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In vitro study on anti-diabetic and antioxidant activities of acetone extracts from the leaves of *Trichosanthes cucumerina*

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Abstract
The main objective of this work is to evaluate the *in vitro* anti diabetic and antioxidant activities of acetone extract from the leaves of *Trichosanthes cucumerina*. The selected plant extract was studied for their effect on inhibition of glycosylation of haemoglobin, glucose transport across yeast cells, inhibition of alpha amylase, alpha glucosidase, inhibition of lipid peroxidation and metal chelating activity. It was found that the percentage increase of the rate of uptake of glucose into yeast cells was linear in different glucose concentrations used. Acetone extract from the leaves of *T. cucumerina* showing the maximum increase in 25mM Glucose concentration i.e. 68.79% at 100µg/mL. In diabetes mellitus higher amounts of glycated hemoglobin indicates poor control of blood glucose levels and inhibition of inhibition of alpha amylase 83.64%, alpha glucosidase 69.33%. The results of the work indicate that the acetone extract from the leaves of *T. cucumerina* possessed considerable *in vitro* anti diabetic activity and further these effects need to be confirmed using *in vivo* models for its effective utilization as therapeutic agents.

Keywords: acetone extract; *T. cucumerina;* alpha amylase; alpha glucosidase

Introduction

Therapeutic plants have been used for the conduct of various ailments for thousands of years. Straight with the initiation of allopathic medicine, using plants for medicinal purposes is still prevalent in many parts of the world. The universal occurrence of diabetes has risen in the past two decades. Type 2 diabetes is extra joint, and its commonness is expected to rise more rapidly in the future because of increasing obesity and reduced activity levels. In spite of several new pharmacologically active agents have been developed for the management of diabetes, the treatment of diabetes with herbal remedies has also been increasing among practitioners. Ancient Indian literature has prescribed various herbs in the treatment of diabetes mellitus. Many indigenous drugs have been used by the practitioners of the Siddha and Ayurvedic system for the treatment of diabetes mellitus in India (Sathesh *et al*., 2009) [10].

Biological actions of the plant products used as alternative medicines to treat diabetes are in relevance to their chemical composition. Herbal products or plant products are rich in flavonoids, phenolic compounds, coumarins, terpenoids and other constituents which help to reduce blood glucose levels (Jalal Bayati *et al*., 2013) [7]. Several species of herbal drugs with potential antidiabetic activity have been described in the scientific literature. Herbal drugs are prescribed due to their good effectiveness, fewer side effects in clinical experience and relatively low costs (Ren *et al*., 1997) [13]. Medicinal and natural herbal plant products are traditionally used from long time in many countries for the treatment of diabetes mellitus.

Type II diabetes mellitus is a heterogeneous disorder due to a combination of inherited and acquired factors that adversely affect glucose metabolism. It is thought that these factors lead to diabetes mainly by affecting β-cell function and tissue insulin sensitivity. If the amount of insulin produced is too little to allow for glucose to be used or stored, or if the insulin being produced does not work effectively, glucose accumulates in the blood. Hyperglycaemia develops when rates of glucose release into the circulation exceed rates of tissue glucose uptake. This may occur because release is increased, because uptake is reduced, or due to a combination of factors such as increased release with a lesser increase in uptake (Gerich, 2000) [4]. In the normal individual, the concentration of glucose in blood is maintained at about 90 mg/dL of plasma. However, fasting blood glucose in diabetics may be 300-400 mg/dL and may even reach 1000 mg/dL (Johnson, 1998).

Type II diabetes is associated with insulin resistance initially and later, as the function of the β-cell decreases, insulin deficiency (Cerasi, 2000) [2]. Type II diabetes is characterized both by abnormalities of insulin secretion progressively leading to secretion failure as well as insulin resistance of all major target tissues (Haring 1999) [6]. Although insulin resistance is important in the early stages of type II diabetes, the failure in adequate β-cell compensation leads to the progression to the diabetic state. Compensation for insulin resistance is through increased secretion per β-cell or by an increase in β-cell mass through neogenesis or replication of the existing β-cells (Withers *et al*., 1998) [13]. Beta-cell mass is normally tightly maintained through a balance of β-cell birth (β-cell replication and islet neogenesis) and β-cell death through apoptosis. Most of the increase in β-cell mass with insulin resistance is probably due to increased β-cell number, but β-cell hypertrophy may also contribute (Weir and Bonner-Weir, 2004) [12].

