

**HYPOGLYCEMIC ACTIVITY OF THE COMBINATION
OF ACTIVE INGREDIENTS ISOLATED FROM
TRIGONELLA FOENUMGRAECUM IN ALLOXAN
INDUCED DIABETIC MICE.**

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Summary

The aim of the study was to evaluate the hypoglycemic activity of two compounds isolated from seeds of *Trigonella foenumgraecum* viz; 4 hydroxyisoleucine and trigonelline in alloxan induced diabetic mice. The compounds were isolated by column chromatography from fenugreek seeds. The combination of 4 hydroxyisoleucine and trigonelline [4HIT, 40:30, 120 mg/kg] was administered orally in alloxan induced diabetic mice. The parameters studied were blood glucose, histology of pancreas, body weight, mortality and acute oral toxicity. 4HIT (120 mg/kg) showed

reduction in blood glucose level within 2h and reduced the peak blood glucose level at 6h during acute study. After 28 days treatment with 4HIT, there was significant decrease in blood glucose level. 4HIT increased the glucose threshold as compared to only alloxan treated group. Histology of pancreas showed formation of new islets near the vicinity of the pancreatic duct. Decreased glycosylated hemoglobin adds to the effect of 4HIT. Glyburide was used as a standard antidiabetic drug and its effect on pancreatic cell was also studied. The pancreatic beta cells of glyburide treated mice did not show any islets in the vicinity of pancreatic duct. Both 4HIT and glyburide arrested the decrease in body weight and mortality of diabetic mice. LD₅₀ was found to be more than 5000 mg/kg. These results suggests that 4HIT showed hypoglycemic effect in alloxan induced diabetic mice. The presence of the pancreatic islets in the vicinity of duct suggested 4HIT might act by regeneration of new islets.

Keywords: 4 hydroxyisoleucine; Trigonelline; Alloxan; Diabetic mice; Blood glucose; Acute toxicity

Diabetes, a chronic progressive disorder characterized by metabolic abnormalities, has reached epidemic proportions worldwide. Statistics reveal that India will be the diabetes capital of world. Worldwide projections suggest that more than 220 million people will have diabetes by the year 2010 (I). Oral hypoglycemic agents used for the treatment of diabetes have side effects on prolonged use. The patients are using herbal medicines

which have less side effects, easy availability and economic for them. Even World Health Organization (WHO) permits the use of plant drugs for different diseases, including diabetes mellitus (II). One such known anti diabetic plant, *Trigonella foenumgraecum* Linn., family: Leguminosae was used for the study. It is called as 'Fenugreek' (Vernacular name: Methi) and commonly used as a spice in cooking. Fenugreek is cultivated in India, Egypt, Middle East and North Africa. The seeds of the plant have been used as a traditional remedy for conditions including gastrointestinal disorders, gout, wound healing and inflammation, hyperlipidemia and diabetes (III). 4 hydroxyisoleucine (4HI) isolated from fenugreek seeds showed hypoglycemic activity (IV). Mishkinsky studied the hypoglycemic effect of trigonelline (T) in alloxan induced diabetic rats (V). However, no reports are available on the use of combination of 4HI and T on blood glucose and pancreatic beta cells. The objective of the present study was to investigate the effect of 4 hydroxyisoleucine and trigonelline combination (4HIT) on blood glucose and to explore its mechanism of action in alloxan induced diabetes in mice.

Materials and methods

Animals

Swiss Albino mice of either sex (25-30g) were obtained from National Toxicological Centre, Pune. Animals were housed under standard condition of temperature ($25 \pm 2^\circ\text{C}$), 12h/12h light dark cycles and fed with standard pelleted diet (Chakan Oil Mills, Sangli) and water was given *ad libitum*. Animal handling was performed as per *Good Laboratory Practice*. A research proposal was prepared according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The Institutional Animal Ethical Committee (IAEC) of Poona College of Pharmacy approved the proposal.

