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ORIGINAL ARTICLE

Novel pentacyclic triterpene isolated from seeds of *Euryale Ferox Salisb.* ameliorates diabetes in streptozotocin induced diabetic rats

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ABSTRACT

The present research was carried out to study the effect of 2β-hydroxybetulinic acid 3β-oleiate (HBAO), a novel compound isolated from the seeds of *Euryale ferox salisb.* on glycemic control, antioxidant status and histopathological morphological alterations in the liver, pancreas, kidney and heart in streptozotocin induced type-2 diabetes in rats. HBAO was isolated from the seeds of *Euryale ferox salisb.* according to Lee. Isolation of the active principle HBAO was performed for the first time. To date there are no reports on the isolation and evaluation of 2β-hydroxybetulinic acid 3β-oleiate (HBAO) from *Euryale ferox salisb.* Assessment of different biochemical parameters like the effect of HBAO on glycemic control, plasma insulin, glycosylated hemoglobin, hepatic glucose-6-phosphate dehydrogenase, glucose-6-phosphatase and fructose-1-6-biphosphatase, hepatic hexokinase, lipid profile, antioxidant marker and histopathology of pancreas, liver and kidney examination was done at the end of the experimentation, i.e. on day 45. HBAO exhibited remarkable improvement in glycemic control, lipid levels, plasma insulin, glycogenic liver enzymes and antioxidant activity in diabetic rats, along with progressive enhancement of distortive histopathological morphology of liver, pancreas and kidney. The results strongly suggest that HBAO could be a potential therapeutic agent in diabetes.

KEY WORDS: Diabetes; *Euryale ferox salisb.*; HBAO

ABBREVIATIONS:

ANOVA: Analysis of Variance, **CAT:** Catalase, **GPx:** Glutathione Peroxidase, **HBAO:** 2β-hydroxybetulinic acid 3β-oleiate, **HDL:** High Density Lipoprotein, **HE:** Hematoxylin and Eosin, **LDL:** Low Density Lipoprotein, **RNS:** Reactive Nitrogen Species, **ROS:** Reactive Oxygen Species, **SOD:** Superoxide dismutase, **STZ:** Streptozotocin, **T2DM:** Type-2 Diabetes Mellitus, **VLDL:** Very Low Density Lipoprotein

Introduction

The prevalence of diabetes is increasing rapidly throughout the world. According to the International Diabetes Federation (IDF) 2015, it was estimated that there were 415 million adult diabetic patients worldwide (IDF Atlas, 2015). The country specific estimates showed India at number 2 position in terms of highest number of people (99.2 million) with diabetes after China with 109.6 million (IDF Atlas, 2015). Multiple pathophysiological defects are currently recognized in type-2 diabetes mellitus (T2DM): insulin resistance, impaired insulin secretion, impaired glucagon suppression, increased lipolysis, exaggerated hepatic glucose production, incretin deficiency, maladaptive renal glucose reabsorption and defects of central nervous system, which may include impaired dopaminergic tone and dysregulation of satiety (King *et al.,* 2012; Suraamornkul *et al.,* 2010; Yoon *et al.,* 2003). The regulation of blood glucose is complex and involves factors affecting the digestion and absorption of dietary

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carbohydrates, the regulation of hepatic glucose uptake and production, and the effectiveness of insulin to stimulate glucose uptake in insulin sensitive tissues, particularly skeletal muscle and adipose tissues. Type-2 diabetes is most commonly associated with obesity and insulin resistance. While the cause of insulin resistance is not fully understood, both genetic and environmental factors play a contributing role. Metabolic factors include intra-abdominal obesity, increased hepatic triglyceride content and increased plasma free fatty acid concentrations (Poretsky, 2010).

Free radicals are atoms or group of atoms that contain an unpaired electron in their valance shell. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are chemically reactive metabolites classified as free radicals owing to the presence of one unpaired electron in an oxygen atom and a nitrogen atom respectively (Maritim *et al.,* 2003). Larger amounts of NADH and FADH are produced in hyperglycemic conditions owing to glucose autoxidation. Diabetes not only increases the rate of ROS production but also lowers cellular anti-oxidant capacity. Earlier researchers demonstrated impaired antioxidant capacity in brains of streptozotocin-diabetic rats (Thannickal & Fanburg, 2000). There is a wealth of evidence to suggest that oxidative stress plays a key role in the development and progression of diabetic complications such as nephropathy, retinopathy, neuropathy, silent myocardial ischemia, or coronary artery disease.

