



Evaluation of Pharmacological Potential of Stem and Root of *Callistemon citrinus* in STZ Induced Diabetes in Rats

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Abstract

The hydroethanolic extracts of stem and root of *Callistemon citrinus* were evaluated for their antidiabetic, analgesic and antihypertensive potentials in Streptozotocin (STZ) induced diabetes in rats for 21 days. The effect of these extracts was also observed on biochemical and oxidative stress parameters (i.e. SOD level, GSH level, nitrite level, protein level) in STZ induced diabetes in rats after 21 days of treatment. In our *in vitro* studies results showed that hydroethanolic stem and root extract of *Callistemon citrinus* inhibit the alpha amylase enzyme in uncompetitive manner whereas inhibit alpha glucosidase enzyme in mixed manner. The results of *in vivo* studies showed that the hydroethanolic stem and root extract of *callistemon citrinus* at a dose of 400 mg/kg have antidiabetic and analgesic potential in STZ induced diabetes in rats. Also, there was increase in levels of protein, nitrite and glutathione; whereas there was decrease in SOD level as compared to that of STZ treated rats after 21 days of extract treatment.

Keywords: *Callistemon citrinus*; Antidiabetic; Nitrite level; Streptozotocin

Introduction

Diabetes is a chronic metabolic disorder of pancreas, that occurs either when the pancreas does not produce enough insulin or when the body does not utilize insulin efficiently produced by it. One of the major hormones that regulate blood sugar level in human body is insulin. Insulin is also helpful for treatment of diabetes [1]. There are number of drugs that possess antidiabetic effect by decreasing blood glucose level through different mechanisms include increasing of peripheral absorption of glucose by biguanides and thiazolidinediones, reduction of hepatic gluconeogenesis by biguanides, stimulation of insulin secretion by sulfonylurea and meglitinides drugs and delay in the absorption of carbohydrates from the intestine by alpha-glucosidase [2]. The alpha amylase act as a catalyst in the reaction involving the hydrolysis of the alpha-1,4-glycosidic linkages of the glycogen, amylose, amylopectin, starch, and numerous maltodextrins those are responsible for digestion of starch [3]. Glucose was produced by maltase or Alpha Glucosidase (AGS) from complex carbohydrates like disaccharides, starch by breaking 1,4-alpha bonds [4]. An herb that has shown potential in recent experimental investigation for the management of several human ailments is *Callistemon citrinus* (*C. citrinus*). *C. citrinus* belongs to family Myrtaceae and is commonly called Bottlebrush. Morphologically, they were bushy or xyloid like tree (nearly 0.5 m to 7 m length) mostly present in South America, Australia and tropical areas of Asia, but also found all over the world in small numbers [5].

When different fractions of *C. citrinus* were analyzed phytochemically it shows presence of different chemical constituents like three benzoic acid derivatives (i.e. Protocatechuic acid, methyl gallate and gallic acid), two alcohols (i.e. 1-tetratriacontanol, blumenol A), two flavones (i.e. 8-demethyleucalyptin and Eucalyptin), one sterol (i.e. sitosterol), eight phenolic compounds along with one sesquiterpene i.e. 2,6,10-bisabolatriene. The major constituents present in essential oil obtained from stem of *C. citrinus* includes following - 1,8-cineole, α -terpineol, α -pinene and limonene [6].

C. citrinus showed a number of pharmacological activities like antimicrobial, antitubercular, cardioprotective, free radical scavenging, anti-Alzheimer, antidepressant, calcium channel blocker, anti-inflammatory, antifungal, anti-caries, antioxidant, spasmolytic, hypoglycemic, hepatoprotective, cytotoxic, anti-*Helicobacter pylori*, and anti-bacterial activity [7]. Various studies have suggested antidiabetic potential of leaves and fruits of *C. citrinus*. But none of the study has reported antidiabetic effect of hydroethanolic stem and root extract of *C. citrinus* as well as its effect on α -glucosidase and α -amylase. Therefore, the present study is focused to explore the effect of

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Received Date: 22 Jul 2020

Accepted Date: 05 Aug 2020

Published Date: 08 Aug 2020

Citation:

Kumar N, Singh P, Singh G, Verma PK. Evaluation of Pharmacological Potential of Stem and Root of *Callistemon citrinus* in STZ Induced Diabetes in Rats. *Am J Pharmacol.* 2020; 3(1): 1027.

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hydroethanolic stem and root extract of *C. citrinus* on the inhibitory potentials of α -glucosidase and α -amylase. The present study is also focused to evaluate the antidiabetic potential, analgesic activity as well as effect on blood pressure of the hydroethanolic extract of stem and root of *C. citrinus* in STZ treated diabetic rats.

Material and Methods

Convoy and marking of plant materials

The stem and root of *Callistemon citrinus* were procured from Maharshi Dayanand University, Rohtak. The plant material was identified and authenticated at Department of Botany, M. D. U., Rohtak (Haryana).

Preparations of *C. Citrinus* root and stem extract

Roots and stems of *C. citrinus* were shade dried and then passed it to grinder for reduction of particle size for a coarser form of powder. The plant homogenate would be sufficient to fill the plant materials in thimble. The ethanol and water were added to a 500 ml flask. The drug would be extracted with solvent till complete extraction is affected (about 40 cycles). After completing the extraction liquid content was vaporized and dried in hot air oven till greenish (stem) and brownish (root) colored semisolid mass will be obtained [8].

Preliminary phytochemical screening

Preliminary phytochemical screening of hydroethanolic extract of root stem will be done for the detection of constituents present in plant like sterols, alkaloids, phenolic compounds and tannins, terpenoids, saponins, flavonoids, monosaccharides and reducing sugars [9].

