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EVALUATION OF ANTIOXIDANT ACTIVITY AND OXIDATIVE STRESS PARAMETERS BY TREATMENT WITH *LEUCAS INDICA* VARIETY *NAGALAPURAMIANA* IN STREPTOZOTOCIN INDUCED DIABETIC RATS

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ABSTRACT

Leucas indica var. nagalapuramiana is a high value medicinal plant of Seshachalam hill range of Eastern Ghats, Andhra Pradesh. The present study was conducted to determine the yield anti-diabetic, antioxidant activity, of the crude extracts of Nagalapuramiana. The extracts were prepared by using methanol solvent and fractionated with toluene, ethyl acetate and n-butanol. The extracts are subjected to test their biological activities by few in vitro tests like 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) assay, estimation of malondialdehyde (MDA), glutathione (GSH), and nitric oxide (NO) levels. The ethyl acetate fraction of Leucas indica (EALI) shows significant antioxidant and anti-hyperglycemic activity.

KEY WORDS

Ethyl acetate fraction, Leucas indica, Nagalapuramiana

INTRODUCTION

Plants with medicinal properties play a huge role in the treatment of diabetes mellitus which is a serious metabolic disorder. Medicinal plants are accounted to have noteworthy anti-diabetic properties with minimal side effects. They are rich sources of compounds that are useful in diabetes, for example, flavonoids, alkaloids, phenolic and tannins that improve the efficiency of pancreatic tissues by increasing the insulin release or decreasing the intestinal absorption of glucose [1]. Even though the pathophysiology of diabetes is not exclusively well known, recent reports suggest that it includes the involvement of free radicals in the pathogenesis of diabetes, and its complications [2]. Free radicals often produce damage to cellular molecules, proteins, lipids and DNA, which leads to the dysfunction and death of the cell. Hence the abnormalities in lipids and proteins physiology play a crucial role for the development of diabetic complications. The free radicals readily oxidize the different lipoproteins, and abnormalities of lipoprotein metabolism in very low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) are evident in diabetes patients [3]. Due to increase in blood glucose levels various extracellular proteins are converted into glycoproteins, which are related with severe diabetic complications [4]. ROS are anticipated in the development of insulin resistance and dysfunction of pancreatic β-cell by different reactions such as non-enzymatic glycosylation**,** electron transport chain in mitochondria and membrane-bound NADPH oxidase [5-8].

ROS are deposited in the tissues and also, advanced glycation end products (AGE's) which are produced by non-enzymatic glycosylation of proteins; this leads to abnormalities of cell and tissue functions [9]. AGEs play a crucial role in vascular permeability in both micro- and macro-vascular structures by sticking to specific macrophage receptors, which leads to free radical

production and endothelial dysfunction and on nucleic acids it leads to altered gene expression and mutation. In diabetes, decrease in the antioxidant properties along with oxidative stress lead to the damaging effects due to free radicals [10, 11]. The phytochemicals like phenylethanoid, glycosides were extracted from the elevated pieces of *Leucas indica* having cancer prevention property [12, 13].

MATERIALS AND METHODS

This study was executed at the Department of Pharmacology, University College of Pharmaceutical sciences, Kakatiya University, Warangal, India. Collecting the whole plant of *Leucas indica variety nagalapuramiana*, were validated and checked by a taxonomist. Streptozotocin (STZ), Thiobarbituric acid (TBA), malondialdehyde (MDA), hematoxylin and eosin stains were procured from Sigma (Bangalore, India). Ellman's reagent and Griess reagent were obtained from Hi-Media (Mumbai, India). The various synthetic compounds used in this examination were of research grade quality procured from local suppliers.

