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Original Article

# ANTIDIABETIC ACTIVITY OF LINALOOL AND LIMONENE IN STREPTOZOTOCIN- INDUCED DIABETIC RAT: A COMBINATORIAL THERAPY APPROACH

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**ABSTRACT**

**Objective:** To demonstrate the efficacy of a protein glycation inhibitor (limonene) and antihyperglycemic plant component (linalool) in combination towards better management of diabetes.

**Methods:** Antihyperglycemic activity was assessed by OGTT and glucose uptake studies on diaphragm tissue. Protein glycation inhibition was studied using BSA as a model protein through fructosamine measurement. *In vivo* effect of mono and combinatorial therapy was assessed through oral treatment on streptozotocin induced diabetic rats.

**Results:** Linalool at 3mM demonstrated 1.3mg/g tissue glucose uptake in 30 min as against 1.5 mg/g uptake by 2 unit insulin. In combination with insulin it showed an uptake of 2.2 mg /g tissue. Limonene demonstrated 85.61 % inhibition of protein glycation at 100 µM while the positive control aminoguanidine demonstrated 88.02% inhibition at 1mM concentration. In vivo studies has demonstrated lowering of blood glucose, improved lipid profile and glycated hemoglobin. Combinatorial therapy demonstrated significantly improved kidney parameters viz. urine glucose, albumin and creatinine levels as compared to diabetic control rats.

**Conclusion:** Combination of an antihyperglycemic agent with a protein glycation inhibitor is more efficient in control of diabetes.

**Keywords:** Linalool, Limonene, Glucose uptake, In vitro protein glycation, Diabetes mellitus, Combinatorial therapy.

## INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder that is characterized by hyperglycemia, relative insulin insufficiency and insulin resistance. According to IDF Diabetes Atlas, 382 million people in the world suffered from diabetes in 2013.The prevalence of diabetes is likely to increase up to 592 million by the year 2035 [1]. The diseasehas becomea real problem of public health in the developing countries, where its prevalence is increasing steadily. Developing countries like India have today become the diabetic capital of the world with over 65.1 million diabetics in 2013 [1].

The control of blood glucose in diabetic patients is achieved mainly by the use of dietary manipulations, oral hypoglycemic agents and insulin [2]. However, these therapies have limited efficacy and have been reported to be associated with undesirable side effects [3]. Intensive insulin therapy carries a high risk of side effects, especially occurrence of severe hypoglycemia and weight gain [4-5].In order to overcome the side effects associated with diabetes treatment, interest has been shifted to the use of alternative medicine. In developing countries 80% of the population is using traditional medicine in primary medical care [6].

One of the major problems of blood glucose control in diabetes is that, despite the best glycemic control over a number of years, diabetes gradually progresses to secondary complications. Glycation of protein is major cause for initiating oxidative stress and immunological signaling leading to tissue damage [7]. Hence inhibition of protein glycation in addition to an effective anti-hyperglycemic agent could prove to be beneficial in better control of blood glucose and help prevent progression of diabetes to secondary complications.

Linalool, a monoterpene, is a major volatile component of the essential oils that possesses antimicrobial, antibacterial and antiviral effects, as well as anti-inflammatory, analgesic and local anesthetic activities [8]. The antidiabetic activity of linalool in diabetic rats has already been reported [9]. Linalool enhanced the peripheral utilization of glucose in the rat diaphragm [10]. Our own studies have demonstrated similar results.

Sugar and primary amino group of protein react through the Maillard reaction [11]. The reaction proceeds through a Schiff base adduct, followed by an Amadori rearrangement, to yield a stable, ketoamine derivative of proteins [12]. Recently our laboratory has demonstrated limonene to be the potent protein glycation inhibitor that acts through stabilization of protein structure [13].It was observed that treatment of limonene can prevent cataract formation and prevent secondary complications.

The current trend in diabetes treatment is towards combinatorial therapy. Generally an insulin secretagogue, an alphaglucosidase inhibitor and an insulin sensitizer are given in combination. However, over a time period dosages continue to increase and establishment of secondary complications are seen. In the present study, it is attempted to study the effect of combinatorial therapy of limonene and linalool through in vivo and in vitro studies.

