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Evaluation of Anti-Diabetic Activity of Otostegia Integrifolia Leaves in Alloxan Induced Diabetic Animal Model

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Abstract
Context: *Diabetes mellitus is chronic disorder of carbohydrate metabolism. Prevalence of diabetes mellitus is rising day by day globally. Since antiquity, diabetes has been treated with plant medicines. In the present research, anti-diabetic activity of Otostegia integrifolia leaves was evaluated in alloxan-diabetic rabbits. Blood glucose levels were determined at two doses of Otostegia integrifolia leaves.*
Objective: *The present study was undertaken to establish scientific base for the anti-diabetic activity of Otostegia integrifolia leaves extracts in different groups of rabbits.*
Materials and Methods*: Fresh leaves of Otostegia integrifolia were collected, shade dried and made into powder by mechanical grinding. The powder obtained thus was extracted by cold maceration method using methanol as solvent. For evaluation of hypoglycemic properties of the plants extracts, methanolic extract were evaluated by oral administration at dose of 100mg/kg and 200mg/kg to alloxan induced diabetic rabbits and Glibenclamide (5mg/kg/day) was orally administered to Alloxan-induced diabetic rabbits for 7 days to serve as standard drug. Blood glucose levels were monitored at 24 hours interval for seven consecutive days along with estimation of biochemical parameters at the end day of the study. All the methods were carried out thrice and the data thus obtained was statistically evaluated by using one-way analysis of variance (ANOVA) using SPSS Version 20.0.*
Results and Discussion*: In our study, it was found that the methanolic extract of Otostegia integrifolia possess anti-hyperglycemic activity, which was significant at $p<0.05$ at 100mg/kg and 200mg/kg in general and at the higher dose (200mg/kg) the effect was highly significant. Hyper-lipidemic activity was statistically significant in reducing cholesterol ($p<0.05$), TG($p<0.05$) and HDL($p<0.05$) effectively.*
Conclusion: *The methanolic extract of Otostegia integrifolia leaves exhibited significant anti-hyperglycemic activity and anti-hyperlipidemic activity at 100mg/kg and 200mg/kg.*
Keywords: *Glibenclamide; Diabetes; Hypoglycaemic; Alloxan; Otostegia integrifolia*

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I. Introduction

There are many hypoglycemic plants known through the folklore but their introduction into the modern therapy system awaits the discovery of animal test system that closely parallel to the pathological course of diabetes in human beings. Hypoglycemic activity has been reported in many plants during the last twenty years. Moreover, their action differs from that of insulin in that they appear to act as antimetabolites, capable of blocking the pathway of oxidation of fatty acids. This depletion of liver glycogen subsequently induces hypoglycemia. Approximately 343 plants of the world have been tested for the blood glucose which found lowering effect in the laboratory experiments. [1] *Otostegia integrifolia* commonly known as Ban tulsi (Hindi), Kadu Tulsa (Marathi), Nalla Toomu (Telgu), Quba mutalamil al-award (Arabic), Ch'endog (Tigrinya) is a shrub from the family *Lamiaceae* and Genus *Otostegia*. *O.integrifolia* is a shrub which grows up to 3 m tall, often with paired spines at the nodes. Its leaves are sessile or shortly petiolate. The blade is bluish greyish-green, oblanceolate to lanceolate shaped, and reaches 2-9 cm long. The plant grows in the wild but is also cultivated in

gardens. It grows on montane bush lands and wood lands over grazed slopes at altitudes ranging from 1, 300 to 2, 800 m. The plant is endemic to Eritrea, Ethiopia and Yemen. [2] Their stems are usually square and they contain abundant flowers which are quite attractive. Many members of the family are used as culinary or medicinal herbs, as sources of volatile oils and in some cases for the preparation of constituents of the volatile oils such as menthol and thymol. [2, 3] It is an herbaceous plant that grows in the wild, but it is also cultivated in the gardens. Five species of this genus have been reported to occur in flora of Ethiopia and Eritrea with various medicinal applications. [2] There are more than 65 new and novel compounds that have been isolated from this genus. Particularly, compounds from *O.integrifolia*, *O.perisca* and *O.fruticosa* were pharmacologically important.

