Effect of ethanolic extract of seeds of *Linum usitatissimum* (Linn.) in hyperglycaemia associated ROS production in PBMNCs and pancreatic tissue of alloxan induced diabetic rats

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**Objective:** To evaluate the effect of ethanolic extract of seeds of *Linum usitatissimum* (EELU) in hyperglycaemia associated reactive oxygen species (ROS) production in peripheral blood mononuclear cells (PBMNCs) and pancreatic antioxidant enzymes in alloxan induced diabetic rat. **Methods:** Diabetes was induced in male Wistar rats by alloxan (120 mg/kg, i.p.). After acute and subacute treatment serum glucose was determined. Oral glucose tolerance test (OGTT) was performed in EELU pretreated animals. ROS production in PBMNCs and pancreatic antioxidant enzymes were measured in alloxan induced diabetic rat. **Results:** Our results showed that treatment of EELU (200 and 400 mg/kg) significantly reduced serum glucose level in acute and subacute study. The antihyperglycaemic effects of EELU showed onset at 4th h (P<0.001) and peak effect at 6th h (P<0.001). The effect was sustained until 24th h with 400 mg/kg. In subacute study, significant antihyperglycaemic effect was observed from 14th day (P<0.001) onwards. In EELU treated rat the body weight was significantly (P<0.001) increased as compared to diabetic group on 21st day onwards. In OGTT, increased glucose utilization was observed. Treatment of EELU 400 mg/kg showed significant reversal in pancreatic GSH (P<0.01) and SOD (P<0.05) indicating antioxidant nature of EELU. Flow cytometric estimation of total ROS production in PBMNCs in diabetic rats was significantly increased (P<0.001), whereas EELU treatment showed significant (P<0.001) decrease in PBMNCs ROS. **Conclusions:** It is concluded from the investigation that EELU showed antihyperglycaemic effect mediated through inhibition of ROS level in PBMNCs and preservation of endogenous antioxidant enzymes in pancreatic tissue in alloxan induced diabetic rat.
generated in pancreas. It is reported that supplementing diet with flax and pumpkin seed mixture partly improved peripheral glucose and restored pancreatic histology in alloxan induced diabetic rats\cite{8}. However there are no reports present on hyperglycemia mediated ROS production in PBMCs and pancreas in alloxan induced diabetic rats.

Dupasquier et al\cite{9} have reported that, flaxseed supplementation prevents the development of hypercholesterolemic atherosclerosis. Hypercholesterolemic atherosclerosis increases the cholesterol content of platelets, polymorphonuclear leukocyte, endothelial cells and peripheral blood mononuclear cells (PBMCNs). It is then further responsible for generation of reactive oxygen species (ROS). Antioxidant nature of SDG has been reported and it is proposed that because of its anti-platelet activating factor (PAF) and antioxidant activity it inhibit the production of ROS by PBMCNs and scavenge the ROS produced. Mordes et al\cite{10} reported that SDG with anti-PAF and antioxidant activity prevent the development of diabetes in diabetic prone BioBreeding rats. These rats develop spontaneous autoimmune insulin-dependent diabetes mellitus (IDDM) that resembles human IDDM. Prasad et al\cite{6} studied antidiabetic potential of SDG in streptozotocin (STZ) induced diabetes and reported that SDG treatment prevent the development of diabetes by 75%. He also reported that STZ induced diabetes is associated with an increase in lipid peroxidation product malondialdehyde (MDA) in serum and pancreas and ROS producing activity of PBMCNs.

Earlier study carried out in our laboratory by Zanwar et al\cite{8}, showed antioxidant potential of ethanolic extract of \textit{L. usitatissimum} (EELU). It is reported that EELU has more DPPH radical scavenging activity, reducing power, hydroxyl radical scavenging and hydrogen peroxide radical scavenging but less superoxide scavenging and metal chelation activity than \textit{a}-tocopherol. Phenolic compounds seem to be the main components responsible for the antioxidant activity.