Inhibitory assay for alpha amylase

This assay was done by using the method given by McCue and Shetty in 2004. In this 250 μ L of extricate (0.31 mg/mL to 10 mg/mL) was poured in a test tube and in this test tube also add 250 μ L of alpha amylase solution which was prepared in sodium phosphate buffer (0.02 M, pH 6.9). This test tube was preincubated for 10 min at 25°C. After that add 250 μ L starch solution (1%) which was prepared in sodium phosphate buffer (0.02 M, pH 6.9). After addition of starch solution incubate the test tube again for 10 min at 25°C. After incubation reaction was started in above test tube which can be stopped by mixing 500 μ L of DNS reagent in it. After that these test tubes were placed on warm water which is boiling for 5 min. After that cool them to room temperature and also add 5 ml of distilled water in it. Then at last absorbance of these test tubes were noted at 540 nm with the help of spectrophotometer. For absorbance of control we follow the same procedure except extricate was replaced by distilled water. The inhibitory potential of alpha-amylase was calculated by % inhibition of alpha amylase which was calculated by using given formula:

$$\% \text{ Inhibition} = \left[\frac{(\text{Abs. of control} - \text{Abs. of extracts})}{\text{Abs. of control}} \right] \times 100$$

The concentration at which 50% of alpha amylase was inhibited is called IC50 of that extricate. It is calculated by using the equation obtained from % inhibition graph.

Manner of alpha amylase inhibition

The alpha amylase inhibition manner was calculated by using minimum IC50 value of plant extricate as in procedure given by Ali in 2004. Firstly add 250 μ L of the extricate (5 mg/mL) with 250 μ L alpha amylase solution in one bunch of test tubes. These tubes were preincubated at 25°C for 10 min. Whereas in other bunch of

test tubes 250 μ L of the extricate (5 mg/mL) with 250 μ L alpha-amylase solution was added and preincubated. After preincubation added different increasing concentrations (i.e. 0.5 mg/mL to 5.0 mg/mL) of 250 μ L starch solution in all test tubes for the beginning of reaction. Then these test tubes were incubated at 25°C for 10 min. After that added the 500 μ L of DNS in above test tubes and boiled them for 5 min for the stoppage of reaction. The quantity of different carbohydrates was calculated by using spectrophotometer and also by utilizing standard curve of maltose from which different velocity of reactions was calculated. A dual reciprocal plot (i.e. between $1/v$ vs. $1/(S)$) here v was the velocity of reaction and (S) was the concentration of substrate shown in the graph. The type of inhibition for the given plant extricate on the activity of alpha amylase was calculated by using Lineweaver-Burk and Michaelis-Menten plots which were also called as dual corresponding plot [10].

Inhibitory assay for alpha glucosidase

The consequences of the given extricate on the function of alpha glucosidase was observed using the procedure given by Kim in 2005. For these 100 μ L alpha glucosidase solutions with 50 μ L plant extricates of different concentrations (i.e. 0.31 mg/ml to 10 mg/ml) were filled in test tubes and preincubated these test tubes 10 min. After preincubation 50 μ L pNPG (in 20 mM phosphate buffer) was poured in above test tubes for beginning of process. Then these test tubes were incubated for 20 min at 37°C after the addition of 2 ml sodium carbonate (0.1 M). Addition of sodium carbonate in above test tubes will cause stoppage of reaction and the function of enzyme was calculated by observing these test tubes at 405 nm by using spectrophotometer. For absorbance of control we follow the same procedure except extricate was replaced by distilled water. The inhibitory potential of alpha glucosidase was calculated by % inhibition of alpha glucosidase which was calculated by using given formula:

$$\% \text{ Inhibition} = \left[\frac{(\text{Abs. of control} - \text{Abs. of extracts})}{\text{Abs. of control}} \right] \times 100$$

The concentration at which 50% of alpha glucosidase was inhibited is called IC50 of that extricate. It is calculated by using the equation obtained from %inhibition graph.

Manner of alpha glucosidase inhibition

The alpha amylase inhibition manner was calculated by using minimum IC50 value of plant extricate as in procedure given by Ali in 2004. Firstly add 50 μ L of the extricate (5 mg/mL) with 100 μ L alpha glucosidase solution in one bunch of test tubes. These tubes were preincubated at 25°C for 10 min. Whereas in other bunch of test tubes 50 μ L of the extricate (5 mg/mL) with 100 μ L alpha glucosidase solution was added and preincubated. After preincubation added different ascending concentrations (i.e. 0.5 mg/mL to 5.0 mg/mL) of 50 μ L PNP solution in all test tubes for the beginning of reaction. Then these test tubes were incubated at 25°C for 10 min. After that add the 500 μ L sodium carbonate solution in above test tubes for the stoppage of reaction. The quantity of different carbohydrates was calculated by using spectrophotometer and also by utilizing standard curve of PNP from which different velocity of reactions was calculated. A dual reciprocal plot (i.e. between $1/v$ vs. $1/(S)$) here v was the velocity of reaction and (S) was the concentration of substrate shown in the graph. The type of inhibition for the given plant extricate on the activity of alpha amylase was calculated by using Lineweaver-Burk and Michaelis-Menten plots which were also called as dual corresponding plot [11].

In vivo studies

Animals: Animals used in our work were Wistar albino rats. Average weight of the animals in our work varies between 130 g to 200 g. Experimental protocol was approved by Institutional Animal Ethical Committee, M.D. University, Rohtak (Reg. No. 1767/RE/S/14/CPCSEA) on 31/08/2017. All our work was carried out under the supervision of ethical committee for animals of M.D.U., Rohtak, Haryana - 124001.

STZ induced diabetes in rats: All animals i.e. rat used in our work were given a solution of STZ at a dose of 60 mg/kg intraperitoneally which was prepared in citrate buffer (0.1 M). This STZ solution produces diabetes in these animals. The plasma glucose level in these animals were noted initially when STZ was not given as well as 72 h after STZ administration. This measurement of plasma glucose level would tell us whether these animals were diabetic or not. The rats in which plasma glucose levels were greater than 200 mg/dl would be taken into our experimental work [12].

Experimental groups

Experimental groups for assessment of the antidiabetic potential of *C. citrinus* includes (n=6)

1. Vehicle treated rats: Receives normal saline (0.9% w/v NaCl) only for 21 days.
2. STZ treated rats: Only one injection of STZ solution in citrate buffer at a dose of 60 mg/kg intraperitoneally was caused diabetes in rats.
3. Glibenclamide treated rats: STZ treated diabetic rats were given glibenclamide at a dose of 10 mg/kg orally with the help of cannula daily for 21 days.
4. Hydroethanolic stem extract of *C. citrinus* given rats: STZ treated diabetic rats were given hydroethanolic stem extract of *C. citrinus* (400 mg/kg, p.o.) with the help of oral cannula daily for 21 days.
5. Hydroethanolic root extract of *C. citrinus* given rats: STZ treated diabetic rats were given hydroethanolic root extract of *C. citrinus* (400 mg/kg, p.o.) using an intragastric tube for 21 days.

