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Citral as a potential antihyperlipidemic medicine in diabetes: a study on streptozotocin-induced diabetic rats

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Abstract

Background: As potential anti-diabetic and anti-obesity agents, glycosidase inhibitors are the subject of numerous studies. Among these enzymes, alpha-amylases are of particular interest, and most of their reported inhibitors have so far been natural compounds. Citral is an isoprenoid compound of various essential oils, and based on its alpha-amylase inhibitory effect, was further studied here in an in vivo model of type 1 diabetes.

Methods: In vitro effect of the compound was assessed on mammalian alpha-amylase activity with the use of the Bernfeld method. In vivo effect of the compound was studied on streptozotocin-induced diabetic rats (wistar). Non-diabetic and diabetic rats received citral at 2, 8, 16 or 32 mg/kg body weight; the compound was dissolved in grape seed oil. The control groups received grape seed oil alone. Treatment was done for 24 days, after what the animals were sacrificed under light ether anesthesia. Measured parameters included: food and water ingestion and urine volume (daily), blood glucose levels (every two days), cholesterol, triacylglycerol, concentrations, and alpha-amylase levels after 24 days .

Results: Citral was found to be a moderate inhibitor of mammalian alpha-amylase, with an IC50 of 120 µM, and caused also a decrease of alpha-amylase levels in vivo. Moderate lowering of postprandial glucose, alongside with normalization of blood lipid profile was observed in diabetic rats upon treatment with the compound. Citral was also found to be able to promote weight loss and to decrease food intake.

Conclusion: Citral could be proposed as a possible antihyperlipidemic agent in diabetes and potential therapeutic in obesity, although further studies are needed to establish its complete profile as potential medication .

Keywords: Citral; 3, 7-dimethylocta-2,6-dienal; alpha-amylase; inhibitor; diabetes; hyperlipidemia, hyperglycemia

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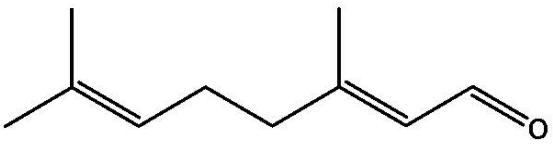
Introduction

Diabetes mellitus, characterized by a raised blood sugar, is estimated by the World Health Organization to cause 5% of all global deaths in year, with the number of patients estimated to reach 366 million in 2030 (http://www.who.int/diabetes/en/).

Among potential therapeutics designed to counteract the deleterious chronic effects of this disease, compounds capable of lowering postprandial glucose, correcting hyperlipidemia and promoting weight-loss are of interest (1,2) since they could also be of use in the prevention of the disease (3). Traditional medicines, including herbal medicine possess great potential in this regard (4, 5), while multiple targeting is achieved by these extracts (e.g. (6)).

Citral (3,7-dimethylocta-2,6-dienal) consists of the E- and Z-isomers of an isoprenoid structure (scheme 1), and is present in a variety of plants essential oil, with the highest concentration existing in *Backhousia citriodora* (Lemon myrtle) oil (7).



Scheme 1 (citral 2D-structure)

It is used as food additive and fragrance, and has been found to possess anti-bacterial (9), anti-fungal, and anti-cancer (10) properties.

As part of a project involving the screening of compounds for their alpha-amylase inhibitory activity, citral was found to be a moderate inhibitor of mammalian alpha-amylase, and as such, further investigated as to its *in vivo* effect on diabetic rats. It should be mentioned here that inhibitors of mammalian alpha-amylase are suggested to have the potential of lowering postprandial glucose (11).

Methods

Chemicals

Citral (3,7-dimethylocta-2,6-dienal), porcine pancreatic alpha-amylase (PPA) (E.C.3.2.1.1), and dinitrosalycilic acid were from Sigma Chemical Co. (St. Louis, MO), Dimethyl sulfoxide, soluble starch, and maltose were obtained from Merck (Darmstadt, Germany). Streptozotocin was from Pharmacia & Upjohn (Kalamazoo, Michigan).