The present investigation is aimed to prove antidiabetic activity of EELU and to provide direct evidence for protective antioxidant role played by EELU in pancreatic tissue and PBMCNs in alloxan treated male Wistar rats.

2. Materials and methods

2.1. Collection and authentication of plant

Authenticated seeds of \textit{L. usitatissimum} (variety NL–97) were obtained from Dr. P. B. Ghorpade, Principal, Scientist and Linseed breeder, Punjabrao Deshmukh Krushi Vidyapeeth, College of Agriculture, Nagpur, India, Maharashtra State, India and voucher specimen was deposited at the institute.

2.2. Drugs and chemicals

Epinephrine hydrochloride, super oxide dismutase (SOD) and MDA were purchased from Sigma Chemical Co., USA. Reduced glutathione (GSH), 5, 5′-dithiobis (2–nitro benzoic acid) (DTNB) and thiobarbituric acid (TBA) were obtained from Hi media, India. glibenclamide (Sanbaxy Pharma. Ltd, India), alloxan monohydrate (Spectrochem, India), glucose estimation kit (glucose oxidase/peroxidase kit) (Accurex Biomedical Pvt. Ltd, India), petroluem ether (60:80 °C) (Merk, India) and D-glucose (S.D. Fine–Chem. Ltd, India) were purchased from respective vendors. All chemicals used were of analytical grade.

2.3. Preparation of ethanolic extract of \textit{L. usitatissimum}

The seeds of \textit{L. usitatissimum} were crushed to get flaxseed cake. These flaxseed cake was defatted by petroleum ether (60–80 °C) in soxhlet apparatus. The marc was then hydrolyzed with 1 mol aqueous sodium hydroxide for 1 h at room temperature by constant rotation, followed by extraction with 50% ethanol. Then solution was acidified to pH 2–4 using 1 mol hydrochloric acid. The filtrate was dried on tray dryer at 50 °C. The yield of the extract was 14.81% w/ w. The powdered ethanolic extract was dissolved in distilled water to prepare desired concentration of drug solution.

2.4. Experimental animals and research protocol approval

Male Wistar rats (100–150 g) were purchased from National Toxicology Centre, Pune, India. Animals were maintained in an air-conditioned room at (22±2 °C and relative humidity of 45% to 55% under 12–h light: 12–h dark cycle. The animals had free access to standard food pellets (Chakan Oil Mills, Pune, India) and water was available \textit{ad libitum}. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) constituted in accordance with the rules and guidelines of the Committee for the Purpose of Control and Supervision on Experimental Animals (CPCSEA), India.

2.5. Induction of experimental diabetes and determination of serum glucose level

The experimental rats underwent fasting for 12 h prior to alloxan administration. Freshly prepared alloxan in phosphate buffer solution (PBS), pH=7.4 was injected intraperitoneally at a dose of 120 mg/kg of body weight. After 48 h, the animals showing serum glucose level above 300 mg/dL were considered as diabetic and selected for the study. Blood samples from the experimental rats were collected by retro-orbital plexus technique using heparinised capillary glass tubes. The collected blood samples were placed in Eppendorf tubes (Tarsons Micro Centrifuge Tube 2 mL, Cat. No. 500020). The serum was separated by centrifugation at 4 °C and run at speed of 7000 rpm for 15 min using Eppendorf high speed cooling centrifuge (model no. 5810, Germany). Ten microliters of serum and 1 mL of working reagent (GOD/ POD) were mixed and incubated for 15 min at 37 °C. The UV–vis spectrophotometer (Jasoe V–530, Japan) reading was adjusted to 0 by measuring the absorbance of blank with distilled water. The absorbance of sample and standard provided by manufacturer (Accurex Biomedical Pvt. Ltd., Mumbai, India) were measured against blank at 505 nm. Glucose concentration was estimated by using the formula:

$$Glucose (mg/dL) = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 100$$

2.6. Effect of EELU on serum glucose level and body weight in diabetic rats

The selected diabetic (alloxan treated) and nondiabetic (vehicle treated) rats were divided into five groups (n=6) viz; Group I– Nondiabetic (vehicle treated, 10 mL/kg p.o.), Group II– Diabetic (alloxan 120 mg/kg, i.p.), Group III– Diabetic +
EELU (200 mg/kg), Group IV– Diabetic + EELU (400 mg/kg), and Group V– Diabetic + glibenclamide (5 mg/kg) were given orally.

