ANTI DIABETIC ACTIVITY OF ETHANOLIC EXTRACT OF *Aconitum Ferox* IN ALLOXAN INDUCED DIASEASED RATS

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ABSTRACT

Diabetes mellitus a metabolic disorder of multiple aetiology characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both. It is common chronic disease in nearly all countries and continues to increase in number and significance, as changing lifestyle lead to reduced activity, and increase in obesity. There are many herbal medicines used in treating diabetes. So, we have found Aconitum ferox. The tuberous roots have the antioxidant property so for that we have done extraction of active components by using ethanol as the main solvent and we conducted some in-vitro and in-vivo studies depending on the preliminary phytochemical screening.

KEY WORDS
Diabetic, Alloxan, Aconitum ferox, Ethanolic extract.

INTRODUCTION

The word diabetic means siphon, which refers the major symptom of the condition, frequent urination. And mellitus means honey, which indicates the presence of the sugar in the urine. The diabetes disease is not the newly born one it has been from the human race but it came to known from the period of 155 B.C. where many Greek and French physicians worked on the diabetes disease and found the nature and which organs are responsible for causing the disease. The history of diabetes is a fascinating one[1,2,3].

In the first century B.C, Aretaeus of Cappadocia the Greek physician termed diabetes, meaning "one who straddles" or "siphon'. But in India the ancient physicians called diabetes as "sweet urine disease" they termed because they used to observe for the ants, if they observe any ants they used to conform as diabetic patient[4].

In 1675 Dr. Thomas Willis an English physician described the sugar taste of the urine in people with diabetes and in 1750's Cullen, a scientist adds, mellitus which is a Latin word means "honey-sweet". During 1900 period Drs. Joseph von Mering and Oskar Minkowski carried out a research on the animals and they found that the diabetes problem is due the pancreas and in 1910 Sir Edward Albert Sharpey-

Schafer found the chemical which is released by the pancreas known as insulin.

In 1922 first insulin injections were used to treat the diabetic patients and for that awarded the noble prize. In 1936 Sir Harold Percival Himsworth distinguished the difference between the type-1 and type-2 diabetes. 1942 The first Sulfonylurea's are identified and in 1977 the radioimmunoassay for insulin was discovered by Rosalyn Yalow and Solomon Berson. In 1988 Dr. Gerald Reaven identified the metabolic syndrome and in 1990 Thiazolidinedione was identified as an effective insulin sensitizer[5-9].

Diabetes is a chronic disorder where is it affecting millions of people every year and it is spreading very fast like scourge. Worldwide about 246 million are affected by the diabetes and it is to reach about 380 million by the year 2025.

Diabetes is the sixth leading cause of deaths in the United States and its risk increases along with the age. About 7 out of 10 countries the diabetes is prevailing in the developing countries. Where India is leading the front with first place follow up by the China, United States. And in some countries like Caribbean and Middle East the percentage of the diabetes population is about 12-20%. The 21 million Americans or 7% of the population are affected by the diabetes and about 41 million people with the

pre-diabetes, where this pre-diabetes condition can lead to the type-2 diabetes, given by the Centre for Disease Control (CDC) [10-14].

Other than the epidemic factors the diabetes is also affected mainly by change in their lifestyle, diet, genetic predisposition. And also by the industrialization, which provides us the leisure time along with decrease in the reduction of physical activity. The type-1 diabetes is seen in only 5-10% of the cases and which is due to genetic factors[15]. In Americas 60 years or older population, about 21% are affected by the diabetes and 2% of population is between the age group of 20-39 years, and 10% of diabetes population is between age group of 40-59 years. Basing on these statistics the US government is spending approximately about $132 billion dollars every year on diabetes disease.[16-18]

Amino acids are the building blocks of the Insulin which contains about 51 amino acids. Insulin is a anabolic peptide hormone secreted by the beta cells of islet of Langerhans. Insulin contains two chains of amino acids namely chain A and chain B. These two chains are connected by the disulfide bond. The chain A contains about 21 amino acids and the chain B contains 30 amino acid residues. This was confirmed by the Cambridge in 1955 by the Sanger's group. The insulin was first crystallized in rhomobohedral in the year 1926[19-22]

