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ANTI-DIABETIC ACTIVITY AND PHYTOCHEMICAL SCREENING OF EXTRACTS OF THE LEAVES OF *TINOSPORA CORDIFOLIA* ON ALLOXAN-INDUCED DIABETIC MICE

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Abstract:
In India, the number of people suffering from diabetes is believed to be rising steadily and the current antidiabetic therapies are frequently reported to have adverse side effects. Ethno medicinal plant use has shown promise for the development of cheaper, cost-effective antidiabetic agents with fewer side effects. Te aim of this study was to investigate the antidiabetic activity and mechanism of action of aqueous leaf extract prepared from Tinospora cordifolia. Since this claim has not been investigated scientifically, the aim of this study was to evaluate the antidiabetic effect and phytochemical screening of alloxan-induced diabetic Mice. The leaves of Tinospora cordifolia have been used in traditional health systems to treat diabetes mellitus. However, the antidiabetic activity of this medicinal plant is not scientifically validated and authenticated. The present study aimed to investigate the in vitro and in vivo anti-diabetic activity of flower crude extract and solvent fractions of Tinospora cordifolia. The in vitro α–amylase inhibition of the crude extract and solvent fractions of Tinospora cordifolia. Blood glucose lowering activity of 80% Ethanolic crude extract and solvent fraction was studied in animal models: Hypoglycemic mice model, oral glucose loaded mice model, dose-treated Alloxan -induced diabetic mice model. The effect of the crude extract on diabetic lipid profile was studied.The acute toxicity study of Tinospora cordifolia leaves extract did not show mortality in the animals at the limit dose during the observation period. The result of α–amylase enzyme inhibition activity was found in a dose-dependent manner, the strongest activity was shown by Crude extract fraction (89.60 % inhibition at 1000 µg/mL) compared to the standard acarbose having 97.19% inhibition at 1000 µg/mL. The crude extract of Tinospora cordifolia showed significant blood glucose-lowering effect on hypoglycemic mice and oral glucose loaded mice. In Alloxan-induced diabetic mice model, the crude extract fraction significantly decreased the fasting blood glucose level after 14 days of treatment.The result demonstrated the beneficial biochemical effects of Tinospora cordifolia extract by inhibiting α–amylase improving serum lipid profile levels. The leaves crude extract are effective in lowering blood glucose levels in diabetic and hypoglycemic mice. The claimed traditional use as antidiabetic has scientific ground.

Key words: *Diabetes mellitus, Herbal medicine, Tinospora cordifolia, Alloxan, Anti diabetic activity.*

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1. INTRODUCTION:

1.1-Diabetes Mellitus (DM):

Diabetes is one of the most common non-communicable diseases and a serious life-long condition appearing worldwide. The etiology of diabetes is a complex interaction of genetic and environmental factors. It is a heterogeneous group of metabolic disorders characterized physiologically by dysfunction of pancreatic beta cells and deficiency in insulin secretion or insulin activity and clinically by hyperglycemia or impaired glucose tolerance and other manifestable disorders. It is an endocrinological syndrome abnormally having high levels of sugar in the blood. This may be either due to insulin not being produced at all, is not made at sufficient levels, or is not as effective as it should be. Diabetes is still a serious health problem all over the world since it is associated with increased morbidity and mortality rate. When compared with the general population, mortality and morbidity increase in diabetes is mainly due to the associated chronic complications both specific (microvascular) and nonspecific (macrovascular). Since the disease prevails in both genders and in all age groups, the general public has a concern about its control and treatment[1].

1.2-Classification of DM

Diabetes is classified by underlying cause. The most common forms of diabetes are categorized as

Type 1, or insulin-dependent diabetes mellitus (IDDM) - an autoimmune disease in which the body's own immune system attacks the pancreatic beta cells, rendering it unable to produce insulin and

Type 2, or non-insulin-dependent diabetes mellitus (NIDDM) - in which there is resistance to the effects of insulin or a defect in insulin secretion. Type 2 diabetes commonly occurs in adults associated with obesity. There are many underlying factors that contribute to the high blood glucose levels in these individuals. An important factor is the resistance to insulin in the body essentially ignoring its insulin secretions. A second factor is the decreased production of insulin by the cells of the pancreas. Therefore, an individual with Type 2 diabetes may have a combination of deficient secretion and deficient action of insulin. In contrast to Type 2 diabetes, Type 1 diabetes most commonly occurs in children and is a result of the body's immune system attacking and destroying the beta cells. The trigger for this autoimmune attack is not clear, but the result is the end of insulin production[2].

Multiple risk factors for the development of Type 2 diabetes mellitus[3]:

- Family history (parents with diabetes).
- Obesity (i.e., ≥20% over ideal body weight or body mass index ≥25kg/m^2).
- Habitual physical inactivity.
- Impaired glucose tolerance.
- Hypertension (≥140/90mm Hg in adults).
- High density lipoprotein (HDL) cholesterol ≤ 35mg/dl and/or triglyceride level ≥ 250mg/dl.