Measurement of blood glucose level: Blood glucose level was monitored after 7, 14 and 21 day of treatment. Different samples of plasma were taken from veins present in rat's tail and these samples helps in determination of plasma glucose level with the help of digital glucometer (i.e. ACCU-CHEK) at weekly interval.

Measurement of body weight: Body weight was measured by digital weighing balance at weekly interval.

Behavioral parameters

Hot plate method: In this method we used hot plates which contain a platform surrounded by glass wall and this platform was warmed by using electricity. The temperature of heating of this plate was maintained between 55°C to 56°C based on the sensitivity of specific animals. The heating platform in this method was made up of either copper or glass. This method was performed by putting each rat on the heating platform and noted the time at which the rat started licking his paws or started jumping on the platform for escape. The paw licking time or jump response time was noted down at weekly interval (i.e. at 7, 14 and 21 day) of the medication.

Tail immersion test: In this test rats were stored in cages which

were store only one rat with their tail moving freely outside of cage. Rats were stored in these cages 30 min. earlier then the start of testing. In this test firstly we marked the spot in lower portion of tail at 5 cm of each rat and then placed this portion of tail in hot water container in which water was heated up to 55°C. After few seconds rat removed the tail from this hot water that time was noted for each rat. The cut off time for this test is 10seconds. After measuring the time for tail flicking each rat tail was fully dried. The tail flick time was noted on 7, 14 and 21 day of treatment [13].

Biochemical and oxidative stress parameters: Plasma was withdrawn from the veins of all group animals' tail after 21 days. Plasma will be separated by centrifugation (2500 rpm at 4°C) for 10 min. It was stored in a refrigerator and processed for estimation of various parameters within 24 h.

Measurement of total protein content: Alkaline copper solution (5 ml) was added to 1 ml of supernatant and kept it for 10 min. After that 0.5 ml of Folin's reagent in diluted form (1:2) was mixed with above solution by vigorous shaking of test tubes containing these solutions. The absorbance was noted at 750 nm spectrophotometrically after 30 min. By using appropriate blank solution. The protein level was measured in mg/ml [14].

Measurement of SOD level: First of all, took 0.1 ml of supernatant by adding 0.8 ml glycine buffer (50 mM) in it by keeping the pH at 10.4 in a test tube and mixed them well by vigorous shaking. After that added 0.02 ml epinephrine in above mixture for the beginning of reaction. The absorbance was noted by using spectrophotometer at 480 nm. The level of SOD was calculated by the formula given below and it was measured in unit/mg of protein [15].

Calculation: The SOD level was measured in units/mg protein. Here one unit of enzyme was equal to % inhibition i.e. how much superoxide dismutase was inhibited by particular amount of given sample. For measurement of SOD level, we first have to calculate % inhibition followed by SOD level. The formulas for these were given as follows:

$$\% \text{ inhibition} = \frac{(\text{Absorbance of control reaction} - \text{Absorbance of test reaction})}{(\text{Absorbance of control reaction})} \times 100$$

$$\text{SOD level} = \frac{\% \text{ inhibition}}{(50 \times \text{vol. of sample used} \times \text{incubation time} \times \text{protein content})} \times 100$$

Measurement of nitric oxide content: first of took the same volumes of Griess reagent and supernatant in a test tube and by shaking mixed them properly. After that the test tubes were placed in incubation for 10 min. at room temperature. At last the absorbance was determined at 540 nm by using spectrophotometer. The standard curve of sodium nitrite was used to determine the amount of nitrite present in the plasma. The nitric oxide level was calculated in micromole/milligram [16].

Glutathione: First of all, added 1ml of 4% sulphosalicylic acid in a test tube containing 1 ml of supernatant obtained from plasma and this test tube was placed in refrigerator at 4°C for cold metabolism for one hour. The given plasma samples were centrifugated at 1200 × g for 15 min. and these samples were kept at 4°C in refrigerator. After that took 1 ml of this centrifugated supernatant with addition of 2.7 ml of phosphate buffer (0.1 M) followed by addition of 0.2 ml DTNB in a test tube. Addition of all chemicals in test tube caused the formation of colored solution which was measured by using spectrophotometer at 412 nm. The glutathione level was measured in μmol/mg protein [17].

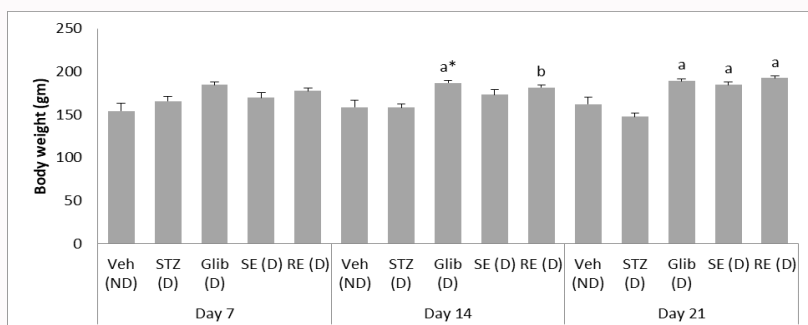


Figure 1: Effect of various treatments on Blood glucose level of rats on 7th, 14th and 21st day of STZ induced diabetes.

Values are expressed as mean \pm S.E.M, n=6 in each group. Data was analyzed by one-way ANOVA followed by Tukey's Post hoc test. a= p<0.001 significant difference from the vehicle treated rats; b= p<0.001 significant difference from the STZ treated diabetic rats.