II. Materials and Methods

Plant Material and Authentication

Fresh leaves of *O.integrifolia* were collected from Zoba Maekel around Asmara a Village called *Kushet*. The leaves were botanically identified and authenticated by Mr. Biniam, botanist from Eritrean Institute of Technology, Asmara, Eritrea and authenticated by ICMR, Belgaum India. (RMRC-1853). The leaves were dried under shade for 7 days. The dried leaves were mechanically grinded and the coarse powder was stored in well closed container until it was used for extraction.

Extraction [4]

The coarse powder (500g) of *O.integrifolia* leaves was macerated in 80% methanol (1:10 leaves powder to solvent ratio) for 72 hours with occasional shaking. This was repeated 3 times until the extract gave faint or no coloration. The extract was then filtered through Whattsman filter paper No.1 and solvent was evaporated to dryness by rota-evaporator and further concentrated by water bath. Then, gummy residue extract was stored in desiccator until used for the experiment.

Phytochemical Screening [4]

Preliminary phytochemical screening was carried out by using reported methods to reveal the phytoconstituents present in the extract.

Pharmacological Screening

Animals used

Twenty New Zealand Rabbits of weight 1-1.2kg (initial) with no prior drug treatment were used for the study. All the animals were purchased from rabbits and pigs breeding center, Ministry of Agriculture (MoA), Paradizo, Asmara. All the rabbits were acclimatized to the experimental condition for seven days before commencing the experiments and fed with their usual food and tap water *ad libitum*. The animals were housed in 12 hours light and dark cycle at room temperature. The experiment was performed after ethical approval was obtained from Ethical Committee of Orotta College of Medicine and Health Sciences, Asmara.

Grouping of Rabbits

The rabbits were randomly divided in to five groups consisting of four rabbits each Group A were normal control group (**NC**), Group B were negative control group (**DC**), Group C were standard drug treated group (**SC**), Group D were low dose 100mg/kg of *O.integrifolia* extract treated group (**LD**) and Group E were high dose 200mg/kg of *O.integrifolia* extract treated group (**HD**).

Temporary Hyperglycemic Rabbits

16 rabbits were made temporary hyperglycemic by administrating 2g/kg of glucose dissolved in normal saline at 50% concentration and were given to the rabbits orally through 5 ml syringe. This state of temporary hyperglycemic was maintained for about 2 hours. The other four were used as normal control.[5]

Experimental induction of Diabetes Mellitus in Rabbits

Diabetes mellitus was induced in overnight fasted rabbits by a single intra-peritoneal injection of freshly prepared solution of Alloxan monohydrate (400mg/kg body weight) dissolved in normal saline. To overcome initial drug induced hypoglycemic mortality 2g/kg of glucose was given orally prior to injecting alloxan monohydrate. Diabetes mellitus was confirmed by measuring the fasting blood glucose concentration 72 hours after injection with Alloxan monohydrate. Rabbits with fasting blood glucose above 150mg/dl were used in the study. [6]

Experimental Design and Treatment Protocol

Acute Oral Toxicity Study

Acute oral toxicity study was performed as per the protocols of Organization for Economic Cooperation and Development (OECD) guidelines 425. Female rabbits were fasted overnight prior to dosing. The crude methanol extracts (2000mg/Kg) of *O.integrifolia* was administered in a single dose. The rabbits were then kept under strict observation for physical and behavioral changes for 24 hours, with special attention during the first 4 hours. These observations continued for further 14 days for any signs of overt toxicity. Hence, 1/20th (100mg/kg) and 1/10th (200mg/kg) of this dose were adopted for further anti-hyperglycemic studies. [7]

Hypoglycemic Effect in Temporary Hyperglycemic

A first blood sample was taken prior to glucose administration and was used as reference then 30 minutes later second blood sample was taken to measure the temporary hyperglycemic state. After confirming the temporary hyperglycemic state rabbits were treated according their group as stated above. After that the third and fourth blood samples were taken to see the effect of the treatment at 60, 90 and 120 minutes from the time where first blood samples were taken.