## MATERIALS AND METHODS

### Chemicals/material

Linalool, limonene, streptozotocinwere purchased from Sigma Chemicals (St Louis, MO, USA). Fatty acid-free bovine serum albumin (BSA), sodium azide, aminoguanidine (AG), Folin-Ciocalteu reagent, tri-chloroacetic acids (TCA), nitro blue tetrozolium (NBT), thiobarbituric acid (TBA), were purchased from Himedia (Mumbai, MS, India). All other chemicals and reagents were of analytical grade and locally purchased.

### Oral glucose tolerance test (ogtt)

For conducting the OGTT, rats were fasted overnight with free access to water. Initial blood glucose of each rat was measured. All rats were fed orally with glucose load of 3 mg/g body weight. They were divided into five groups (n=3) as normal control, diabetic control, positive control (Glicazide 1.6 mg/kg body weight), diabetic rat with linalool and the diabetic rats with limonene. Blood samples were withdrawn from the tail vein at intervals of 30, 60 and 120 min of glucose administration. Glucose levels were estimated with glucose oxidase-peroxidase (GOD-POD) method using commercial kit (BIOLAB Diagnostics).

**Experimental animals**

Male Wistar rats weighing about 180–200 g were used in the experiments. All the animals were maintained under laboratory conditions and were allowed free access to food (Amruth, Pune) and water *ad libitum*. Experiments were carried out according to the guidelines of the Animal Ethical Committee of the Institute after due approval of the protocols (registration no. 233/CPCSEA).

**Glucose uptake by isolated rat hemi-diaphragm**

Glucose uptake by rat hemi-diaphragm was estimated by the methods described by Walass and Chattopadhyay with some modification [14-15]. Albino rats of either sex weighing between 180-200 g were selected. The animals were maintained on a standard pellet diet and water *ad libitum*, and fasted overnight. The animals were sacrificed and diaphragms were dissected out quickly with minimal trauma, divided into two halves and weighed. The hemi-diaphragms were then rinsed in cold Kreb's Ringer bicarbonate buffer (without glucose) to remove any blood clots and were placed in small culture tubes containing Kreb's Ringer bicarbonate buffer with 5.55mM glucose and incubated for 30 minutes at $37^0$ C.

**Group 1:** Kreb's Ringer bicarbonate buffer with 5.55 mM glucose.

**Group 2:** Kreb's Ringer bicarbonate buffer with 5.55 mM glucoseandrapid acting insulin (2 units).

**Group 3:** Kreb's Ringer bicarbonate buffer with 5.55 mM glucose andlinalool 1.5 mM.

**Group 4:** Kreb's Ringer bicarbonate buffer with 5.55 mM glucose and linalool 3 mM.

**Group 5:** Kreb's Ringer bicarbonate buffer with 5.55 mM glucose, linalool 1.5 mM and insulin (2 units).

**Group 6:** Kreb's Ringer bicarbonate buffer with 5.55mM glucose, linalool 3 mM and Insulin (2 units).

Following incubation, the hemi-diaphragms were taken out. The glucose content of the incubated medium was measured by GOD-POD method. The uptake of glucose was calculated in mg/g tissue. Glucose uptake per gram of tissue was calculated as the difference between the initial and final glucose content in the incubated medium.

***In vitro* glycation inhibition study using bovine serum albumin-glucose model system**

Each 5 ml reaction mixture contained 20 mg/ml of bovine serum albumin in 50 mM phosphate buffer (pH 7.4), 300 mM D-glucose, and 0.01% sodium azide [16]. Test molecules limonene was included at concentrations 25µM, 50µM and 100µM. Reaction mixtures containing aminoguanidine at 1 mM served as positive control. Each reaction mixture was pre-incubated with test molecule for 10 min before addition of glucose. Reaction mixtures were incubated in the dark at $37^0$C for 3 weeks. At the end of the incubation, unbound glucose was removed by extensive dialysis against the same buffer. Protein concentration was determined by Lowry's method using BSA as standard [17].