1.4-Epidemiology[6]

Present status projects that incidence of diabetes is on rise. Present number of diabetics worldwide is 150 million and according to new estimates from researchers at the World Health Organization (WHO), there will be an increase of about 300 million or more by the year 2030 (Warner, 2004). Only in year 2001, about 441,004 deaths were registered and 49,855 of them provoked by diabetes, representing 11.2% of the total population. In United States, diabetes is the sixth leading cause of death. The prevalence of diabetes mellitus is rapidly increasing worldwide and India is estimated to have 31 million diabetics from the total population of the world. Diabetes is predicted to become one of the most common diseases in the world within a couple of decades, affecting at least half a billion people.

The driving force behind the high prevalence of diabetes is the rise of obesity, sedentary lifestyle, consumption of energy rich diet, etc. The diabetes epidemic is accelerating in the developing world, with an increasing proportion of affected people in younger age groups.

The prevalence of Type 2 diabetes is now at epidemic proportions. Type 2 diabetes has a significant impact on the health, quality of life, and life expectancy of patients, as well as on the health care system. Type 2 diabetes accounts for about 90-95 % of population while Type 1 diabetes accounts for about 5 -10% of the total population. In the past, Type 2 was rarely seen in the young, but recent reports describe Type 2 diabetes being diagnosed even in children and adolescent[7].

1.5-Sugar Regulation: Carbohydrate, protein and lipid metabolism[8]

Glucose is an essential fuel for the body and is the main source of energy for the tissue cell. The amount of glucose in the blood is controlled mainly by the hormones insulin and glucagon. The rise in blood glucose following a meal is detected by the pancreatic beta cells, which respond by releasing insulin. Glucose is transported into the beta cell by Type 2 glucose transporters (GLUT2). As glucose metabolism proceeds, ATP is produced which closes ATP-gated potassium channels in the beta cell membrane. Positively charged potassium ions (K^+) are now prevented from leaving the beta cell. The rise in positive charge inside the beta cell causes depolarization thereby opening the voltage-gated calcium channels and allowing calcium ions (Ca^{2+}) to flood into the cell. The increase in intracellular calcium concentration triggers the secretion of insulin via exocytosis.

Insulin increases the uptake and use of glucose by tissues such as skeletal muscle and fat cells. Once inside the cell, some of the glucose is used immediately via glycolysis. Any glucose that is not used immediately is taken up by the liver and muscle where it can be converted into glycogen (glycogenesis). When glycogen stores are fully replenished, excess glucose is converted into fat in a process called lipogenesis by increasing the number of glucose transporters (GLUT4) expressed on the surface of the fat cell, causing a rapid uptake of glucose. Glucose is converted into fatty acids that are stored as triglycerides for storage[9].

The rise in glucose also inhibits the release of glucagon, inhibiting the production of glucose from other sources, e.g., glycogen break down. Glucose may also indirectly contribute to protein synthesis by synthesis of amino acids. Glucagon is the main hormone opposing the action of insulin and is released when food is scarce. Glucagon also helps the body to switch to using resources other than glucose, such as fat and protein during starvation[10].

MATERIALS AND METHODS:

Instruments

Following instruments were required for the study:

Table No: 6.1- List of Instruments used for study

Name of the instrument	*Source*
Centrifuge	Dolphin
Digital weighing balance	Horizon
Glucometer	Horizon
Heating mantle	ASGI®
Refrigerator	Videocon
Soxhlet extractor	ASGI®
Condenser	ASGI®
Burette stand	Dolphin
Round bottom flask	ASGI® , Amar
Mixer	Videocon
Oven	ASGI®
Water bath	ASGI®
Stirrer/glass rod	ASGI®
Watch glass	ASGI®
Whatmann filter paper	Manipore microproducts, Ghaizabad.
Butter paper	ASGI®
Spatula	ASGI®
Rubber pipes	ASGI®

a) Plant collection

The leaves s of *Tinospora cordifolia* was collected from Local market.

b) Preparation of coarse powder and Extraction technique

The leaves were shade dried at room temperature for 10 days. Then these were milled into powder by mechanical grinder. This powder was sequentially extracted to their increasing polarity with Petroleum ether, Ethyl acetate, Ethanol respectively. About 500gm of powdered leaves was uniformly packed into a thimble in a Soxhlet apparatus and extracted with 1000ml Petroleum ether, Ethyl acetate and Ethanol, respectively. Constant heat was provided by Mantox heater for recycling of the solvent. The process of extraction continues for 1-2 hours for each solvent. The excess solvent was evaporated and the dried extracts were kept in refrigerator at 4ºC for their future use in phytochemical analysis and pharmacological screenings.

