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Original Article

CITRONELLOL: A POTENTIAL ANTIOXIDANT AND ALDOSE REDUCTASE INHIBITOR FROM *CYMBOPOGON CITRATUS*

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ABSTRACT

Objective: The present study deals with identification of bioactive component from the distillate of *Cymbopogon citratus* plant and to evaluate its aldose reductase inhibitory and antioxidant activity.

Methods: The aldose reductase enzyme was semi purified by ammonium sulphate precipitation from chicken eye lens. Differential solvent extraction of *Cymbopogon citratus* was carried out and by using HPLC, HPTLC and Gas chromatography active component responsible for aldose reductase inhibition was identified. The Oral Glucose Tolerance Test was carried by induction of diabetes to the male Wistar rats with streptozotocin. The sorbitol content was measured by sorbitol dehydrogenase enzyme. The antioxidant activity was studied using DPPH and FRAP methods.

Results: Differential solvent extraction of the distillate revealed presence of aldose reductase inhibitory activity in the petroleum ether extract of the plant. Citronellol was identified as bioactive component by HPLC, HPTLC and gas chromatography. Citronellol demonstrated 90.4% inhibition of aldose reductase with an IC_{50} value of 19.6 ±0.8 µg/ ml as against 92.3 % inhibition by the standard drug epalrestat with an IC_{50} value 9.8 ±0.43 µg/ ml. It demonstrated better lowering of sorbitol (44%) than epalrestat (28%) in the eye lens studies as compared to the diabetic control. Kinetic studies using Lineweaver Burk Plot revealed citronellol to be a mixed type of inhibitor. Docking studies revealed the binding of citronellol at a site similar to epalrestat.

Conclusion: Citronellol from *C. citratus* is found to possess potent aldose reductase inhibitory activity with mixed type of enzyme inhibition and a very good antioxidant potential.

Keywords: Diabetes mellitus, Aldose reductase inhibition, Citronellol, Antioxidant activity, Molecular docking.

INTRODUCTION

Diabetes Mellitus is a chronic metabolic disorder characterized by hyperglycemia which is associated with long-term damage; dysfunction and failure of various organs of the body [1]. According to IDF Diabetes Atlas; 382 million people suffer from diabetes and has recorded about 5.1 million deaths [2]. Chronic hyperglycemia is a major determinant in the development of secondary complications of diabetes. Increased availability of glucose to insulin independent tissues such as lens, erythrocytes, kidney and nerve leads to an inrush of glucose and upregulation of several intracellular pathways. Polyol pathway is one of the major pathways that is activated [3]. Aldose reductase (NADP oxidoreductase, EC 1.1.1.21) is the first key enzyme of polyol pathway which converts D-glucose into D-sorbitol using NADPH as a cofactor; further sorbitol is converted into fructose by sorbitol dehydrogenase enzyme using NAD^+ as a cofactor, impacting NADPH/$NADP^+$ ratio. NADPH is essential for regeneration of reduced form of intracellular antioxidant, glutathione [4]. Sorbitol accumulation, oxidative stress, and few other factors are responsible for establishment of secondary complications in diabetes. Inhibition of aldose reductase reported to prevent establishment of secondary complications in diabetes [5]. Several plant products have been studied for aldose reductase inhibitory activity which includes flavonoids such as quercetin, desmanthin [6] and tannoids from *Emblica officinalis* [7].

Cymbopogon citratus commonly known as lemon grass is widely used in tropical countries for the treatment of hypertension [8], epilepsy [9] and other disorders of the central nervous system, gastrointestinal disorders. In India, tea prepared from lemon grass is used as a sedative [10]. In Thailand and other countries, it is used in several cuisines and brews. Various studies have shown the presence of bioactive components with medical utility in essential oils produced by this herbal plant [11]. The essential oil of the plant contains constituents such as Citral 13 %, Limonene 2.25%, Citronellol 11.52 %, and Geraniol 19.03 % [12].