Animals

2.5 months aged male adult wistar rats weighting 200 ± 15g were used. Rats were housed as six per cage at room temperature (22-24° C). Lights were on from 08:00 to 20:00 h. Standard rodent diet was used as follows: maintenance diet Letica, Panlab S.L., Barcelona, Spain; 61.4%(w/w) carbohydrate (100%starch), 3.9% fibre, 15.1% protein and 2.7% fat, and tap water. Food and water were ad libitum.

Diabetes Induction

Diabetic condition was induced by the use of a single dose of streptozotocin (STZ) at 70mg/kg body weight. STZ was administered intraperitoneally on the first day of experiment (12), and blood glucose levels measured after 2 days. The experiments conditions were approved by the ethical committee of the Science and Research Branch of Islamic Azad University, Tehran.

Treatment with citral

Rats were divided into "non diabetic rats" (ND) and "diabetic rats" (D), and each class was subsequently divided to five groups, as defined below:

(I) Non diabetic control group (NDS): this group was administered grape seed oil (O) orally during 24 day with use of a gastric cannula in single doses of 0.5ml at 8:30 AM. (II) Diabetic control group (DS): this group was administered grape seed oil (O) during 24 day with the use of a gastric cannula in single doses of 0.5ml at 8:30 AM. (III) Non diabetic group receiving citral (Ci): citral was administered at 2, 8, 16, 32 mg/kg body weight (respectively NDCi2, NDCi8, NDCi16, NDCi32) dissolved in grape seed oil, orally, during 24 day with the use of a gastric cannula in single doses of 0.5ml at 8:30 AM. (IV) Diabetic groups receiving citral (Ci): citral was administered at 2, 8, 16, 32 mg/kg body weight (respectively DCi2, DCi8, DCi16, DCi32) dissolved in grape seed oil, orally, during 24 day through a gastric cannula in single doses of 0.5ml at 8:30 AM. Throughout the manuscript, citral doses of 2, 8, 16, 32 mg/kg body weight are shown as Ci2, Ci6, etc.

Measured parameters

Ingestion of food and water and urine volume were measured every morning at 9:00 AM. Blood glucose levels were measured in blood samples extracted from the tail of the animal

every two days on mornings at 9:00 AM with a glucometer (One Touch Profile, Life Scan). Body weight of the rats was measured after day 24, and the animals were then sacrificed under light ether anesthesia. Blood samples collected from rats hearts were placed on ice and centrifuged within 15min after blood collection at 3000g for five minutes. Cholesterol, Triacylglycerol, high-density lipoproteins (HDL, low-density lipoproteins (LDL) concentrations, and alpha-amylase activity were measured with standard biochemical kits. Estimates of Very Low Density Lipoprotein (VLDL) were calculated from the formula VLDL-C = Triacylglycerol /5 (13).

Statistical analysis

Results are expressed as mean±SD. The data were analyzed by one-sample Kolmogrov-Smirnov test and Levene's test. One way analysis of variance (ANOVA) followed by Turkey's post hoc test for multiple comparisons were used to compare difference between experimental groups. The criterion for statistical significant was $P<0.05$.

Enzyme inhibition experiment

Enzyme assay was performed with the use of the Bernfeld method (14). The substrate was soluble starch and the activity of alpha-amylase was defined as one unit being the amount of enzyme able to release one micromole of maltose from starch per minute. Remaining activity in presence of different concentrations of citral was calculated as a percentage by comparison with the control sample activity.

Results

A limited number of non-proteinaceous inhibitors of alpha-amylase which could be orally taken_ have been reported, from which polyphenols are recently attracting more attention (15). With the aim of finding other inhibitory scaffolds, a variety of compounds were tested on a model mammalian alpha-amylase, from which citral came as a moderate inhibitor, with an IC_{50} of 120 µM. In order to assess its *in vivo* effects, the compound was subsequently tested in a model of streptozotocin-induced diabetes which showed in average 398.6±40.8mg/dl of blood glucose in the beginning of the experiment, in comparison with the control non-diabetic group with blood glucose levels of 107.3±12.3 mg/dl ($P<0.001$).

