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Antihyperglycemic Effect of *Annona squamosa* Leaf and Oleanolic Acid Combination in Diabetic Albino Rats

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Abstract

Since form ancient time, plant derived product has been used as holistic approach in the control of diabetes mellitus. The object of the study is evaluation of the synergistic anti-hyperglycemic effect of medicinal plant *Annona squamosa* leaf extract (ASLE) and plant-derived oleanolic acid (OA) in streptozotocin (STZ)-induced diabetic rats. Thirty six number of Wistar rats are taken and divided within six groups (n = 6). The group 1 is normal control, group 2 is diabetic control induced with STZ, groups 3, 4, 5, and 6 treated with 50 mg/kg of ASLE, 50 mg/kg of OA, the combination of both 25 mg/kg of ASLE and 25 mg/kg of OA, and 0.1 mg/kg of glimepiride respectively for 28 days. The parameters like body weight, blood glucose, insulin, glycosylated hemoglobin, C-peptide, serum biochemical, lipid profiles and liver antioxidant parameters were evaluated. Results shows significant (p < 0.05) restoration of body weight, insulin and C-peptide levels. The animal groups treated with the combination of both ALSE and OA have experienced a remarkable restoration of serum biochemical, lipid profiles, antioxidant parameters, and C-peptide level as compared to the above groups due to the synergistic effect. These findings conclude that combination of both ASLE and OA had a synergistic action and it shows more potent anti-hyperglycemic effect.

Keywords: Oleanolic acid, *Annona squamosa*, streptozotocin, diabetes, C-peptide, antioxidant.

Introduction

The insufficient release of insulin hormone from the β-cells of the pancreas is one of the principal contributing patho-physiology factors in the disposition of hyperglycemic disorder or type-2 diabetes mellitus (1). In addition, increased oxidative impact and lipid peroxidation play a vital role in the occurrence of diabetes conditions. In this regard, extensive investigations have been performed using plants parts or plant-derived products for the control of diabetes mellitus and its related complications. The plant-derived products like flavonoids and triterpenoids like oleanolic acid have shown significant effects in control of pathological involvement of diabetic complications (2). Further, the literature cited the improved beneficial effects of the combination of two or more plant-derived plant products in the management of hyperglycemic conditions either by modulating insulin secretion or preventing insulin resistance (3). Based on the literature review, two agents, one medicinal plant *Annona squamosa* leaf and another natural product oleanolic acid (OA) were

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combined to perform the synergistic evaluation of their hypoglycemic effect in streptozotocin (STZ) induced diabetic rats.

Annona squamosa Linn. Generally called as custard apple, belongs to the family Annonaceae, and is distributed in the tropical as well as in the Eastern region of India. The whole plant including the leaves, bark, roots, and fruits, has a wide span of medicinal and nutritional effects, as they carry a significant amount of vitamin C, thiamine, riboflavin, and nutrients like amino acids, potassium, calcium, and dietary fibers. The leaf extract possesses different pharmacological effects like antioxidant, antitumor, anti-malarial, wound healing, hepato-protective and anti-diabetic activity. The phytochemical investigation of leaf extract of *A. squamosa* revealed the presence of annonaine alkaloid, flavonoids and acetogenins which possess significant anti-diabetic activity by maintaining the plasma insulin level, lipid profiles and decreasing the blood glucose (4-6).

Oleanolic acid (OA) is a pentacyclic triterpenoid, a plant-produced bio-active constituent originating mainly from the leaves and fruits of *Olea europaea* L (2). OA shows a potential biological role in the management of diabetes, neurological disorders, anti-inflammatory, antioxidant, lipid-lowering effect, antimicrobial, hepatoprotective and antiatherosclerotic activity (2, 7-9). It suggests that both natural products have anti-diabetic properties. However, the combination of both of these forms has not been studied to date. Therefore, the aim of this experiment is to envisage the synergetic anti-diabetic potential of the combination form of *A. squamosa* leaf and OA in the STZ induced hypoglycemic rat model.