***Invitro* antidiabetic activity of *Tinospora cordifolia* leaves extracts**

Alpha-amylase inhibition assay

The a-amylase inhibition assay was performed using the 3,5-dinitrosalicylic acid (DNSA) method.50 The crude and solvent fractions of *Tinospora cordifolia* were dissolved in buffer (($Na2HPO4$/ $NaH2PO4$ (0.02 M), NaCl (0.006 M) at pH 6.9) to give concentrations ranging from 50 to 1000 mg/mL. A volume of 200 mL of a-amylase solution (Molychem) (2 units/mL) was mixed with 200 mL of the extract and was incubated for 10 minutes at 30 C. Thereafter, 200 mL of the starch solution (1% in water w/v) was added to each tube and incubated for 3 minutes. The reaction was terminated by the addition of 200 mL DNSA reagent (12 g of sodium potassium tartrate tetrahydrate in 8.0 mL of 2 M NaOH and 20 mL of 96 mM 3,5-DNSA solution) and was boiled for 10 minutes in a water bath at 85°C. The mixture was cooled to ambient temperature and was diluted with 5 mL of distilled water, and the absorbance was measured at 540 nm using a UV-visible spectrophotometer (Agilent Technologies). The blank with 100% enzyme activity was prepared by replacing the plant extract with 200 mL of the buffer. A blank reaction was similarly prepared using the plant extract at each concentration in the absence of the enzyme solution. A positive control sample was prepared using acarbose (Bayer) and the reaction was performed similarly to the reaction with plant extract as mentioned above. The inhibition of a-amylase was expressed as percentage of inhibition and was calculated by the following equation: Inhibition (%) ¼ [(Ac -Acb) (As Asb) / (Ac -Acb)] × 100, where Ac is the absorbance of control; Acb is the absorbance of control blank; As is the absorbance of sample; and Asb is the absorbance of sample blank. The % a-amylase inhibition was plotted against the extract concentration and the IC50 values were obtained from the graph.

RESULTS:

a) Appearance and percentage yield of EETC (Ethanolic Extract of *Tinospora cordifolia* Leaves)

Table 7.1: a-Amylase Inhibitory Activities of the Crude Extract and Solvent Fractions.

	Percentage inhibition				
Concentration (mg/mL)	**Chloroform fraction**	**Ethyl acetate fraction**	**Aqueous fraction**	**Crude extract**	**Acarbose**
50	6.41 + 0.1	15.82 + 0.35	29.16 + 1.11	34.91 + 0.36	57.65 + 0.79
100	11.64 + 0.69	20.04 + 0.11	35.71 + 0.82	41.05 + 1.42	68.10 + 0.46
200	23.14 + 0.45	27.16 + 1.92	42.12 + 0.46	61.19 + 0.98	76.93 + 1.53
400	29.65 + 0.50	46.90 + 0.15	54.81 + 0.53	73.34 + 0.76	88.51 + 0.17
600	38.01 + 0.99	54.14 + 0.64	68.93 + 0.92	81.92 + 0.24	93.06 + 0.26
800	45.15 + 0.81	65.54 + 0.49	75.50 + 0.76	86.41 + 0.19	96.27 + 0.17
1000	53.34 + 0.76	74.77 + 0.12	83.19 + 0.81	89.60 + 0.74	97.19 + 0.92
IC50	31.14 + 0.12	21.80 + 0.71	14.24 + 0.64	7.21 + 0.91	3.34 + 0.14

Abbreviation: IC50, half maximal inhibitory concentration.
Each value of percentage inhibition of a-amylase is presented as means + standard error of the mean (SEM), n ¼ 3.