Drugs

4HI and T were isolated and identified from *Trigonella foenumgraecum* by column chromatography. The combination formulation (4HIT) was prepared by mixing powder of 4 hydroxy

isoleucine (4HI) and Trigonelline (T) in the ratio of 40:30. The solution of 4HIT was prepared in distilled water and administered in the doses of 30, 60 and 120 mg/kg, p.o. Glyburide an oral antidiabetic drug was obtained as gift sample from Ranbaxy Pvt. Ltd., New Delhi. Appropriate dilutions were made for preparation of the suitable dose so as to administer *per orally* 0.1 ml / 10 g of body weight of mice.

Chemicals

Alloxan monohydrate was purchased from Spectrochem, India.

Induction of diabetes and estimation of blood glucose:

Alloxan monohydrate (70 mg/kg, i.v) was used to induce diabetes in mice. Blood was withdrawn by retro orbital plexus technique and blood glucose was estimated by glucose oxidase peroxidase (GOD/POD) method using kit obtained from Accurex Biomedicals, Mumbai.

Normal mice were made diabetic by giving them alloxan monohydrate (70 mg/kg, i.v). After 48 hours, the blood was withdrawn and blood glucose was estimated. The animals showing blood glucose more than 200 mg/dl were called 'diabetic' and were selected for the study (VI). The animals were divided into four groups (diabetic) and one group of non diabetic animals each containing 6 animals. Group I - Control (non diabetic), Group II - Only alloxan (70 mg/kg, i.v), Group III – Only glyburide (10 mg/kg), Group IV– 4HIT (120 mg/kg). The rationale behind the selection of dose of 4HIT: 4HIT was administered at three dose levels (30, 60 and 120 mg/kg). In the present study, the dose of 120 mg/kg was selected because lower dose i.e 30 and 60 mg/kg did not decrease the blood glucose significantly.

Acute study:

Animals were fasted overnight before commencing the experiment. Blood glucose was estimated before giving any drug

and the readings were noted as 0 h reading. Drugs were administered to respective groups orally and blood glucose was estimated at 2h, 4h, 6h and 24h for acute study (VII).

Sub acute study:

The drugs were administered daily for 28 days at a prefixed time (7). Blood glucose was estimated on weekly intervals. At the end of 28 days the drug administration was stopped and a rest period of 7 days was given to the animals to study effect of drug treatment on blood glucose after 7 days.

Histology of pancreas

After seven days rest period, the animals were sacrificed and pancreas was isolated to study regenerative ability of pancreas. Histological examinations was carried out using Haematoxylin

Determination of glycosylated hemoglobin (HbA_{1c})

At the end of the study, the blood samples were taken from the animals. Glycosylated hemoglobin was estimated by using the chromatographic spectrophotometric ion exchange method using the kit obtained from Biosource S.A, Spain.

Intraperitoneal glucose tolerance test (IPGTT) in diabetic mice

Diabetic animals were fasted overnight and blood glucose was determined before commencing the experiment, which was considered as normal. Drug was administered to the animals and half an hour later glucose (2.5g/kg) was administered intraperitoneally to the animals. Blood glucose was estimated immediately after administration of glucose which was

considered as 0 min reading and further at 30, 60 and 120 min (VIII).

Body weight and mortality

During the study period of 35 days, animals were weighed daily and their body weight was recorded. Death of animals was noted and percentage mortality was calculated.

Determination of LD₅₀

Acute oral toxicity and LD₅₀ determination were carried out (IX).

Statistical analysis

Data was expressed as mean \pm S.E.M and statistical analysis was carried out by One Way ANOVA followed by post-hoc Tukey's test using GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego California USA, www.graphpad.com (X).

Results

Effect of 4HIT on blood glucose in alloxan induced diabetic mice

Glucose levels measured in normal and alloxan induced diabetes in mice are shown in Table 1. Alloxan (70 mg/kg) treated animals showed significant (P<0.001) increase in blood glucose as compared to the normal animals.

Acute study:

The onset of blood glucose decrease in 4HIT (120 mg/kg) and glyburide was seen at 2h. The peak decrease in blood glucose appeared to be at 6h incase of 4HIT and glyburide (127.05 and 279.45 mg/dl). 4HIT showed more reduction in blood glucose than glyburide treated group at all hours. (Table I)

Table I: Effect of 4HIT on blood glucose in alloxan induced diabetic mice (Acute Study).