Veh (ND): Vehicle Treated Non-Diabetic Rats; STZ (D): Streptozotocin Treated Diabetic Rats; Glib (D): Glibenclamide Treated Diabetic Rats; SE (D): Stem Extract Treated Diabetic Rats; RE (D): Root Extract Treated Diabetic Rats

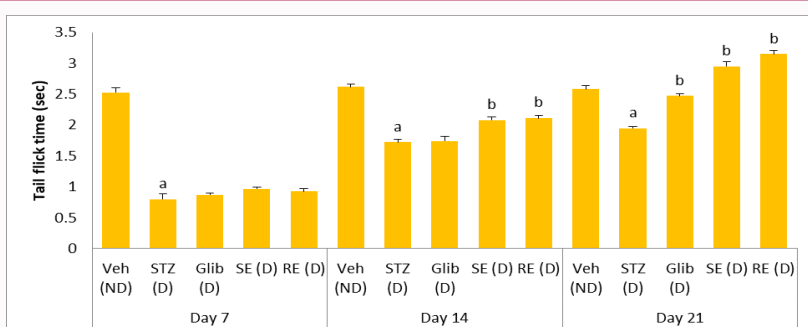


Figure 2: Effect of various treatments on body weight of rats on 7th, 14th and 21st day of STZ induced diabetes.

Values are expressed as mean \pm S.E.M, n=6 in each group. Data was analyzed by one-way ANOVA followed by Tukey's Post hoc test. a= p<0.001 significant difference from the STZ treated diabetic rats. a'= p<0.01 significant difference from the STZ treated diabetic rats. b= p<0.05 significant difference from the STZ treated diabetic rats.

Veh (ND): Vehicle Treated Non-Diabetic Rats; STZ (D): Streptozotocin Treated Diabetic Rats; Glib (D): Glibenclamide Treated Diabetic Rats; SE (D): Stem Extract Treated Diabetic Rats; RE (D): Root Extract Treated Diabetic Rats

Tail cuff method: This method was used for measuring the pressure exerted by blood on the arteries wall non-invasively i.e. without threatening the animals. In this it contains an instrument that is composed of a number of small parts and each of them play significant role in blood pressure measurement. It consists of a photoelectric sensing material that was used for measurement of end point. This sensing material is composed of small autofocus light beam and resistive photocell that was inserted in a long expansive cuff made up of rubber with a length of 50 mm. The rats were placed in individual restrainers by keeping their tail free before 30 min of blood pressure measurement so that they can familiarize with the environment and become stress free also. This method could be done by maintaining constant temperature at 27°C. After that cuff made up of rubber was tied on tail of rat individually for measurement of plasma pressure. Photocell passed the heartbeat signals to a stereo system (Model 47, IIT Inc) for maintenance of strength of the light as well as procure and counterbalance of it. An organized electro sphygmomanometer was also attached to the cuff made up of rubber for maintenance of constant expansiveness and diminution rates. This constant maintenance of diminution and expansive rates would cause change in pressure which was shown by photoconductive cell. This instrument also contains an ink writing recorder which helps in recording of signals that come from the cuff photoconductive cell as well as from photocell stereo system. Contractile blood pressure (i.e. in mm of Hg) was measured after 7, 14, 21 day of treatment [18].

Statistical analysis

Examination of data was done with the help of GraphPad InStat. Values were expressed as mean \pm SEM. The data was analyzed using one-way ANOVA. The mean of different groups used in our work were compared by using Tukey's Post Hoc test. If P Values were less than 0.001 then results were considered significant.

Results

Percentage yield

Coarse powder of *C. citrinus* stem and root was extracted with hydroethanolic solvents. *C. citrinus* root dark reddish-brown semisolid and stem was of light brown semisolid consistencies were obtained.

Preliminary phytochemical screening

Preliminary phytochemical screening was conducted to determine the phytoconstituents present in semisolid extract obtained from coarse powder of stem and root of *C. citrinus*. The phytochemical screening of stem and root extract of *C. citrinus* for detection of various chemical constituents.

In vivo studies

Effect on blood glucose level: As shown in Figure 1 glucose level in blood was significantly (p<0.001) increased in STZ treated diabetic rats (367 mg/dl \pm 8.5mg/dl) as compared to vehicle treated rats (165.8

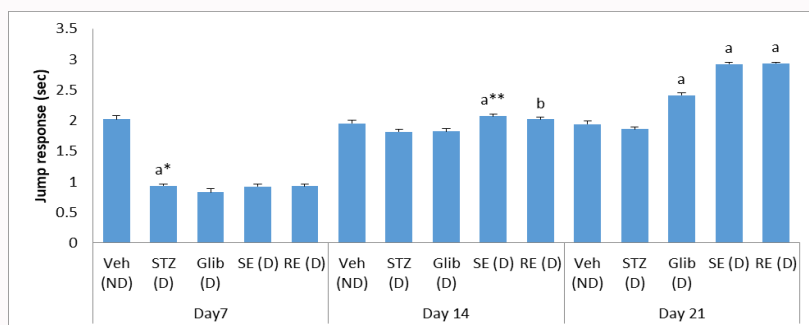


Figure 3: Effect of various treatments on tail flick latency (in sec) of rats on 7th, 14th and 21st day of STZ induced diabetes.

Values are expressed as mean \pm S.E.M, n=6 in each group. Data was analyzed by one-way ANOVA followed by Tukey's Post hoc test. a= p<0.001 significant difference from the vehicle treated rats. b= p<0.001 significant difference from the STZ treated diabetic rats.

Veh (ND): Vehicle Treated Non-Diabetic Rats; STZ (D): Streptozotocin Treated Diabetic Rats; Glib (D): Glibenclamide Treated Diabetic Rats; SE (D): Stem Extract Treated Diabetic Rats; RE (D): Root Extract Treated Diabetic Rats

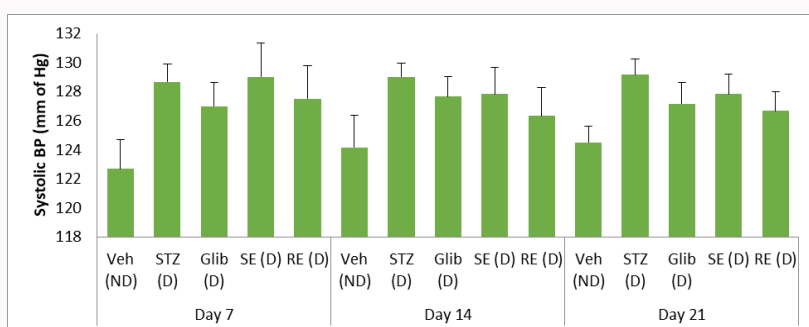


Figure 4: Effect of various treatments on jump response (in sec) of rats on 7th, 14th and 21st day of STZ induced diabetes.