Snake gourd is scientifically called as *Tricosanthes cucumerina* is a well-known plant, the fruit of which is mainly consumed as a vegetable. It is an annual climber

belonging to the family Cucurbitaceae. It is commonly called as snake gourd, viper gourd, snake tomato or long tomato. The fruit is usually consumed as a vegetable due to it is good nutritional value. The fruit is a good source of Vitamin A, Vitamin B and Vitamin C. It improves the appetite and acts as a tonic and stomachic and cures biliousness. The wild bitter forms are used in many ayurvedic preparations. The fruits of cultivated forms also have medicinal uses and are useful for people suffering from blood pressure, heart diseases, rheumatism and psoriasis. The plant is richly constituted with a series of chemical constituents like flavonoids, carotenoids, phenolic acids which makes the plant pharmacologically and therapeutically active. It has a prominent place in alternative systems of medicine like Ayurveda and Siddha due to it is various pharmacological activities like antidiabetic, hepatoprotective, cytotoxic, anti-inflammatory, larvicidal effects. Snake gourd has been mooted to help lessen the effects of diabetes. Although Chinese therapy regularly includes snake gourd in the treatment of diabetes, the vegetable is generally a lowcalorie food. This makes it the ideal food to help keep weight under control, yet provide the proper nutrition to people with Type-II diabetics (Sathesh *et al*., 2009) [10].

Materials and Methods

Collection and preparation of plant extracts

The leaves of *Trichosanthes cucumerina* were collected from Government Siddha Medical College Herbal Garden, Arumbakkam, Chennai - 600 106. The plants authenticated (Voucher Number. GSMC/MB-345/21) identification done by Dr. S. Sankaranarayanan, Asst. professor, Department of Medicinal Botany, Government Siddha Medical College, Arumbakkam, Chennai, Tamil Nadu, India. The *Trichosanthes cucumerina* (100 g) was crushed using food masher then extracted with sterile water twice at room temperature for 1 h. The extracts partition with ethyl acetate and concentrated under rotary evaporator at 55°C. After evaporation, freeze dryer was applied to remove the moisture from extracts. The dry extracts were stored at −20°C until analysis. The measurements in this study were done in triplicate and the biological activities of food samples were determined at a concentration of 25, 50, 75 and 100 from 1 mg/mL.

Phytochemical Analysis

The aqueous leaves extract of *Trichosanthes cucumerina* 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 (Harborne 1973) [5].

The Partial Characterization of Thin Layer Chromatography

The acetone extract of *Trichosanthes cucumerina* leaves was loaded on to pre coated TLC (60 F_2 54) and it was developed using solvent system in the ratio of Petroleum ether, Chloroform and methanol (1:0.5:0.1, V/V/V) was used for the development of the exudates on silica gel plates silica gel 60 F_{254} (10x20 cm, 0.2mmlayer). Visible and the non-visible spot given and it is fluorescent with UV light at 360nm and 240nm.

Glucose Uptake in Yeast Cells

The commercial baker's yeast in distilled water was subjected to repeated centrifugation (3,000×g, 5 min) until clear supernatant fluids were obtained and a 10% (v/v) of the suspension was prepared in distilled water. Various concentrations of acetone extract of *T. cucumerina* (25-100µg/mL) were added to 1mL of glucose solution (25 mM) and incubated together for 10 min at 37 °C. Reaction was started by adding 100 µL of yeast suspension followed by vortexing and further incubation at 37 °C for 60 min. After 60 min, the tubes were centrifuged (2,500 × g, 5 min) and amount of glucose was estimated in the supernatant (Cirillo, 1962). Metronidazole was used as standard drug. The percentage increase in glucose uptake by yeast cells was calculated using the following formula:

$$\text{Increase in glucose uptake (\%)} = \frac{\text{Abs sample} - \text{Abs control}}{\text{Abs sample}} \times 100$$

Where, Abs control is the absorbance of the control reaction (containing all reagents except the test sample) and Abs sample is the absorbance of the test sample. All the experiments were carried out in triplicates.