Preparation of *the Leucas indica var. nagalapuramiana*

The whole plant of *Leucas indica var. nagalapuramiana* were collected and dried at room temperature. The dried plant were ground into a fine powder and sieved through a 40 mm work strainer. The acquired powder was kept in water/air evidence plastic sacks. 100 gm of each powdered leaves test was extracted with 400 ml of methanol with occasional shaking for 7 days by maceration. The move was shifted into a clean conical flask and sieved through a Whatman's channel paper into another funnel shaped jar. The methanolic extract of the whole plant was blended with 1000 ml of water freely and fractioned with toluene, ethyl acetate and n-butanol. The solvents were emptied from the parts under decreased strain to yield the relating extract. The ethyl acetate parts of leaves were sifted, dried in a rotavapour and used for further examinations.

***In vitro* study of selected medicinal plants**

DPPH assay for antioxidant potential of *Leucas indica var. nagalapuramiana*: The free radical scavenging action of the considerable number of concentrates was evaluated by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) as showed by the method [15, 16]. Quickly, 0.1 mM arrangement of DPPH in methanol was prepared and 1 mL of this solution was added to 3 ml of the arrangement of all moves in methanol at different fixations (50, 100, 200, 400 and 800 µg/mL). The blends were shaken energetically and allowed to stay at room temperature for 30 minutes. By then the absorbance was evaluated at 517 nm using an UV-visible spectrophotometer. Ascorbic acid was used as the reference. Lower absorbance estimations of response blend show higher free radical rummaging activity. The limit of rummaging the DPPH radical was controlled by using the accompanying condition.

$$\text{DPPH scavenging effect (\% Inhibition)} = \frac{A_0 - A_1}{A_0} \text{ X } 100$$

Where, A_0 is the absorbance of the control response, and A_1 is the absorbance in presence of all the concentrate tests and reference. Every one of the tests was performed in triplicates and the results were averaged and shown in Figure 1.

Experimental Animals

Animal's investigations were executed according to the CPCSEA guidelines of the organization of India. Animal's conventions were affirmed by the Institutional Animal Ethics Committee of the University College of Pharmaceutical Sciences (IAEC/10/UCPSC/KU/2016). Male Sprague Dawley (SD) rats were used for the high fat diet (HFD) notwithstanding STZ (HFD+STZ) induced type II diabetes model and for assessment of protective effect of *Leucas indica*. Animals were acclimatized to the conditions one week before the tests. Animals were kept up at standard conditions, 12 h day/light cycle, 50-60% relative moistness, nourishment, and water provided not indispensable.

Experimental design

- Group I- Normal Control,
- Group II- HFD STZ, (Diabetic Control)
- Group III- EALI (low dose of *Leucas indica* 10 mg/kg
- Group IV- EALI (Mid dose of *Leucas indica*) 30 mg/kg
- Group V- EALI (high dose of *Leucas indica*) 100 mg/kg
- Group VI- Metformin 10 mg/kg (Standard drug).

Treatment methodology: For the diabetogenesis and pancreatic beta cell security, rats were treated with selected extract at the dose of 10, 30 and 100 mg/kg orally. Regardless of the preliminary rats from normal control rats were nourished with HFD for around fourteen days. Following 2 weeks post HFD supporting, rats were induced with STZ to impel diabetes and after that extracts and standard treatment was started on one day post STZ organization. A month of time for

testing we have considered in the present work is post STZ treated period. The extracts were suspended in 0.5% carboxy methyl cellulose (CMC) and given orally for 28 days and first treatment was started on one day after STZ administration. The volume of extracts controlled was kept as 10 ml/kg body weight. Basically, the standard medication of diabetic drug metformin was given orally at the portion of 10 mg/kg.

Induction of type II diabetes: For induction of type II diabetes, male SD rats were from the start nourished with high fat eating regimen routine for around fourteen days (pre investigative period), trailed by low dose of streptozotocin (STZ) 35 mg/kg was mixed intra-peritoneally (i.p.) on around fourteen days present HFD as per well-established method. HFD quantity was fixed as 58% fat, 25% protein and 17% starch. In the wake of beginning the metabolic issue like condition due to HFD, rats were injected with single administration of STZ at the dose of 35 mg/kg, i.p. Required proportion of STZ was taken in individual Eppendorf tubes and dissolved in chilled Citrate buffer pH 4.5. Citrate buffer was added to each individual syringe just before administration to rats. 48 h after the STZ induction, blood glucose levels were evaluated by glucometer. The non-fasting blood glucose levels more than 250 mg/dl were considered as diabetic rats. The incidence of diabetes was furthermore attested by polyphagic and polydipsic lead of the rats.