Hypoglycemic Effect on Alloxan induced Diabetic Rabbits

Rabbits were randomly divided in to five groups consisting of four rabbits each as explained earlier. Blood glucose, HbA1c level and the lipid profile were first measured on overnight fasted rabbits then they were treated according to their group as stated above for seven consecutive days. At the end of treatment blood glucose, HbA1c and lipid profile were again measured.

Biochemical Analysis

Blood samples were collected from the marginal ear vein of all the rabbits from each and were used for determining the following parameters.

Determining of Blood Glucose Level

Blood sample taken from the marginal ear vein of rabbits were measured using glucometer.

Determining of HbA1c Level

To determine the level of HbA1c, blood samples were collected in vacationers and the analysis of HbA1c was performed at biochemical laboratory, Sembel hospital, Sembel, Asmara.

Determining of Lipid Profile

Blood samples collected in vacationers were sent to biochemical laboratory, Sembel hospital, Sembel, Asmara to determine lipid profile.

Histopathological Examination

From each group rabbits was sacrificed to acquire the pancreas instantly after accumulating blood under ether anesthesia. Minor fragments of the rabbit pancreas from each group were fixed in 10% formalin solution and the histopathological study was carried out at biochemical laboratory, Sembel Hospital, Sembel, Asmara.

Statistical Analysis

The statistical analysis in this study was performed using SPSS version 20.The relationship between dose and time after intervention was evaluated using one-way ANOVA; and Post Hoc was performed using Tukey, where applicable data was presented as mean with standard error of mean (± SEM) / standard deviation (SD). Statistical significance was set at the $p \leq 0.05$ levels.

III. Results And Discussion

Percentage Yield

The 80% methanol leaf extract of *O.integrifolia* gave a greenish brown semi-solid product with a percentage yield of 17.25% w/w.

Preliminary Phytochemical Screening

Preliminary phytochemical screening indicated that the extract contains phenols, flavonoids, terpenes, saponins, glycosides. Alkaloids and tannins were absent in the same extract.

Acute Oral Toxicity Test

No sign of toxicity or mortality was observed in rabbits after oral administration of the methanolic leaf extract of *O. integrifolia*, at dose as high as 2000mg/kg, signifying that the oral LD_{50} was greater than 2000 mg/kg.

Anti-hyperglycemic Effects in Temporarily Hyperglycemic Rabbits

Initial there was no significant difference in the mean glucose level of all groups. After 30 min of glucose administration a significant difference was observed between the normal control and the remaining four other groups. At 60 min both the standard drug and high dose treated group show a significant decrease in the mean glucose level. Although there was no significant difference between the low dose treated group and the temporary hyperglycemic control group at the same time the mean blood glucose level of the low dose treated group and standard drug treated group shows no significant difference. At 90 min, all treated group's shows a significant difference with the untreated group then at time 120 min it was observed no significant difference in the mean blood glucose level.

Groups	Mean glucose level ± SD				
	0 min	30min	60 min	90 min	120 min
Normal control (**NC**)	111±8.88	113.33±8.5	108.66±10.5	108.66±7.37	106.66±7.09
Diabetic control (**DC**)	113.66±6.65	194.66±2.08*	191±3.46	178.33±2.51	118.33±5.5
Standard (**SC**)	113.33±8.08	195±6.55*	170.33±5.03*	142.33±4.04*	113.33±6.8
Low dose (**LD**)	114±6.55	195.33±8.5*	175.66±3.51	147.66±4.04*	115.66±6.8
High dose (**HD**)	112.33±13.61	194.33±4.93*	170.66±2.08*	144.66±3.51*	114.33±1.52

Table 1 Mean blood glucose level ± SD of Normal and Temporary Hyperglycemic Rabbits