Different in vitro and animal studies have shown that dietary antioxidants have beneficial effects on glucose metabolism and can help to prevent diabetes or related disorders (Ahmed *et al.,* 2015[a]). Such studies have attracted the attention of many researchers to develop and identify potential antioxidants. Several Indian medicinal plants are considered to have great antioxidant potential.

Euryale ferox salisb. is a perennial plant of the Nymphaeaceae family, which is native to eastern Asia. It is found from India to Korea and Japan (Ahmed *et al.,* 2015[b]). The seeds of *Euryale ferox salisb.* are considered for the treatment of circulatory, respiratory, digestive, excretory and respiratory system disorders (Ahmed *et al.,* 2015[b]). Some important research work has shown the beneficial properties of seeds of *Euryale ferox salisb.,* such as tonic, astringent properties, expectorant, emetic, cardiac stimulant and immune stimulant properties (Ahmed *et al.,* 2015[b]). *Euryale ferox* has been used as a crucial cash crop and as a valuable nutritive tonic in ancient medication for hundreds of years (Rai *et al.,* 2002). Eurayle ferox seed exhibited positive potency in trating spermatorrhea, diarrhea, xerostomia and polydipsia. A review of several literature data has confirmed that the alcoholic as well as aqueous extract of Eurayle ferox holds antioxidant activity (Sun *et al.,* 2011).

In our previous research work (Ahmed *et al.,* 2015[b]) we isolated another novel molecule 2β-hydroxybetulinic acid 3β-caprylate (HBAC) from *Euryale ferox salisb.* which depicted a prominent activity against distorted glycemic control, lipid level, hepatic gluconeogenic enzymes and corrected the deformed histopathological alterations in liver, pancreas and kidney. This directed us to isolate another active principle from seeds of *Euryale ferox salis.*

The aim of our present research work was to isolate a novel and imperative active principle from seeds of *Euryale ferox salisb.* and to study its diabetic, antioxidant, antihyperlipidemic, as well as hepatic and pancreatic protective action.

Materials and methods

General experimental procedure

The melting point was recorded on a melting point apparatus, Veego, Model No. MPI. NMR spectra were collected on Bruker Advance II 100 NMR spectrophotometer in DMSO, utilizing TMS as internal standard. Mass spectra were recorded on VG-AUTOSPEC spectrometer. UV spectra were determined on Shimadzu double-beam 210A spectrometer. FT-IR (in 2.0 cm^{-1}, flat, smooth, Abex) were determined on Perkin Elmer – Spectrum RX-I spectrophotometer.

Chemicals and reagents

Streptozotocin (STZ) was purchased from Sigma Aldrich (MO, USA). Span Diagnostics (Surat, India) had kindly gifted us free samples of kits, namely Glucose Kit, Triglyceride Kits and Cholesterol Kit. Silica gel for column chromatography was purchased from Nicholas, India. Glibenclamide was a generous gift from Ranbaxy, Gurgaon, India. All reagents/solvents used were of analytical grade.

Plant material

Seeds of *Euryale ferox Salisb.* were collected in Allahabad district (India) in March-April 2014. With respect to voucher number: 11098/BOT/DOP/FHS/2014, seeds of *Euryale ferox salisb.* were botanically recognized by a botanist at the Department of Pharmaceutical Science, Faculty of Health Sciences, SHIATS, where the specimen voucher was deposited.

Extraction and isolation

The sun dried and ground seed powders of *Euryale ferox salisb.* (2 kg) were extracted according to Lee (Lee *et al.,* 2002). The extraction of powdered seed was done at 80 °C in 70% methanol for 3 hours. Filtration of the extract was accomplished with rotary evaporator (Buchi, India) under low pressure. The resultant residue was freeze dried at -80 °C. Then the whole extracts were extracted using separating funnel with dichloromethane, n-hexane, n-butanol and ethyl acetate in a stepwise method. The ethyl-acetate extract (27 g) was connected to a silica gel segment column chromatography and eluted with expanding measures of methanol to outfit the part containing compound 2β-hydroxybetulinic acid 3β-oleiate (HBAO). Fractions of *Euryale ferox salisb.* were collected by removing the solvents with rotary evaporator.