Materials and Methods

Drugs and chemicals

Oleanolic acid and ST were procured from Sigma-Aldrich, USA. The rest of the solvents and chemicals used for the experiment belonged to the analytical grade obtained commercially.

Collection of plant material and extract preparation

Mature leaves of A. squamosa plant were gathered from the local areas near Chinsurah town, Hooghly district, West Bengal, India in October-November 2021 and verified by Dr. Pratap Chandra Panda, Taxonomist, Centre of Biotechnology, Bhubaneswar, Odisha with a voucher number 2247 The leaf extract was prepared by the Soxhlet extraction method. A. squamosa leaves were washed and shade dried for about one week at room temperature. Theseleaves were crushed into a powder of fine particles with the help of a mixer grinder. The leaf powder was weighed and extracted using Methanol as the solvent. The solvent was then evaporated under lower pressure at room temperature to find an extract of reddish-brown color. The obtained leaf extract (ASLE) was kept at a temperature of 2-8ºC for further use.

Experimental animals

Albino Wistar rats of 150–200 g weight were employed in this experiment after getting approval from Institutional Animal Ethical Committee (IAEC), Centurion University, Bhubaneshwar, India with approval no. 111. The experimental rats were classified into six groups (n = 6) and kept in polypropylene cages at the temperature of 25°C ± 2°C with 55% of relative humidity in 12 h of the light/dark cycle. During this experiment, the experimental rats were provided with the food pellet with water *ad libitum*. Before this experiment, these selected rats were reserved for one week to acclimatize to the identical laboratory situations.

Experimental protocol

The experimental rats were kept fasting overnight prior to the induction of diabetes. Freshly prepared STZ (0.05 M in citrate buffer solution, pH 4.5) at the dose of 65 mg/kg body weight was administered intraperitoneally to the experimental animals for the induction of diabetes. The initiation of diabetes was examined by measuring the blood glucose concentrations

(≥225 mg/dl) later 72 h of administration of STZ were regarded as diabetic (10). Then the rats were classified to the following six groups and treated for the next 28 days. Group 1 was normal control supplied with normal (0.9% w/v) saline. Group 2 was diabetes control induced with single dose of STZ. Groups 3, 4, 5 and 6 were treated with ASLE - 50 mg/kg body weight, OA - 50 mg/kg of body weight, ASLE - 25 + OA 25 mg/kg of body weight and glimepiride - 0.1 mg/kg body weight (reference) respectively after induction with STZ.

Determinations

The body weight was noted regularly with the use of an electronic weighing machine before the initiation till the end of the treatments. Blood glucose was monitored with a glucometer at 0, 7, 14, 21, 28 days. On cessation of the experiment, the rats were kept fasted overnight and sacrificed by cervical disruption on the 29th day. The blood was assembled with or without anticoagulants to isolate the portion of serum and plasma respectively for evaluation of serum insulin, C-peptide, glycosylated hemoglobin, serum biochemical parameters such as serum glutamate-pyruvate transaminase (SGPT)/alanine aminotransferase (ALT), serum glutamate-oxaloacetate transaminase (SGOT)/aspartate aminotransferase (AST), serum alkaline phosphatase (ALP), serum lipid parameters. The animals were then immolated by cervical dislocation for the estimation of liver antioxidative biochemical parameters viz. lipid peroxidation i.e., malondialdehyde (MDA), reduced glutathione (GSH), glutathione peroxidase (GSH-Px), catalase (CAT) and superoxide dismutase (SOD). All biochemical determinations were performed by employing commercially procured reagent kits (Span Divergent Ltd., Surat, India).

Statistical analysis

The data were studied by one-way ANOVA (SPSS ver. 19.0), which was followed by Dunnett's multiple tests of comparison and expressed as mean ± SEM. The $p < 0.05$ was considered as statistically significant.