Effect of citral on blood glucose levels

The control groups which ingested only the carrier (grape seed oil) maintained an almost constant blood glucose level of average 398.6-402.3mg/dl and 111.5-109.1mg/dl for DS and NDS groups respectively throughout the experiment duration. However, treatment with citral resulted in decreased final blood glucose levels of the diabetic group as: DCi2 349.8±35.8, DCi8 313.6± 33.6, DCi16 295.5±31.5, and DCi32 302.1±31.8 ($P<0.01$). Similar results were found in the non-diabetic group as follows: NDCi2 96.5±10, NDCi8 83.9±9.1, NDCi16 81.8±9.5, and NDCi32 83.5 ±10.1 ($P<0.01$). In all groups, the rate of decrease in blood glucose concentrations was faster until day 6 and slowed down afterwards (Fig.1).

Effect of citral on blood lipid levels

Upon becoming diabetic, serum lipid levels of rats presented a marked elevation as follows: cholesterol (43% increase), TG (65% increase), very low density lipoproteins (VLDL) (65% increase), and LDL (81% increase), while reduced concentration of HDL also occurred (35% decrease). Treatment with citral showed an interesting potential of the compound, with the dose of 16 mg/kg being able to practically restore normal blood lipid levels in the diabetic rats (Table 1). A dose-dependent effect is observed in the range of 2-16 mg/kg of citral. Another interesting result is the decrease of cholesterol, triglycerides and LDL levels in the non-diabetic rats, which occurs however to a lesser extent in comparison with diabetic rats, and reaches its maximum effect in the dose of 8mg/kg.

Effect of citral on serum alpha-amylase

As mentioned above, citral was found to inhibit mammalian alpha-amylase *in vitro*. In order to assess its effect *in vivo*, serum alpha-amylase levels were also measured in the rats. In this case too, both diabetic and non-diabetic rats showed decreased levels of alpha-amylase (Fig.2). As represented in Fig.2, a significant difference is starting to appear from the dose of 8mg/kg in non-diabetic rats, where the 2mg/kg dose was already showing significant difference in diabetic rats.

Effect of citral on body weight and food intake

Citral intake results in a decrease of food consumption, as well as a marked lowering in

the weight of the rats which were receiving the treatment (Fig.3). The effect is similarly observed in both diabetic and non-diabetic rats, but is slightly more pronounced in the diabetic group. As observed in Fig.3, the untreated diabetic group presented higher food intake, associated with lower body weight in comparison with the non-diabetic rats, which would be a characteristic of the disease state.

Effect of citral on water intake and urine volume

Both water intake and urine volume was increased in the diabetic control group, again as a logical characteristic of the disease (Fig.4). Treatment with citral resulted in a decrease of both of these parameters in the diabetic rats, even though normalization was not reached (Fig.4). Here, a dose-dependent relationship could be observed in the range of 2-16 mg/kg doses. In the non-diabetic rats, however, citral did not have any significant effect on these parameters.