**Measurement of fructosamine**

The fructosamine assay was performed by the method of Johnson with slight modification [18]. The reaction mixture which contained 0.2 ml glycated sample and 0.8 ml nitro blue tetrozolium (NBT) reagent (300 mM) in sodium carbonate buffer (100 mM, pH 10.3) was incubated at room temperature for 15 min, and the absorbance was read at 530 nm against a blank. The results were expressed as fructosamine nM $mg^{-1}$ protein using an extinction coefficient of 12,640 $M^{-1}cm^{-1}$ for monoformazan [19].

**Experimental design for prolonged treatment**

Diabetes mellitus was induced by single intraperitoneal injection of freshly prepared streptozotocin (70 mg/kg body weight) in 0.1 M citrate buffer (pH 4.5). After 14 days, rats with fasting blood glucose above 200 mg/dl were considered diabetic and included in the study. The following groups were formed for prolonged treatment. Each group had six animals (n= 6):

**Group I:** Normal untreated rats were given vehicle 0.5 ml of 0.01% DMSO;

**Group II:** Streptozotocin-induced diabetic control rats.

**Group III:** Diabetic rats treated with linalool (20 mg/kg body weight).

**Group IV:** Diabetic rats treated with limonene (20 mg/kg body weight).

**Group V:** Diabetic rats treated with linalool (10 mg/kg body weight) and limonene (10 mg/kg body weight) orally, in 0.5 ml of 0.01% DMSO twice a day.

The animals were treated for 45 days. Fasting blood glucose of all the rats was monitored after each week of treatment. After 45 day rats were sacrificed by cervical dislocation, blood was collected and used for serum parameter analysis.

**Determination of blood/serum parameters**

Blood glucose was determined by the glucose oxidase-peroxidase method using a glucometer (Accuchek, Roche Diagnostics, and Mannheim, Germany). Glycated hemoglobin was measured from whole blood by using the ion exchange methodby using commercially available kit (Span Diagnostics, India). Total triglycerides, HDL, Serum creatinine, blood urea nitrogen (BUN),and total cholesterol were measured by diagnostic kit (Creast Biosynthesis, Biolab Diagnostics, India). VLDL and LDL were calculated from triglyceride, total cholesterol and HDL cholesterol values.

**Determination of urine parameters**

Urine samples of 24 hours were collected in metabolic cages at the end of the $45^{th}$ day treatment. The urine samples were used to calculate urine glucose [20], urinary creatinine, and urinary albumin [21].

**Preparation of homogenate and estimation of antioxidant status**

Kidney and liver were homogenized in 100 mM of Tris–HCL buffer pH 7.4 inteflon homogenizer. Thiobarbituric acid reactive substances (TBARS) were estimated [22] in a part of the homogenate and the remaining was centrifuged at 8000 g for 20 min at $4^0$C. Supernatant used for the estimation of catalase (CAT), superoxide dismutase (SOD). Protein content was determined by Lowry method. CAT was determined in tissue supernatant by method of Aebi [23]. The change in optical density at 240 nm per unit time was taken as a measure of catalase activity. SOD was estimated using the principle of inhibition of riboflavin auto oxidation by the method of Mishra and Fridovich [24]. A unit activity of SOD is defined as 50% inhibition of riboflavin auto oxidation per min.

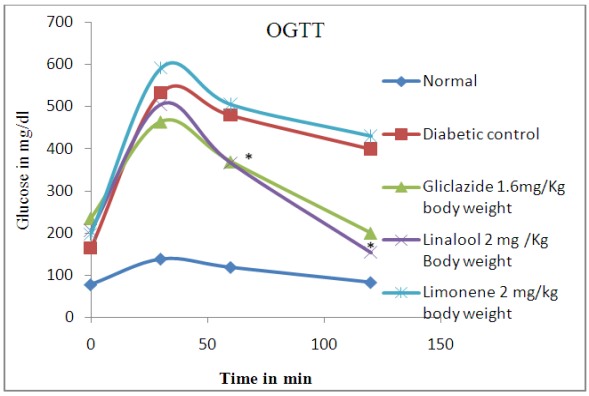


**Fig. 1: Oral Glucose Tolerance Test**

Values are given as mean S.E.M for groups of three rats in each value expressed as means ±SEM, (n = 3). *Values significant at P < 0.05 as against diabetic control.