Compound (2β-hydroxybetulinic acid 3β- oleiate) HBAO

2β-hydroxybetulinic acid 3β-oleiate: 2β-hydroxybetulinic acid 3β-oleiate was obtained as yellow amorphous powder. $[\alpha]^{20}$-3°. IR max (KBr): 3419, 3275, 2919, 2850, 1725, 1687, 1635, 1464, 1388, 1376, 1273, 1238, 1194, 1108, 1032, 883, 720 cm^{-1}, ^{1}HNMR ($DMSO_6$):δ 5.32 (1H m1, H2-9'), 5.30 (1H, m1, H-10'), 4.68 ($1H_1$ brs, H_2-29b) 4.22 (1H, d, J=5.3 Hz., H-3α), 3.76 (1H, ddd, J=5.3, 5.5, 8.5 Hz, H-2α), 2.27 ($2H_1$, t, J=7.6 Hz., H_2-2), 1.68 (3H, brs, Me-30), 1.25 (20H, brs, 10x CH_2), 1.23 (3H, brs, Me-23), 0.92 (3H, brs, Me-24), 0.87 (3H, brs, Me-25), 0.84 (3H, t, J=6.3 Hz, Me-1'8), 0.77 (3H, brs, Me-27), 0.65 (3H, brs, Me-27), 2.96-1.30 ($29H_1$, m_1, 12xCH_2, 5xCH. ESI MS mHz (rel. int.) 736 $[M]^+$ ($C_{48}H_{80}O_5$) (12.6), 471 (57.5), 455, (71.3), 281 (8.4), 265 (8.7) (Table 1).

The compound, named 2β-hydroxybetulinic acid 3β-oleiate (HBAO) (Figure 1), showed positive test for triterpenoids and IR absorption bands for hydroxyl group (3419 cm^{-1}), ester function (1725 cm^{-1}), carboxylic group (3275, 1687 cm^{-1}), unsaturation (1635 cm^{-1}) and long aliphatic chain (720 cm^{-1}). It had molecular ion peak at mHz 736, established on the basis of mass and 13C NMR spectral data analysis which was consistent with the molecular formula of a triterpenic ester $C_{48}H_{80}O_5$. The ion fragments generated at mHz 265 $[CO(CH_2)_7$ CH=CH-$(CH_2)_7CH_3]$+, 281 $[OCO(CH_2)_7CH$=CH $(CH_2)_7CH_3]$+, 471 (M-265]+ and 455 {M-281]+ indicated that a pentacyclic triterpenoid was esterified with oleic acid. The 1HNMR spectrum of the compound displayed two one-proton multiplets at δ5.32 and 5.30, assigned to vinylic H-9' and H-10' proton respectively. Two one-proton broad singlets at δ 4.68 and 4.55 were ascribed to methylene H2-29 protons. A one-proton doublet at δ 4.22 (J=5.3 Hz) and a one-proton triplet doublet at δ3.76 (J=5.3, 5.5 8.5 Hz) were attributed to α- oriented carbinol H-2 and oxygenated methine H-3 protons respectively. A two-proton triplet at δ 2.27 (J=7.6 Hz) was due to methylene H2-2' protons adjacent to the ester function. Six broad singlets at δ 1.68, 1.23, 0.92, 0.87, 0.77 and 0.65 and a triplet at δ 0.84 (J=6.5 Hz) , all

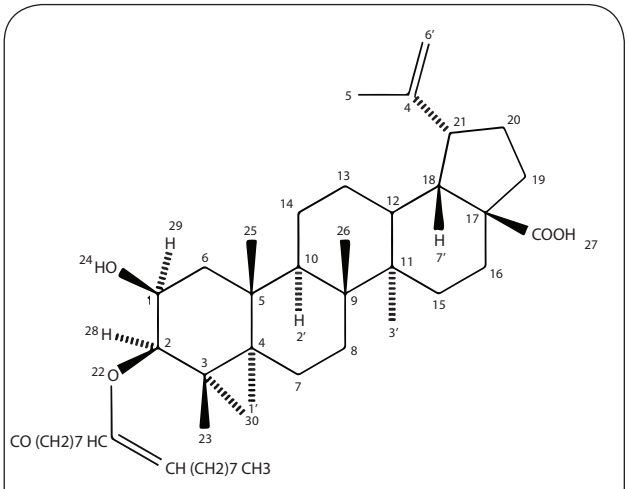

Figure 1. Chemical structure of isolated compound (HBAO).