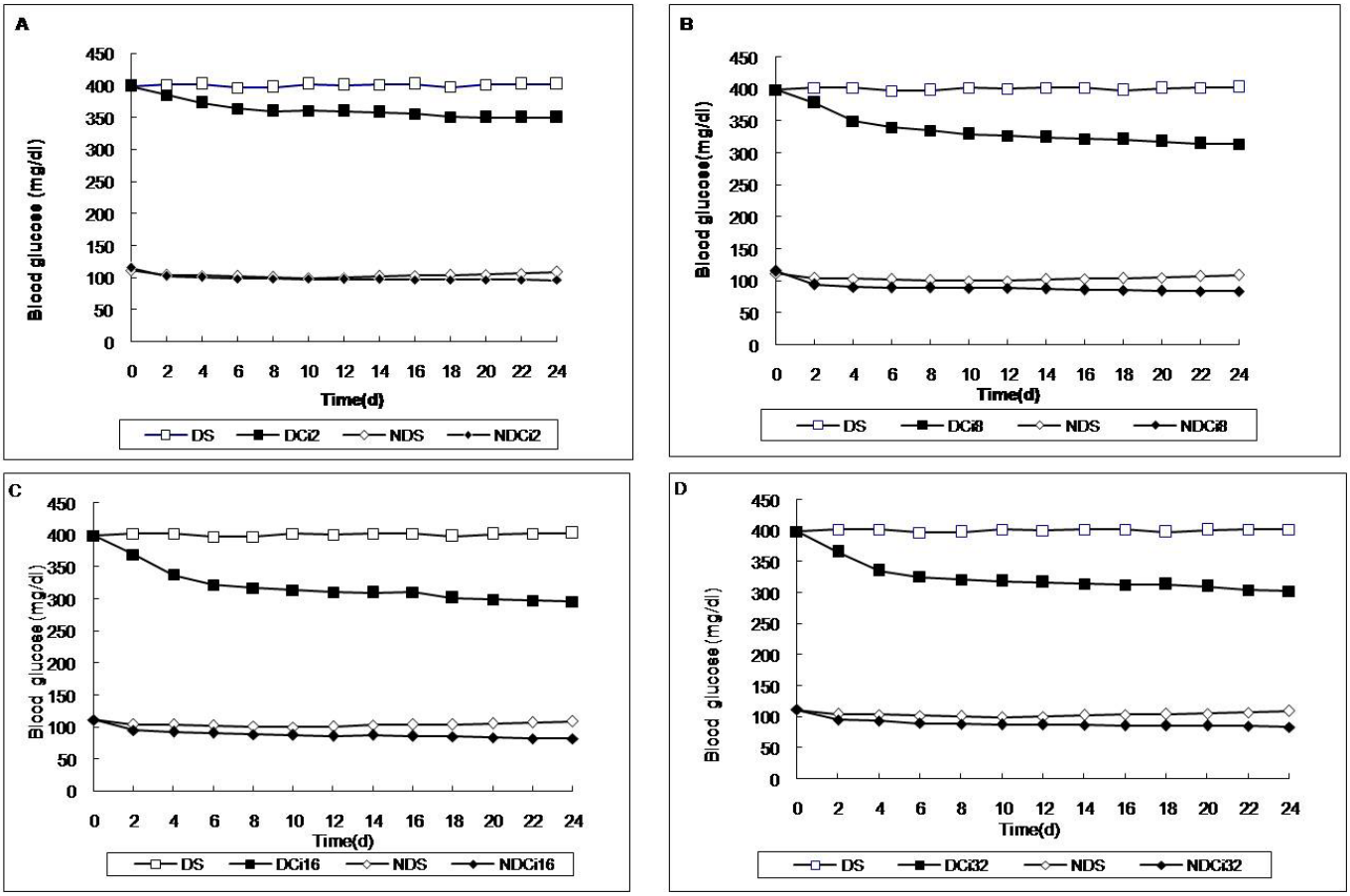


Fig. 1. Blood glucose values measured in the control group of diabetic (DO) and non-diabetic rats (NDO), and groups receiving citral. Values are represented as means with standard deviation. **A :** The effect of administration of 2mg/kg body weight of citral in diabetic and non-diabetic rats (DCi2 and NDCi2 respectively), **B :** The effect of administration of 8mg/kg body weight citral in diabetic and non diabetic rats (DCi8 and NDCi8 respectively), **C :** The effect of administration of 16mg/kg body weight citral in diabetic and non diabetic rats (in diabetic and non diabetic rats (DCi8 and NDCi8 respectively) , **D :** The effect of administration of 32mg/kg body weight citral in diabetic and non diabetic rats (in diabetic and non diabetic rats (DCi32 and NDCi32 respectively).