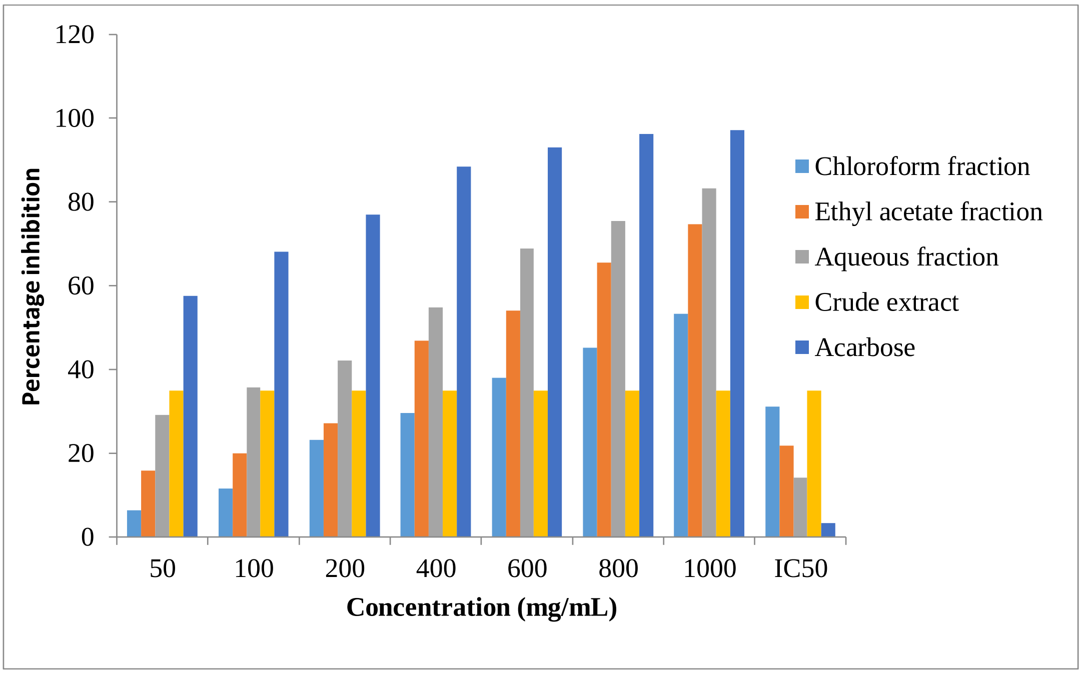

Figure 7.1:a-Amylase inhibitory activity of the ripe crude extract and solvent fractions of *Tinospora cordifolia*

In Vitro a-Amylase Inhibition Activity of Crude Extract and Solvent Fractions *In vitro* a-amylase inhibitory study evaluating the percent of a-amylase inhibition as a function of extract concentrations and the IC50 values were calculated (Figure). Concentration dependent inhibitions were observed for various concentrations of the tested extracts and the standard. Among the extracts, the crude extract exhibited the lowest IC50 of 67.21 + 0.91 mg/mL and the IC50 values of water fraction, ethyl acetate fraction, and the chloroform fraction were 14.24 + 0.64, 21.80 + 0.71, and 31.14 + 0.12 mg/mL, respectively. The standard positive control acarbose showed an IC50 of 3.34 + 0.14 mg/mL (Table).

Minimum % Inhibition was found *Tinospora cordifolia* leaves which resemblance to %Inhibition of positive control, So Ethanolic extract of *Tinospora cordifolia* contain active constituents of antidiabetic.

Phytochemical studies

Table No 7.2: Results of Ethanolic extract of *Tinospora cordifolia* leave

Class of compounds	Tests performed	Results
Carbohydrates	Molisch's test Fehling's test	-
Phenols	Phosphomolybdic acid test	+
Flavonoids	Shinoda test Lead acetate test	+ +
Tannins	Braemer's test	-
Alkaloids	Wagner's Mayer's Draggendrof's test	+ + +
Glycosides	Legal's test Brontranger's test	+ +
Saponins	Foam test	+
Sterols	Salkowski's test	-
Amino acids	Ninhydrin test	-
Terpenoids	Lieberman Burchardt test	+

+Present in moderate amount
-Absence

The phytochemical studies results revealed that the Molisch's test no characteristic observation indicated the absence of carbohydrates, by phosphomolybdic acid test Blue coloration of the spot indicated the presence of phenols. Shinoda test and Lead acetate test gave pink or red coloration of the solution indicated the presence of flavonoids Flocculent white precipitate also indicated the same. There is no dark blue or greenish grey coloration of the solution indicated the absence of of tannins in the drug. No characteristic observation for steroids and dark pink or red coloration of the solution indicated the presence of Terpenoids. Orange coloration of the spot indicated the presence of alkaloids. Yellow or reddish brown precipitation indicated the presence of alkaloids. Pink to red colour solution indicates the presence of glycosides. No layer of foam formation indicates the absence of Saponins. If the response to the test is indicated table-1high it can be noted or which indicates that the particular group is present as the major class. If the response is average then note it as indicates the presence in moderate quantity and note it as indicating the presence of only in traces. If no response is then negative.

TABLE 7.3: Hypoglycemic Test

TREATMENT	DOSE mg/kg	BLOOD GLUCOSE LEVEL (mg/dl)		
		0 min	**30min**	**1hr**
CONTROL Carboxymethyl Cellulose (CMC)	0.5%	69.15±2.451	68.14±4.320	71.19±2.129
Positive Control Glibenclamide	2	67.24±3.209	50.15±1.492**	30.96±3.298***
Aqueous Ethanolic Extract of *Tinospora cordifolia*	200	66.87±1.251	57.91±3.482*	55.14±2.101*
Aqueous Ethanolic Extract of *Tinospora cordifolia*	400	66.18±3.420	50.19±3.281**	34.2+±1.921***

The glucose levels were analyzed by using glucometer and each value is the mean ± standard error (n= each group consist of 6 animals)($p<0.05$)*, ($p<0.001$)**& ($p<0.0001$)*** as compared to control & positive control group evaluated by one way, ANOVA followed by Dunnet 't' test.