α- Amylase Inhibition Activity

Alpha amylase is an enzyme that hydrolyses alpha-bonds of large alpha linked polysaccharide such as glycogen and starch to yield glucose and maltose. Alpha amylase inhibitory activity was based on the starch iodine method that was originally developed by (Pant *et al*., 2013) [8] and later employed by others for determination of amylase activity in plant extracts with some modifications. In alpha amylase inhibition method 1ml substrate- potato starch (1% w/v), 1 ml of acetone extract of *T. cucumerina* of different concentration such as 25-100 µg/ml, 1ml of alpha amylase enzyme (1% w/v) and 2ml of acetate buffer (0.1 M, 7.2 pH) was added. NOTE- Potato starch solution, alpha amylase solution and drug solution was prepared in acetate buffer (820.3 mg Sodium acetate and 18.7mg sodium chloride in 100ml distilled water).

$$\text{Inhibition of alpha- Amylase (\%)} = \frac{\text{Abs sample} - \text{Abs control}}{\text{Abs sample}} \times 100$$

α- Glucosidase Inhibitory Activity

The α-glucosidase inhibitory activity was assessed by the standard method (Vennila *et al*., 2015) [11], with slight modifications. Briefly, a volume of 60µl of sample solution and 50 µl of 0.1 M phosphate buffer (pH 6.8) containing α-glucosidase solution (0.2 U/ml) was incubated in 96 well plates at 37 ºC for 20 min. After pre-incubation, 50 µl of 5 mM *p*-nitrophenyl-α-D-glucopyranoside (PNPG) solution in 0.1 M phosphate buffer (pH 6.8) was added to each well and incubated at 37 ºC for another 20 min. Then the reaction was stopped by adding 160 µl of 0.2 M $NaCO_3$ into each well, and absorbance readings (A) were recorded at 405 nm by micro-plate reader and compared to a control which had 60 µl of buffer solution in place of the extract. For blank incubation (to allow for absorbance produced by the extract), enzyme solution was replaced by buffer solution and absorbance recorded. The α-glucosidase inhibitory activity was expressed as inhibition % and was calculated as follows:

$$\text{Inhibition Percentage-} \frac{A_0-A_1}{A_0} X100$$

Where, ACO is absorbance of the control and at is absorbance of the sample the concentration of inhibitors required for inhibiting 50% of the α-glucosidase activity under the assay conditions was defined as the IC_{50} value.

Inhibition of Lipid Peroxidation

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 acetone extract of *T. cucumerina* leaves 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.

Metal Chelating Activity

Metal chelating capacity of acetone extract of *T. cucumerina* was measured according to Dinis *et al*., (1994) [3]. 1 ml of different concentrations of acetone extract was added to 0.05 ml of 2 mM ferric chloride solution. The reaction was initiated by the addition of 0.2 ml of 5 mM Ferrozine and the mixture was shaken vigorously. After 10 min, the absorbance was measured at 562 nm against blank. All readings were taken in triplicate and ascorbic acid was used as standard. The % inhibition of ferrozoine-Fe^{2+}complex was calculated by following equation.

% Inhibition of ferrozoine-Fe2+complex=$[(A_0-A_1)/A_0] \times 100$

Where A_0 was the absorbance of control and A_1 was the absorbance of flavonoid rich fraction.

Results and discussion

Phytochemical screening

The phytochemical screening of the *T. cucumerina* leaves were studied showed the presence of alkaloids, flavonoids, tannins, terpenoids, glycosides and phenols (Table -1). Recent interest in plant secondary metabolites has focused on their potential benefits to human health. The polyphenols, tannin, alkaloids are capable not only to reduce oxidative stress but also to inhibit carbohydrate hydrolyzing enzymes and thus preventing hyperglycemia (Kirti *et al.,* 2008).

Table 1: Phytochemical screening of aqueous extract of *T. cucumerina*

Sl. No.	Phytochemical Constituents	Observation	Aqueous leaves extract *T. cucumerina*
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	+

-- = Negative (absent); + = Positive (present)

TLC profile of acetone extract of *T. Cucumerina*

The acetone extract of *T. cucumerina* leaves 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 antidiabetic compound it is used for further studies. The developed plate was viewed under UV 240nm and 360nm (Table-2 and Fig-1).