Assessment of diabetogenesis in rats: The event of diabetes was assessing by the plasma glucose levels every week. The blood glucose levels were assessed by means of glucometer by collecting the drop of blood from rat's tail. Rats with blood glucose levels >250 mg/dl were considered as diabetic.

Effect of Leucas indica variety Nagalapuramiana extract treatment on blood glucose levels: The blood glucose levels were estimates during a month of preliminaries in all the investigation groups, at the basal levels (2 weeks post HFD sustaining), there was a 1.35 fold increments in the blood glucose levels in all the HFD experimental animals, it is average, in perspective on high vitality substance accomplished mellow hyperglycemia. Upon STZ organization, noteworthy increments in the blood glucose levels were seen in HFD+STZ control when contrasted with control rodents. The glucose levels supposedly were continuing 3.65-fold increasing till the test length of about a month (28 days). The hyperglycemic condition found in diabetic control animals was conditionally brought down in concentrate treated creatures. The ethyl acetate fraction of leaves extract at 100 mg/kg passed on exceedingly significant decrease 2.30-fold in the blood glucose levels before 28 days of treatment. The high portion of concentrate indicated hypoglycemic impacts like standard drug metformin 2.85-fold. Low 1.21 folds and mid 1.66-fold portion of concentrate treatment came about smooth to direct control in blood glucose levels. Figure 1 clarifies the blood glucose profile of various groups for 28 days post STZ organization and impact of focus and standard medication on glucose profiles.

Estimation of biochemical parameters: The plasma profile for metabolic parameters like triglycerides and total cholesterol in the plasma was evaluated in all the experimental groups. These estimations were done by using economically open biochemical packs got from Accurex, Biomedical (Mumbai, India).

Estimation of oxidative and nitrosative stress parameters in pancreas: After completion of the assessment (28 days), rats were sacrificed, pancreas was separated, and weights were recorded. In the pancreatic tissue the oxidative stress marker like malondialdehyde (MDA) levels and glutathione (GSH) levels and estimation of tissue nitrosative stress were assessed.

Estimation of MDA levels in pancreas: Pancreas was homogenized in 5 volumes of ice-cold PBS (PH 7.4) by using tissue homogenizer. The total homogenate was used for the malondialdehyde (MDA) estimation and reduced glutathione (GSH) was assessed in tissue homogenate supernatants. Estimation of thiobarbituric acid reactive substances (TBARS) is a procedure for choosing the lipid per-oxidation in biological samples. TBARS are evaluated as MDA levels. The colorimetric technique for estimation was sought after for MDA estimation. Rapidly, 100 µl tissue homogenate was blended with 100 µl of sodium dodecyl sulfate (SDS) (8.1 %), 750 µl of thiobarbituric acid (TBA) (0.8 %) and 750 µl of 20 % glacial acidic acid (GAA) (pH 3.5), final substance volume was made up to 2 ml with refined water. Substance were warmed at 95°C for 1 h, then cooled to room temperature and centrifuged to disconnect supernatants and absorbance was evaluated in supernatants at 532 nm. Lipid peroxidation was determined from the standard bend using the 1, 1, 3, 3 tetra-ethoxy propane (97 %) and imparted as nM

MDA/mg protein. Protein concentration in tissues tests was estimated by the Lowry method [17].