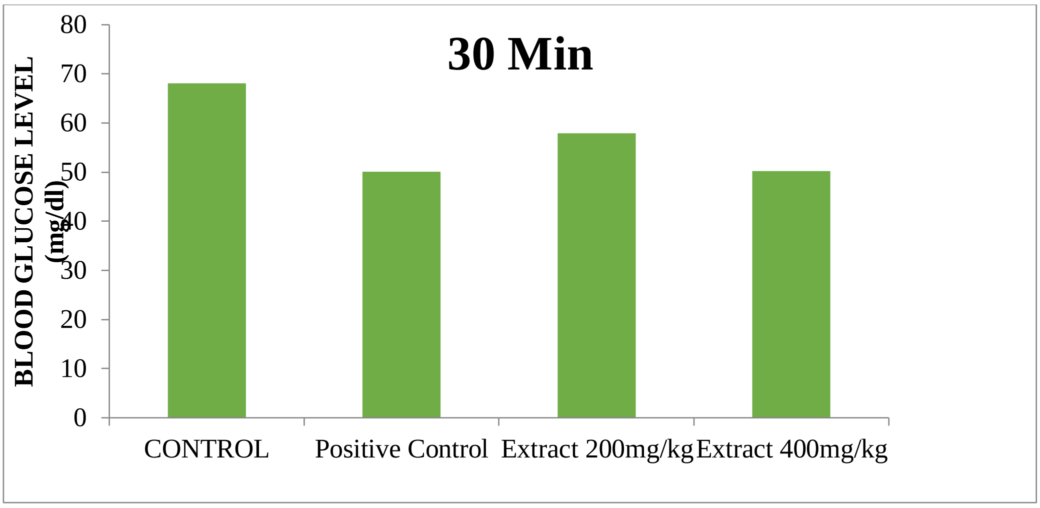

Fig 7.2: Blood glucose level 30min

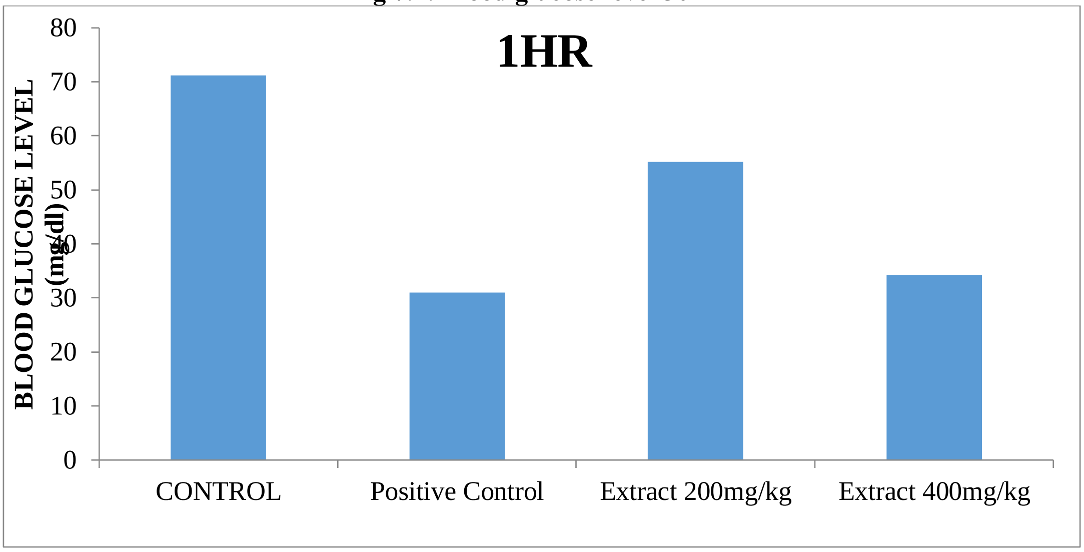

Fig 7.3: Blood glucose level 1hr

The hypoglycemic test results have shown Table No: which indicated aqueous ethanolic extract of *Tinospora cordifolia* treated animals 200 & 400, significantly decreased in blood glucose level when compared to control and positive control.

g) *Invivo* antidiabetic study

Table 7.4: Results of the effects of Ethanolic extract on blood Glucose levels

TREATMENT	BLOOD GLUCOSE LEVEL (mg/dl)		
	0 min	30min	1hr
Normal control 10 ml/kg P.O	77.29±3.104	73.1±3.219	72.2± 3.917
Negative control	261.1±2.91	267.2±4.1	271.3±2.1
Positive control (Glibenclamide 2mg/kg) P.O	251.18±3.156	136.98±2.4***	113±1.1***
EETC 200 mg/kg P.O	256±2.1	245.1±2.154**	241.2±1.209**
EETC 400 mg/kg P.O	260±1.10	170.2±1.72***	158.1±2.9***

(The values were expressed as Mean ± S.E.M. (n=6 animals in each group).