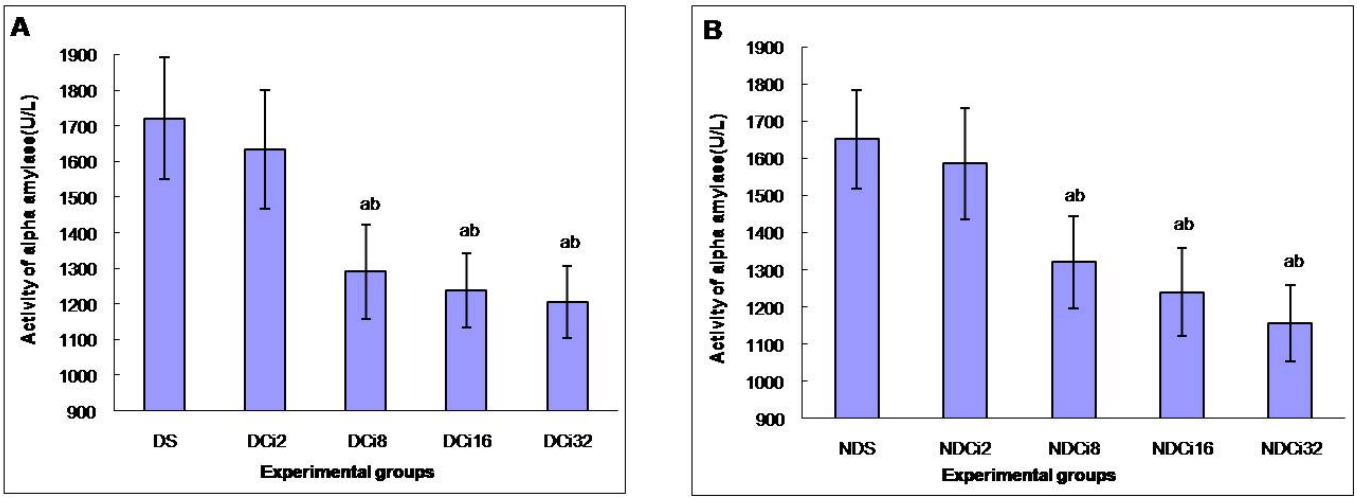


Fig. 2. A. Serum alpha-amylase activity in diabetic rats; the diabetic control group is shown as DS ; a: significantly different from DS, b: significantly different from DCi2. B. Serum alpha-amylase activity in non-diabetic rats; the non diabetic control group is shown as NDS; a: significantly different from NDS, b: significantly different from NDCi2.