Results and Discussion

Effect on body weight

The changes in body weight from initial to final body weight of ASLE alone, OA alone, and the combination of both ASLE and OA was exhibited remarkably less weight gain contrasted to the normal control animals of group 1. The groups 3 and 4 have shown significant gain in weight ($p < 0.05$) in contrast to the diabetic control animals. Additionally, diabetic rats treated with the combination of both ASLE and OA (group 5) exhibited significantly improved weight gain ($p < 0.05$) due to the synergistic effect similar to standard group 6 (Table 1).

Table 1: Effect of ASLE and OA on body weight in STZ-induced hyperglycemic rats

Treatment Groups	Initial body weight (Mean ± SEM)	Final body weight (Mean ± SEM)
Group 1	158.3 ± 4.3	169.3 ± 5.1
Group 2	162.4 ± 5.6	156.0 ± 4.1
Group 3	164.3 ± 4.9	175.2 ± 5.9
Group 4	171.9 ± 6.3	178.5 ± 7.2
Group 5	160.6 ± 5.7	179.8 ± 8.3
Group 6	159.2 ± 4.1	167.2 ± 4.5

Each value is expressed as mean ± SEM ($n = 6$). *$p < 0.05$ compared with normal control and **$p < 0.05$ compared with STZ control group (group 2).

Effects on blood glucose level

The blood glucose level measured in normal control and treated groups on 0, 7, 14, 21, 28 days of the experiment and found to be remarkable ($p < 0.05$) increase in blood glucose in STZ-treated group in comparison to group 1. Nevertheless, the blood glucose level declined significantly ($p < 0.05$) in groups 3 and 4 animals. Simultaneously, due to the combined effect of ASLE and OA, animals of group 5 experienced a sharp fall in blood glucose ($p < 0.05$) similar to that of group 6 animals treated with standard drugs (Figure 1).

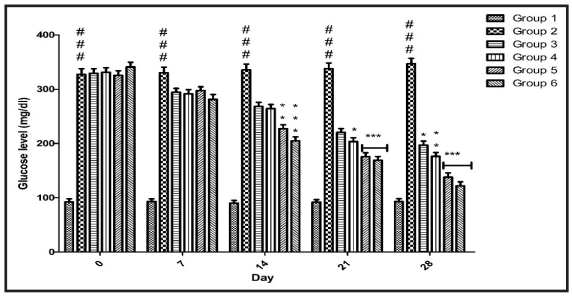


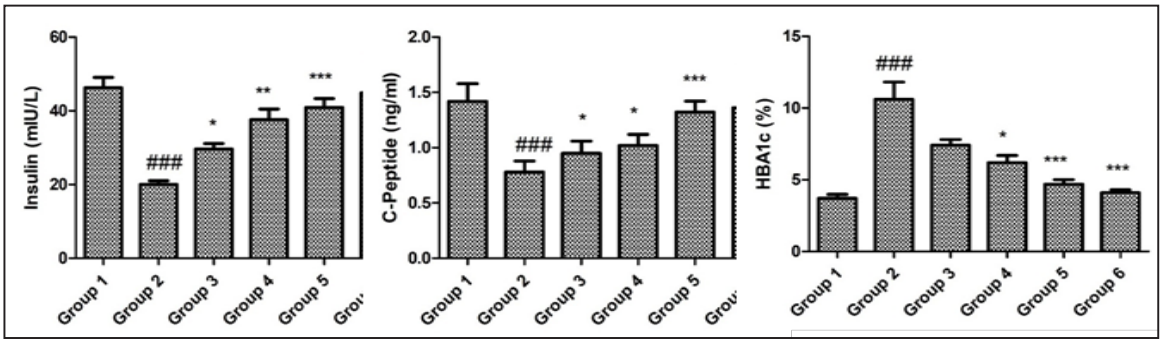
Figure 1. Mean blood glucose levels. Each value is expressed as mean ± SEM (n = 6). The values signed with '###' represents the remarkable increase in blood glucose level (P < 0.05) in group 2 treated with STZ whereas '***' represents the sharp fall in blood glucose (p < 0.05) in group-5 (combination of ASLE and OA) similar to that of group 6 animals treated with reference drug.