## RESULTS AND DISCUSSION

### OGTT

The anti-diabetic activity of linalool and limonene was analyzed by OGTT as shown in the Fig 1. The OGTT analysis of linalool showed a very prominent blood glucose lowering activity as compared to the diabetic control. It has been suggested earlier that linalool has insulin mimetic activity [10].Glicazide was used as a positive control. Limonene does not show any reduction of blood glucose up to 2 hr.

### *In-vitro* glucose uptake studies

The uptake of glucose using rat hemi diaphragm is a commonly employed and reliable method for *in vitro* study of peripheral uptake of glucose [25]. It can be observed from Fig.2 that linalool demonstrated a dose dependent uptake of glucose. Linalool at 3mM demonstrates an uptake that is almost equivalent to 2 units of insulin. Further it demonstrates an additive effect with insulin showing an increase in glucose uptake to 2.3 mg / g tissue in 30 min. It is likely that linalool being a membrane permeable molecule may have post receptor action leading to an enhancement in glucose uptake with insulin. Its detailed mechanism of action needs to be validated.

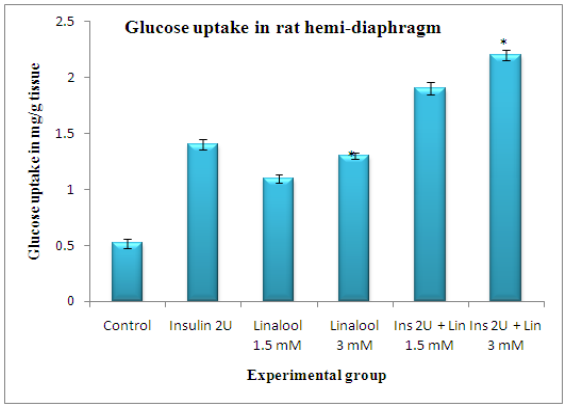


**Fig. 2: In vitro glucose uptake in rat hemi-diaphragm.**

Each value expressed as means ±SEM, (n = 3). *Values significant at P < 0.05 as against control.

### *Invitro* protein glycation

Fructosamine content is used as a measure for short-term control of blood glucose level in diabetic patients. Fig.3 shows the *in vitro* inhibition of fructosamine formation. It can be observed that limonene can reduce the fructosamine formation in a dose dependent manner. The dose at 100 μM is comparable to the positive control aminoguanidine at 1 mM concentration (showing a tenfold reduction in inhibitor concentration) aminoguanidine is known to act as a competitive inhibitor of protein glycation and can mop up the dicarbonyls formed [26]. Limonene on the other hand is known to inhibit protein glycation through stabilization of protein structure and preventing alpha beta transitions [13].

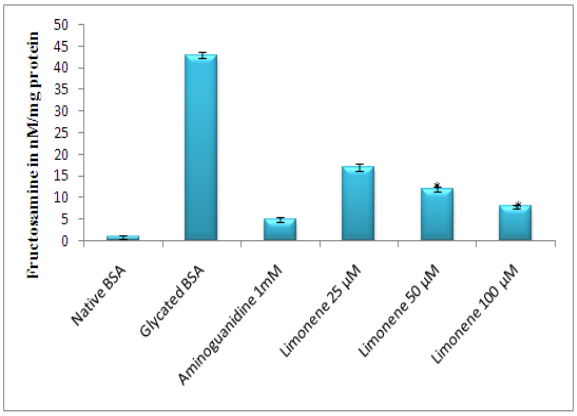


**Fig. 3: The levels of fructosamine formed after 21 days of glycation.**

Each value expressed as means ±SEM, (n = 6). *Values significant at P < 0.05 as against glycated BSA