Table 2: TLC Rf Value of acetone extract of *T. cucumerina*

S.No	UV 240 nm RF value	UV 360 nm RF value
1	-	0.75
2	0.63	0.63
3	0.42	0.42
4	0.38	0.38

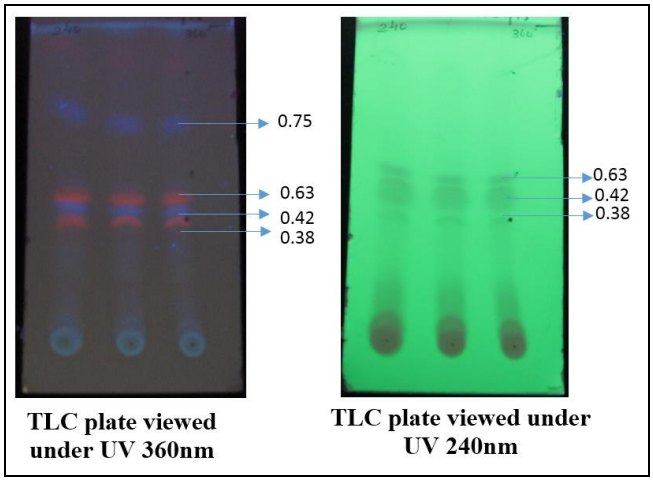

Fig 1: TLC plate viewed UV Light

Glucose Uptake in Yeast Cells of Acetone Extract of *T. Cucumerina*

The rate of glucose transport across cell membrane in yeast cells system is presented in Fig-2. The amount of glucose remaining in the medium after a specific time serves as an indicator of the glucose uptake by the yeast cells. The rate of uptake of glucose into yeast cells was linear in all the three glucose concentrations.

The acetone extract of *T. cucumerina* leaves exhibited significantly higher activity than at all concentrations. However the highest uptake of glucose was seen in 25mM Glucose concentration. The result showed the lower uptake of glucose by the yeast cells which conformed the highest activity.

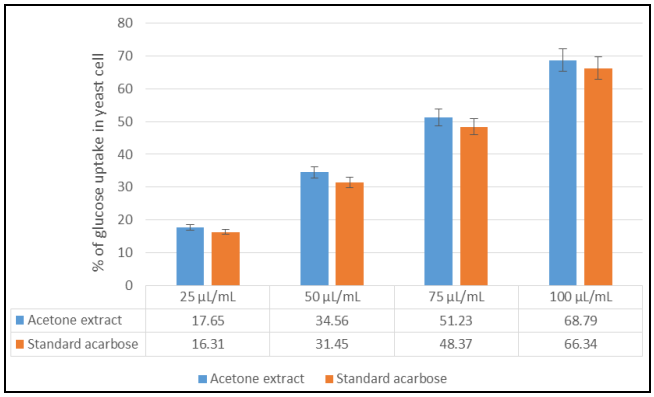

Fig 2: Glucose Uptake in Yeast Cells of Acetone Extract of *T. Cucumerina*

α- Amylase inhibition of acetone extract of *T. Cucumerina*

Alpha amylase is an enzyme that hydrolyses alpha-bonds of large alpha linked polysaccharide such as glycogen and starch to yield glucose and maltose. Alpha amylase inhibitors bind to alpha- bond of polysaccharide and prevent break down of polysaccharide in to mono and disaccharide. In present experimental study it was observed that acetone extract of *T. cucumerina* demonstrated inhibition of alpha amylase. But the result of acetone extract of *T. cucumerina* significant inhibition of alpha amylase activity as compared to standard drug acarbose (Fig-3).

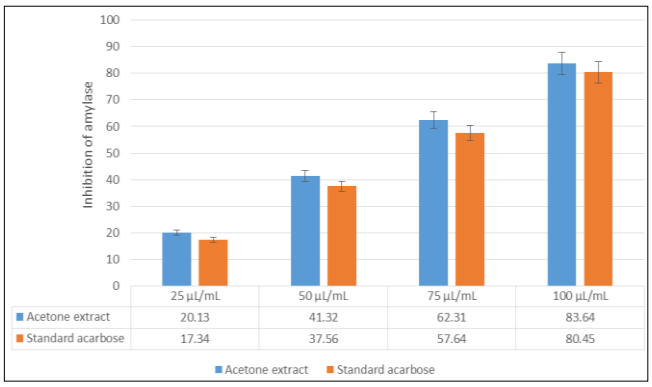

Fig 3: α- Amylase inhibition of acetone extract of *T. Cucumerina*

α- Glucosidase inhibitory activity of acetone extract of *T. Cucumerina*

The results of *in-vitro* α-glucosidase inhibitory study are showed in Fig-4. The acetone extract of *T. cucumerina* showed a concentration-dependent inhibition of enzyme. The highest concentration of 100 µl/ml tested showed a maximum inhibition of nearly 69.31% in acetone extract of *T. cucumerina* seems to be less potent in α-glucosidase inhibitory potential compared to Acarbose. It may be that α-glucosidase is more sensitive towards acarbose with the concentration required for 50% inhibition (EC_{50}) found to be 72.31µg/ml.