Acute study involved determination of serum glucose level at 0, 1, 2, 4, 6, 8 and 24 h after EELU and glibenclamide administration. Subacute study involved repeated administration of EELU and glibenclamide for 21 days (once a day) at predetermined time and serum glucose level were determined in samples withdrawn after 6th h of EELU and glibenclamide administration on 7th, 14th and 21st day. At the end of 21st day the EELU and glibenclamide administration were stopped and a rest period of 7 days was given to the animals. Serum glucose level was determined on 28th day. During the study period of 28 days, the rats were weighed daily and their body weights were recorded. From this data, mean change in body weight and SEM were calculated. The data was represented as mean serum glucose level ± standard error of mean (SEM).

2.7. Effect of EELU on oral glucose tolerance test (OGTT) in diabetic rats

The oral glucose tolerance test was performed in diabetic rats. Animals were divided into four groups, each consisting of six rats. Group I– Diabetic (vehicle treated); Group II– glibenclamide (5 mg/kg); Group III– EELU 200 mg/kg and Group IV– EELU 400 mg/kg, respectively. D-glucose (2.5 g/kg, p.o.) was administered in all 4 groups at 6th h after pretreatment with respective drugs. Serum glucose level were determined before and 2nd h after glucose loading[11]. The data was represented as mean serum glucose level and standard error of mean (SEM).

2.8. Isolation of PBMNCs from rat blood

On the last day of experiment 3 mL blood were collected from Wistar male rat on EDTA coated vacutainers (Becton & Dickinson India Pvt. Ltd., Gurgaon, India) before decapitation and were carefully layered on a double gradient of Ficoll Paque Plus (Histopaque) solution (Sigma Diagnostics, Cat. No. 1077, St. Louis, MO, USA) with equal volumes[12]. After centrifugation (30 min, 3000 rpm, 4 °C), the MNCs layers (found at the interface between plasma and Ficoll Paque Plus solution) were collected. Residual erythrocytes were lysed by hemolytic shock using (1 ×) FACS lysing solution (Cat. No. 349202, Becton & Dickinson, San Diego CA, USA). PBMNCs were then washed twice with PBS (pH=7.4), centrifuged and resuspended in 1 mL of Hanks balanced salt solution (HBSS). For the flow cytometry assay PBMNCs were then washed with PBS and adjusted to 106 cells/mL with HBSS.

2.9. ROS production in PBMNCs by H2DCFDA assay using flow cytometry

ROS production was quantified by the H2DCFDA method according to Lawler et al[13], based on the ROS–dependent oxidation of DCFH–DA to DCF according to the method described elsewhere[14]. Briefly MNCs (10^6/mL) were preincubated for 15 min with 10 μL of 10 mmol H2DCFDA in a dark condition. H2DCFDA diffuses into cells and is hydrolyzed into nonfluorescent 2′-7′-dichlorofluorescin (DCF). DCF fluorescence was assayed at 530 nm after excitation of cells at 488 nm. Acquisition and analysis of the processed samples was performed on flow cytometer by using CELL Quest software (Becton & Dickinson, San Diego CA, USA).

2.10. Effect of EELU on endogenous antioxidant enzymes in pancreas

Pancreas from rats were isolated and weighed. Pancreas sample from animals were cut into small pieces, placed in chilled 0.25 mol sucrose solution and blotted on a filter paper. The tissues were then homogenized in 10% chilled tris hydrochloride buffer (10 mmol, pH 7.4) by tissue homogenizer (Remi Motors, Mumbai, India 400 058) and centrifuged at 7500 rpm for 15 min at 0 °C using Eppendorf 5810–R high speed cooling centrifuge. The clear supernatant was used for the assays of lipid peroxidation (MDA content), endogenous anti-oxidant enzymes superoxide dismutase (SOD), reduced glutathione (GSH) and total protein. SOD and GSH was determined by the method of Misera and Fridovich[15] and Moron et al[16]. Lipid peroxidation or malondialdehyde (MDA) formation was estimated by the method of Slater and Sawyer[17].