The essential oil obtained from the leaves of this plant is reported to possess effective antioxidant potential [13]. Oxidative stress and free radicals are associated with induction of diabetic state or progression of diabetic complications [14]. Therefore, use of anti-diabetic agents having antioxidant activity is a prominent strategy for the amelioration of diabetes. There are two major classes of aldose reductase inhibitors characterized by a structural or functional group such as spirohydantoins and carboxylic acids [15]. Inhibition and computational studies suggest that these compounds bind at a site independent of the substrate and coenzyme binding site [16]. The docking of 3-D-databases of organic molecules into the crystal structures of enzymes provides an efficient way of rationally selecting small subsets for biological testing [17].

In the present study, attempts are made to isolate and identify the bioactive component responsible for aldose reductase enzyme inhibition and evaluate its antioxidant activity. Kinetic studies and *in silico* studies are carried out to evaluate the nature of inhibition and possible binding sites on the enzyme.

MATERIALS AND METHODS

Chemicals and reagents

Petroleum ether, Chloroform, DichloroMethane, Xylose, NADPH, $LiSO_4$, DPPH all these chemicals were locally purchased. Standard Citronellol (95 % pure) was purchased from Sigma-Aldrich (St Louis, MO, USA).

Plant material

C. citratus or lemon grass was collected from Kolhapur District, Maharashtra State, India and was identified by the Botany

Department of Shivaji University, Kolhapur, India. A voucher specimen of this plant material was deposited under the number ADJ1 in the Herbarium of the Department of Botany, Shivaji University, Kolhapur.

Extract preparation

The plant material was air dried; finely powdered and 10 gm powders was subjected to steam distillation at 60°C. The distillate was further processed by successive solvent extraction with petroleum ether (Pet ether), chloroform and dichloromethane. The respective solvents were evaporated and the residue was weighed. The dry extracts were stored at 4°C.

HPLC Profiling of *C. citratus* pet ether fraction

The HPLC analysis was carried out using (JASCO autosampler) with an automatic sample injector (autosampler), 10 µl of standard citronellol and pet ether extract of plant was applied on C18 column (4.6 mm × 250 mm) by high pressure gradient (HPG2) with run time 10 min. A mobile phase consisting of acetonitrile - methanol (90:10 v/v) and flow rate of 1.0 ml/min was used and the detection was carried out at detection wavelength 210 nm with an UV-VIS detector.

HPTLC analysis of pet ether extract

To identify the components present in the pet ether extract HPTLC was performed using CAMAG HPTLC system (Switzerland). Standard citronellol and pet ether extract were applied on the pre-coated silica gel plates (HPTLC silica gel 60 F 254, Merck, Germany) by micro syringe using spray gas nitrogen sample applicator (Linomat V, CAMAG, Switzerland). The parameters for plate were set as 8 mm bands, 10 mm apart from Y-axis, first application position 13 mm from left edge. The solvent system used was benzene: ethyl acetate (9:1). The chamber was pre-equilibrated with developing solvent and plate was developed by placing in the chamber containing pre-conditioning solvent until the desired running distance is reached. After development, densitometric evaluation was carried out at 254 nm wavelengths using deuterium and tungsten lamp respectively with slit dimension of 4 × 0.30 mm using CAMAG TLC Scanner-3. The chromatograms were integrated using HPTLC win CATS evaluation software (Version 1.4.4.6337).

Gas Chromatography analysis

GC analysis was carried out using GC system (Agilent Technologies 7820 A) from Institute of Chemical Technology, Mumbai, India, with HP-5 (5% Phenyl Methyl Siloxane), with 30 m x 320 µm x 0.25 µm column, FID detector with detector temperature 300°C and the programme was run at inlet temperature 250°C, spilt ratio: 80:1, oven temp. Initial temperature 80°C for 1 min, Ramp 1-10°C/min till 300°C with nitrogen as a carrier gas having flow rate 1 ml/min.

***In vitro* aldose reductase enzyme activity**

Aldose reductase enzyme was isolated from chicken eye lens and the enzyme assay was performed as previously described by Hayman and Kinoshita [18] with some modifications. Fresh chicken eyeballs were obtained from the slaughter house and immediately transported to the laboratory at 0–4°C. The lenses were removed by extra capsular extraction. One gram lenses was washed with saline and homogenized in 3 volumes of saline containing 10 mM β-mercaptoethanol. The homogenate was subjected to centrifugation for 15 min at 10,000 rpm at 4°C. Partial purification of enzyme was carried out by ammonium sulphate precipitation in 3 stages at 40, 50, 75 % saturation. Then the precipitated enzyme was dissolved in 0.05 M sodium chloride and dialyzed overnight. Dialyzed enzyme was used for determination of aldose reductase (AR) activity and the enzyme activity was expressed in µM of NADPH oxidized/min/gm tissue.