The main precursor of the insulin synthesis is preproinsulin in the rough endoplasmic reticulum, where this preproinsulin gets migrated to the Golgi apparatus and it undergoes proteolytic cleavage. The proinsulin and insulin plus are formed. This insulin plus is a fragment where its function is unclear called as C-peptide. The C-peptide is of variable length peptide consisting of 26-31 residues, which gets linked up with carboxy terminal of the B chain to the amino terminal of A chain via two dibasic links Arg-Arg and Lys-Arg. At these dibasic links the proinsulin gets cleaved by the enzyme called Trypsin separates the two chains of insulin along with free C-peptide[23]. The formed insulin is stored in the B-cells granules. The main primary function of the insulin is the stimulation of the glucose uptake from the systemic circulation. And it suppresses the hepatic gluconeogenesis, there by serving primary role in glucose homeostasis and preventing the metabolic disorder diabetes mellitus[24].

MATERIALS AND METHODS:

Chemically induced diabetes is the most commonly used animal model of diabetes. Chemical agents which induce diabetes can be classified into three categories. Specifically damage β-cell, Cause temporary inhibition of insulin production and secretion, Diminish the metabolic efficacy of insulin in target tissue.

Mainly two chemicals are use in inducing diabetes, they are: Alloxan induced diabetes and Streptozotocin induced diabetes.

Alloxan and Streptozotocin are the most prominent diabetogenic chemicals in diabetic research. The Alloxan and Streptozotocin are the toxic glucose analogues that preferentially accumulate in the pancreatic β-cell via the GLUT2 transporter[25].

Alloxan can generate the reactive species (ROS) in a cyclic reaction with its reduction product, dialuric acid. Chemical redox reaction between the Alloxan dialuric acid and protective actions of cytoprotective enzymes. The reduction of Alloxan to the dialuric acid requires the suitable thiol, tripeptide glutathione (GSH). The triketone structure of the Alloxan is important for the reaction with the glutathione. This in turn generates Alloxan radical as intermediate product. Other thiols such as cysteine, which are present at lower concentrations in the cell, dithiols and ascorbic acid are also suitable reducing agents and may therefore contribute to Alloxan reduction[26].

The major oxidation pathway of dialuric acid, a chain reaction dependent upon superoxide radicals and it is inhibited by the superoxide dismutase (SOD). In the absence of thiols such as GSH the Alloxan is not cytotoxic. When in the plasma membrane if there is presence of the thiols then the Alloxan reacts with the thiols and forms the ROS in the redox cycle. Thus these free radicals damage the DNA of β-cells and cause the cell death. And also Alloxan has the ability to react with the protein SH groups, especially the membrane protein like glucokinase on the β-cells, finally resulting in the cell necrosis[27].

Selection of Animals: About 40 Sprague Dawley was provided by the Sai Nath Agencies, Himayatnagar, Hyderabad, and they were divided into 10groups each of having four animals. This is divided based on their body weights.

All the S.D rats were feed a normal laboratory chew diet (Nutrilab Rodent Feed, PROVIMI) containing (W/W) of 21.88% crude proteins, 52.15% carbohydrates and 5.97% crude fat and they were housed under a 12:12hour light: dark cycle with 22-25°C.

The average body weights of the rats were 240-290±2. Before injecting the single dose of the Alloxan to animals they were kept overnight fasting and

immediately in next day the STZ mixed in 0.1M citrate buffer is administered to the animals with in the 30min.

About 442.48mg of Alloxan is dissolved in the 26ml of citrate buffer this dose is prepared as 45mg/kg bodyweight of the animals.

Collection of Plant: collection of plant tuberous roots from local market Afzal Gunj, Hyderabad, they were dried and pulverised. The powdered form is then used for the extraction process using Ethanol as the main solvent. The extracted compound is further used for the work.

Preliminary phytochemical screening: After the completion of the extraction process the obtained ethanolic extract is to be identified for the presence of the phytochemicals. The known quantity of ethanol extract of *Aconitum Ferox* (EEAF) is taken and used for the preliminary studies.