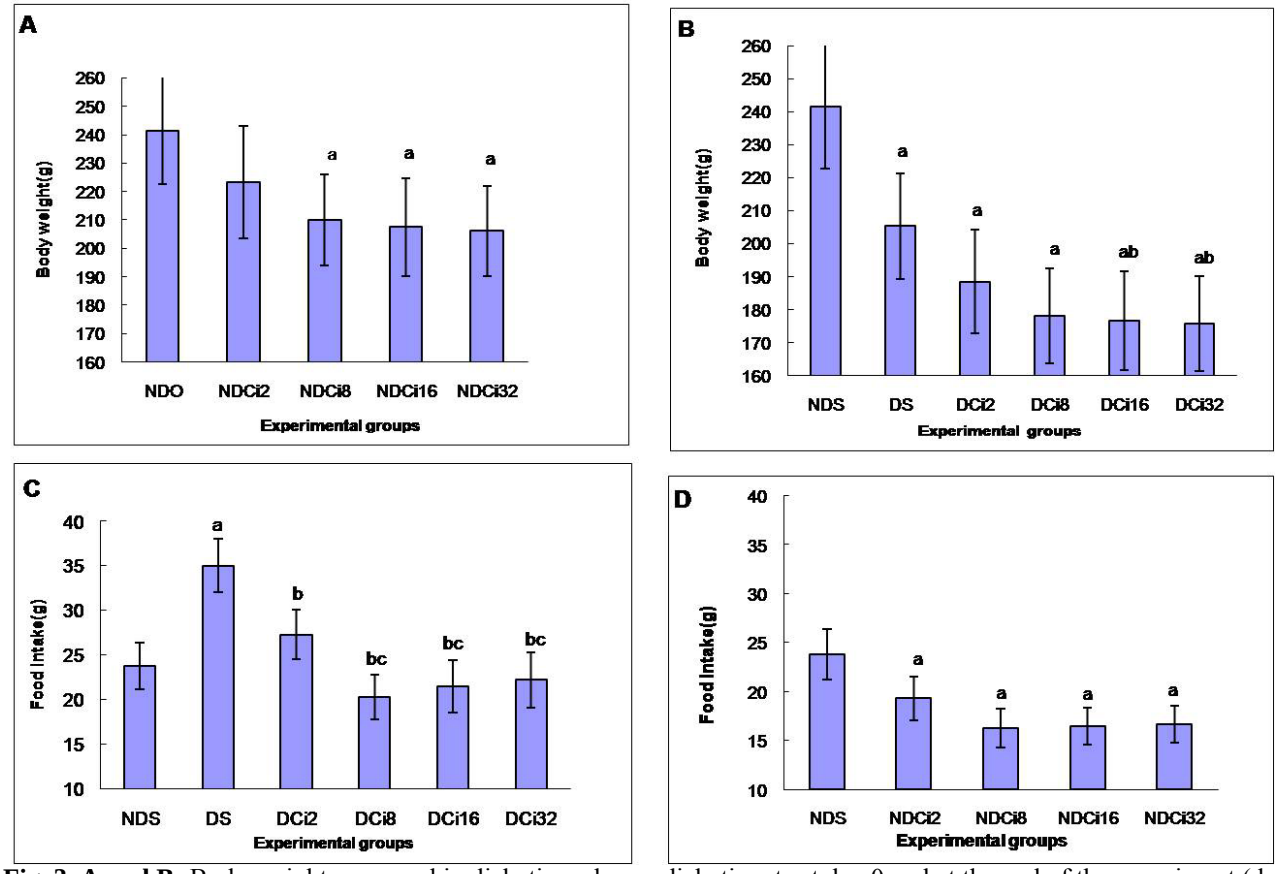


Fig. 3. A and B: Body weight measured in diabetic and non- diabetic rats at day 0 and at the end of the experiment (day 24). **A:** Body weight in non diabetic control group (NDS), diabetic control group (DS) and diabetic rats receiving citral from 2 to 32 mg/kg. a: significantly different from NDS; b: significantly different from DS; c: significantly different from DCi2 using one way ANOVA with Tukey-Kramer test at $P<0.05$. **B:** Body weight in non-diabetic control group (NDS) and non-diabetic rats (receiving citral from 2 to 32 mg/kg. a: significantly difference from NDS using one way ANOVA with Tukey-Kramer test at $P<0.05$. **C and D:** Food intake in diabetic and non-diabetic groups. **C:** Food intake in diabetic rats. a: significantly different from NDS; b: significantly different from DS using one way ANOVA with Tukey–Kramer test at $P<0.05$. **D:** Food intake in non diabetic rats. a: significantly different from NDS using one way ANOVA with Tukey-Kramer test at $P<0.05$.