Values are expressed as mean \pm S.E.M, n=6 in each group. Data was analyzed by one-way ANOVA followed by Tukey's Post hoc test. a= p<0.001 significant difference from the STZ treated diabetic rats. a'= p<0.001 significant difference from the vehicle treated non diabetic rats. a''= p<0.01 significant difference from the STZ treated diabetic rats. b= p<0.05 significant difference from the STZ treated diabetic rats.

Veh (ND): Vehicle Treated Non-Diabetic Rats; STZ (D): Streptozotocin Treated Diabetic Rats; Glib (D): Glibenclamide Treated Diabetic Rats; SE (D): Stem Extract Treated Diabetic Rats; RE (D): Root Extract Treated Diabetic Rats

mg/dl \pm 5 mg/dl) after 7 days of STZ induced diabetes. Vehicle treated group does not showed any significant difference from STZ treated rats. Drug treated groups i.e. glibenclamide (282 mg/dl \pm 2.8 mg/dl), stem (299.1 mg/dl \pm 1.7 mg/dl) and root extract (303.8 mg/dl \pm 4.7 mg/dl) of *C. citrinus* were significantly (p<0.001) decreased the blood glucose level as compared to STZ treated diabetic rats (367 mg/dl \pm 8.5 mg/dl).

Glucose level in blood was significantly (p<0.001) increased in STZ treated diabetic rats (334.6 mg/dl \pm 6.7 mg/dl) as compared to vehicle treated rats (149 mg/dl \pm 6.6 mg/dl) after 14 days of STZ induced diabetes. Vehicle treated group does not showed any significant difference from STZ treated rats. Drug treated groups i.e. glibenclamide (230.8 mg/dl \pm 2.5 mg/dl), stem (248.6 mg/dl \pm 3.4 mg/dl) and root (258.8 mg/dl \pm 7.7 mg/dl) extract of *C. citrinus* were significantly (p<0.001) decreased the blood glucose level as compared to STZ treated diabetic rats (334.6 mg/dl \pm 6.7 mg/dl).

Glucose level in blood was significantly (p<0.001) increased in STZ treated diabetic rats (311.6 mg/dl \pm 4.7 mg/dl) as compared to vehicle treated rats (144 mg/dl \pm 3.2 mg/dl) after 21 days of STZ induced diabetes. Drug treated groups i.e. glibenclamide (166.6 mg/dl \pm 3.8 mg/dl), stem (193 mg/dl \pm 2.5 mg/dl) and root (193.5 mg/dl \pm 3.2 mg/dl) extract of *C. citrinus* were significantly (p<0.001) decreased the blood glucose level as compared to STZ treated diabetic rats (311.6 mg/dl \pm 4.7 mg/dl). Vehicle treated group does not showed any significant difference from STZ treated rats.

Effect on body weight

As shown in Figure 2 after 7 days of STZ induced diabetes, body weight of drug treated groups i.e. glibenclamide, stem and root extract of *C. citrinus* was improved as compared to STZ treated diabetic rats but there was no significant difference between drug treated groups and STZ treated diabetic rats. Vehicle treated group does not showed any significant difference from STZ treated rats.

After 14 days of STZ induced diabetes, body weight of glibenclamide treated group (187 g \pm 2.6 g) was significantly (p<0.01) improved as compared to STZ treated diabetic rats (158 g \pm 4.6 g). Body weight of root extract of *C. citrinus* treated group (181.5 g \pm 2.7 g) was significantly (p<0.05) improved as compared to STZ treated diabetic rats (158 g \pm 4.6 g). Body weight of stem extract of *C. citrinus* treated group was improved as compared to STZ treated diabetic rats but there was no significant difference between them. Vehicle treated group does not showed any significant difference from STZ treated rats.

After 21 days of STZ induced diabetes, body weight of drug treated groups i.e. glibenclamide (189.1 g \pm 2.2 g), stem (184.3 g \pm 3.5 g) and root (192.5 g \pm 2.5 g) extract of *C. citrinus* were significantly (p<0.001) increased as compared to STZ treated diabetic rats (147.5 g \pm 4.1 g). Body weight of STZ treated rats were decreased as compared to vehicle treated rats but there was no significant difference between them. Vehicle treated group does not showed any significant difference from STZ treated rats.

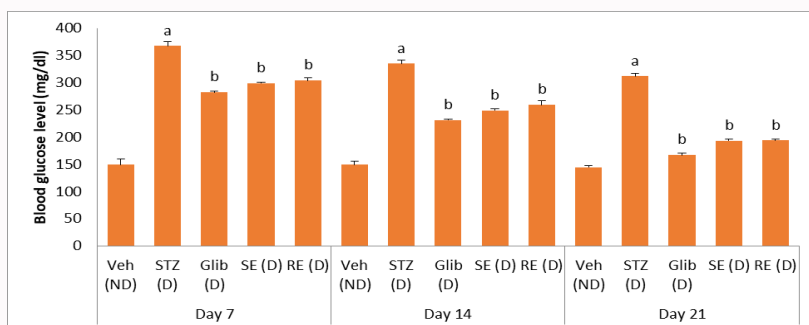


Figure 5: Effect of various treatments on the systolic blood pressure (mm of Hg) of rats on 7th, 14th and 21st day of STZ induced diabetes. Values are expressed as mean \pm S.E.M, n=6 in each group. Data was analyzed by one-way ANOVA followed by Tukey's Post hoc test. Veh (ND): Vehicle Treated Non-Diabetic Rats; STZ (D): Streptozotocin Treated Diabetic Rats; Glib (D): Glibenclamide Treated Diabetic Rats; SE (D): Stem Extract Treated Diabetic Rats; RE (D): Root Extract Treated Diabetic Rats