Effect on serum insulin and C-peptide

Result showed that both serum insulin and C-peptide in diabetes control group 2 decreases significantly in contrast to the normal control group 1 (p < 0.05). After administration of ASLE and OA, the serum insulin and C-peptide were increased significantly when compared with diabetic rats (p < 0.05). Similarly, the animals of group 5 treated with the combination of both ASLE and OA exhibited a significant rise in serum insulin and C-peptide (p < 0.05), which could be due to the synergistic effect (Figure 2A and 2B).

Effect on glycosylated hemoglobin levels

The result shows significant increased level of glycosylated hemoglobin in hyperglycemic rats. However, the value was reduced significantly (p < 0.05) in groups 1, 3, and 4 animals and more remarkably declined (p < 0.05) in group 5 animals receiving the combination of ASLE and OA similar to that of group 6 animals (Figure 2C).



2.A. Serum insulin levels 2.B. C-peptide levels 2.C. Glycosylated hemoglobin percentages

Figure 2. Each value is expressed as mean ± SEM (n = 6). The values signed with '###' represents the significant level (p < 0.05) in group-2 treated with STZ whereas '***' represents the significant level (p < 0.05) in group 5 (combination of ALSE and OA) similar to that of group 6 animals treated with reference drug.

Effect on serum biochemical parameters

The result indicated a significant (p < 0.05) elevated level of serum AST, ALT, and ALP in diabetic control group contrasted to normal control (group 1). The diabetic rats administered with groups 3 and 4 exhibited a significant (p < 0.05) decrease in AST, ALT and ALP in contrast to the diabetic control animals. Moreover, rats treated with the combination of both ALSE and OA in group 5 exhibited considerable (p < 0.05) lowering of AST, ALT and ALP in comparison to reference group 6 might be due to the synergistic effect (Figure 3). Similarly, the effects of ASLE, OA, and a combination of both on serum lipids profiles level show increased level. of TC, TG, LDL-C and VLDL-C but a reduced level of HDL-C in group 2 when compared to

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normal control ($p < 0.05$). The high levels of TC, TG and LDL-C with decreased HDL-C levels are the collective dyslipedemic symptoms of diabetes in STZ induced rats. The diabetic rats administered with ASLE and OA demonstrated significant ($p < 0.05$) fall in serum TC, TG, LDL-C and VLDL-C but an increase in HDL-C contrast to the diabetic control animals. Moreover, rats treated with the combination of both ASLE and OA exhibited a significant lowering of serum TG, TC, LDL-C and VLDL-C ($p < 0.05$) and an elevation in HDL-C, which could be due to the synergistic effect of the extracts and OA (Figure 4).