Aldose reductase inhibitory activity

Assay mixture (total 2 ml) containing 0.2 ml of freshly prepared enzyme, 0.067 M phosphate buffer (pH 6.2), 0.125 mM NADPH, 400 mM $LiSO_4$, 40 mM xylose (substrate) and 75 µg/ml of pet ether extract was taken in a cuvette. The decay of absorbance (due to the NADPH oxidation) was measured at 340 nm for 5 min. at 37°C using a UV-Visible spectrophotometer [19].

Kinetic studies of AR enzyme inhibition by citronellol

For the study of enzyme kinetics, the values of K_m and V_{max} were determined and the type of enzyme inhibition by citronellol was studied from Lineweaver-Burk plot using 12.5 and 25 µg/ml concentrations of citronellol with increasing concentrations of substrate (xylose) 40-200 mM [20].

Determination of sorbitol content

The goat eyeballs were obtained immediately after sacrification from a slaughterhouse and were transported to the laboratory at 0–4°C. The lenses were removed by extracapsular extraction and incubated in modified M-199 medium by the method of Kumar et al [21]. The lenses were divided into following groups (n= 6 in each group) - Group I - 5.5 mM glucose (normal control), group II- 50 mM glucose (diabetic control), group III- glucose 50 mM + Epalrestat 50 µg/ml, group IV- glucose 50 mM+ Pet ether extract 75 µg/ml, group V- glucose 50 mM + Citronellol 50 µg/ml. These lenses were incubated for 72 hrs at 37°C. The sorbitol content was determined by the method used by Hye- Lim Noh et al [22] with some modifications. After 72 hr incubation the lenses were homogenized and de proteinised by perchloric acid followed by neutralization then 2 ml assay mixture contained 0.1 ml of homogenate, 0.2 M glycine -NaOH buffer pH 9.4, 200 µM NAD^+. Reaction was started by addition of 0.7 U/ ml sorbitol dehydrogenase enzyme and the absorbance was measured at 340 nm after 10 min.

Induction of diabetes in experimental animals

The animal experiments were carried out according to the guidelines of animal ethical committee of the Institute and CPCSEA (Registration no. 233/CPCSEA). Normal male Wistar rats weighing 190-200 gm were fasted overnight and diabetes was induced by intraperitoneal administration of freshly prepared streptozotocin (65 mg/kg body weight) in 0·1 M citrate buffer (pH 4·5) [23]. After 14 days, the animals showing stable blood glucose values above 200 mg/dl were considered as diabetic and used for further experiment.

Oral glucose tolerance test

The oral glucose tolerance test was carried out as described by Andrade-Cetto et al [24] with some modifications. After overnight fasting of rats the initial blood glucose was measured using ACCU-CHECK glucometer. Animals were divided into five groups as normal control, diabetic control epalrestat 2.5 mg/kg Body Weight (BW), standard citronellol (2 mg/kg BW) and pet ether extract (10mg/kg BW). The components were given orally and after a gap of 10 min, glucose solution (3 mg/gm BW) was administered orally. Blood samples at intervals of 30 min up to 120 min were taken and blood glucose was measured using ACCU-CHECK glucometer.

***In-vitro* antioxidant activity of citronellol**

DPPH radical scavenging activity

The antioxidant potential of the extract was measured by using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay [25]. The percent inhibitory activity of plant extract was determined by using different concentrations as 40, 80, 120, 160 and 200 µg/ml of the extract. The different concentrations of extract were allowed to react with 3 ml of 0.25 mM of DPPH prepared in methanol; the mixture was left to stand at room temperature for 30 min in the dark. The absorbance was measured spectrophotometrically at 517 nm. A standard curve was then prepared by plotting the percentage of free radical scavenging activity of ascorbic acid versus its concentration. The final result was expressed as percent radical scavenging activity (% RSA).