Estimation of GSH levels: Reduced glutathione (GSH) levels were assessed in the supernatants of the tissue homogenates as indicated by the recently announced procedure with specific changes. Tissue homogenates were centrifuged at 700 g for 10 min and the supernatant was blended with 5 % sulphosalicylic acid and set in ice for 20 min then the precipitated proteins were centrifuged at 10000 g for 5 min at 4°C and the supernatant was used for the GSH estimation. Ellman's (5, 5'- dithiobis-(2-nitrobenzoic acid) reagent (1.5 ml, 0.1 mM) was added to the above supernatant and incubated at 37°C for 10 min sought after by estimation of digestion at 412 nm using diminished GSH as a standard and levels were conveyed as µM/mg protein.

Estimation of nitric oxide (NO) levels in pancreatic tissue: After homogenizing the pancreatic tissue, homogenate was centrifuged at 10000 rpm and supernatant was used for the NO estimation according to the Griess procedure [18]. The Griess Reagent technique relies upon a compound reaction that uses sulfanilamide and N-1-naphthylethylenediamine dihydrochloride. 100 µl of tissue homogenate supernatants were mixed with 100 µl of Griess reagent incubated at room temperature for 10 min, and after that the absorbance at 540 nm using a microplate reader. The nitrite levels were evaluated as µM/mg protein. Sodium nitrite was used as standard.

Statistical Analysis: All the investigative values were communicated as mean ± standard error of mean (SEM). The statistical significance among the groups was investigated by one-way examination of change (ANOVA) trailed by Tukey's multiple comparison test. P value <0.05 is considered as statistically significant.

RESULTS

DPPH assay for antioxidant potential of *Leucas indica variety nagalapuramiana*: The free radical scavenging effects of two different extracts of *Leucas indica* was evaluated by DPPH assay. In case of the toluene fractions of *Leucas indica,* we observed a 49.96% scavenging activity at 62.5 µg/mL, whereas it doses dependently increased up to 60.36% at 250 g/mL. In case of the ethyl acetate fractions, we found 56.66% scavenging activity at 62.5 µg/mL. However, there was a slight improvement at 250 µg/mL with scavenging activity of 61.61% at 250 µg/mL and the free radical scavenging activity of ascorbic acid was found to be 64.50% at 62.5 µg/mL. Hence, the ethyl acetate fraction of the *Leucas indica* was found to be better compared to the toluene fraction as its radical scavenging activity was slightly higher and shown in Figure 3 and Table 1.

Effect of *Leucas indica variety nagalapuramiana* on pancreatic oxidative stress: The MDA levels which are markers of lipid peroxidation incited by oxidative stress were remarkably increased in HFD+STZ control pancreatic tissues appeared differently in relation to non-diabetic control pancreas. There was a 2.70 folds increase in the MDA levels in diabetic control groups appeared differently in relation to normal control rodents. Further, treatment with plant concentrate showed at low dose 1.10-fold, mid dose 1.28-fold and high dose 1.62 folds declined, exclusively in 10, 30 and 100 mg/kg groups appeared differently in relation to diabetic control pancreatic tissues. In addition, metformin treatment showed 2.0-fold diminishes in the MDA levels and shown in Figure 4.

Of course, the endogenous antioxidant GSH was seen to be 2.34-fold fundamentally diminished in HFD+STZ control pancreatic tissues stood out from normal chow diet fed control rodents. Treatment with extracts at 10, 30 and 100 mg/kg doses realized 1.12, 1.55- and 2.10-folds addition in the GSH levels. The high dose of plant concentrate made equivalent kind of addition like standard medicine metformin 2.16 folds and shown in Figure 5.

Effect of *Leucas indica variety nagalapuramiana* extract on pancreatic nitrosative stress: The NO levels which is a marker of reactive nitrogen species status, prescribed that there is reduction in the nitrosative stress in diabetic control pancreas compared to non-diabetic control rats. There was a 2.37-fold augmentation in the NO levels in pancreatic tissues of HFD+STZ treated groups appeared differently in relation to normal pancreas. Further, treatment with the blood glucose levels were estimates during a month of preliminaries in all the investigation groups, doses restrictively produced abatement in pancreatic nitrosative stress, with low dose 1.09 fold, mid dose 1.16 fold and high dose groups rats showed 1.60 fold decline in the NO levels appeared differently in relation to diabetic control pancreas and shown in Figure 6 and Table 2.