Groups	Blood glucose in mg/dl				
	0 h	2 h	4 h	6 h	24 h
Control	116 ± 6.9	121 ± 5.74	127.67 ± 5.04	134.83 ± 3.86	119 ± 3.24
Only alloxan (70 mg/kg)	431.79 ± 10.96	434.69 ± 10.80	443.04 ± 13.15	452.43 ± 13.83	452.75 ± 14.57
Glyburide (10 mg/kg)	442.51 ± 6.99	344.75 ± 25.38	292.04 ± 31.36 ^b	279.45 ± 28.20 ^b	429.65 ± 31.06
4HIT (120 mg/kg)	384.52 ± 29.16	221.17 ± 35.75 ^{b,c}	147.83 ± 20.94 ^{b,d}	127.05 ± 17.40 ^{b,d}	265.29 ± 38.10 ^{b,c}

Values are mean ± S.E.M, n=6 in each group; Statistical analysis by one – way ANOVA followed by Tukey's test using Graphpad Instat software; P values < 0.01^a, P < 0.001^b compared to only alloxan group and P<0.01^c, P<0.001^d as compared to glyburide group. Drugs were administered orally.

Sub acute study:

Results indicated gradual significant decrease in blood glucose on continued administration of 4HIT and Glyburide. Maximum decrease in blood glucose (182.88 and 172.42 mg/dl) was observed after rest period of seven days in the animals treated with 4HIT and glyburide respectively. 4 HIT showed significant decrease in blood glucose as compared to glyburide group for 7th, 14th and 21st day. These results indicated that maximum reduction occurred after seven days of rest period may be due to the action

of drug on pancreas. The blood glucose reduction was sustained during the study period (Table II).

Table II: Effect of 4HIT on blood glucose in alloxan induced diabetic mice (Sub acute Study).

Groups	Blood glucose in mg/dl					
	0 day	7 th day	14 th day	21 st day	28 th day	After 7 days rest period
Control	116 ± 6.9	126.17 ± 1.2	120.33 ± 3.8	116.67 ± 3.6	123.67 ± 2.8	129.5 ± 2.05
Only alloxan (70 mg/kg)	431.79 ± 10.96	475.87 ± 7.39	474.22 ± 8.78	493.94 ± 11.97	503.26 ± 8.51	515.67 ± 6.74
Glyburide (10 mg/kg)	442.51 ± 6.99	427.55 ± 30.70	380.95 ± 12.74 ^a	336.01 ± 24.55 ^b	229.58 ± 32.02 ^b	172.42 ± 16.87 ^b
4HIT (120 mg/kg)	384.52 ± 29.16	323.51 ± 32.05 ^{b,c}	235.80 ± 31.27 ^{b,d}	206.49 ± 23.20 ^{b,d}	197.08 ± 27.60 ^b	182.88 ± 24.65 ^b

Values are mean ± S.E.M, n=6 in each group; Statistical analysis by one – way ANOVA followed by Tukey's test using Graphpad Instat software; P values < 0.01^a, P < 0.001^b compared to only alloxan group and P<0.05^c, P<0.001^d, as compared to glyburide group. Drugs were administered orally.

Histology of pancreas

The pancreatic sections of non diabetic (Group I) showed normal population of islets in the vicinity of the duct

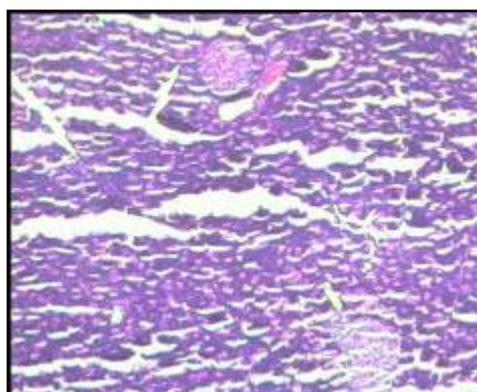


Figure I: Pancreatic section of control animals showing normal population of islets. X 200