Table 1. ^{13}C NMR spectral data for compound 2β-hydroxybetulinic acid 3β-oleiate (HBAO).

Carbon (position)	13C NMR (DMSO6)
1	38.45
2	69.75
3	76.73
4	38.23
5	55.37
6	18.89
7	33.88
8	41.95
9	49.91
10	37.54
11	24.47
12	25.04
13	39.04
14	46.58
15	31.67
16	36.68
17	54.87
18	48.49
19	46.56
20	150.24
21	30.06
22	36.31
23	29.04
24	15.75
25	17.93
26	15.67
27	13.92
28	177.18
29	109.58
30	20.42
1′	174.43
2′	33.62
3′	31.66
4′	28.92
5′	28.84
6′	28.75
7′	27.55
8′	31.30
9′	129.66
10′	127.70
11′	30.89
12′	29.17
13′	29.17
14′	28.92
15′	27.12
16′	26.58
17′	22.09
18′	14.33

integrating for three protons each, were accounted correspondingly to C-30 methyl located on a vinylic carbon C-20, tertiary C-23, C-24, C-25, C-26 and C-27 methyl and primary C-18' methyl protons. The other methylene and methine protons were from δ 2.96 to 1.30.

The ^{13}C NMR spectrum of compound exhibited signals for ester carbon at δ 177.18 (C-28), oxygenated methine carbons at δ 69.75 (C-2) and 76.33 (C-3), vinylic carbons at δ 150.24 (C- 20), 109.58 (C-29), 129.66 (C-9') and 127.70 (C-10'), and methyl carbon between δ 29.04-13.92. The ^{1}H and ^{13}C spectral data of the triterpenic carbon framework were comparable with the lupe type triterenic spectral values. On the basis of the above discussion, the structure of the compound was characterized as lup-20 (29)-en-2β-ol-3β-octadec-9'-enoate-28-oic acid. This is a new teriterpenic ester.

Animals

All animals used as part of this experimentation got altruistic care in consistence with the standards of creature care defined by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Male wistar rats (200-250 g) were taken for this research. All the animals were kept under standard conditions of temperature (25±1 °C), relative humidity (55±10%), 12hr/12hr light/dark cycle. The animals were fed standard pellet diet (Amrut rat feed, Pune) and water *ad libitum*. The trial convention was affirmed by the Institutional Animal Ethical Committee of Adina Institute of Pharmaceutical Sciences, Sagar, MP, India (IAEC Reg. no. 1546/PO/a/11/CPCSEA).

Table 2. Experimental groups.

Group	Dose plan
Group 1	Normal control
Group 2	Normal + HBAO (60 mg/kg p.o.) continued for 45 days.
Group 3	Diabetic control (Infused with STZ 60 mg/kg i.p)
Group 4	Diabetic + HBAO (20 mg/kg p.o.) and continued for 45 days
Group 5	Diabetic + HBAO (40 mg/kg p.o.) and continued for 45 days.
Group 6	Diabetic + HBAO (60 mg/kg p.o.) and continued for 45 days
Group 7	Diabetic + Glibenclamide (10mg/kg p.o.) and continued for 45 days

Experimental design

The male albino wistar rats were divided into experimental groups shown in Table 2.

Every morning the drugs were given orally to the animals using a catheter. The rats were categorized as per above distribution and on the 45th day of dosage, which was the end of the research plan, all the animals were fasted overnight with free access to water.

Induction of diabetes

Wistar rats were infused intraperitoneally with STZ disintegrated in newly prepared 0.1 M citrate buffer at 60 mg/kg (Ahmed *et al.,* 2017) (pH=6.5). Animals of the control group received equal volume of vehicle. Blood glucose concentration was examined in all rats after 2 days of STZ administration. The rats were categorized as Diabetic with the blood glucose level exceeding 250 mg/dl.