### Blood parameters estimation

It can be observed from Table 2that after 45 days of treatment in the drug-treated animals, blood glucose levels were found to decline. However, it was observed that while it has taken linalool to reduce blood glucose to 157 mg/dl in 45 days, combination of linalool and limonene could demonstrate similar lowering in 28 days. The combinatorial therapy could lower blood glucose to 126 mg/dl in 45 days. In an earlier report [10], a lowering of blood glucose level to 107 mg/dl in 45 days treatment using 25 mg/kg body weight of linaloolwas reportedwhile in the present study we were able to demonstrate lowering of blood glucose to 157 mg/dl using 20 mg/kg body weight of linalool. This could be further reduced to 126 mg/dl at 10 mg dose of linalool when used in combination with limonene. It is interesting to note that limonene was unable to lower blood glucose in OGTT however its impact on reduced protein glycation that can lower the inflammatory markers may have led to further effective action of linalool proving the efficacy of a combinatorial therapy and synergistic effect. This is also reflected in lower glycated Hb levels demonstrating a consistent glucose control. Linalool has glucose lowering property and hence can maintain a better lipid profile. However it can be observed from Table 3 that the combinatorial treatment demonstrated a lipid profile that is almost on par with normal controls. The serum creatinine and BUN levels are also significantly improved in the treated animals.

### Antioxidant parameter

Reduction in antioxidant status was observed in the liver and kidneys (Table 3) of diabetic rats. Treatment with linalool, limonene and their combination significantly increased the activity of antioxidant enzymes, viz SOD and CAT as compared to diabetic control. Oxidative stress as indicated by reduction in antioxidant enzymes and increase in lipid peroxidation (Table 3) was observed in liver and kidneys of diabetic rats. Treatment with linalool, limonene,and linalool and limonene combination significantly reduced the formation of TBARS. Combinatorial therapy showed levels similar to normal control.

**Table 1: Weekly levels of fasting blood glucose, in control and experimental groups of rats after 45 days of treatment.**

| Day | Group I | Group II | Group III | Group IV | Group V |
|---|---|---|---|---|---|
| **0th** | 73±2 | 255±4 | 219±4 | 230±5 | 234±5* |
| **7th** | 73±4 | 261±3 | 212±4 | 219±4 | 225±4* |
| **14th** | 75±3 | 267±5 | 201±6 | 212±6 | 203±6* |
| **21th** | 77±7 | 287±5 | 194±3 | 196±4 | 175±3* |
| **28th** | 80±3 | 297±8 | 187±5 | 187±7 | 158±7* |
| **35th** | 76±2 | 302±3 | 171±3 | 182±6 | 148±3* |
| **42th** | 75±6 | 340±3 | 165±5 | 175±3 | 133±6* |
| **45th** | 79±3 | 355±6 | 157±6 | 171±4 | 126±4* |

Each value expressed as means ±SEM, (*n* = 6). *Values significant at *P < 0.05* as against diabetic control.

**Table 2: The levels of cholesterol, triglyceride, HDL, LDL, VLDL, GHb, creatinine, BUN in control and experimental groups of rats after 45 days of treatment.**

| Parameters | Group I | Group II | Group III | Group IV | Group V |
|---|---|---|---|---|---|
| Cholesterol (mg/dl) | 77±2.6 | 130±5.0 | 115±4.0 | 114±4.56 | 100±3.0* |
| Triglyceride (mg/dl) | 71±2.5 | 126±3.6 | 111±2.5 | 104±3.0 | 93±5.5* |
| HDL (mg/dl) | 55±2.2 | 20±3.3 | 39±2.0 | 40±3.1 | 45±3.0* |
| LDL (mg/dl) | 7.8±2.0 | 84.8±3.01 | 53.8±1.0 | 53.2±3.4 | 36.4±2.4* |
| VLDL (mg/dl) | 14.2±1.1 | 25.2±0.6 | 22.2±0.4 | 20.8±1.0 | 18.6±0.5* |
| GHb (%) | 5.8±0.2 | 13±1.1 | 9.1±0.7 | 7.9±0.25 | 7.7±0.38* |
| Creatinine (mg/dl) | 0.5±0.13 | 2.3±0.1 | 1.5±0.22 | 1.53±0.14 | 1.2±0.11* |
| BUN (mg/dl) | 23±2.1 | 57±1.5 | 37±1.9 | 36±2.2 | 31±1.07 |

Each value expressed as means ±SEM, ($n$ = 6). *Values significant at $P < 0.05$ as against diabetic control.