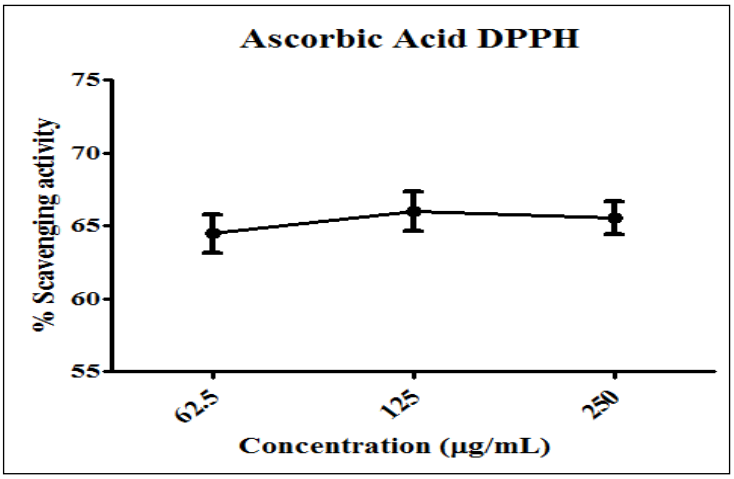

Figure 1: DPPH free radical scavenging activity of Ascorbic acid (Standard)

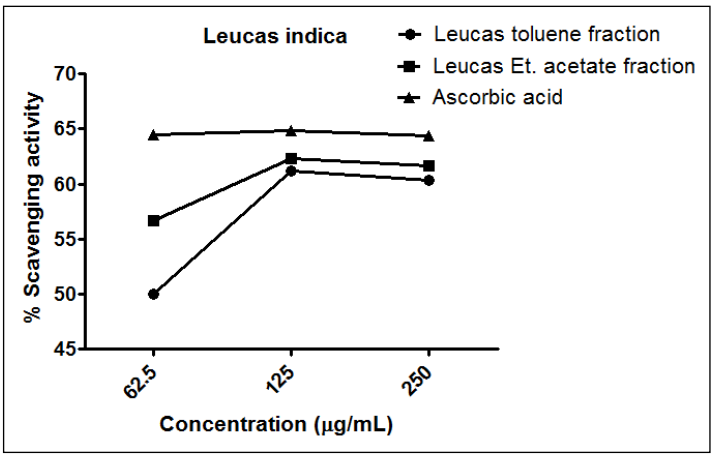

Figure 2: DPPH free radical scavenging activity of Leucas indica extracts

Table 1: Effect of selected plant extracts on free radical scavenging property

Extract	62.5 µg/ml	125 µg/ml	250 µg/ml
Ascorbic acid	64.32±1.00	64.80±1.55	68.63±1.43
TLI	51.14±1.09	62.25±1.01	60.01±1.53
EALI	57.41±0.78	61.28±0.99	61.72±1.44

% Scavenging property of selected extracts and each value represents mean± SEM (n=3)