FRAP assay

The ferric-reducing antioxidant power (FRAP) was performed as described by Panaskar et al [26]. The working FRAP reagent prepared by mixing 300 mM acetate buffer (pH 3.6) 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3 \cdot 6H_2O$ in 10:1:1 ratio prior to use and heated to 37°C in the water bath for 10 min. Pet ether extracts of various concentrations as 10 - 50 µg/ml were allowed to react with 2.7 ml of the FRAP reagent. The final volume of the reaction mixture was made up to 3 ml with distilled water. The reaction mixture was

kept in dark for 30 min. The readings of the colored product (ferrous tripyridyltriazine complex) were then taken at 593 nm and results were expressed as mM Trolox equivalent gm^{-1} dry weight of sample.

Patch dock analysis of aldose reductase with citronellol

Patch dock is a geometry-based molecular docking algorithm. Generally, docking programs predict the protein-ligand complex structures with reasonable accuracy and speed [27]. Docking studies were performed by using aldose reductase enzyme and citronellol as ligand using online patchdock server (http: //bioinfo3d. cs. tau. ac. il/PatchDock/). The pdb structures of aldose reductase from human lens (pdb ID 1PMW) and citronellol were obtained from RCSB (http: //www. rcsb. org/pdb/explore/explore. do?structureId=2QMJ).

Statistical analysis

Results were expressed as mean value ± standard error mean (SEM) of six replicate experiments (n=6). Statistical analysis was performed using one-way analysis of variance (ANOVA). P-values at α less than 0.05 were considered to be statistical significant.

RESULTS AND DISCUSSION

As the plant distillate demonstrated good aldose reductase inhibitory action it was subjected to differential solvent extraction. The yield of pet ether, chloroform, dichloromethane fractions obtained with respect to the dry powder was 0.101 gm (1.01%), 0.03 gm (0.3%), 0.06 gm (0.6%). Analysis of the different solvent extracts revealed that the pet ether extract at 75 µg/ml concentrations possesses 82% aldose reductase inhibitory activity.

Active component in pet ether extract of *C. citratus* was identified as citronellol by HPLC method. It can be seen from the HPLC chromatogram in (Fig. 1) that the *C. citratus* pet ether fraction demonstrated a major peak at retention time 4.99 min comparable with standard citronellol at a retention time of 5.02 min. along with three minor peaks at retention time 1.95, 3.80, 4.14 min.

This finding is confirmed through HPTLC analysis (fig. 2) and GC analysis (fig. 3) demonstrating citronellol as the major component on comparing with the standard compound.