In vitro **Studies:**

Determination of free radical scavenging activity on DPPH radical: The EEAF of 5mg was weighed and dissolved in the 1ml of the DMSO solution. And further six serial dilutions were made 5mg/ml, 2.5mg/ml, 1.25mg/ml, 0.625mg/ml, 0.3125mg/ml, and 0.15625mg/ml. Then take 96 well micro-plate was taken and 25µl of test sample which is dissolved in the DMSO, to it add 125µl of 0.1M tris-HCL buffer of p^H 7.4and also add 125 µl of 0.5mM DPPH which is dissolved in the ethyl alcohol is used. This mixture is shaken and incubated for 30min. The absorbance was read at 517-nm spectrophotometrically.

The obtained absorbance was seen against the control group which is prepared without the test sample.

The test samples were measured against the Ascorbic acid as standard. And the serial dilutions are made as that made for the test samples.

The free radical scavenging activity was determined by using the formula **(A-B/A) × 100**[28].

Where A is the absorbance of the DPPH solutions without the test sample i.e., control sample and the B denotes the absorbance of the DPPH solution with the test sample, The SC_{50} values were calculated by using test sample values.

α-Glucosidase inhibitory assay:

About 5mg of the EEAF was taken and dissolved in the 1ml of the distilled water and further six serial dilutions were made like 5mg/ml, 2.5mg/ml, 1.25mg/ml, 0.625mg/ml, 0.3125mg/ml and 0.15625mg/ml. About 10µl of the sample was taken and dissolved in the DMSO solution and incubated for 5ml with 50µl of yeast α-glucosidase enzyme prepared in the 100mM phosphate buffer p^H 7.4. After 5min of incubation, 50µl substrate (5mM, p-nitrophenyl-α-D-glucopyranoside) prepared in the 100mM phosphate buffer.

The pre-substrate and 5min post substrate addition absorbance was measured at 405nm spectrophotometrically. The increase in absorbance from pre-substrate addition to post-substrate is observed.

The control was taken without the extract and compared with the extract.

The ascorbic acid is taken as the standard or reference drug against the test samples. The ascorbic acid sample is prepared same as the test sample preparation and six serial dilutions are made. And this standard is also incubated for pre and post addition of the substrate absorbance for 5Min.

The percentage of α-Glucosidase inhibition was measured by **(1-B/A) ×100**.

Where A is the absorbance of control and B is the absorbance of samples containing extracts.

The inhibitory concentration of the extract required to inhibit the activity of the enzyme by 50% (IC50) was calculated.

In vivo **methods: OGTT**

The oral glucose tolerance test (OGTT) measures the body's ability to use a type of sugar, called glucose, which is the body's main source of energy. An OGTT can be used to diagnose prediabetes and diabetes.

Before doing OGTT animals are fasted over night, and then the blood was collected by retro-orbital method. The blood is collected in the effendrof tubes which contains the EDTA (20%), mix the blood. This is done to avoid the blood coagulation. The collected blood is centrifuged for plasma. The blood is centrifuged for 5-10min at an rpm of 4000. Then the basal reading of the blood glucose was taken and then the oral glucose is given to the animals basing on their bodyweights. And the blood glucose levels are checked for 30min, 60min, 120min, 240min, and 480min respectively. The glucose levels are compared with the fasting glucose.

GROUP	TREATMENT
Group I	Control rats given only Citrate buffer (p.o.)
Group II	Diabetic rats treated with Alloxan (45mg/kg/i.p)
Group III	Standard rats treated with Alloxan (45mg/kg/i.p)+Acarbose (180µg / kg /day p.o.)
Group IV	Test rats treated with Alloxan (45mg/kg/i.p)+ EEAF (100mg / kg / day p.o.)
Group V	Test rats treated with Alloxan (45mg/kg/i.p)+ EEAF (200mg / kg / day p.o.)
Group VI	Test rats treated with Alloxan (45mg/kg/i.p)+ EEAF (400mg / kg / day p.o.)