The experimental results have indicated on Table the negative control group glucose levels were significantly increased when compared to each other groups. All the groups of animals were affected in diabetes, which indicated blood glucose levels were slight changes in the blood glucose level for normal control group at 7th and 14th days. On day 7th glucose levels were significantly decreased Glibenclamide 2mg/kg treated group when compared with control group at 7th and 14th days. The Ethanolic leaves extract of *Tinospora cordifolia* treated groups 200 & 400 mg/kg were dose dependent manner decreased when compared with control group but positive control have more anti diabetic activity at 7th day. The aqueous Ethanolic leaves extract of *Tinospora cordifolia* at the dose level 400mg/kg have equipotent activity when compared with positive control at 7th day. The Ethanolic leaves extract of *Tinospora cordifolia* 200 & 400 mg/kg have been expressed dose dependent anti diabetic action when compared to control and positive control. On day 14th, Ethanolic leaves extract of *Tinospora cordifolia* treated animals 200 & 400 mg/kg significantly decreased and maintain the blood glucose level when compared to control and positive control.

Table 7.5: Oral Glucose Tolerance Test

Treatment	DOSE mg/kg	Blood Glucose Level (mg/dl)						
		0 min	0.5hr	1hr	1.5hr	2hr	2.5hr	3hr
Control (CMC)	0.5%	65.01±2.164	140.1±1.352	185.1±2.151	170.1±12.41	154.2±4.121	151.0±1.194	130.1±10.81
Positive Control Glibenclamide de	2	69.10±0.18	102.0±2.181**	110.1±3.24 ***	91.21±3.287 ***	81.20±1.921 **	75.01±1.259* **	71.51±2.910 ***
EETC	200	68.14±5.101	125.1±2.014	144.1±2.115 *	134.1±0.181 *	125.1±0.126 *	111.14±0.26**	105.0±3.214 **
EETC	400	68.00±1.159	113.1±0.181**	121±4.142 **	101.1±4.296 ***a	91.30±1.365 *** a	84.21±2.06* **a	80.21±316 ***a