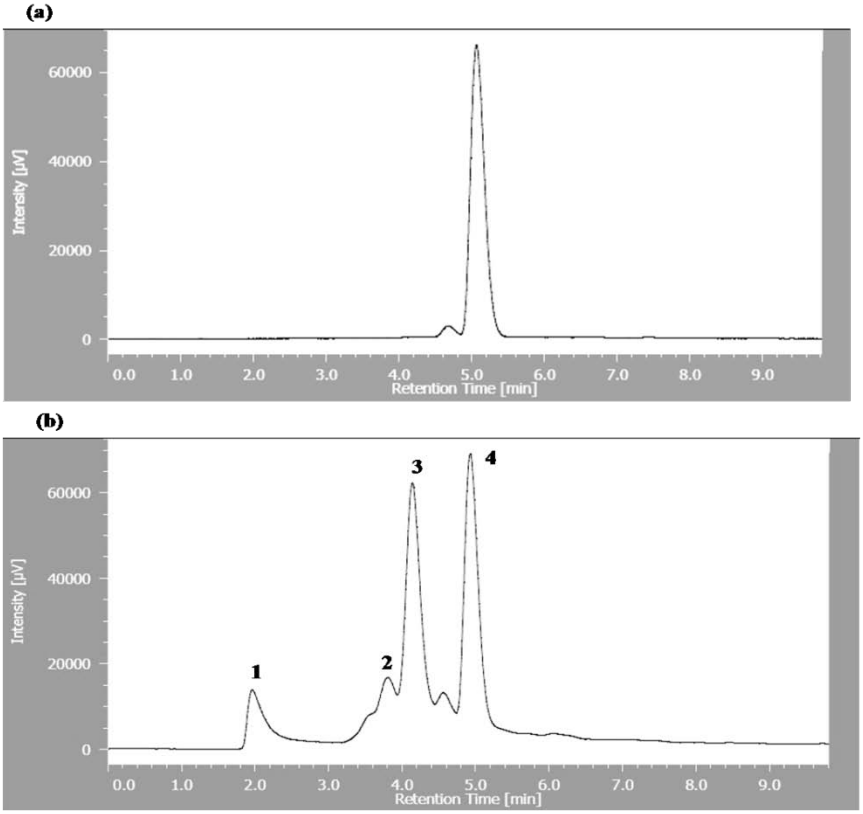

Fig. 1: HPLC profile of (a) standard citronellol and (b) pet ether extract

Citronellol along with other known components present in the extract viz. geraniol, citral and limonene (commercially purchased) were evaluated for aldose reductase inhibition. Citronellol demonstrated 90.4% inhibition, while geraniol showed 48% inhibition and the other two components did not demonstrate any such activity. It can be observed from (Table 1) that as compared to the pet ether extract standard citronellol demonstrated higher inhibitory activity almost on par with the standard commercially used drug epalrestat. The advantage of citronellol as a potent inhibitor is that it is a regularly used food components with no reported toxicity at the quantity used for inhibition. The oral LD_{50} in rats (3.45 g/kg) and a 12-week subchronic study conducted in rats produced no toxicity even on a daily consumption of 50 mg/kg body weight [28].

Table 1: Aldose reductase inhibitory activity

Components	Concentrations (µg/ ml)	% Inhibition	IC_{50} value (µg/ ml)
Epalrestat	20	92.3 ± 1.3	9.8 ±0.43
Pet ether extract	75	82 ± 0.8	27 ±0.67
Std. Citronellol	50	90.4 ± 0.9	19.6 ±0.8

Each observation expressed as mean value ± standard error mean (SEM) of six replicate experiments (n=6) and $P < 0.05$