Table 1: Grouping of animals after diabetes

Statistical Analysis: Data obtained from our study were statistically analysed by using Graphpad Instant Demo version 3.0. The values were expressed as mean ± SEM for each group. Significant difference between groups was determined using one-way ANOVA followed by Dunnet multiple comparison test.
 A p value less than 0.05 were considered significant

RESULTS AND DISCUSSION

Qualitative phytochemical analysis of Ethanolic extract of *Aconitum ferox* showed the presence of majority of the compounds including alkaloids, glycosides, carbohydrates, steroids, sterols, Flavonoids, tannins, proteins and amino acids.

Tests	Observation	Result
Killer Killiani's test	Reddish brown layer is seen.	+
Legal's test	Pink red colour is formed.	+
Baljet's test	Yellow to orange color is observed.	+

Table: 2 Test for Glycosides

Test	Observation	Result
Shinoda test	Red color was produced	+

Table: 3 Test for flavonides

In-Vitro Studies: Determination of free radical scavenging activity on DPPH radical.

And the absorbance of the different test and standard samples are noted and the SC_{50} values were calculated by using test sample values.

Control	Absorbance	% scavenging activity
	0.828	

Concentration (mg/ml)	Absorbance (test)	% scavenging activity
5	0.161	80.5
2.5	0.290	64.9
1.25	0.361	56.8
0.625	0.527	36.3
0.31	0.684	17.8
0.156	0.751	8.5

Concentration (mg/ml)	Absorbance (standard)	% scavenging activity
5	0.115	86.1
2.5	0.221	73.3
1.25	0.299	63.3
.625	0.352	57.2
0.31	0.427	48.4
0.156	0.505	39.00

Table: 4 Free radical scavenging activity of the DPPH radical
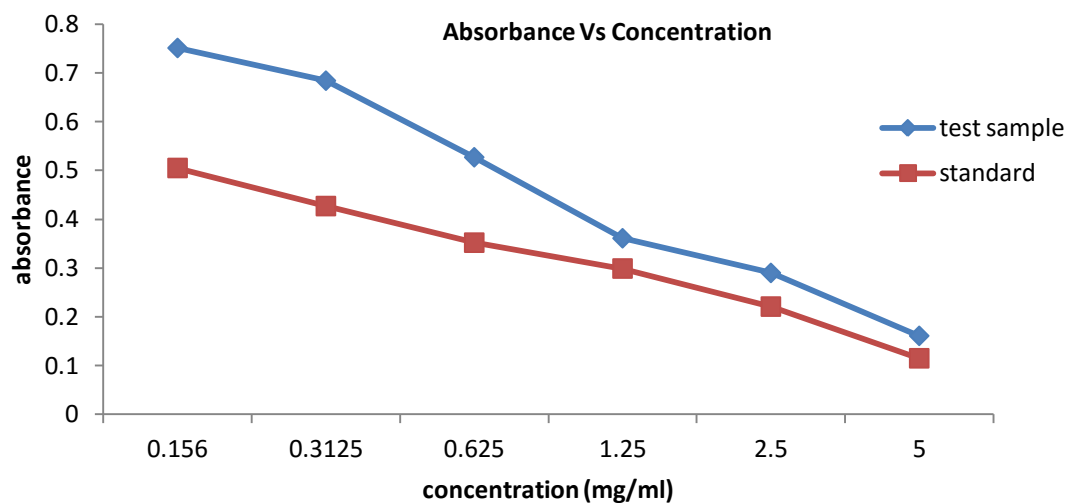
Absorbance Vs Concentration