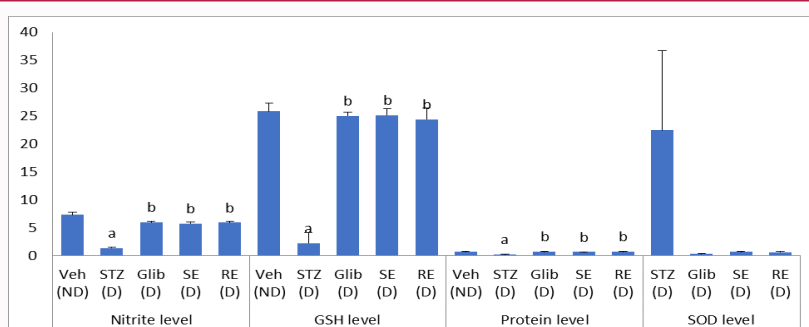


Figure 6: Effect of various treatments on Nitrite level (μmol), GSH level ($\mu\text{g/ml}$), Protein level (mg/ml), SOD level of rats after 21 days of STZ induced diabetes. Values are expressed as mean \pm S.E.M, n=6 in each group. Data was analyzed by one-way ANOVA followed by Tukey's Post hoc test. a= $p < 0.001$ significant difference from the vehicle treated rats. b= $p < 0.001$ significant difference from the STZ treated diabetic rats. Veh (ND): Vehicle Treated Non-Diabetic Rats; STZ (D): Streptozotocin Treated Diabetic Rats; Glib (D): Glibenclamide Treated Diabetic Rats; SE (D): Stem Extract Treated Diabetic Rats; RE (D): Root Extract Treated Diabetic Rats

Effect on tail flick time

As shown in Figure 3 tail flick latency of drug treated groups i.e. glibenclamide, stem and root extract of *C. citrinus* was improved as compared to STZ treated diabetic rats but there was no significant difference between drug treated groups and STZ treated diabetic rats. Tail flick latency was significantly ($p < 0.001$) decreased in STZ treated diabetic rats ($0.8 \text{ sec} \pm 0.08 \text{ sec}$) as compared to vehicle treated rats ($2.5 \text{ sec} \pm 0.07 \text{ sec}$) after 7 days of STZ induced diabetes.

Tail flick latency was significantly ($p < 0.001$) decreased in STZ treated diabetic rats ($1.7 \text{ sec} \pm 0.05 \text{ sec}$) as compared to vehicle treated rats after 14 days of STZ induced diabetes ($2.6 \text{ sec} \pm 0.04 \text{ sec}$). Tail flick latency of drug treated groups i.e. stem ($2.0 \text{ sec} \pm 0.05 \text{ sec}$) and root ($2.1 \text{ sec} \pm 0.03 \text{ sec}$) extract of *C. citrinus* was increased significantly ($p < 0.001$) as compared to STZ treated diabetic rats ($2.6 \text{ sec} \pm 0.04 \text{ sec}$). Tail flick latency of glibenclamide treated groups was improved as compared to STZ treated diabetic rats but there was no significant difference between glibenclamide treated groups and STZ treated diabetic rats.

Tail flick latency was significantly ($p < 0.001$) decreased in STZ treated diabetic rats ($1.9 \text{ sec} \pm 0.01 \text{ sec}$) as compared to vehicle treated rats ($2.5 \text{ sec} \pm 0.05 \text{ sec}$) after 21 days of STZ induced diabetes. Tail flick latency of drug treated groups i.e. glibenclamide ($2.4 \pm 0.04 \text{ sec}$), stem ($2.9 \text{ sec} \pm 0.07 \text{ sec}$) and root (3.1 ± 0.05) extract of *C. citrinus* were increased significantly ($p < 0.001$) as compared to STZ treated diabetic rats ($1.9 \text{ sec} \pm 0.01 \text{ sec}$). Vehicle treated group does not showed any significant difference from STZ treated rats.

Effect on jump response time

As shown in Figure 4 jump response time drug treated groups i.e. glibenclamide, stem and root extract of *C. citrinus* was decreased as compared to STZ treated diabetic rats but there was no significant difference between drug treated groups and STZ treated diabetic rats. Jump response time was significantly ($p < 0.001$) decreased in STZ treated diabetic rats ($0.94 \text{ sec} \pm 0.02 \text{ sec}$) as compared to vehicle treated rats ($2.0 \text{ sec} \pm 0.05 \text{ sec}$) after 7 days of STZ induced diabetes.

Jump response time of stem extract of *C. citrinus* treated group ($2.1 \text{ sec} \pm 0.03 \text{ sec}$) was increased significantly ($p < 0.01$) as compared to STZ treated diabetic rats ($1.8 \text{ sec} \pm 0.04 \text{ sec}$). Jump response time of root extract of *C. citrinus* treated group ($2.0 \text{ sec} \pm 0.03 \text{ sec}$) was increased significantly ($p < 0.05$) as compared to STZ treated diabetic rats ($1.8 \text{ sec} \pm 0.04 \text{ sec}$). Jump response time of glibenclamide treated group was increased as compared to STZ treated diabetic rats but there was no significant difference between glibenclamide treated group and STZ treated diabetic rats. Jump response time was decreased in STZ treated diabetic rats as compared to vehicle treated rats after 14 days of STZ induced diabetes but there was no significant difference between them.

After 21 days of STZ induced diabetes, jump response time of drug treated groups i.e. glibenclamide ($2.4 \text{ sec} \pm 0.03 \text{ sec}$), stem ($2.9 \text{ sec} \pm 0.03 \text{ sec}$) and root ($2.9 \text{ sec} \pm 0.02 \text{ sec}$) extract of *C. citrinus* were significantly ($p < 0.001$) increased as compared to STZ treated diabetic rats ($1.8 \text{ sec} \pm 0.03 \text{ sec}$). Jump response time of STZ treated rats were decreased as compared to vehicle treated rats but there was no

significant difference between them. Vehicle treated group does not showed any significant difference from STZ treated rats.

Effect on systolic BP

As shown in Figure 5 after 7 days of STZ induced diabetes, systolic BP of STZ treated diabetic rats were improved as compared to vehicle treated rats but there was no significant difference between them. Systolic BP was decreased in glibenclamide and root extract of *C. citrinus* treated diabetic rats as compare to STZ treated diabetic rats but there was no significant difference between them. Systolic BP was increased in stem extract of *C. citrinus* treated diabetic rats as compare to STZ treated diabetic rats but there was no significant difference between them.

After 14 days of STZ induced diabetes, systolic BP of STZ treated diabetic rats were improved as compared to vehicle treated rats but there was no significant difference between STZ treated diabetic rats and vehicle treated rats. Systolic BP was decreased in drug treated groups i.e. glibenclamide, stem and root extract of *C. citrinus* treated diabetic rats as compared to STZ treated diabetic rats but there was no significant difference between drug treated groups and STZ treated diabetic rats.