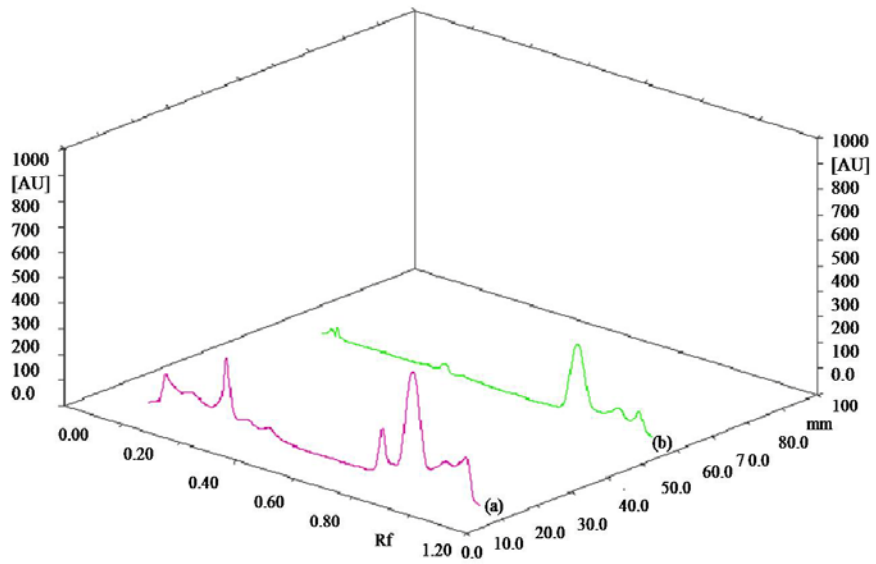

Fig. 2: HPTLC 3D profiling of (a) Pet ether extract (b) standard citronellol

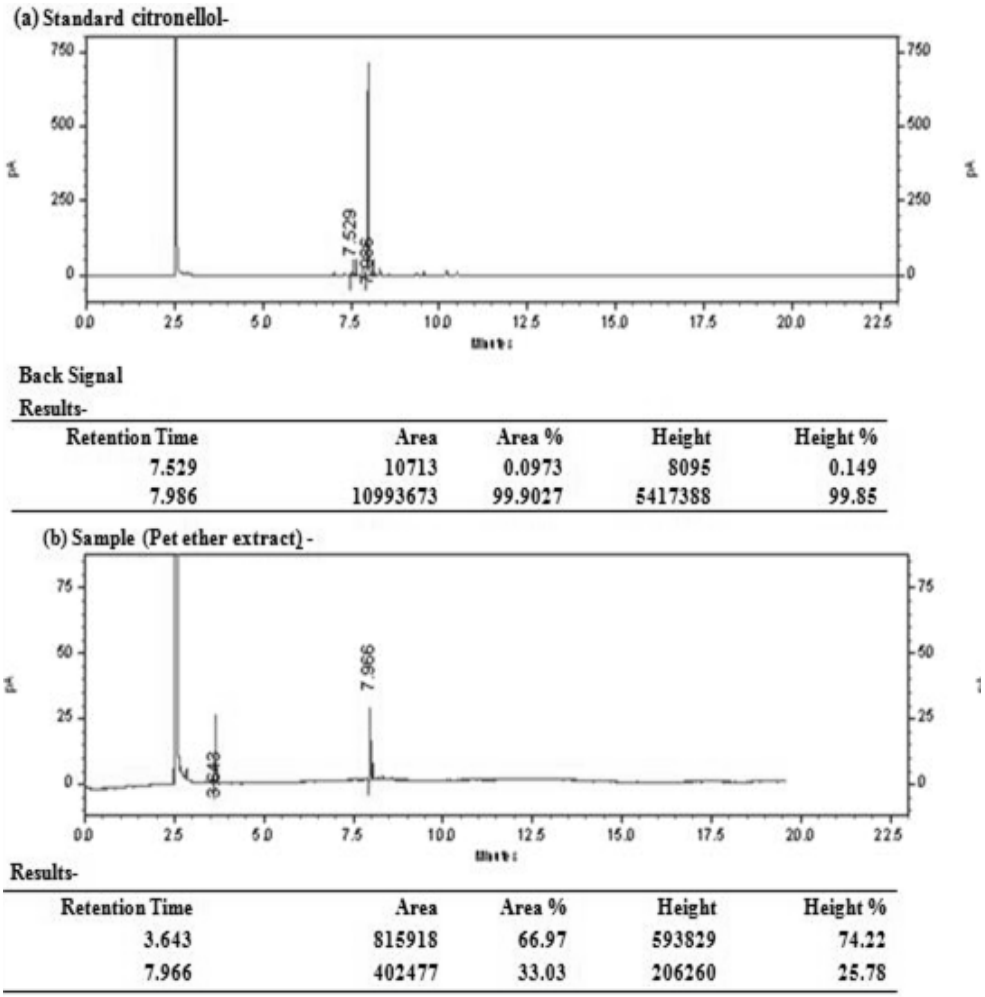

Fig. 3: Gas chromatogram of (a) standard citronellol and (b) pet ether extract

Activation of aldose reductase leads to accumulation of sorbitol in the eye lens enhancing osmotic pressure and is suggested to be a major cause of cataract formation [29]. The effect of citronellol on sorbitol content in goat eye lens tissue revealed that as against the control diabetic value citronellol could reduce sorbitol formation by 44% which is even better that epalrestat that could reduce sorbitol formation by 28.2% (Fig. 4). This has implications in prevention of cataract formation and establishment of retinopathy in diabetic animals.

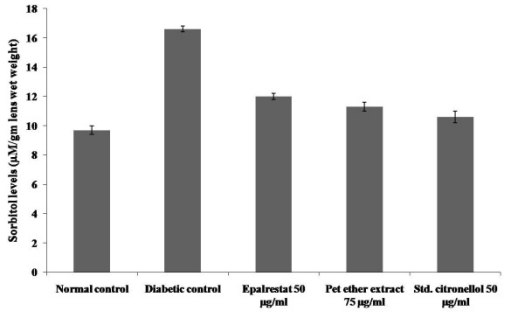

Fig. 4: Sorbitol content of lenses after 72 hr. incubation Each observation expressed as mean value ± standard error mean (SEM) of six replicate experiments. Values were significantly different from that of diabetic control group (*p* <0.05)