To estimate the impact of HBAO on STZ induced diabetes mellitus rats, a few biochemical estimations were performed in all groups of tentatively instigated diabetic rats for the estimation of plasma glucose, plasma insulin, serum cholesterol, serum triglycerides, glycated hemoglobin (A1c), hepatic hexokinase, hepatic glucose-6-phosphate dehydrogenase, hepatic fructose-1-6-biphosphatase, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx).

Histological changes

Pancreas and hepatic tissues were removed from the body and preserved by fixing them in 10% neutral-buffered formalin, dehydrated by passing through series of alcohol and followed by sectioning into 4 µm slices with the help of semi-automated rotary machine (model RM2245, Leica Microsystems, Wetzlar, Germany). Hematoxylin and eosin (HE) staining was done. Histological observation

Table 3. Effect of HBAO on plasma glucose level (mg/dl) and glycosylated hemoglobin (A1c) (in percentages) level in normal control and STZ-induced diabetic treated rats.

Groups	Plasma glucose level at different periods of time in normal control and STZ-induced diabetic treated rats			Glycosylated Hemoglobin (A1c) (%) at the end of therapy
	At start of therapy	On 22nd day of therapy	At the end of therapy (on 45th day)	
Group 1	93.78±1.03	96.45±1.003	97.80±1.61	7.09±0.02
Group 2	92.64±0.86	93.54±0.93	94.34±1.34	7.13±0.02
Group 3	290.6±1.13[a]	294.5±1.22[a]	326.9±1.6[a]	12.51±0.15[a]
Group 4	286.4±1.17	265.4±0.87	244.5±0.98	11.52±0.13
Group 5	284±0.7	244.2±2.14	153.8±1.42*	9.606±0.12*
Group 6	282±0.66	206.1±0.75	114.7±1.46*	8.42±0.18**
Group 7	280.3±0.7	197.6±1.14	104.3±1.66*	7.46±0.18***

The data are expressed as mean ± SEM. The comparisons were made by one way ANOVA followed by Dunnett's test. *p<0.05 is considered significant when compared to the control group (0 h); **p<0.001 is considered very significant when compared to the control group (0 h); ***p<0.001 is considered extremely significant when compared to the control group (0 h). [a] – compared to normal; * – compared to diabetic control

was done to rectify the potency of HBAO against diabetes (Ahmed *et al.,* 2014). Imaging software for laboratory microscopy (Model No. DXIT 1200, Nikon, Japan) was used for photomicrography of each tissue.

Statistical analysis

Data were given as the mean ± SEM. All the data were statistically analyzed by one-way analysis of variance ANOVA, further followed by DUNNET's 't'test. $p<0.05$ was considered to be significant.

Results

Effect of HBAO on glycemic control

The data resulting from Table 3 and Figure 2 show that blood glucose level fed by normal diet (group 1) was constant in Wistar rats. In comparison to the normal group, the glucose level in STZ induced diabetic rats (group 3) was increased to a significant level ($p<0.001$). Furthermore, the level of glucose in (group 2), i.e. HBAO administered, was steady throughout 45 days, indicating that no hypoglycemic effect occurred with administration of the maximum dose of HBAO. In addition, the increasing dose of HBAO administered to the diabetic group showed that the lowering in blood glucose level in the drug treated group receiving HBAO=60 mg/kg p.o. was the best when compared to the diabetic control group, as well as in HBAO group receiving doses of 20 mg/kg p.o., 40 mg/kg p.o. and the group receiving the standard drug Glibenclamide.

Effect of HBAO on glycosylated hemoglobin (A1c)

Glycosylated level was found to be normal with the group receiving HBAO 60 mg/kg without induced diabetes (Table 3). On the other hand, the group with STZ received diabetes when given HBAO doses in increasing manner, it depicts significancy level of ($p<0.01$) lowering in glycosylated hemoglobin in the group that received HBAO with dose of 60 mg/kg p.o. as compared to the group 3, group 4, group 5 and the group received Glibenclamide (group 7).