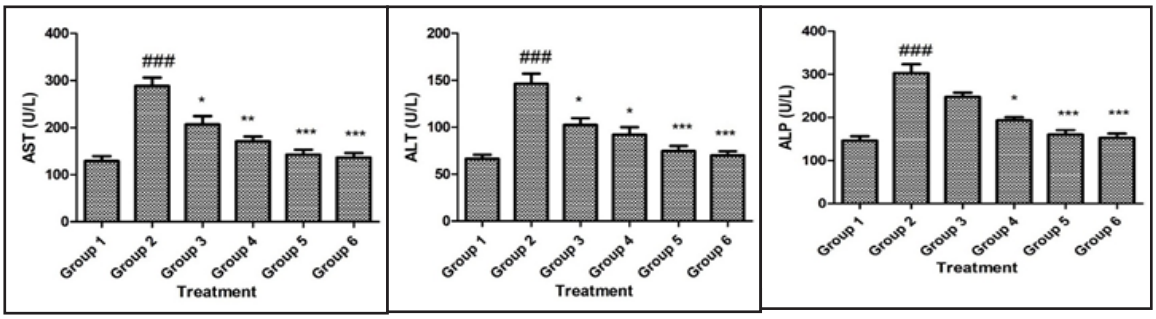

Figure 3. Serum biochemical parameters. Each value is expressed as mean ± SEM ($n = 6$). The values signed with '###' represents the remarkable rise in level of serum AST, ALT, and ALP ($p < 0.05$) in group 2 treated with STZ whereas '***' represents the sharp fall in serum AST, ALT and ALP levels ($p < 0.05$) in group 5 (combination of ASLE and OA) similar to that of group 6 animals treated with reference drug.

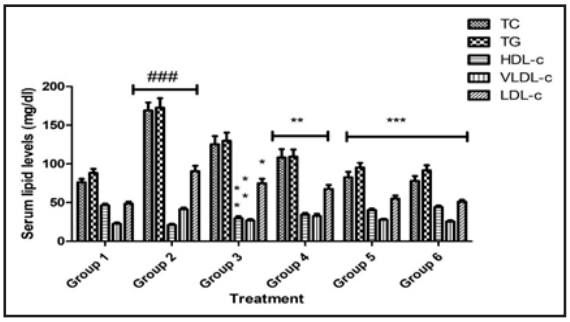

Figure 4. Serum lipid parameters. Each value is expressed as mean ± SEM ($n = 6$). The values signed with '###' represents the remarkable rise in level of serum lipid parameters such as TC, TG, LDL-C, and VLDL-C but reduced level of HDL-C ($p < 0.05$) in group 2 treated with STZ whereas '***' represents the sharp fall in serum lipid profiles ($p < 0.05$) in group 5 (combination of ASLE and OA) similar to that of group 6 animals treated with reference drug.

Effect on liver biochemical parameters

Lipid peroxidation in the pancreas because of diabetes was determined by MDA formation. The hypoglycemic rats of group 2 exhibited increased MDA level ($p < 0.05$) compared to group 1. This condition was significantly altered in groups 3 and 4 treated with ASLE and OA respectively with decreased levels of MDA compared with the rats of group 2. Additionally, animals treated with the combination of both ASLE and OA in group 5 revealed a significant ($p < 0.05$) reduction in MDA formation.

The concentration of non-enzymatic antioxidants like GSH and enzymatic antioxidants like CAT, SOD and GSH-Px were reduced significantly ($p < 0.05$) in group 2, in comparison to the normal control. This finding proved that the tissues were experiencing oxidative stress. Interestingly, administration of ASLE and OA was significantly ($p < 0.05$) capable to maintain the level of all non-enzymatic and enzymatic antioxidants showing more activities of GSH, SOD, CAT and GSH-Px in groups 3 and 4 in contrast with the group 2. Simultaneously, the animals treated with the combination of both ASLE and OA in group 5 showed a prominent rise in GSH, SOD, CAT and GSH-Px concentrations (Figure