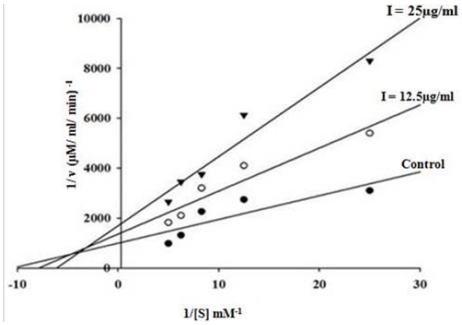

Fig. 5: Lineweaver–Burk plot of aldose reductase inhibition with variable substrate concentrations (40–200 mM) at 12.5 µg/ml and 25 µg/ml concentrations of citronellol.

The nature of inhibition was evaluated using the Lineweaver Burk plot. It was found that citronellol is a mixed type of inhibitor as observed in (fig.5). The values of Vmax decreased and that of Km was found to increase, suggesting that the binding of inhibitor lowers the affinity of the enzyme for the substrate. It is likely that the inhibitor binds to a site other than the active site. The other advantage of a mixed type of inhibitor as against a competitive inhibitor is that it is needed in small concentration to demonstrate a similar inhibition.

It was attempted to study the blood glucose lowering effect of these components in which neither pet ether extract of plant *C. citratus* nor citronellol do not significantly reduces blood glucose level in diabetic rats (Fig. 6) significantly. But there are also reports showing the decrease in blood glucose levels using the whole extract of this plant [30]. It may be likely that the glucose lowering activity may reside in other solvent extracts. Epalrestat, which is currently available in market as an aldose reductase inhibitor, was also investigated for glucose lowering activity in rat. It also could not lower the blood sugar of animals significantly.

Table 2: Antioxidant activity of different components using DPPH radical scavenging study

Components	40 µg/ml	80 µg/ml	120 µg/ml	160 µg/ml	200 µg/ml
Ascorbic acid	35±1.4	61±1.8	70.4±0.7	87±0.8	96±0.8
P. E. extract	25±1.2	49.5±1.2	58.5±1.1	66.4±0.8	79±0.9
Std. citronellol	28±1.3	52±2.2	63.2±1.3	76.1±2.2	86±2.1
Epalrestat	21±1.0	43.3±0.7	54±1.8	62±1.4	74.4±0.8

Each observation expressed as mean value ± standard error mean (SEM) of six replicate experiments (n=6) and $P < 0.05$

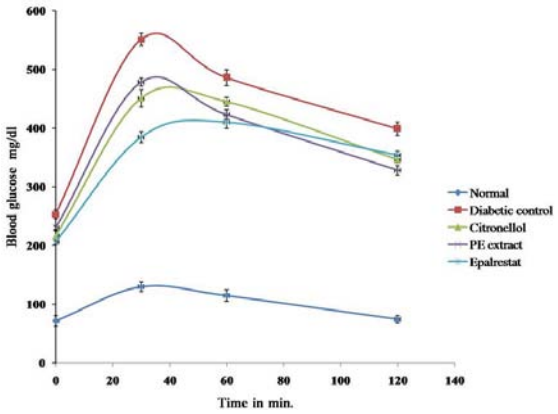

Fig. 6 Oral Glucose Tolerance Test of different components in streptozotocin-induced diabetic rats, Each observation expressed as mean value ± standard error mean (SEM) of six replicate experiments (n=6) and *P* < 0.05

The radical scavenging effect of pet ether extract of plant, standard citronellol and eparlestat at different concentrations is represented in (table 2) and (table 3) showing that the antioxidant activity is concentration dependent.

Pet ether extract of plant and standard citronellol showed higher antioxidant potential than that of epalrestat. Citronellol had demonstrated good radical scavenging activity (86 %) as compared to ascorbic acid (96 %).

However it has demonstrated about 50% of antioxidant activity as compared trolox in the FRAP assay. Nevertheless this could prove to be a valuable contribution to lowering oxidative stress along with aldose reductase inhibition making it a potent therapy option to combat establishment of secondary complications in diabetes.