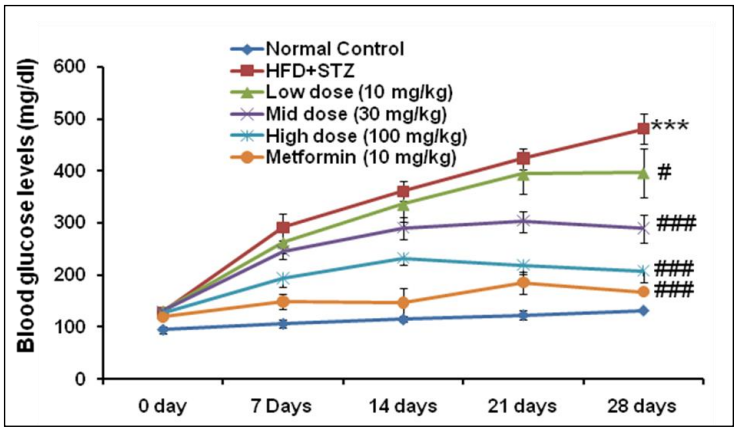

Figure 3: Effect of Oral administration of *Leucas indica* var. Nagalapuramiana extract on glucose levels evaluated in HFD+STZ induced type II diabetes model. Data was represented as mean±SEM (n=6). *** P<0.001 vs normal control group, # P<0.05 and ### P<0.001 vs HFD+STZ group

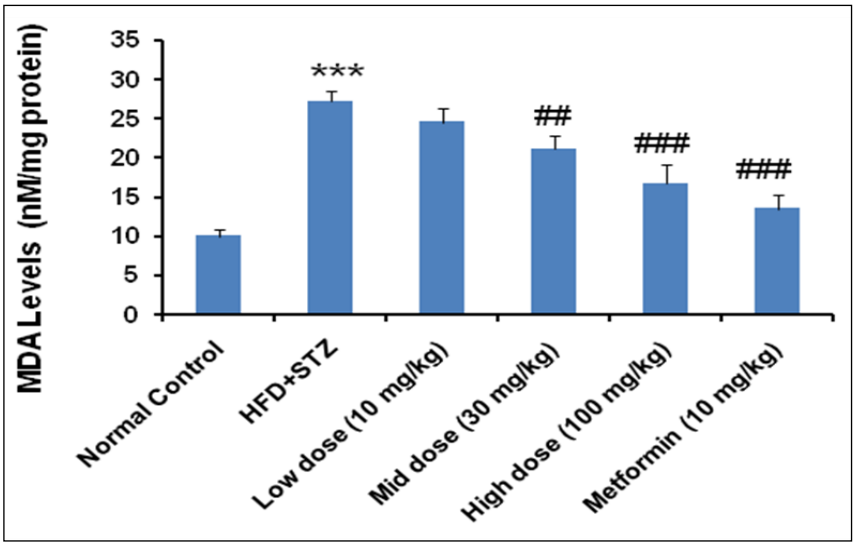

Figure 4: Effect of Oral administration of *Leucas indica var. Nagalapuramiana* extract on pancreatic lipid peroxidation (MDA) levels at the end of 28 days evaluated in HFD+STZ induced type II diabetes model.
Data was represented as mean±SEM (n=6). *** P<0.001 vs normal control group, ## P<0.01 and ### P<0.001 vs HFD+STZ group.

Table 2: Effect of Oral administration of *Leucas indica* (EALI) on various parameters in HFD+STZ induced type II diabetes model

GP	MDA	GSH	NO
I	10.07±1.90	304.80±4.01	19.03±2.04
II	27.22±3.16	129.95±3.09	45.18±4.95
III	24.59±4.06	146.76±3.67	41.26±2.03
IV	21.17±3.63**	202.64±1.47	38.93±1.84
V	16.75±5.27***	274.4±2.03*	28.09±6.95***
VI	13.56±4.03***	281.89±4.04*	22.45±4.77***

Data was represented as mean±SEM (n=6). Statistically significant * P <0.05, **P <0.01 and *** P<0.001 vs Normal control group.