Effect of HBAO on level of plasma insulin

Table 4 and Figure 3 show that the insulin levels of untreated diabetic rats were considerably lower than those in normal rats. The increasing levels of HBAO administered results in significant enhancement in the level of plasma insulin in STZ induced diabetic rats. The maximum enhancement in the plasma insulin level was observed in HBAO treated rats that received a dose of 60 mg/kg p.o for 45 days in comparison to all other HBAO receiving groups and also including the standard group receiving Glibenclamide.

Effect of HBAO on hepatic glucose-6-phosphate dehydrogenase, glucose-6-phosphatase and fructose-1-6-biphosphatase

Table 5 and Figure 4A and 4B state clearly the activities of gluconeogenic enzymes with levels of glucose-6-phosphatase and fructose-1-6-biphosphatase significantly

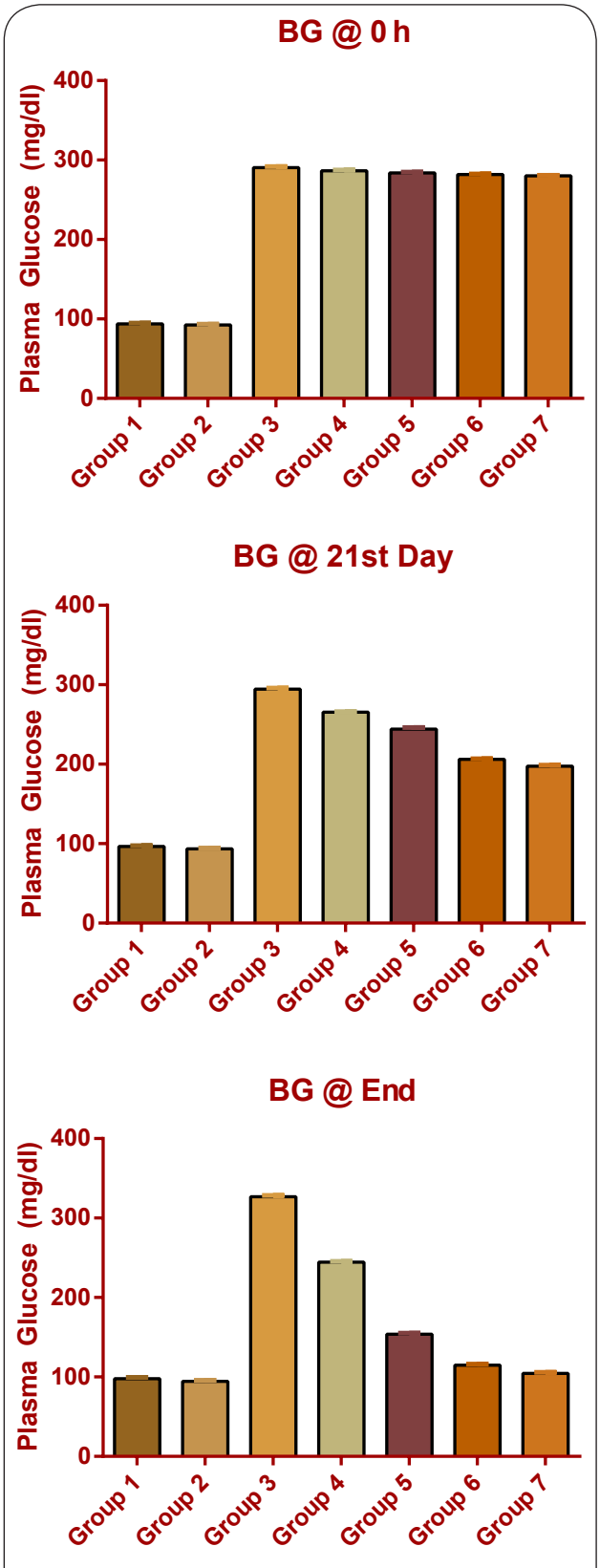

Figure 2. Effect of HBAO on Blood Glucose at different time interval of therapy: Group 1: Normal control; Group 2: Normal + HBAO (60 mg/kg p.o.); Group 3: Diabetic Control (Infused with STZ 60 mg/kg i.p); Group 4: Diabetic + HBAO (20 mg/kg p.o.) & continue till End; Group5: Diabetic + HBAO (40 mg/kg p.o.) & continue till End; Group6: Diabetic + HBAO (60 mg/kg p.o) & continue till End; Group 7: Diabetic + Glimepiride (10mg/kg p.o.) & continue till End. END = 45 Days.