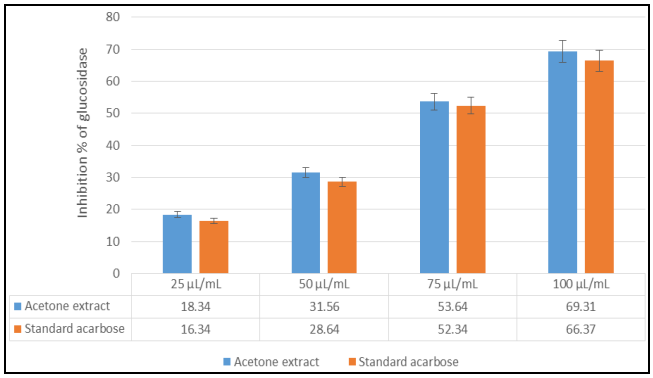

Fig 4: α- Glucosidase inhibitory activity of acetone extract of *T. Cucumerina*

Inhibition of Lipid Peroxidation Activity

The acetone extract of *T. cucumerina* also inhibited the lipid peroxidation induced by ferrous sulfate in egg yolk homogenates. Maximum inhibition was recorded in acetone extract of *T. cucumerina* leaves (73.21%) and lowest inhibition percentage of ascorbic acid was found in 70.32% (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).

In the present study, egg yolk was used as substrate for free radical mediated lipid peroxidation, which is a non-enzymatic method. Normally, the mechanism of phenolic compounds for antioxidant activity includes neutralizing lipid free radicals and preventing decomposition of hydroperoxides into free radicals.

Table 3: Inhibition of lipid peroxidation activity acetone extract of *T. cucumerina*

Different concentration of extract	Inhibition percentage of lipid peroxidation	
	Acetone extract of *T. cucumerina*	Standard Vitamin-C
25 µl/ml	16.34±1.78	19.64±0.89
50 µl/ml	35.32±3.21	33.65±1.78
75 µl/ml	54.23±0.89	51.48±2.89
100 µl/ml	73.21±2.56	70.32±1.45
EC_{50} Value	56.32	60.32

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

Metal Chelating Activity of Actone Extract of *Trichosanthes Cucumerina*

The metal chelating property of acetone extract of *T. cucumerina* was displayed as per Table-5. Acetone extract of *T. cucumerina* were evaluated for their ability to compete with ferrozine for ferrous iron in the solution. In this evaluation, the acetone extract of *T. cucumerina* hindered the formation of ferrous and ferrozine complex, signifying that they have chelating activity and are capable of Capturing ferrous iron before ferrozine.

The acetone extract of *T. cucumerina* reduced the greenish blue color complex immediately and showed the highest chelating activity 77.32 % than positive control. The iron (II) chelating activity of herbal extracts is of great significance, because it has been proposed that the transition metal ions contribute to the oxidative damage in neurodegenerative disorders and balance of diabetics (Aparadh *et al*., 2012) [1].

Table 4: Metal chelating activity of acetone extract of *T. cucumerina*

Different concentration of extract	Percentage of Metal chelating activity	
	Acetone extract of *T. cucumerina*	Standard Vitamin-C
25 µl/ml	20.31±2.39	17.32±2.45
50 µl/ml	38.64±2.54	30.56±2.89
75 µl/ml	58.23±1.78	48.63±1.56
100 µl/ml	77.32±2.48	67.32±0.28
EC_{50} Value	51.23	63.34

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

Conclusion

In conclusion, the potential anti-diabetic effect of acetone extract of *T. cucumerina* was well established and the possible underlying mechanisms of this effect may be related with that acetone extract of *T. cucumerina* could inhibit α-amylase and α- glucosidase activity to decrease the absorption of carbohydrates from food; and also, acetone extract of *T. cucumerina* could directly stimulate insulin secretion and preserve the function of β-cell to ameliorate glucose metabolism. So, further investigations are deserved to elucidate specific components and their mechanisms of acetone extract of *T. cucumerina* for its anti-diabetic effect.

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