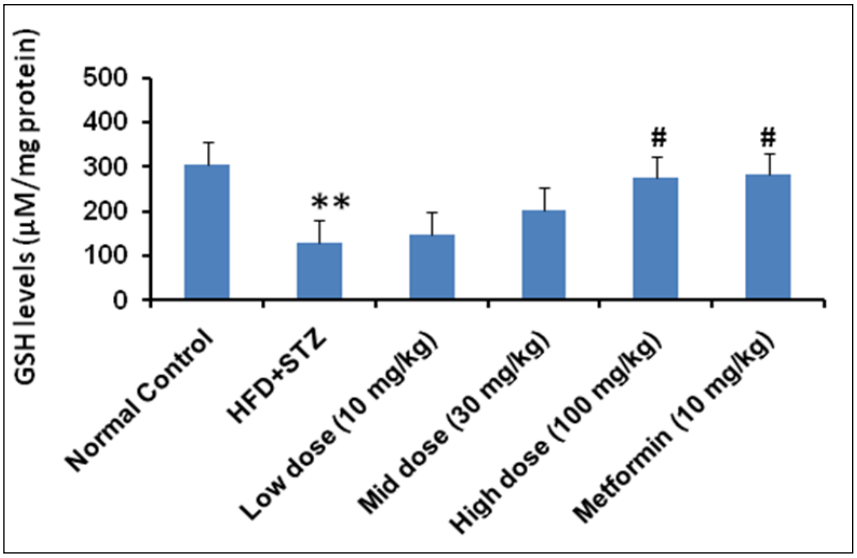

Figure 5: Effect of Oral administration of *Leucas indica var. nagalapuramiana* extract on pancreatic antioxidant (GSH) levels at the end of 28 days evaluated in HFD+STZ induced type II diabetes model.
Data was represented as mean±SEM (n=6). ** P<0.01 vs normal control group and # P <0.05 vs HFD+STZ group.

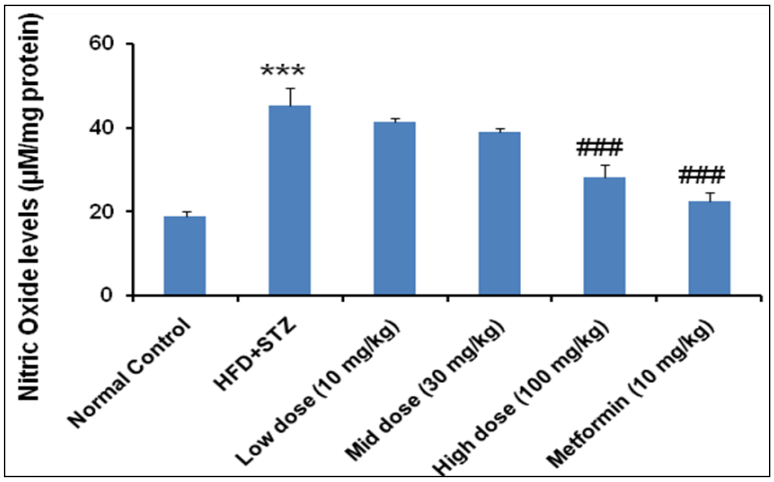

Figure 6: Effect of Oral administration of *Leucas indica var. Nagalapuramiana* extract on pancreatic Nitrosative stress (Nitric Oxide) levels at the end of 28 days evaluated in HFD+STZ induced type II diabetes model.
Data was represented as mean±SEM (n=6). *** P<0.001 vs normal control group and ### P<0.001 vs HFD+STZ group.

DISCUSSION

In the present investigation we have made an effort to assess the protective role of *Leucas indica* extract in animal experimental model of type 2 diabetes. The active constituent *Leucas indica* was extracted productively by extraction and these extracts were described by GC-MS and other analytical techniques. The *Leucas indica* extract found to have promising antioxidant properties assessed by DPPH test [15, 16]. The plasma glucose levels were found to be considerably decreased in leaves extract treated animals, it is noticeable from the figure that leaves extract productively controlled the hyperglycemic conditions in time dependant and dose dependant way. Essentially, the percentage level of diabetes induction and diabetes incidences were diminished in leaves extract treated animals contrasted with untreated diabetic control animals.