Nitrile was estimated in the pancreatic homogenate using the Greiss reagent and served as an indicator of nitric oxide production. A measure of 500 μL of Greiss reagent (1:1 solution of sulphanilamide in 5% phosphoric acid and 0.1% naphthylamine diamine dihydrochloric acid in water) was added to 100 μL of pancreatic homogenate and absorbance was measured at 546 nm by the method of Green et al[18]. Nitrite concentration was measured using a standard curve for sodium nitrite. Nitrite levels were expressed as μg/mL.

2.11. Statistical analysis

Data was expressed as mean±SEM and statistical analysis was carried out by One–way ANOVA with post hoc Tukey’s test for antioxidant enzymes and H2DCFDA fluorescence data and Two–way ANOVA followed by post hoc Bonferroni tests for BSL and change in body weight data. Analysis was performed using GraphPad InStat version 5.00 for Windows VistaTM BASIC, GraphPad Software, San Diego California USA. Flow cytometric analysis was performed using CELL Quest software (Becton & Dickinson, San Diego CA, USA). P value was considered significant when less than 0.05.

3. Results

3.1. Effect of EELU on acute and subacute serum glucose level

Single dose administration of EELU 200 mg/kg, p.o. significantly reduced serum glucose level at 2nd (P<0.01), 4th (P<0.001) and 6th (P<0.001) h, whereas EELU 400 mg/kg, p.o. showed significant (P<0.001) reduction in serum glucose level from 4th to 8th h. The onset of antihyperglycaemic effect of EELU 200 and 400 mg/kg was observed at 4th h; peak effect at 6th h whereas, the antihyperglycaemic effect waned at 24th h. The onset of glibenclamide (5 mg/kg) was observed at 2nd h; peak effect at 6th h. antihyperglycaemic effect of glibenclamide (5 mg/kg) was waned at 24th h (Figure 1). In the chronic study, repeated administration of EELU...
200 mg/kg body weight ($P<0.05$, $P<0.001$, $P<0.001$, $P<0.001$) and 400 mg/kg body weight ($P<0.001$) once a day for 21 days showed significant reduction in the serum glucose level on day 7, 14, 21, and 28 respectively when compared to alloxan treated diabetic group (Figure 2).

3.2. Effect of EELU on change in body weight

Alloxan treated diabetic rat shows significant ($P<0.01$, $P<0.001$, $P<0.001$) decrease in body weight on day 7, 14 and 21 respectively when compared with nondiabetic group. EELU treatment at 200 mg/kg/body weight ($P<0.01$, $P<0.001$) and 400 mg/kg/body weight ($P<0.001$, $P<0.001$) doses given to alloxan induced diabetic rats caused significant increase in body weight on day 21st and 28th respectively when compared to diabetic group. There was significant ($P<0.05$, $P<0.001$, $P<0.001$) increase on day 14, 21 and 28 in body weight of diabetic rat, whereas EELU 200 mg/kg showed nonsignificant decrease in body weight (Figure 3).

3.3. Effect of EELU on oral glucose tolerance test (OGTT)

In OGTT, EELU (200 and 400 mg/kg body weight) and glibenclamide (5 mg/kg body weight) administration produced significant ($P<0.01$, $P<0.001$, $P<0.001$) increase in glucose utilization at 6th h in diabetic rats respectively (Table 1).

3.4. Effect of EELU on ROS production in PBMNCs by H2DCFDA method using flow cytometry

Intracellular ROS was measured with 2, 7–dichlorofluorescein diacetate by triple–color analysis using CELL Quest software on flow cytometry. Alloxan induced diabetic rat showed significantly enhanced intracellular levels of ROS in the form of H2DCFDA fluorescence intensity ($P<0.001$). Treatment of EELU 200 and 400 mg/kg shows significant restoration of H2DCFDA fluorescence intensity ($P<0.001$) when compared with diabetic group.