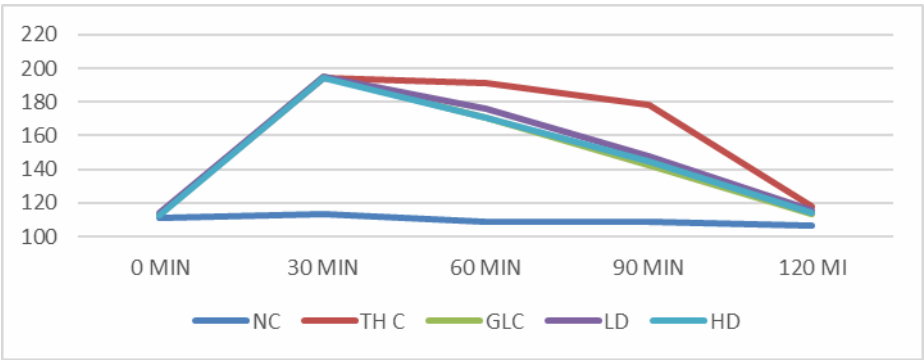

Figure 1 Mean blood glucose level of normal and temporarily hyperglycemic rabbits

Hypoglycemic Activity in Alloxan Induced Diabetic Rabbits

The standard drug treated group and the high dose treated groups shows their significant effect after two hour of administration and the low dose treated group shows its significant effect at the fourth hour of administration but there was no significant difference in the mean blood glucose level of the low dose treated group when compared to the high dose treated group and standard drug treated group at the second hour of treatment. The significance of these three treated group was maintained at the sixth hour and seventh day of treatment.

GROUPs	Mean blood glucose level ± SD						
	0hr	0.5hr	1hr	2hr	4hr	6hr	7day

NC	109±10.8	109±11.91	111.25±5.73	108.5±9.98	108.25±13.3	108.5±8.69	111.5±9.32
DC	204±11.22	207.5±13.07	207.25±13.4	203.75±6.8	204.25±15.6	205.25±9.25	232.5±7.58
SC	205.25±17.	200.25±6.65	185.25±13.12	164±13.34*	154.64±13.5*	148±4.69*	140.25±18.9*
LD	204.5±17.0	201.75±10.7	191.25±10.46	183.25±9.5	162±4.96*	1599.25±8.1*	151.75±6.1*
HD	212±10.39	205±13.29	189.5±9.32	172.5±9.46*	161.25±8.05*	155.5±10.01*	145.25±9.25

Table 2 Mean blood glucose ±SD of Normal and Diabetic Rabbits

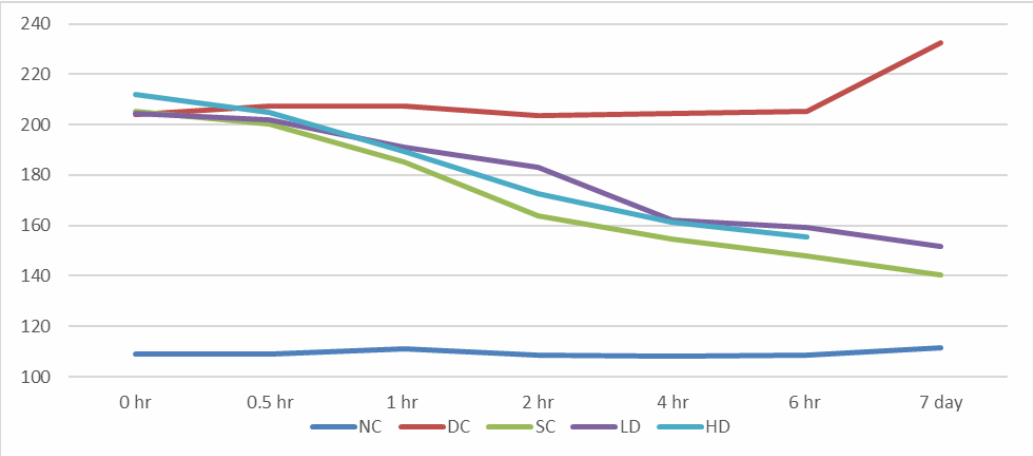

Figure 2 Mean blood glucose level of normal and diabetic rabbits

The standard drug treated group and the high dose treated groups shows their significant effect after two hour of administration and the low dose treated group shows its significant effect at the fourth hour of administration but there was no significant difference in the mean blood glucose level of the low dose treated group when compared to the high dose treated group and standard drug treated group at the second hour of treatment. The significance of these three treated group was maintained at the sixth hour and seventh day of treatment.