The diabetic animals (Group II) showed occasional islets, which were negligible in the vicinity of the duct. It also showed depleted population of islets and the islets were very scanty and small in size

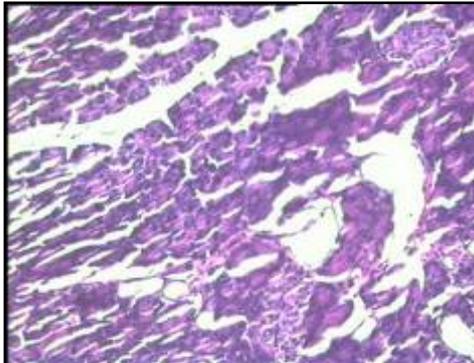


Figure II: Pancreatic section of alloxan treated mice showing occasional Islets. Islets are depleted, scanty and small in size. X 200

The pancreatic section of glyburide (Group III) treated animals showed no islets

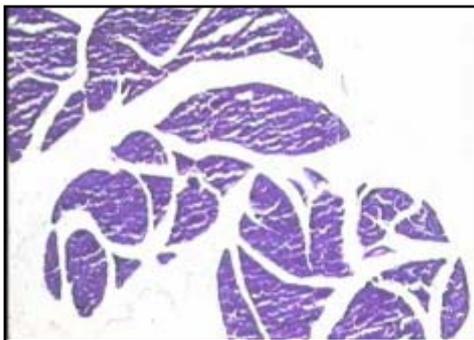


Figure III: Pancreatic section of Glyburide showing no islets as the drug exhausts pancreas. X 200

In 4HIT treated animals (Group IV) pancreatic section had many islets occasionally around the duct (Figure IV).

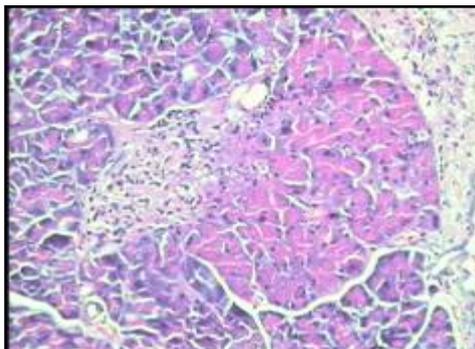


Figure IV: Pancreatic section of 4HIT showing many islets occasionally around the duct indicating pancreatic regeneration X 200.

Histology indicated that 4HIT treatment enhanced the pancreatic regeneration compared to other alloxan or glyburide treated groups.

Glycosylated hemoglobin (HbA_{1c})

The HbA_{1c} level in normal animals was 6.28 g %. In alloxan induced diabetic mice the levels of HbA_{1c} increased to 10.56 g%. HbA_{1c} was decreased significantly in glyburide (P<0.01) and 4HIT (P>0.05) treated group (Table III).

Table III: Effect of 4HIT on glycosylated hemoglobin (HbA_{1c})

Group	Glycosylated hemoglobin (g%)
Control	6.28 ± 0.128 ^b
Only Alloxan (70 mg/kg)	10.56 ± 0.372
Glyburide (10 mg/kg)	6.96 ± 1.06 ^b
4HIT (120 mg/kg)	8.19 ± 0.25 ^a

Values are mean ± S.E.M, n=6 in each group; Statistical analysis by one – way ANOVA followed by Tukey's test using Graphpad Instat software; P values < 0.05^a, < 0.01 compared to only alloxan group. Drugs were administered orally.

IPGTT in diabetic mice

Pretreatment of the animals with 4HIT and glyburide for half an hour improved the glucose tolerance, decreasing the blood glucose in alloxan diabetic mice. 4 HIT and glyburide showed non significant decrease in blood glucose 0, 30 and 60 min. On administration of 4HIT and glyburide significant (P<0.01) decrease in the blood glucose (279.58 and 283.00 mg/kg respectively) was seen at 120 min when compared to only glucose group (Table IV).