Further, i*n silico* docking studies were carried out to identify the binding residues of the aldose reductase enzyme to citronellol in comparison with standard aldose inhibitor epalrestat. The 3D structure of the enzyme revealed as a $(\beta/\alpha)_8$ barrel fold [31]. The potential binding residues of aldose reductase with epalrestat and citronellol are as shown in (Fig.7).

Table 3: Antioxidant activity of different components using FRAP assay

Concentration (µg/ml)	Standard trolox	Pet ether extract	Citronellol	Epalrestat
10	0.31± 0.04	0.12 ± 0.03	0.16 ± 0.02	0.10 ± 0.03
20	0.81 ± 0.07	0.31 ± 0.04	0.30 ±0.02	0.21 ±0.05
30	1.21 ± 0.09	0.64 ± 0.04	0.61± 0.04	0.53 ±0.06
40	1.61 ± 0.04	0.83 ± 0.03	0.89 ± 0.07	0.79 ±0.04
50	1.72 ± 0.10	1.31 ± 0.05	1.49 ± 0.05	0.98±0.04

Each observation expressed as mean value ± standard error mean (SEM) of six replicate experiments (n=6) and $P < 0.05$

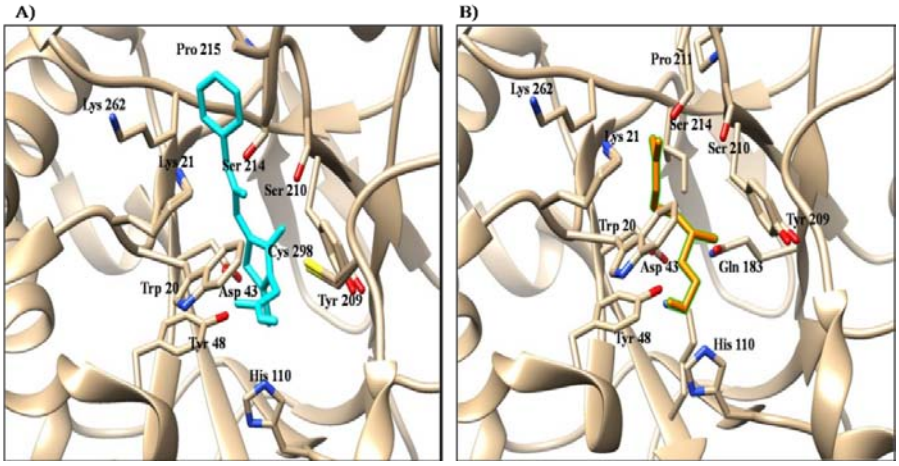

Fig. 7: Binding site of (A) Epalrestat (B) Citronellol with aldose reductase enzyme and its binding residues

It can be observed that with the exception of one amino acid epalrestat and citronellol show a very similar binding pattern showing amino acid Glutamine 183 for citronellol binding and Cystine 298 for epalrestat binding. This binding site is apart from the substrate binding site. It is likely that interaction at this site may impact binding of substrate to the catalytic site lowering the enzyme activity. This is also reinforced by our finding of a mixed type of inhibition in the kinetic studies.

CONCLUSION

Citronellol a component of the common lemon grass plant *C. citratus* is found to possess potent aldose reductase inhibitory and antioxidant activity. It was found to lower the sorbitol content and is a mixed type of inhibitor. Docking studies have revealed that citronellol binds to a site similar to epalrestat. The major advantage of citronellol is that it is a commonly used component with no reported toxicity and hence may prove to be a potent component that can prevent the progression of secondary complications of diabetes such as neuropathy, nephropathy and retinopathy.

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ABBREVIATION

AR: aldose reductase, BW: Body weight, DPPH: 2, 2-diphenyl-1-picrylhydrazyl radical, FRAP: ferric-reducing antioxidant power, HPTLC: High Performance Thin Layer Chromatography, $LiSO_4$: Lithium sulphate, mM: millimolar, NADPH: Nicotinamide Adenine Dinucleotide Phosphate Hydrogen, TPTZ: 2, 4, 6-tripyridyl-s-triazine.

CONFLICT OF INTERESTS

Authors declare that there is no conflict of interest.

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