Groups		CHOL	TG	HDL	LDL
NC	DAY 1	**Effect of the extract on Lipid Profile**	138.50±4.12	50.50±6.60	54.25±10.99
	DAY7	60.50±6.02	75.75±6.50	51.25±4.34	61.75±16.45
DC	DAY1	156.5000±11.03	150.75±4.92	31.25±4.78	153.75±9.06
	DAY7	169.75±4.11	161.00±5.94	23.25±6.65	155.25±10.21
SC	DAY1	155.0000±9.96	152.50±7.18	31.25±3.09	154.00±7.02
	DAY7	120.50±8.06	110.25±8.46	43.75±8.99	120.50±6.60
LD	DAY1	158.50±9.74	149.25±3.09	32.50±3.87	156.00±5.94
	DAY7	137.25±4.27*	142.00±2.16*	38.25±6.44*	148.50±3.69*
HD	DAY1	162.75±5.90	155.50±6.02	31.50±3.51	157.00±4.96
	DAY7	128.50±9.74*	113.75±8.05*	42.25±5.90*	133.75±11.95*

Table 3 Mean lipid profile ± SD of normal and diabetic rabbits

Histopathological examination

The Histopathological changes were observed in pancreas of rabbits from each group and a good control on recovery of the cells was observed with the group treated with high dose of the plant extract (Figure 3). Group A were normal control group (**NC**), Group B were negative control group (**DC**), Group C were standard drug treated group (**SC**), Group D were low dose 100mg/kg of *O.integrifolia* extract treated group (**LD**) and Group E were high dose 200mg/kg of *O.integrifolia* extract treated group (**HD**).