Figure 1: The graph plotted between the concentration Vs absorbance

By performing the DPPH scavenging activity the test sample also showed the radical scavenging activity with that of the standard which is ascorbic acid. The ascorbic acid is vitamin C, L-ascorbic acid, which function as the antioxidant. The DPPH free radical scavenging is due to the hydrogen donating ability. DPPH is the nitrogen cantered free radical. This scavenges the phenolic hydroxyl group of the C-ring flavonoid structure. The phenolic compounds show the redox properties. The DPPH radical contains an odd electron which is responsible for absorbance at 515-517nm and also for a visible deep purple colour. When DPPH accepts an electron donated antioxidant compound, the DPPH is decolourised. From this result it showed the radical scavenging activity of the test samples at the different concentrations.
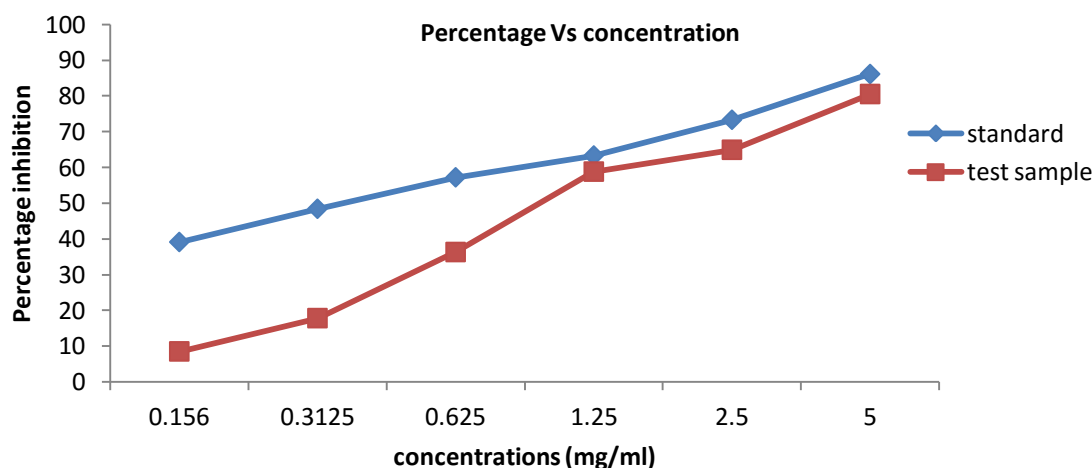
Percentage Vs concentration

Figure 2: The graph plotted between concentration Vs percentage inhibition

The scavenging activity is may be due to the presence of the flavonides in the EEAF.

The SC_{50} value was calculated by 50% of the free radical scavenging activity.

The SC_{50} value was found to be 0.9375mg/ml.

α-Glucosidase inhibitory assay:

Concentrations (mg/ml)	Absorbance	Percentage inhibition
Control	2.201	
0.156	2.003	9
0.312	1.802	19
0.625	1.654	25
1.25	1.316	41
2.5	0.812	64
5	0.475	81.9

Concentrations (mg/ml)	Absorbance (standard)	Percentage inhibition
0.156	1.945	12
0.312	1.712	23
0.625	1.387	37
1.256	1.157	48
2.5	0.742	67
5	0.262	89