3.5. Effect of EELU on endogenous antioxidant enzymes in pancreas

The pancreatic tissue MDA content was significantly elevated in the diabetic rats after induction of hyperglycemia by alloxan (120 mg/kg) compared to the nondiabetic group ($P<0.001$). Whereas treatment of EELU 200 and 400 mg/kg showed significant ($P<0.01$, $P<0.001$) decrease in MDA content respectively. Pancreatic tissue GSH and SOD content were significantly ($P<0.001$, $P<0.001$) higher in diabetic group when compared with nondiabetic group. A significant restoration was observed after EELU (200 and 400 mg/kg) treatment in the level of GSH ($P<0.05$, $P<0.01$) respectively, on the other hand pancreatic SOD was significantly ($P<0.05$) restored after the treatment of EELU 400 mg/kg.

### Table 1
Effect of EELU on OGTT in alloxan induced diabetic rat.

| Groups          | GSH (µg/mg protein) | MDA (nmol/mg protein) | SOD (unit/mg protein) | Nitrite (µg/mL) |
|-----------------|---------------------|-----------------------|-----------------------|-----------------|
| Diabetic        | 24.6±1.15           | 2.97±0.16             | 13.48±1.58            | 119.74±26.63    |
| EELU 200 mg/kg  | 16.7±0.80           | 4.86±0.24             | 6.10±0.48             | 362.58±60.40    |
| EELU 400 mg/kg  | 21.1±1.10           | 3.62±0.12             | 7.95±0.79             | 219.04±28.34    |
| EELU 400 mg/kg  | 22.96±1.17          | 3.03±0.16             | 10.25±0.44            | 185.62±19.98    |

Values are mean±SEM, ($n=6$) in each group; * $P<0.05$, ** $P<0.01$, *** $P<0.001$, ns—non significant vs. diabetic group.

### Table 2
Effect of EELU on pancreatic GSH, MDA, SOD and nitrite levels in alloxan induced diabetic rat.

| Group          | GSH (µg/mg protein) | MDA (nmol/mg protein) | SOD (unit/mg protein) | Nitrite (µg/mL) |
|----------------|---------------------|-----------------------|-----------------------|-----------------|
| Diabetic        | 23.46±1.5           | 2.97±0.16             | 13.48±1.58            | 119.74±26.63    |
| EELU 200 mg/kg  | 16.7±0.80           | 4.86±0.24             | 6.10±0.48             | 362.58±60.40    |
| EELU 400 mg/kg  | 21.1±1.10           | 3.62±0.12             | 7.95±0.79             | 219.04±28.34    |
| EELU 400 mg/kg  | 22.96±1.17          | 3.03±0.16             | 10.25±0.44            | 185.62±19.98    |

Values are mean±SEM, ($n=6$) in each group; * $P<0.05$, ** $P<0.01$, *** $P<0.001$, ns—non significant vs. diabetic group.
Nitrite levels were significantly ($P<0.01$) increased in the diabetic pancreas compared to the nondiabetic group. EELU (200 and 400 mg/kg) treatment showed significant ($P<0.05$ each) decrease in nitrite levels as compared to the diabetic group (Table 2).

4. Discussion

Alloxan induced diabetes mellitus produce irreversible destruction of the pancreatic beta cells causing degranulation and decrease in insulin secretion. It is proposed that alloxan induced diabetogenic effect is due to excess production of ROS leading to the toxicity in pancreatic cells\[19\]. There are some evidences which indicate that the DNA of the pancreatic $\beta$-cells is primary target of ROS produced by alloxan treatment, which ultimately causes DNA strand breaks\[20\]. Increase of cytosolic Ca$^{2+}$ also plays an important role in the development of alloxan induced diabetes, in relation to ROS generation and fragmentation of DNA. It is then further responsible for disturbances in synthesis and release of insulin as well as affecting the other organs like liver, kidney, and hematopoietic system\[21\].