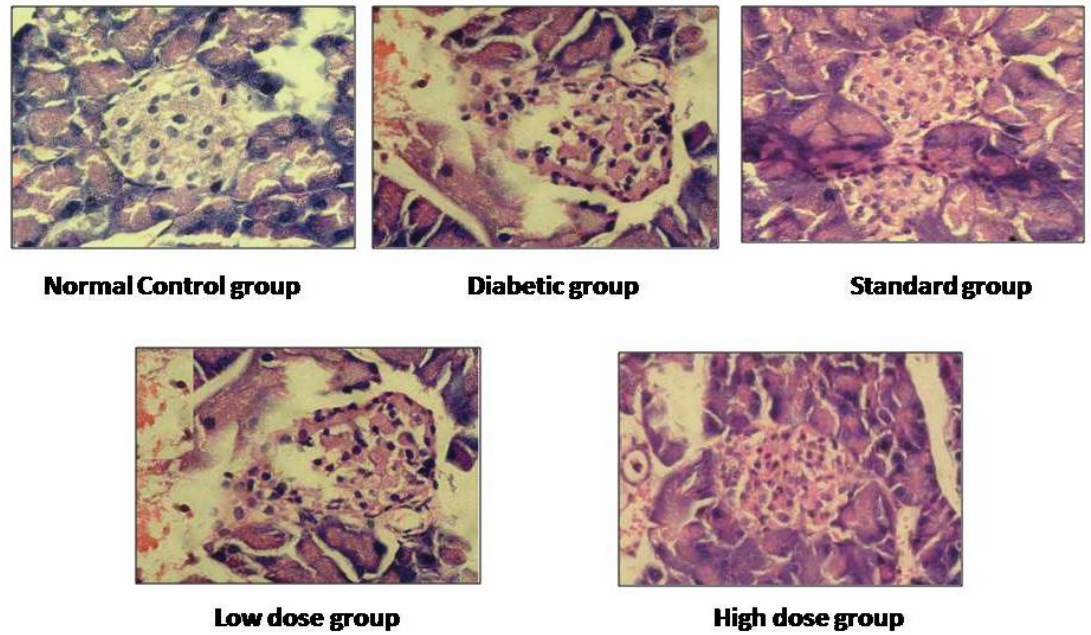

Figure 3 Histopathological examinations of the animals from each group

IV. Discussion

The percent yield of 80% methanolic extract was 17.25% which is similar to the percent yield obtained by earlier researchers. [8, 9]

In the phytochemical screening, it was found that the extract contains phenols, flavonoids, terpenoids, saponins, glycosides which are the same to the results obtained by earlier researchers [8, 9] where they found that the extract contains phenolic compounds, saponins, and flavonoids while alkaloids, tannins and steroidal compounds were absent the same was true in our case that alkaloids and tannins were absent but we didn't perform test for steroidal compounds.

In the acute toxicity study, we found out that the LD_{50} is greater than 2000mg/kg. In other studies, it was also found that the LD_{50} is greater than 5000mg/kg which indicates that the doses administered to the experimental rabbits were on the safe margin. [8]

The effect of extract in lowering the mean blood glucose level of temporary hyperglycemic was similar to that of the result found the current literature available, [8, 9] where 200mg/kg and the standard drug glibenclamide have quick onset of action, they show their effect at the first hour of extract administration, and there was no significant difference in their effect ($p< 0.05$) [9] which is consistent to the result we obtained. However, the 100mg/kg treated groups don't show effect at all, where as in our case they show gradual effect.

Similar, results were also found in the diabetic rabbits, where the higher dose and the Glibenclamide treated group shows their significant ($p<0.05$) effect within 2 hours while the low dose treated group gives its effect at the 4th hour of treatment. Earlier researchers have found that 200mg/kg and glibenclamide shows their effect at the 3 hour and the 100 mg/kg gives its effect at the 4 hours. [9]

We saw a significant difference from 0.5 hour to 1 hour in the SC, LD and HD treated groups. From 1hour to 2 hour the SC and HD treated groups show significant difference. [9] In addition to this, the mean difference between the 2 hour and 4 hour of the LD treated group was significant. The NC and DC didn't show significant difference in their mean blood glucose level throughout the experimental period except that the DC shows significant difference from 6th hour to 7th day, which indicates the progression of the disease.

In this study, the death associated with the induction of diabetes mellitus using Alloxan monohydrate was only 10% which is much lower than the expected rate. The reason behind this could be the use of glucose prior to injection. In several literatures, it was documented that Alloxan monohydrate results to transient hypoglycemia at the first 30 minutes of administration which is the main reason for mortality of experimental animals such phenomena can be reduced by giving 2 g/kg of glucose before injection. [10]

Alloxan monohydrate not only induce diabetes but also increases the triglycerides, cholesterol, LDL level and decreases level of HDL, however no effect was seen on the HbA1c level, as hemoglobin takes time to get glycated.

The phytochemical investigation reveals that the extract contains flavonoids that suppressed the glucose level, reduced plasma cholesterol and triglycerides probably by enhancing the insulin release from pancreatic islets,[11] Phenols which increase insulin secretion and utilization. [12] Terpenes, stimulates the release

of insulin and blocks the formation of glucose in the blood stream. [12] These may be the reasons for the hypoglycemic activity.

V. Conclusion

From this investigation, it was possible to conclude that the methanolic extract of *O.integrifolia* has anti-hyperglycemic activity at 100mg/kg and 200mg/kg in general and at the higher dose (200mg/kg) the effect was more. Moreover, the methanolic extract of *O. integrifolia* also possesses anti-hyperlipidemic activity. The present research supports the folklore use of the plant under investigation for the treatment of diabetes. However, comprehensive phyto-pharmacological investigations are to be conducted to reveal how this novel herb is exerting anti diabetic activity.

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