Table 5: Showing the absorbance and the percentage inhibition of the α-Glucosidase inhibitory assay of the EEAF
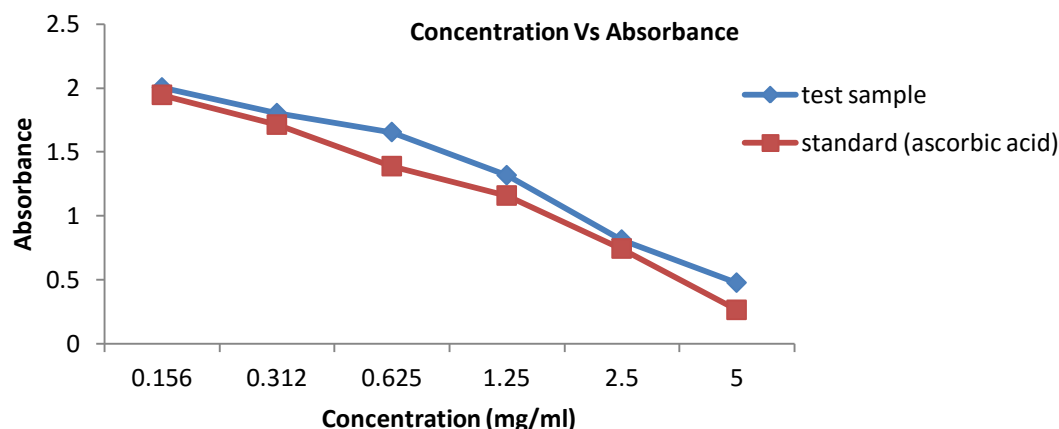
Figure 3: The graph plotted between the concentration Vs absorbance.
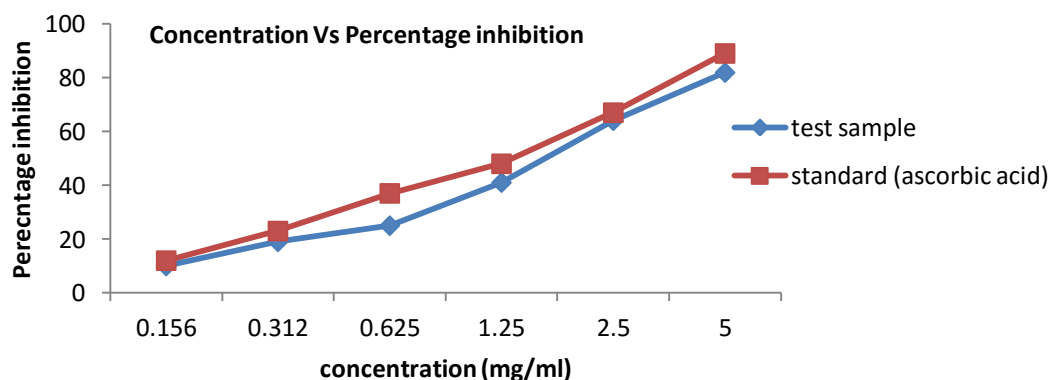

Figure 4: The graph plotted between the concentration VS percentage inhibition

Postprandial hyperglycemia (PPHG) is an independent risk factor for the development of macrovascular complications of diabetes mellitus The α-glucosidase is an enzyme that catalyses the final step in the digestive process of carbohydrate, and hence

inhibition of it could retard the use of dietary carbohydrate to suppress PPHG.

The absorbance was observed at the 405nm and compared with the control (Acarbose). The percentage inhibition of α-Glucosidase was found using the following formula.

The percentage of α-Glucosidase inhibition was measured by **(1-B/A) × 100.**

Where A is the absorbance of control and B is the absorbance of samples containing extracts.

The inhibitory concentration of the extract required to inhibit the activity of the enzyme by 50% (SC_{50}) was calculated.

***In vitro* Studies:**

Oral Glucose Tolerance Test (OGTT) On 7ᵗʰ day;

Effect of Ethanolic extract of *Aconitum ferox* on blood glucose levels of normal and diabetic fasted rats after a glucose load (2 g/kg). The blood glucose level was increased rapidly 1/2 h after administration of glucose, and thereafter decreased gradually. Standard drug Acarbose and three different doses of the extract (100mg/kg, 200 mg/kg & 400 mg/kg) were given orally 30 min before the glucose administration. In normal animals gradual rise in blood glucose level after the glucose load was observed and maximum at 2 hrs. Diabetic control animals showed a gradual rise in blood glucose level and stabilized at 2hr. 200 mg/kg of EEAF showed minimum fall in blood glucose concentration (232 mg/dl) but in 400 mg/kg showed maximum fall in blood glucose concentration (215mg/dl, $p<0.0001$). The values were expressed as Mean ± S.E.M and statistically analysed with the help of Graph pad prism software and values are compared using one way ANOVA.

Blood glucose levels (mg/dl) on 7ᵗʰ

CONDITION	0hr	30min	1hr	2hr	3hr
Normal	70±2	73±2	70±2	71±2	72±2
Diabetic	298±2	311±2	297±2	286±2	280±2
standard	222±2	250±2	241±2	216±2**	208±2**
100mg/kg	276±2	296±2	284±2	270±2	265±2
200mg/kg	256±2	276±2	265±2	250±2*	232±2*
400mg/kg	224±2	232±2	228±2	221±2**	215±2**

Table 6: The plasma glucose (mg/dl) values done on the 7ᵗʰ day after the treatment by OGTT

Values are expressed as mean ± S.E.M; n=6

One way ANOVA followed by Dunnet Multiple comparison test using Graph pad INSTANT version 5.