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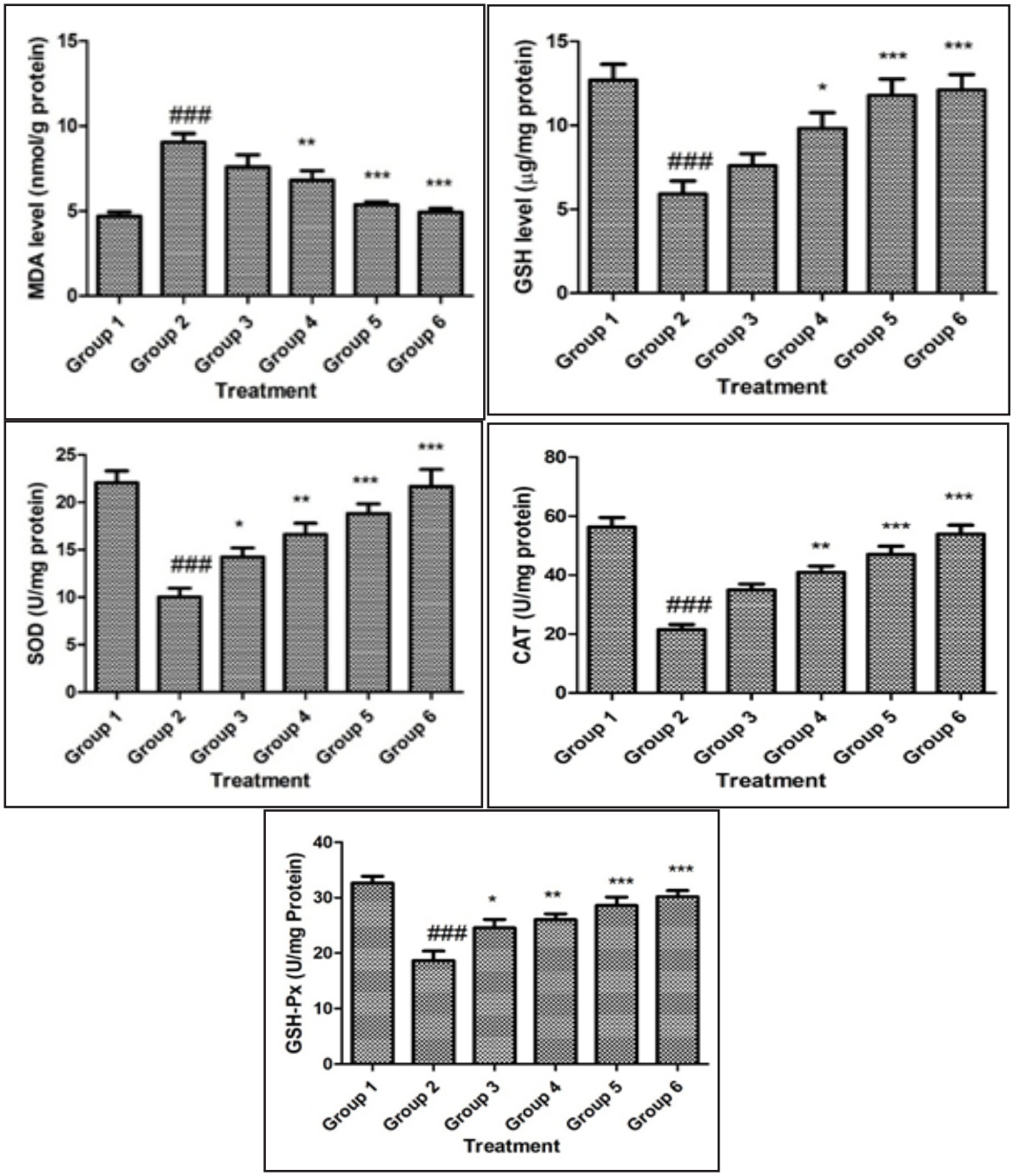

Figure 5. Liver biochemical parameters. Each value is expressed as mean ± SEM (n = 6). The values signed with '###' represents significant rise in level of liver biochemical parameters MDA and decreases in GSH, SOD, CAT, GSH-Px level ($p < 0.05$) in group 2 treated with STZ whereas '***' represents the sharp fall in MDA level and rise in GSH, SOD, CAT, and GSH-Px ($p < 0.05$) in group 5 (combination of ASLE and OA) similar to that of group 6 animals treated with reference drug.

The present series of experiments were designed to explore the hypoglycemic effect of the combined ASLE and OA in STZ induced hypoglycemic rats. The result of the present study shows that the combination of ASLE 25 mg and OA 25 mg/kg body weight restored the blood sugar, levels of C-peptide, glycosylated hemoglobin, serum and hepatic biochemical parameters towards reference values.