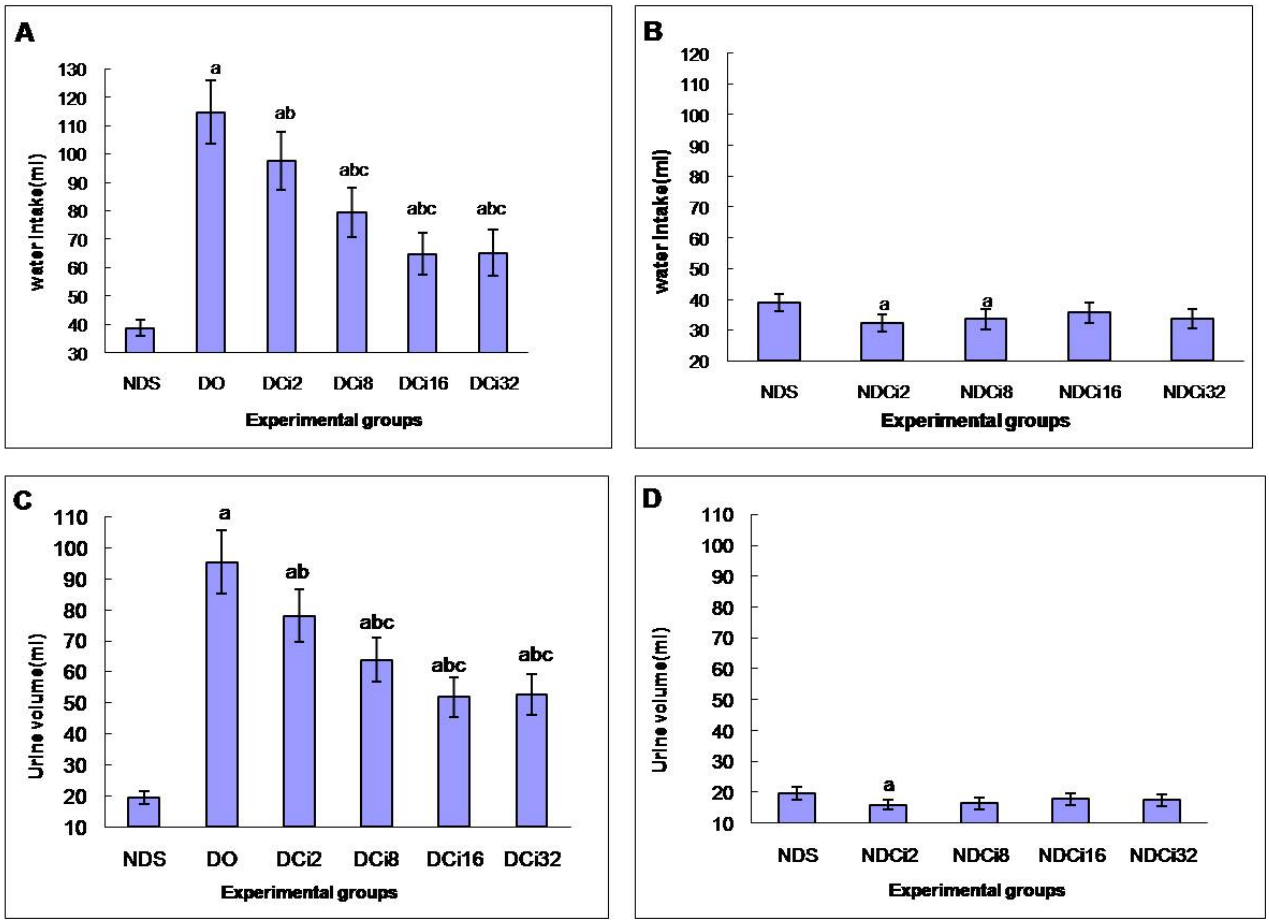


Fig 4. A and B: Water intake in diabetic and non diabetic arts. **A:** Water intake in diabetic rats. a: significantly different from NDS; b: significantly different from DS; c: significantly different from DCi2 using one way ANOVA with Tukey-Kramer test at P<0.05.**B:** Water intake in non diabetic rats; a: significantly different from NDCi2 using one way ANOVA with Tukey-Kramer test at P<0.05.**C and D:** Urine volume in diabetic and non diabetic groups. **C:** Urine volume in diabetic rats. a: significantly different from NDS; b: significantly different from DS; c: significantly different from DCi2 using one way ANOVA with Tukey-Kramer test at P<0.05 **D:** Urine volume in non diabetic rats. a: significantly different from NDS using one way ANOVA with Tukey- Kramer test at P<0.05.

Table 1. Effect of citral treatment on serum lipids of streptozotocin (STZ) – induced diabetic rats

Serum level (mg/dl)	DS	DCi2	DCi8	DCi16	DCi32
Cholesterol	126.5±14.3	101.2±13.3[a]	87.8±10.6[a]	87.3±9.4[a]	88.1±9.2[a]
Triglycerides	131.2±14.5	114.3±12.0	97.0±11.0[a]	80.2±9.4[a,b]	81.2±9.5[a,b]
VLD - C	26.2±2.9	22.9±2.4	19.4±2.2[a]	16.0±1.9[a,b]	16.2±1.9[a,b]
LDL - C	58.8±6.8	44.3±4.8[a]	33.0±3.7[a,b]	34.3±3.8[a,b]	34.2±4.3[a,b]
HDL - C	30.9±3.7	35.2±4.2	43.1±5.0[a]	44.1±5.4[a,b]	43.5±5.2[a,b]

Data are expressed as mean ±SD for six rats. a: significantly different from diabetic control group (DS); b: significantly different from DCi2 using one way ANOVA with Tukey – Kramer test at P<0.05.

Table 2. Effect of citral treatment on serum lipids of non diabetic rats

Serum level (mg/dl)	NDS	NDCi2	NDCi8	NDCi16	NDCi32
Cholesterol	88.5±9.9	76.5±8.7	71.6±8.4[a]	72.1±8.0[a]	72.5±7.7[a]
Triglycerides	79.5±8.4	67.3±8.0	64.0±7.1[a]	60.3±6.9[a]	61.3±7.0[a]
VLDL - C	15.9±1.7	13.5±1.6	12.8±1.4[a]	12.1±1.4[a]	12.3±1.4[a]
LDL - C	32.5±4.4	27.6±3.4	24.8±3.4[a]	24.5±3.3[a]	24.6±3.0[a]
HDL - C	47.5±5.2	47.8±5.3	46.1±5.7	47.2±5.3	49.0±5.4

Data are expressed as mean ±SD for six rats. a: significantly different from non diabetic control group (NDS); using one way ANOVA with Tukey – Kramer test at P<0.05.