Blood glucose level from different treatments is compared with standard drug.
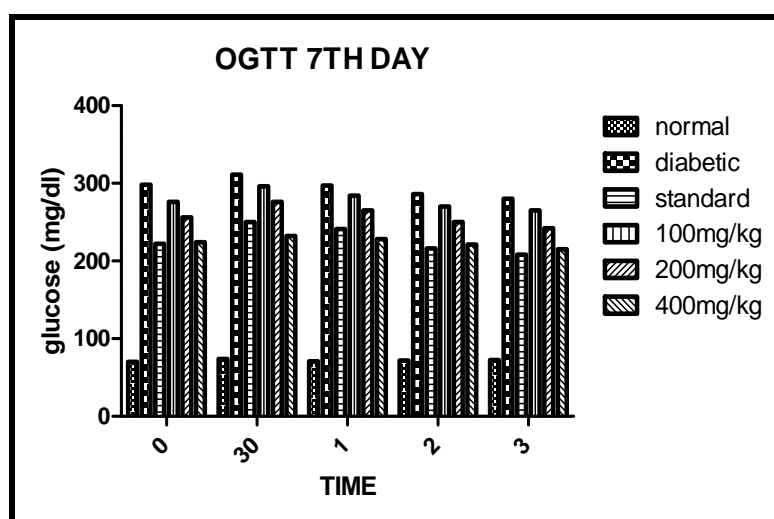
$P<0.05$ is considered as significant.

Figure 5: The graph plotted between the time and plasma glucose (mg/dl), after zero hour immediate oral glucose is given so, at 30min slight increase in plasma glucose levels and later decreased because of the elimination of glucose.
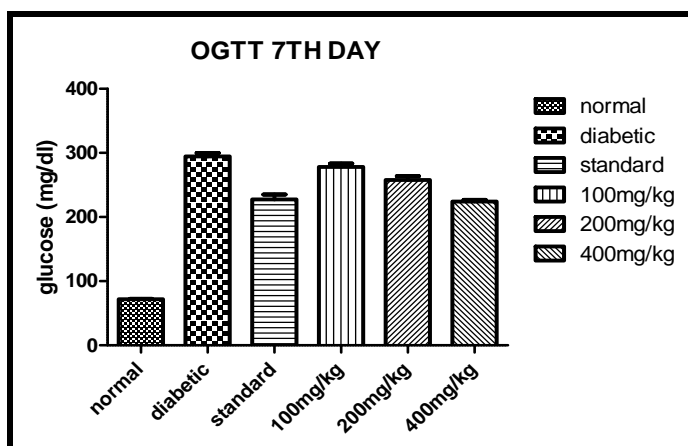
Figure 6: The graph showing the comparison between the normal, diabetic, standard, 100mg/kg extract, 200mg/kg extract and 400mg/kg extract doses.

Oral Glucose Tolerance Test (OGTT) on 21TH day: Effect of EEAF on blood glucose levels of normal and diabetic fasted rats after a glucose load (2 g/kg). The blood glucose level was increased rapidly ½ h after administration of glucose, and thereafter decreased gradually. Standard drug Acarbose and three different doses of the extract (100mg/kg, 200 mg/kg & 400 mg/kg) were given orally 30 min before the glucose administration. In normal animals gradual rise in

blood glucose level after the glucose load was observed and maximum at 2 hrs. 200 mg/kg of EEAF showed minimum fall in blood glucose concentration (179 mg/dl) but in 400 mg/kg showed maximum fall in blood glucose concentration (136 mg/dl, $p < 0.0001$). The values were expressed as Mean ± S.E.M and statistically analysed with the help of Graph pad prism software and values are compared using one way ANOVAs.