After 21 days of STZ induced diabetes, systolic BP of STZ treated diabetic rats were improved as compared to vehicle treated rats but there was no significant difference between STZ treated diabetic rats and vehicle treated rats. Systolic BP was decreased in drug treated groups i.e. glibenclamide, stem and root extract of *C. citrinus* treated diabetic rats as compared to STZ treated diabetic rats but there was no significant difference between drug treated groups and STZ treated diabetic rats.

Effect on nitrite level

As shown in Figure 6 nitrite level of STZ treated diabetic rats ($1.3 \mu\text{mol} \pm 0.14 \mu\text{mol}$) were significantly ($p < 0.001$) decreased as compared to vehicle treated rats ($7.2 \mu\text{mol} \pm 0.42 \mu\text{mol}$). Nitrite level of drug treated groups i.e. glibenclamide ($5.9 \mu\text{mol} \pm 0.18 \mu\text{mol}$), stem ($5.7 \mu\text{mol} \pm 0.24 \mu\text{mol}$) and root ($5.8 \mu\text{mol} \pm 0.23 \mu\text{mol}$) extract of *C. citrinus* were significantly ($p < 0.001$) increased as compared to STZ treated diabetic rats ($1.3 \mu\text{mol} \pm 0.14 \mu\text{mol}$). Vehicle treated group does not showed any significant difference from STZ treated rats.

Effect on GSH level

As shown in Figure 6 GSH level of STZ treated diabetic rats ($2.2 \mu\text{g/ml} \pm 1.9 \mu\text{g/ml}$) were significantly ($p < 0.001$) decreased as compared to vehicle treated rats ($25.8 \mu\text{g/ml} \pm 1.4 \mu\text{g/ml}$). GSH level of drug treated groups i.e. glibenclamide ($25 \mu\text{g/ml} \pm 0.6 \mu\text{g/ml}$), stem ($25.1 \mu\text{g/ml} \pm 1.0 \mu\text{g/ml}$) and root ($24.3 \mu\text{g/ml} \pm 2.0 \mu\text{g/ml}$) extract of *C. citrinus* were significantly ($p < 0.001$) increased as compared to STZ treated diabetic rats ($2.2 \mu\text{g/ml} \pm 1.9 \mu\text{g/ml}$). Vehicle treated group does not showed any significant difference from STZ treated rats.

Effect on protein level

As shown in Figure 6 protein level of STZ treated diabetic rats ($0.14 \text{ mg/ml} \pm 0.04 \text{ mg/ml}$) were significantly ($p < 0.001$) decreased as compared to vehicle treated rats ($0.72 \text{ mg/ml} \pm 0.02 \text{ mg/ml}$). Protein level of drug treated groups i.e. glibenclamide ($0.66 \text{ mg/ml} \pm 0.01 \text{ mg/ml}$), stem ($0.62 \text{ mg/ml} \pm 0.03 \text{ mg/ml}$) and root ($0.64 \text{ mg/ml} \pm 0.03 \text{ mg/ml}$) extract of *C. citrinus* were significantly ($p < 0.001$) increased as compared to STZ treated diabetic rats ($0.14 \text{ mg/ml} \pm 0.04 \text{ mg/ml}$). Vehicle treated group does not showed any significant difference from STZ treated rats.

Effect on SOD level

As shown in Figure 6 SOD levels of drug treated groups i.e. glibenclamide ($0.23 \text{ units/min mg of protein} \pm 0.08 \text{ units/min mg of protein}$), stem ($0.64 \text{ units/min mg of protein} \pm 0.13 \text{ units/min mg of protein}$) and root ($0.59 \text{ units/min mg of protein} \pm 0.15 \text{ units/min mg of protein}$) extract of *C. citrinus* were non significantly decreased as compared to STZ treated diabetic rats ($22.4 \text{ units/min mg of protein} \pm 14.2 \text{ units/min mg of protein}$). SOD level of stem and root extract of *C. citrinus* treated diabetic rats was improved as compared to glibenclamide treated diabetic rats but there was no significant difference between them.

Discussion

The results of the present study showed that the administration of the STZ (60 mg/kg, i.p.) alone at a single dose induce diabetes in rats. Previously it has been reported the single intravenous dose of STZ is sufficient to induce diabetes in adult rats, but in some cases larger doses of STZ were used [19]. STZ showed therapeutic effects post intraperitoneal injection at a same or larger dose. Pancreatic β -cells took STZ inside it with the help of transporters of glucose i.e. GLUT2. When STZ act through intracellular surface of pancreatic β -cells then it causes the degradation of their DNA [20]. A lot of studies nowadays said that STZ causes the death of β -cells due to its DNA alkylation by STZ [21]. The DNA alkylation due to STZ injection was specifically due to the presence of nitrosourea at the 6th position oxygen of guanine. Quantities of different purines of methylate in different tissues of rats were increased due to STZ administration to them [22]. One of the major reasons for DNA degradation by STZ is that STZ increases the NO level in pancreatic cells due to which these cells are destroyed. However, the results from several experiments showed that STZ does not produce only NO molecule that has toxic effects on body cells but it also produces a number of ROS that damages the DNA of different cells by causing their death [23]. Poly ADP ribosylation was activated in STZ induced diabetic rats [24]. From this ATP molecules will be decreased due to degradation of NAD⁺ present in different cells [25]. Whereas, further degradation causes insulin deficiency due to less insulin secretion and production [26]. STZ induced the diabetes in rats by differing the plasma glucose and insulin levels in β -cells of pancreas. After two hours of STZ injection, plasma glucose level was increased with simultaneously plasma insulin level decreases. However, after six hours of STZ injection, plasma glucose level was decreased simultaneously plasma insulin level was increased. At last plasma glucose level was increased whereas plasma insulin was decreased [27]. β -cells dysfunction can be characterized by differing the levels of insulin and glucose in plasma. Diabetes induced by STZ inhibits the oxidation of glucose and due to which STZ causes the degradation of insulin secretion and production and used to induce both type-1 and type-2 diabetes in rodents.