Table IV. IPGTT of 4HIT in alloxan induced diabetic mice.

Sr.No	Groups	Blood glucose (mg/dl)				
		Before glucose	After glucose administration			
			0 min	30 min	60 min	120 min
I.	Glucose (2.5%)	314.07 ±28.98	446.64 ± 42.05	467.58 ± 10.39	415.28 ± 21.25	445.45 ± 9.4
II.	Glyburide (10 mg/kg)	310.04 ±18.88	379.23 ± 19.02	400.60 ± 7.77	372.25 ± 16.31	283.00 ± 35.64 ^a
III.	4HIT (120 mg/kg)	318.83 ± 28.93	346.76± 20.80	416.29 ± 22.08	362.36 ± 42.54	279.58 ± 52.41 ^a

Values are mean ± S.E.M, n=6 in each group; Statistical analysis by one – way ANOVA followed by Tukey's test; P values < 0.05^a, compared to Group I and P < 0.05^b as compared to group II. Glucose was administered intraperitoneally and other drugs were given orally.

Body weight of animals

Alloxan gradually decreased the body weight of mice during the period of 28 days. It was observed that after administration of both glyburide and 4 HIT, the body weight of the animals did not decrease further. On the other hand, mice showed significant gain in body weight as compared to only alloxan treated group indicating that both glyburide and 4HIT were effective in preventing further loss of body weight (Table V).

Table V: Effect of 4HIT on body weight of diabetic mice.

Groups	Body weight in g					
	0 day	7 th day	14 th day	21 st day	28 th day	After 7 days rest period
Control	29.0 ± 0.44	30.67 ± 0.84	31.67 ± 1.2	32.67 ± 1.11	33.83 ± 1.01	34.50 ± 1.02
Only alloxan (70 mg/kg)	30.17 ± 0.47	26.33 ± 0.76	24.33 ± 0.95	22.33 ± 0.66	17.83 ± 0.6	17.50 ± 0.8
Glyburide (10 mg/kg)	30.33 ± 0.95	30 ± 0.81 ^a	30 ± 1.03 ^c	30.16 ± 1.53 ^c	29.33 ± 1.6 ^c	29.5 ± 1.64 ^c
4HIT (120 mg/kg)	28.16 ± 0.74	31 ± 0.81 ^b	31.33 ± 0.8 ^c	32.83 ± 0.6 ^c	33 ± 0.44 ^c	32.83 ± 0.74 ^c

Values are mean ± S.E.M, n=6 in each group; Statistical analysis by one – way ANOVA followed by Tukey's test using Graphpad Instat software; P values < 0.05^a, P < 0.01^b, P < 0.001^c compared to only alloxan group.

Mortality of animals

Administration of alloxan resulted in death of 57 % of the total animals during 28 days study period. Administration of both glyburide and 4HIT reduced mortality to 45% and 35% respectively. It was thus apparent from the results that when no

drug was administered progression of diabetes resulted in mortality of mice, while the antidiabetic effect of 4HIT prevented mortality.

Determination of LD₅₀

LD₅₀ determination was done as per OECD guidelines using AOT425 software. LD₅₀ of 4HIT and glyburide was found to be greater than 5000mg/kg.

Discussion

Fenugreek is a well known anti diabetic plant. Its hypoglycemic properties have been reported (XI). The hypoglycemic properties of fenugreek seeds in experimentally induced diabetic rats, dogs, mice and healthy volunteers (both IDDM and NIDDM) have also been reported (XII). The hypoglycaemic effect of fenugreek seeds was studied on the serum glucose level (XIII). The usual practice is to soak one teaspoonful of seeds overnight in water and consume the water next day and add the seeds to wheat flour or dal. Using column chromatography, two compounds were isolated viz; 4 hydroxyisoleucine (4HI) and trigonelline (T). 4HI is an amino acid mainly found in trigonella species (XIV). Broca reported the essential structural features required for insulinotropic activity of 4HI involved in fenugreek seeds antidiabetic property (XV). The experiments were performed on isolated exvivo glucose perfused rat pancreas. 4HI is the most potent insulinotropic agent (XVI). Sauvaire reported the glucose-induced changes in blood glucose after administration of 4HI on isolated islets of Langerhans from both rats and humans (IV). Similarly, trigonelline was first studied by Mishkinisky (V). Trigonelline, another component showed hypoglycemic effect in healthy non diabetic volunteers but some studies showed no effect on fasting or postprandial glucose levels in non diabetic volunteers (III). Mishkinisky (V) proved that trigonelline had some hypoglycemic effect in both alloxan induced diabetic rats and diabetic patients. Thus, combination (4HIT) of 4 Hydroxy isoleucine: Trigonelline (40:30) was used for the study. Acute study showed that 4HIT had rapid onset i.e less than 2h and