Discussion

This *in vivo* study was planned in order to test the ability of a moderate *in vitro* alpha-amylase inhibitor to have actual therapeutic potential in a rat diabetic model.

The decrease in hyperglycemia that was observed in diabetic rats upon treatment with citral reached a maximal value of about 26% with a dose of 16 mg/kg (DCi16). This can be considered as a "postprandial" hyperglycemia, and as so, the alpha-amylase inhibitory effect of citral could have a role in this regard. According to the *in vivo* assay experiment, this compound possesses definitely this property. However, the effect of citral appears to be one of a partial inhibitor, and does not present any significant increase after the dose of 8mg/kg. What is also in accordance with the effects of a well-known alpha-amylase inhibitor (a peptidic one from white beans) (12), is the effect of this compound on weight loss.

The effect that may be related to another mechanism of action is the anorexigenic property of the compound, which appears again markedly from the dose of 8 mg/kg. Anorectic effect has been reported for a variety of peptides (e.g. nesfatin-1 (16), pro-opiomelanocortin (17)) but also for chemicals such as metformin which acts via an increase in the central sensitivity to leptin (18).

Water intake and urine volume of diabetic rats is also showing a remarkable decrease with the use of 16mg/kg of citral, which is in accordance with its effect on blood glucose, and interesting in terms of the therapeutically benefits that it could have on these discomforting consequences of diabetes in patients. This effect is not observed in normal rats, which points out this compound as a potential preventive therapeutic which could lessen food intake and lower postprandial hyperglycemia in normal individuals who could be at risk of developing signs of metabolic syndrome.

The most prominent effect of citral treatment on diabetic rats was the normalization of blood lipids upon use of the 16 mg/kg dose. As shown in Table 1, cholesterol level of 126.5 mg/dl (on average) in diabetic rats becomes 87.3 mg/dl (on average) after the treatment which is comparable with the 88.5 mg/dl (on average) observed in normal rats. Similar results are obtained for triglyceride.

Diets containing high levels of fats and carbohydrate have proven effects on inducing glucose intolerance and predisposition to diabetes (19). In recent years, the importance of lipotoxicity is attracting more attention, and the necessity of shifting the current point of view toward a "lipocentric approach" in metabolic syndrome is becoming evident (20). On the other hand, elevation of blood lipid levels is correlated with complications in diabetic conditions (21, 22). Thus, compounds that would normalize blood lipid levels become of more importance. Control of blood glucose concentrations in diabetic patients could result in normal levels of blood lipids (23), but here, the anti-hyperglycemic effect of citral is much less pronounced than its anti-hyperlipidemic effect, suggesting the possibility of an independent effect. Hypolipidemic properties have been reported for a range of plant extracts or natural compounds (6, 24-27), and these effects have been related to an influence of the tested compounds in the various stages of lipids synthesis and metabolism. A report that should be mentioned here is a study involving lemongrass oil _containing citral and geraniol_ that was found to be effective to some extent in hypercholestrolemic subjects (28). Given the fact that isoprenoid compounds (such as farnesyl acetate) could inhibit HMG CoA reductase levels (29), the observed effect of citral in the current study could be suggested to be mediated via interaction with the mevalonate pathway, as suggested to be the case in the anti-cancer action of isoprenoids (30). An interesting point is the fact that citral effect on normal rats' blood lipid levels is less significant, in comparison with diabetic rats, suggesting an effect on a possibly disrupted pathway.

Overall, this study has shown the potential of citral as an effective hypolipidemic agent in diabetes, with moderate hypoglycemic property. The proposed dose for further studies would be the 16 mg/kg (DCi16) one, which showed on average the best results, although the DCi8 dose was also effective, with slightly lower results for some parameters. Citral could also be viewed as a potential lead compound for inducing weight loss and lowering postprandial glucose levels and as such, could be used in obesity and metabolic syndrome complications.

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