Glibenclamide is one of the major recommended oral hypoglycemic agent [28] belongs to the category of the sulfonylureas, acts by increasing the release of insulin from the pancreatic β -cells. This drug binds to the sulfonylurea receptor 1 (SUR 1) [29], which is a subtype of KATP in the pancreatic β -cells of islets of langerhans [30]. From that impairment depolarization of cell membrane will be occurred which causes the opening of calcium channels which are voltage dependent. It increases intracellular calcium concentration in β -cells and subsequently stimulates the release of insulin [31]. Thus, the single medicine glibenclamide increases the secretion of insulin and decreases formation of glucose in liver in clinical diabetes. In

addition to this direct action, it also shows extra pancreatic effects. Hypoglycemia is the most common side effect of glibenclamide treatment along with a greater risk of cardiovascular diseases [32]. The results of the present study showed that the administration of the glibenclamide to the diabetic rat's results in the attenuation of the biochemical and physiological parameters in STZ induced diabetic rats.

α -glucosidase and α -amylase are the hydrolyzing enzymes that hydrolyses the complex carbohydrate in simple carbohydrate. So, the α -glucosidase and α -amylase inhibitors are one of the main classes of drug for treatment of diabetic individuals. These inhibitors decrease the post meal increased plasma glucose level by inhibiting these enzymes. There are a number of plant molecules obtained from different medicinal plant showed a good inhibitory potential for α -glucosidase and α -amylase. Among the important constituents terpenoids' (i.e. 1,8-cineole, oleanolic acid, ursolic acid, p-cymene, 1-(S)- α -pinene), flavonoids, (i.e. myricetin, kaempferol, quercetin), and phenolics (i.e. ellagic acid, tannins, gallic acid) have good α -amylase inhibitory potentials. Whereas proanthocyanidins, polyphenols, anthocyanins have potent α -glucosidase inhibitory potential [33]. Some study showed chemical constituents like 1,8-cineole inhibits α -glucosidase in competitive form whereas α -pinene, limonene inhibits α -glucosidase in uncompetitive manner. Present study showed the *C. citrinus* inhibits the enzyme α -glucosidase and α -amylase. *C. citrinus* also decreases the postprandial increase in the blood glucose levels. However, *C. citrinus* extract inhibits the α -glucosidase and α -amylase in mixed and uncompetitive manner respectively.

Callistemon citrinus contains two alcohols (i.e. n-tetracontanol and blumenol A), one sterol (i.e. β -sitosterol), three benzoic acid derivatives (i.e. protocatechuic acid, gallic acid and methyl gallate) and one sesquiterpene (i.e. 2,6,10-bisabolatriene) [6]. *C. citrinus* contains three triterpenoids (Betulinic acid, Pyracrenic acid, Arjunolic acid), together with catechin and piceatannol [34]. The essential oil obtained from stem of *C. citrinus* contains hydrocarbons of monoterpene and oxygenated monoterpenes in 36.18%, 62.05% amounts respectively. The major constituents present in essential oil obtained from stem of *C. citrinus* includes following - 1,8-cineole, α -terpineol, α -pinene and limonene [7]. Eight known triterpenoids i.e. ursolic acid, ursolic acid 3-o-caffeate, aliphilic acid, lupenone, 3-acetoxy-olean-18-en-28-oic acid, moronic acid 3-o-caffeate, betulinic acid 3-o-caffeate and betulinic acid with a new triterpenoid i.e. 30-hydroxyaliphilic acid were isolated from *C. citrinus*. *C. citrinus* aerial parts had showed presence of six flavonoids i.e. syzalterin, 8-demethyleucalyptin, eucalyptin, sideroxylin, quercetin and 4,5-dihydroxy-6,8-dimethyl-7-methoxyflavanone. Phytochemical analysis of different parts of *C. citrinus* showed different chemical constituents as shown above by different analysts. Further our results of preliminary phytochemical screening showed presence of alkaloids, saponins, triterpenoids, quinines, flavonoids, phenolic compounds and carbohydrates in hydroethanolic stem and root extract of *C. citrinus*. These isolated flavones lowered the blood glucose in rats which are made diabetic by STZ [35]. In the present work the administration of the root and the stem extract of the *C. citrinus* to the diabetic rats' results in the significant reduction in the physiological and the plasma related parameters in rats which are made diabetic by STZ. Previously Nazreen et al. [14] had also shown that chloroform, ethanolic and petroleum ether fraction of *C. citrinus* extract have hypoglycemic potential in STZ treated rats [16]. Das et al. showed that daily oral treatment with methanol fruits extract with dose 400 mg/kg have

spectacular antihyperglycemic activity in STZ treated diabetic rats.

Traditionally *C. citrinus* were also used for treatment of diabetes in India. GSH levels as well as lipid peroxidation level along with antioxidant activity of different enzymes were regained when STZ treated diabetic rats were treated by methanol fruits extract of *C. citrinus* [16]. *C. citrinus* showed increase in level of insulin present in serum due to its free radical scavenging activity. The work also showed that ethanolic *C. citrinus* extricates have free radical scavenging activity on superoxide radicals, hydroxyl, and nitric oxide and also on DPPH [36]. Nazreen et al. [14], work showed a notable decrease in level of GSH in STZ treated diabetic rats in comparison to the rats that are only treated with STZ. When *C. citrinus* different fractions like chloroform or ether along with glibenclamide were given to STZ treated diabetic rats then these drugs take the high GSH level to normal GSH level. A number of enzymes are present in body for procurement of inhibited cell working, pathology of diabetic patient as well as in oxidative reactions but out of them CAT and SOD are more functional. The amount of CAT and SOD enzymes in the STZ treated diabetic rats were notably decreased as compared to normal vehicle treated rats. The amounts of these enzymes i.e. CAT; SOD was increased by giving ethanolic extract of *C. citrinus*. So, this shows that the ethanolic extract with its fractions have free radical scavenging as well as antioxidant activity.

It has been concluded that stem and root extract of *C. citrinus* exerted α -amylase and α -glucosidase inhibitory potential. It also concludes that stems and root extract of *C. citrinus* exerted significant antidiabetic potential as well as significant analgesic activity in STZ induced diabetic rats. Whereas the stem and root extract of *C. citrinus* treated diabetic groups were not showed any significant change in systolic BP as compared to STZ treated diabetic group. So, stem and root extract of *C. citrinus* does not exert antihypertensive effect in STZ treated diabetic rats.

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