longer duration of action upto 6 h. The blood glucose increases after 6h. Sub acute study decreased blood glucose from 7th day and blood glucose reduction was seen even after the drug administration was stopped for a week in contrast to the studies done by us with single isolated compounds (XVII). Glyburide is a potent, second-generation, oral sulfonylurea antidiabetic agent used as an adjunct to diet to lower blood glucose levels in patients with diabetes mellitus. The hypoglycemic action of glyburide is due to stimulation of pancreatic islet cells, which results in an increase in insulin secretion. Sulfonylureas are insulin secretagogues, since they control blood glucose levels by directly stimulating first-phase insulin secretion in the pancreatic beta cells. Prolonged administration of glyburide also produces extrapancreatic effects that contribute to its hypoglycemic activity (XVIII). Glycosylated hemoglobin (HbA_{1c}) test indicates the average blood glucose concentration over the past three months in humans and is being suggested as screening tool for diabetes and increased risk of heart disease and overall mortality (XIX). In case of diabetic animals as the mortality rate was high in our study we have studied the glycosylated hemoglobin levels at the end of one-month study. The glycosylated hemoglobin levels in 4HIT treated group showed significant decrease as compared to alloxan treated group that could be due to improvement in insulin secretion (XX). Intraperitoneal Glucose Tolerance Test (IPGTT) in diabetic animals increased the glucose threshold on treatment with 4HIT as compared to only alloxan treated group. Reduction in the body weight in diabetic animals as well as humans is a well known effect. In the present investigation, both glyburide and 4HIT treated animals showed improvement in the body weights. In case of diabetes, the body weight will increase when normal glycemic levels is achieved which is particularly seen in sulfonylureas or insulin. Weight gain is not seen in acarbose, trioglitzone or metformin as these drugs do not stimulate pancreas and do not elevate insulin levels. Increase in body weight in diabetic animals, probably due to improvement in insulin secretion and glycemic control. Increase in the body weight indicated the hypoglycemic action of 4HIT would be useful in preventing further weight loss. Further evidence to support this hypothesis was obtained from histological examination, which showed that in 4HIT treated pancreatic sections many islets were seen occasionally around the

duct. Alloxan treated animals showed occasional islets with fewer islets in the vicinity of pancreatic duct. This study thus indicated that 4HIT treatment enhanced the pancreatic regeneration. Administration of 4HIT showed less mortality (35%) as compared to untreated diabetic animals (57%) or glyburide (45%). It appears that hypoglycemic effect of trigonelline may be contributing to reduction in mortality. The acute oral toxicity indicated trigonelline has good safety profile as the LD₅₀ was greater than 5000 mg/kg. It is thus apparent that, 4HIT possesses hypoglycemic activity which may be due to enhanced pancreatic regeneration. It can be a very useful drug for the use of diabetic patients. It is concluded that oral administration of 4HIT (120 mg/kg) showed hypoglycemic effect probably by formation of new beta cells. The acute toxicity study indicated its high safety.

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Abbreviations:

4HI: 4 hydroxyisoleucine

T: Trigonelline

4HIT: 4 hydroxyisoleucine and trigonelline

IPGTT: Intraperitoneal Glucose Tolerance Test

HbA_{1c}: Glycosylated hemoglobin

GOD/POD: glucose oxidase peroxidase

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