The possible explanation for this protective role of pancreatic beta cell damage by active constituents present in leaf extract antioxidant and anti-inflammatory effects [19, 20]. Curiously, constant oral induction of *Leucas indica* extract produced empowering beta cell ensuring hypoglycemia effect. These anti-hyperglycemic effects are equivalent to the standard anti-diabetic metformin treated animals. The information from glucose levels and diabetes rate unmistakably recommend that *Leucas indica* extract has protective role against HFD+STZ induced pancreatic injury. Since, leaves extracts are protecting the pancreatic beta cells; the OGTT likewise further exhibited improved glucose tolerance. The possible mechanism for anti-diabetic activity of *Leucas indica* is its antioxidant and anti-inflammatory properties. Antioxidant effect of plant-based medicine is a highly sought property in pre-clinical research. A large number of extracts have been studied for their antioxidant profiles with the major aim to study their pharmacological effects against various acute and chronic disorders. The reactive oxygen species (ROS) are the major stimulants of oxidative stress in a number of clinical diseases like diabetes, arthritis, cardiovascular disorders etc. Hence, in the current work we selected three potential plant candidates for the evaluation of their efficacy in scavenging of free radicals. TheDPPH assay is a widely used screening assay to evaluate the free radical scavenging activity of potential antioxidants. To this end, we studied various solvent with different fractions of *Leucas indica*. Interestingly, all the three extracts exhibited promising antioxidant profile as evident from the results of DPPH free radical scavenging effects. In fractions of ethyl acetae, toluene n-butanol fractions of *Leucas indica* were found to best good free radical scavenging effect. Among the three fractions, ethyl acetate fractions *Leucas indica* of showed the best antioxidant activity and may be screened for pharmacological effects against ROS driven disorders.

It is noticeable from the MDA examination that there is tremendous increment in the oxidative stress in the diabetic control animals, a few investigations additionally revealed increased oxidative pressure and in metabolic disorder conditions and STZ known to deliver plentiful increment in oxidative pressure conditions in pancreatic tissues [21]. In comparable lines, past examinations additionally showed the defensive impacts of leaves extracts against pancreatic tissue harm

Notwithstanding increased oxidative pressure, the endogenous antioxidant levels seriously lowered in diabetic pancreatic tissues, which additionally observed in our present examination in which GSH levels were seen as many times diminished in HFD+STZ control pancreatic tissues. The nitrosative stress additionally observed to cause pancreatic damage, which may be the situation in our present investigation, where, we had seen high NO levels in diabetic

control rats, in addition, extract treatment came about promising impacts in the nitrosative stress conditions.

The source of increased NO levels in pancreatic tissues of diabetes groups is over expressed inducible nitric oxide synthesis (iNOS), which may be the possible mechanism for raised nitrosative stress [22]. Besides, *Leucas indica* concentrate resulted in outstanding decrease in the NO levels. Since, NO reacts with superoxides to form peroxynitrites which are profoundly reactive free radical agents, it is significant to control both oxidative and nitrosative stress so as to control the oxidative stress mediated pancreatic beta cell damage [23]. These extracts appear to deliver beta cell protection by controlling the oxidative and nitrosative stress. In light of the biochemical profiles in pancreas and plasma biochemical parameters, it is clear that there is an increase in glucose levels which is for the most part because of damage of the pancreatic tissue.

Therefore, our extract may be compelling in the treatment of diabetic complications fundamentally vascular complications [24].

Further, future work is justified to investigate these pharmacological actions. However, we have shown promising pancreatic defensive and anti-diabetic impacts of *Leucas indica* extracts, the doses utilized in the present investigations are in moderately higher range, in this manner, further examinations might be required to surpass dose related issues. Taken together our tests confirm that it is possible to treat type 2 diabetes. Along these lines, potential outcomes for clinical interpretation could be investigated for further investigation of such a fascinating plant extract with demonstrated health advantages.

CONCLUSION

Streptozotocin diabetic rats have a severe oxidative stress condition, as shown by the high plasma levels of thiobarbituric acid reactive substances and nitric oxide levels, reduced glutathione levels. It further reflects a hope for the development of many more novel therapeutic agents or in future may serve to produce synthetically improved pharmacological agents. It also provides a base to elucidate molecular pharmacokinetic mechanism.

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