STZ is a nitrosourea derivative used to mimic diabetic disorders in rodents. The possible mechanism of induction of diabetes is the formation of free radicals, which leads to oxidative stress and ultimately causes damage to the β-cells of the pancreas, leading to abnormal levels of serum insulin. The hyperglycemic condition characterizes by the symptoms of loss of body weight, polyuria, and polydipsia, due to the inadequate energy released during carbohydrate breakdown (10, 11). In the current study it was found that, the decrease in the body weight in diabetic control animals, indicated gradual proteolysis caused due to disorder in the metabolism of carbohydrates. The administration of the combined ASLE and OA orally at the dose of 25 mg/kg body weight restores the body weight significantly towards the normal group as well as the standard group. The significant improvement in body weight suggests the combination ofASLE and OA could function in blocking the waste of muscles by regulating the glycemic level.

C-peptide shows insulin-mimetic activity by stimulating the receptor of insulin that promotes the synthesis of glycogen and amino acid uptake. The result showed a low level of serum insulin and C-peptide along with hyperglycemic conditions in STZ induced diabetic control animals, however, the combination of ASLE and OA at the dose of 25 mg/kg reverts these levels towards normal. It suggested that, combined treatment of ASLE and OA could be responsible for more glycogen formation or amino acid uptake or induction of insulin production by pancreatic β-cells, consequently antihyperglycemic effect in diabetic animals (11).

The glycosylated hemoglobin is a sign of ambient glycemia that is made by the non-enzymatic interaction of hemoglobin with high blood glucose (12). Current study shows an increased level of glycosylated hemoglobin in STZ induced diabetic control group, which was abridged strikingly by the combined treatment of ASLE and OA in group 5 animals and eventually reduces the level towards normal, suggests the synergistic effect of the combination of both products.

An increased level of serum biochemical parameters was found to occur in STZ-induced diabetic animals, suggesting abnormal liver functions indicating hepatic damage (13, 14). The results of present experiments show restoration of biochemical parameters like AST, ALT, and ALP as well as serum lipid profiles towards normal levels after treatment with ASLE- and OA for 28 days, which suggests the combination of therapy could protect the liver as well prevent cholesterol synthesis.

The study report cited that, STZ-induced diabetic condition induces oxidative stress significantly, leading to enhanced generation of reactive oxygen/nitrogen species (ROS/RNS) and lipid peroxidation. The aggravated lipid peroxidation in STZ induced diabetic animals represents a diminished antioxidant defense mechanism and tissue injury and which is usually measured in the MDA assay (15). Administration of a combination of ALSE and OA at the dose of 25 mg/kg body weight reverts the MDA concentration to the normal level, suggesting inhibition of oxidative stress in STZ-induced diabetic animals. Similarly, the endogenous non-enzymatic as well as enzymatic antioxidants namely GSH, GSH-Px, SOD and CAT were restored to normal levels after administration of ASLE+OA in diabetic rats, indicating that the combination of treatments might have enhanced the cellular antioxidant system.

Previous studies showed the prevalence of alkaloids, flavonoids, acetogenin, phenolic compounds in ASLE (see the introduction

section) as well as OA, a potential natural antioxidant, might be responsible for restoring the antioxidant defense mechanisms *in vivo*.

Conclusion

Medicinal plants and their constituents have a profound impact on healthcare practices.. The current investigation explored the effect of ALSE and OA on blood glucose, insulin, C-peptide, glycosylated hemoglobin, serum biochemical and lipid profiles, liver antioxidative biochemical parameters in STZ-induced diabetic animals. The results indicated that, treatment with combined ASLE and OA produced a significant hypoglycemic effect by restoring the above-mentioned parameters to the normal level. The discussion highlighted the possible mechanism of the hypoglycemic effect that might be due to the abundance of flavonoids, acetogenins, phenolic compounds. In addition, the combination of the products might act as a synergistic formulation for the hypoglycemic effect. However, the molecular mechanism is necessary to establish the potential mode responsible for the hypoglycemic action.

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Conflict of interest

The authors declare that there is no conflict of interest.

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