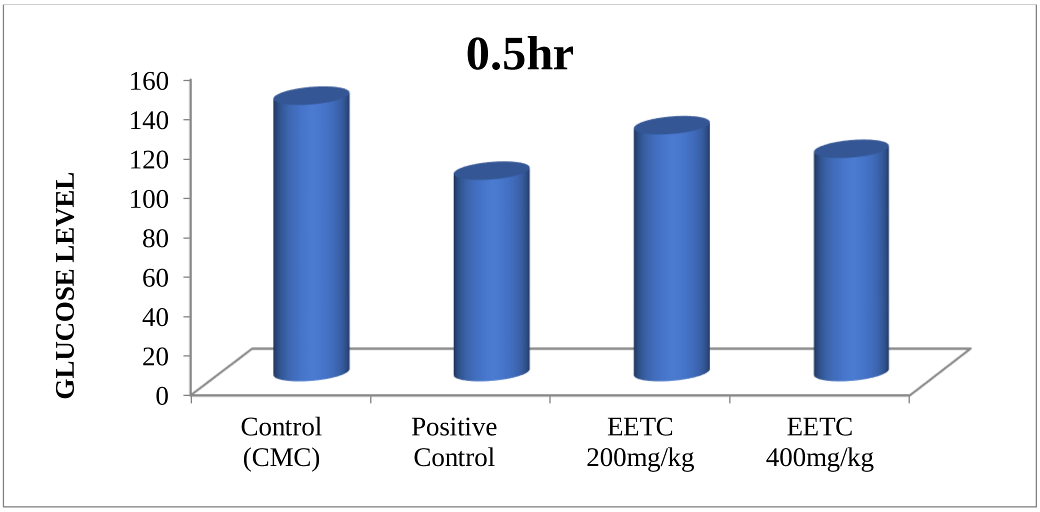

Fig 7.4: Blood Glucose Level (mg/dl) 0.5hr

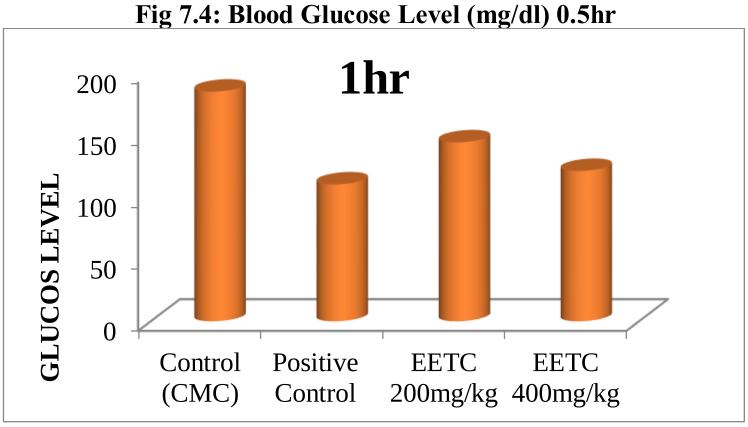

Fig 7.5: Blood Glucose Level (mg/dl) 1hr

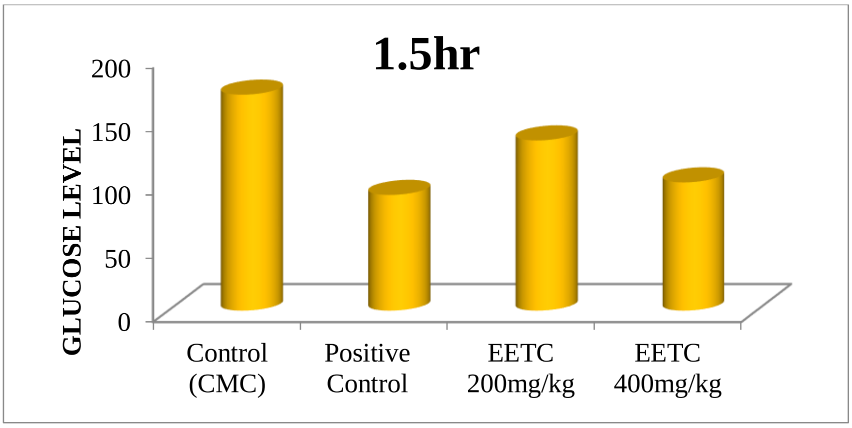

Fig 7.6: Blood Glucose Level (mg/dl) 1.5hr

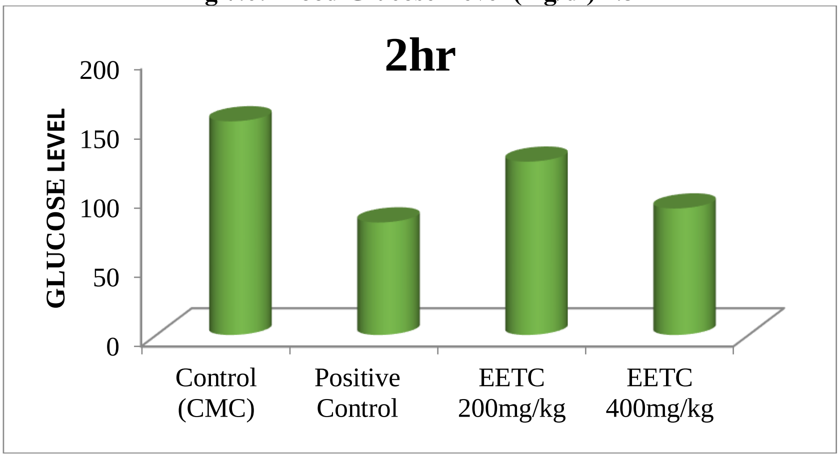

Fig 7.7: Blood Glucose Level (mg/dl) 2hr

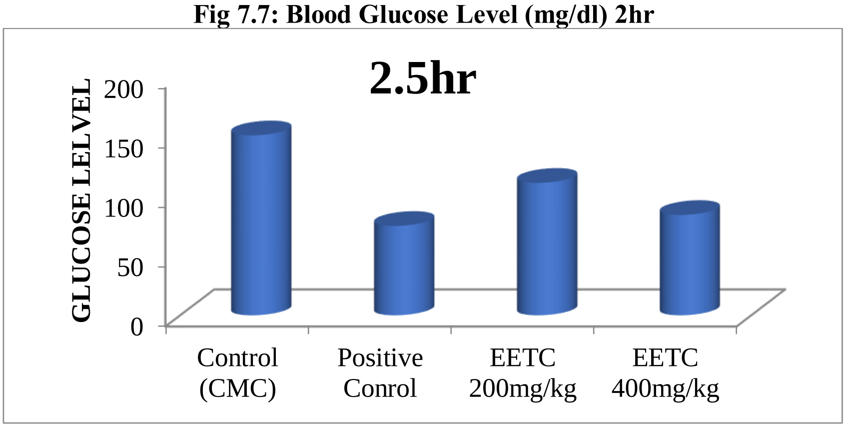

Fig 7.8: Blood Glucose Level (mg/dl) 2.5hr

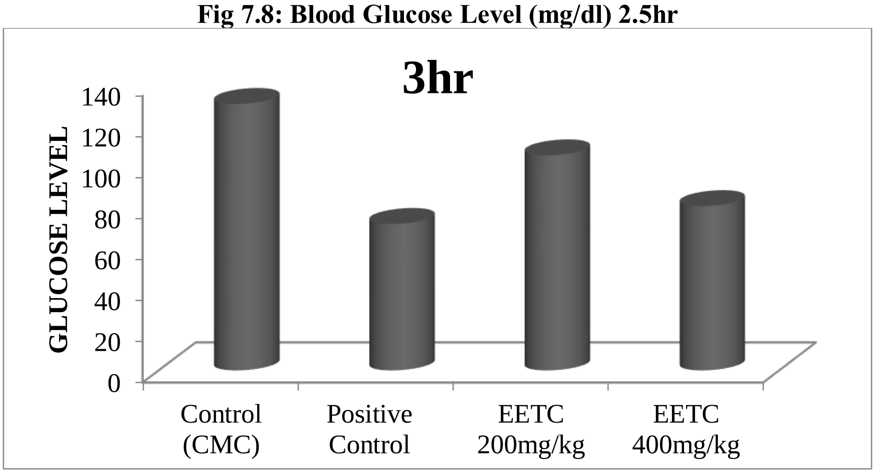

Fig 7.9: Blood Glucose Level (mg/dl) 3hr

Oral Glucose Tolerance Test (OGTT) results have been expressed on Table. Half hour after the glucose treatment, all the groups of animal blood glucose levels were significantly increased. The blood glucose levels were significantly decreased for, Ethanolic extract of *Tinospora cordifolia* 200 & 400 mg/kg when compared to control and positive control at 1hour and each and every ½ hour blood glucose levels (200 mg/kg were changes in the dose dependent manner extract treated group of animals compared to control and positive control but 400mg/kg produce the equipotent activity.

DISCUSSION:

Invitro study is on the principle of Inhibition of α-amylase, enzyme that plays a role in digestion of starch and glycogen are considered a strategy for the treatment of disorders in carbohydrate uptake, such as diabetes. Pancreatic αamylase is a key enzyme in the digestive system and catalyses the initial step in hydrolysis of starch to a mixture of smaller. Sequential extraction was done according to increasing polarity order. Each extracts were tested for α-amylase inhibition to get the extraction with minimum IC50 value. As per the above mechanism all the extract have concentration dependent affinity towards the inhibition of α-amylase. Finally acarbose extract was observed as more active extract.

In this present study acute toxicity study was carried out in Mice. The procedure was followed by using OECD 423 (Acute Toxic Class Method). The acute toxic class method is a step wise procedure with three animals of a single sex per step. Average two to three steps may be necessary. The method used to defined doses (2000, 1000, 500, 50, 5 mg/kg body weight, Up-and-Down Procedure). Observe for signs for toxicity and were noted for 14 days. The onset of toxicity and signs of toxicity also noted. Hence, 1/10th (200mg/kg) and 1/5th (400mg/kg) of this dose were selected for further study. The principle involved in the Alloxan induced diabetes mellitus in Mice, Alloxan, a cytotoxic, diabetes induced chemical but wide variety of animal species by damaging the insulin secreting cells of the pancreas.