Blood glucose levels (mg/dl) on 21th day

CONDITION	0hr	30min	1hr	2hr	3hr
Normal	68±1	75±1	72±1	65±1	67±1
Diabetic	284±2	296±2	287±2	290±2	277±2
Standard	164±1	178±1	160±1	144±1**	133±1
100mg/kg	236±2	239±2	228±2	231±2	219±2
200mg/kg	215±2	221±2	213±2	207±2*	198±2*
400mg/kg	162±1	179±1	169±1	155±1**	136±1**

Table 7: The plasma glucose (mg/dl) values collected on the 21st day after the treatment by OGTT
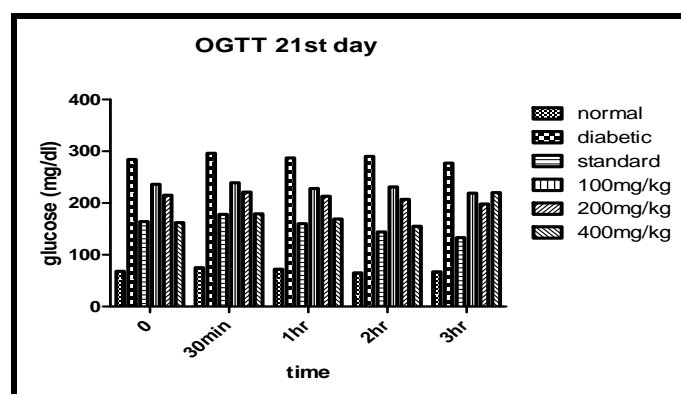

Figure 7: The graph plotted between the time and plasma glucose (mg/dl), after zero hour immediate oral glucose is given so, at 30min slight increase in plasma glucose levels and later decreased because of the elimination of glucose.
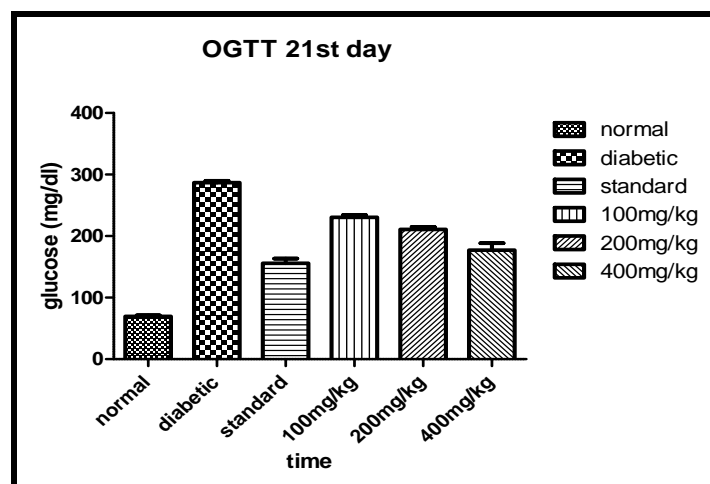
Figure 8: The graph showing the comparison between the normal, diabetic, standard, 100mg/kg extract, 200mg/kg extract and 400mg/kg extract doses.

By comparing the glucose levels of the Ethanolic extract and standard drug found some inhibition from day 7 to the 28 day. The standard drug showed the 54% inhibition. 100mg/kg of EEAF showed 29.9% and 200mg/kg of EEAF showed 33.2% and 400mg/kg showed 51.5% inhibition.

CONCLUSION

From the present study, It supports that therapy with alpha-glucosidase inhibitors could be an interesting adjunctive pharmacological approach to the treatment of type 2 diabetes. In this regard, the protection determined by EEAF clearly suggests the potential usefulness of this plant.

We concluded that the EEAF produced a significant decrease of plasma glucose level. These results suggest that the effectiveness of the drugs depend probably, on the accumulative effect of active principles. In contrast, the significant increase in plasma glucose levels of untreated diabetic rats may be due to a progressive severity of untreated diabetes.

In conclusion, EEAF showed strong inhibitory activity against α-glucosidase *in vitro* and *in vivo*. Further study need to identify the active component responsible for the inhibition of α-glucosidase is strongly recommended.

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