.36% with EC_{50} 62.34(Table-4). As it is identified that lipid peroxidation is the net result of any free radical attack on membrane and other lipid components present in the system, the lipid peroxidation may be enzymatic (Fe/NADPH) or non-enzymatic (Fe/ascorbic acid). Normally, the mechanism of flavonoid compounds for neutralizing lipid free radicals and preventing decomposition of hydroperoxides into free radicals (Miller, 1996).

Table 4: Inhibition of lipid peroxidation activity of Ethyl acetate extract of stem of *T. cardifolia*

Different concentration of extract	Inhibition percentage of Lipid peroxidation	
	Ethyl acetate extract of *Crataeva religiosa*	Standard Vitamin-C
25 µl/ml	16.32±2.34	14.23±1.34
50 µl/ml	31.24±1.78	28.34±1.63
75 µl/ml	53.34±0.89	51.23±2.37
100 µl/ml	74.23±2.14	70.36±1.48
EC_{50} value	57.32	62.34

[a] Results are expressed as percentage inhibit of lipid peroxidation with respect to control. Each value represents the mean+SD of three experiments.

Nitric Oxide Radical Scavenging

Ethyl acetate extract of stem of *T. cardifolia* showed a strong nitric oxide scavenging activity which was comparable to the standards ascorbic acid and rutin. The EC_{50} value 69.32 of ethyl acetate extract of stem of *T. cardifolia* was less than ascorbic acid 74.32. Percentage of Nitric oxide radical scavenging activity ethyl acetate extract of stem of *T. cardifolia* and standards were presented in Table-5. In the current study, nitrite was produced by incubation of sodium nitroprusside in standard phosphate saline buffer at 25°C was reduced by ethyl acetate extract of stem of *T. cardifolia*. Significant scavenging activity may be due to the antioxidant property of flavonoid, compounds present in ethyl acetate extract of stem of *T. cardifolia*, which compete with oxygen to react with nitric oxide, leading to less production of nitric oxide.

Table 5: Nitric oxide radical scavenging assay of ethyl acetate extract of stem of *T. cordifolia*

Different concentration of extract	Percentage of Nitric oxide radical scavenging activity	
	Ethyl acetate extract of stem of *T. cardifolia*	Standard Vitamin-C
25 µl/ml	16.32±1.47	14.23±0.78
50 µl/ml	31.23±2.89	33.64±2.89
75 µl/ml	45.32±2.45	42.34±1.36
100 µl/ml	66.37±1.23	63.32±1.45
EC_{50} value	69.32	74.32

[a] Results are expressed as percentage of Nitric oxide radical activity with respect to control. Each value represents the mean+SD of three experiments.

Reducing power activity of ethyl acetate extract of stem of *t. Cardifolia*

Reducing power assay method is based on the principle that substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric-ferrous complex that has an absorption maximum at 700 nm. The reducing power of the Ethyl acetate extract of stem of *T. cardifolia* and standard increases with the increase in amount of sample and standard concentrations. The reducing power shows good linear relation in both standard (R2 = 0.981) and sample extract (R2 = 0.970) (Michalak *et al*., 2006) [5].

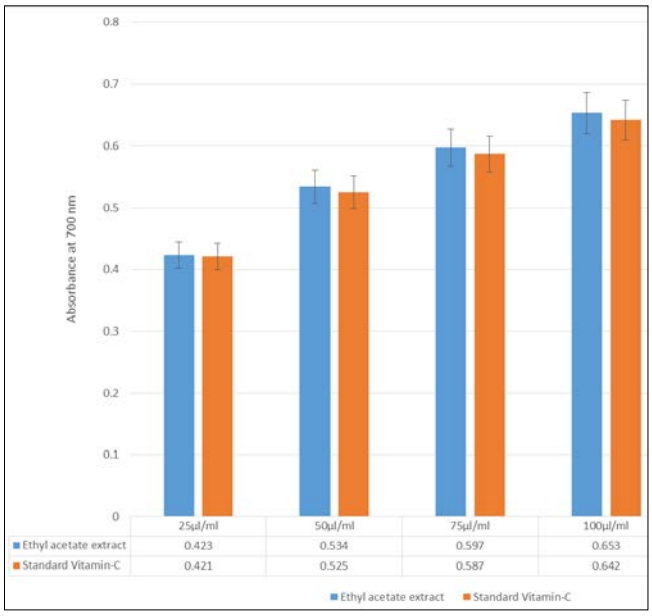

Fig 1: Reducing power activity of ethyl acetate extract of stem of *t. cardifolia*

Conclusions

Our results suggest natural resources that possess strong antioxidant and pancreatic lipase inhibitory activities with potential applications in the treatment and prevention of obesity and overweight problem. The Ethyl acetate extract of stem of *T. cardifolia* have shown possessing strong antioxidants and lipase inhibition potentials. However, future studies are needed for screening in-depth phytochemical, clinical, and possible studies on molecular mechanism of action and identification of the constituents responsible for the antioxidant and lipase inhibition activities.

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