The present investigation demonstrated that the EELU (p.o.) had an antihyperglycemic potential associated with ROS preventing ability in pancreatic tissue as well as PBMCs in alloxan induced diabetic rats. Study showed that, alloxan injection (120 mg/kg i.p.) produced significant hyperglycemia in all the animals. This result is consistent with earliar studies\[22-31\]. EELU 400 mg/kg showed peak antihyperglycemic effect at 6th h indicating a lag period of 5 to 6 h before the peak effect was reached. The subacute study showed that a period of two weeks is required for attaining a steady state concentration of EELU in the blood to reveal antihyperglycemic and antioxidant effect. Glibencamide has reported as a potent, second generation, oral sulfonylureas antidiabetic agent. The hypoglycemic action of glibencamide is due to stimulation of pancreatic islets cells, which results in an increase in insulin secretion\[32\]. Results showed that onset of action of glibencamide is short and duration of action is about 6 h. The subacute treatment with glibencamide was effective in reducing blood sugar after 7 days of treatment and thereafter. Subacute treatment for 28 days with the EELU and glibencamide brought significant improvement in body weights of alloxan treated diabetic rats indicating its beneficial effect in preventing loss of body weight in diabetic condition. Potential of EELU to protect against body weight loss seems to be due to its ability to reduce hyperglycemic condition.

The immune system is especially vulnerable to oxidative damage, because many immune cells, such as polymorphonuclear cells and mononuclear cells produce ROS as part of defense mechanism of body to destroy invading xenobiotics and pathogens. It is reported that blood cells can be collected and utilized conveniently to evaluate the status of oxidative stress and anti-oxidative action\[33\]. PBMCNs were found to be more sensitive than neutrophils in oxidative damage induced by intense exercise and useful tool as marker reflecting the systemic symptoms of oxidative stress under physical and mental stimulation\[34\]. The present investigation showed increased ROS generation in PBMCNs of alloxan treated diabetic rat, which may be because of the increased utilization of these antioxidant enzymes to counteract the ROS generated by alloxan.

Induction of alloxan induced diabetes in rat results in increasing oxidative stress biomarkers. Lipid peroxidation measured in the form of MDA levels (an indicator of lipid peroxidation) and nitrite levels were significantly higher in pancreatic homogenate from diabetic rats in comparison to EELU group. These results were consistent with the previous reports\[35,36\] where MDA was reported to be a marker of lipid peroxidation in alloxan induced diabetic cells. In contrast, EELU treatment significantly decreased MDA and nitrite levels in pancreatic homogenate. Enhanced level of ROS and nitric oxide is known to sensitize pancreatic cells. Moreover, unfeathered production of nitric oxide coupled with deficient superoxide dismutase leads to the production of notorious peroxynitrite, which is several times multiple of its parent\[37\]. In the present study EELU treatment significantly decreased GSH and SOD levels.

Our findings are in agreement with other studies which reported decreased levels of lipid peroxidation in pancreas of diabetic rats after treatment with SDG isolated from flaxseed\[6\]. Particularly in pancreatic cells, increased stress level in the form of lipid peroxidation, nitrite, SOD and GSH can alter the structure of cell membrane lipids and compromising the cell viability. Our investigation indicated that EELU possessed antihyperglycemic activity as well as protective effect against lipid peroxidation and antioxidant reserve.

Based on the oxidative stress hypothesis of alloxan action, it was considered as an adequate model for investigating the role of free radicals in the pathology of hyperglycemia and diabetes mellitus. Our results give support to the traditional use of EELU as an antidiabetic herbal medicine. The antihyperglycemic activity of EELU may be due to inhibition of ROS generation in PBMCs as well as by peroxidative damage in pancreatic tissues, thereby preserving pancreatic $\beta$ cell function. The present study demonstrated that EELU, a potent antioxidant, may offer a promising natural and safe new treatment for diabetes.

Conflict of interest statement

We declare that we have no conflict of interest.

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