**Table 3: The levels of liver and kidney SOD, catalase, TBARS, in control and experimental groups of rats after 45 days of treatment**

| Parameter | Group I | Group II | Group III | Group IV | Group V |
|---|---|---|---|---|---|
| | | **Liver** | | | |
| SOD(U/mg protein) | 10±0.2 | 2.7±0.31 | 5.8±0.11 | 7±0.28 | 7.6±0.17* |
| Catalase(U/mg protein) | 235±5 | 93±6.6 | 143±4.5 | 203±4.9 | 219±3.3* |
| TBARS(nm/mg protein) | 0.4±0.03 | 2.9±0.055 | 1.5±0.01 | 1.4±0.034 | 0.7±0.027* |
| | | **Kidney** | | | |
| SOD(U/mg protein) | 18±1.1 | 7.7±0.43 | 9.2±0.62 | 9.3±0.33 | 12±0.8* |
| Catalase(U/mg protein) | 115±6.5 | 43±3.4 | 59±2.9 | 92±3.9 | 113±4.3* |
| TBARS(nm/mg protein) | 0.61±0.05 | 3.4±0.045 | 2.3±0.073 | 1.5±0.047 | 1.2±0.02* |

Each value expressed as means ±SEM, ($n$ = 6). *Values significant at $P < 0.05$ as against diabetic control.

**Kidney parameters**

At the end of the experimental period, there was a significant increase in the mean urinary glucose, albumin and creatinine observed in the diabetic control group compared asto the normal control group (Table 4). 45 days of treatment with linalool, limonene and linalool and limonene combinatorial group elicited a significant decrease in urinary glucose, albumin and creatinine compared to diabetic control group. The urinary glucose of diabetic control rats after 45 days was 1.4 g/day, while in those rats receiving for linalool, limonene and linalool and limonene combinatorial group was 0.64 g/day, 0.49 g/day and 0.23 g/day, respectively.

In addition, the marked elevated albumin in diabetic rats was decreased significantly in treated rats. Glycation of the kidney matrix proteins leads to changes in the kidney architecture and increased permeability of the basement membrane leading to nephropathy [27]. These are slow changes and accumulate over years ultimately leading to kidney failure. Prevention of protein glycation can prove to be very beneficial in preventing establishment of such changes. Moreover, in the present study we also did not observe cataract formation in the treated groups, while the diabetic controls showed cataract formation by 20 days (results not shown).

**Table 4: Effect of treatment for 45 days on urine parameters**

| Parameter | Group I | Group II | Group III | Group IV | Group V |
|---|---|---|---|---|---|
| Glucose (g/day) | 0.087±0.006 | 1.4±0.07 | 0.64±0.05 | 0.49±0.025 | 0.23±0.024* |
| Albumin (mg/day) | 4.37±0.4 | 125±1.9 | 54±2.4 | 40±2.8 | 22±1.6* |
| Creatinine($mg.dl^{-1}$/day) | 52±2.5 | 12.5±1.5 | 21.5±2.1 | 22.5±2.5 | 35.5±1.8* |
| Volume (ml/day) | 14±2 | 78±3.2 | 45±4.2 | 34±2.9 | 27±1.8* |

Each value expressed as means ±SEM, ($n$ = 6). *Values significant at $P < 0.05$ as against diabetic control.

**CONCLUSION**

Combinatorial approach is best suited for disorders such as diabetes mellitus that have multiple targets for drug action. Currently, combination of insulin sensitizers, secretagogues and / or alpha glucosidase is prescribed. However, it is observed that with time dosages increase and secondary complications such as neuropathy, nephropathy, retinopathy and CVD manifest. Increase in blood glucose leads to protein glycation that activates oxidative stress and inflammatory markers. The latter enhances insulin resistance neccesiting higher dosages of anti-hyperglycemic drugs. A more rational approach would be to treat with an anti-hyperglycemic agent that can lower blood glucose and a non-specific protein glycation inhibitor that can prevent progression of the disorder to secondary complications. We have demonstrated in the present study that a combination of limonene and linalool can function very effectively. Further the concentration of linalool needed in combination is half that needed in mono therapy. It appears limonene and linalool act in synergistic manner.

This is a novel approach being tested for the first time in an effective manner and can lead to development of new therapy options in treatment of diabetes mellitus.

**CONFLICT OF INTERESTS**

Declared None

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