Literature sources indicate that the Alloxan induced Mice are hyperglycaemic. The treatment of lower doses of Alloxan (100mg/kg b.w.) produced partial destruction of pancreatic β-cells even though the animals become permanently diabetic.

Thus these animals have surviving β cells and regeneration is possible. It is well known that the sulfonylurea's act by directly stimulating the β-cells of the islets of Langerhans to release more insulin and these compounds are active in mild Alloxan induced diabetes. *Invivo* anti diabetic screening was performed for the confirmation of above mechanism of action was undergone the Ethanolic extract of *Tinospora cordifolia* biological system (Which was already resulted for α-amylase inhibitory activity.

At the end of the Ethanolic extract of *Tinospora cordifolia* (200 mg/kg p.o, 400 mg/kg p.o.) showed statistically significant decrease in blood glucose levels. So the Ethanolic extract of *Tinospora cordifolia* showed antidiabetic activity. This work will be useful for further diabetes mellitus and its related diseases research worker to develop new entity for the treatment of diabetes mellitus.

CONCLUSION:

This study revealed that the crude extract and solvent fractions of *Tinospora cordifolia* have showed significant lowering of blood glucose level on diabetic, Hypoglycemic and oral glucose loaded mice and not permitted bodyweight loss of diabetic. The results also verified that inhibition of intestinal α-amylase by the extracts may contribute to the antihyperglycemic activity. The results give scientific support for the use of the plant in folk medicine for the management of diabetes and its associated complications. *Tinospora cordifolia* would be promising for further clinical studies in the management of DM. Further studies to find out the mechanism of this plant for its antidiabetogenic effect and there is a need for bioactivity guided investigation to isolate the lead compound responsible for the antidiabetic activity.

The present study suggested that the isolation of active constituents from Ethanolic extract of *Tinospora cordifolia* leaf and characterize the compounds by using preliminary phytochemical studies.

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