Table 4. Effect of HBAO on plasma insulin level in normal control and STZ-induced diabetic treated rats.

Groups	Plasma insulin (μIU/ml) level at different periods of time in normal control and STZ-induced diabetic treated rats		
	At start of therapy	At 22 days of therapy	At the end of therapy (on 45th day)
Group 1	14.42±0.20	15.37±0.18	16.19±0.13
Group 2	14.71±0.15	14.67±0.33	16.90±0.34
Group 3	4.18±0.004[a]	4.81±0.04[a]	10.38±6.65[a]
Group 4	4.29±0.01	4.86±0.18	8.5±0.16*
Group 5	4.34±0.02	6.79±0.06*	10.5±0.14**
Group 6	4.49±0.02	9.67±0.17**	13.66±0.17***
Group 7	4.82±0.07	11.42±0.19**	14.84±0.27***

The data are expressed as mean ± SEM. The comparisons were made by one way ANOVA followed by Dunnett's test. *p<0.05 is considered significant when compared to the control group (0 h); **p<0.001 is considered very significant when compared to the control group (0 h); ***p<0.001 is considered extremely significant when compared to the control group (0 h). [a] – compared to normal; * – compared to diabetic control

Table 5. Effect of HBAO on hepatic gluconeogenic enzymes in normal control and STZ-induced diabetic rats.

Groups	Biochemical estimations of hepatic enzymes			
	Glucose-6-phosphate dehydrogenase (units/min/mg of protein)	Glucose-6-phosphatase (units/min/mg of protein)	Fructose-1-6-biphosphatase (units/min/mg of protein)	Hepatic hexokinase (units/min/mg of protein)
Group 1	0.15±0.005	0.03±0.0007	0.01±0.0003	0.24±0.004
Group 2	0.15±0.003	0.03±0.001	0.017±0.0007	0.212±0.003
Group 3	0.04±0.001[a]	0.07±0.0003[a]	0.07±0.002[a]	0.11±0.005[a]
Group 4	0.05±0.0008	0.07±0.0005	0.065±0.001	0.13±0.006
Group 5	0.07±0.001	0.18±0.11	0.046±0.001	0.16±0.005
Group 6	0.09±0.002**	0.04±0.0003**	0.02±0.001*	0.20±0.001**
Group 7	0.12±0.008***	0.03±0.002***	0.02±0.001***	0.21±0.001***

The data are expressed as mean ± SEM. The comparisons were made by one way ANOVA followed by Dunnett's test. *p<0.05 is considered significant when compared to the control group (0 h); **p<0.001 is considered very significant when compared to the control group (0 h); ***p<0.001 is considered extremely significant when compared to the control group (0 h). [a] – compared to normal; * – compared to diabetic control

(p<0.01) increased in the group of diabetes induced STZ. On contrast, the activity of glucose-6-phosphate, dehydrogenase was significantly reduced in diabetic control rats. Moreover, HBAO given in the dose of 60 mg/kg depicts the best output compared to other doses.

Effect of HBAO on level of hepatic hexokinase

Diabetic group induced with STZ showed a prominently lower level of hexokinase. All other diabetic groups when given HBAO with increasing dose, i.e. 20, 40 60 mg/kg p.o., showed a marked (p<0.01) increment in the level of hexokinase. The best activity was observed at the dose of 60 mg/kg p.o. of HBAO as compared to the other groups (Table 5 and Figure 4C).

Effect of HBAO on levels of serum total cholesterol

The results from Table 6 and Figure 5A show that when STZ diabetic rats were treated with HBAO in increasing doses, i.e. 20, 40 60 mg/kg p.o., there was a significant (p<0.01) decrease in serum cholesterol level as compared to other groups and standard group having Glimepiride. The most favorable result was deduced with the group receiving HBAO with 60 mg/kg p.o., compared to the other groups.

Effect of HBAC on levels of serum HDL cholesterol

An increment with significant extent of (p<0.01) in serum HDL was seen when given to STZ diabetic rats with novel isolated HBAO, as compared to diabetic group as well as standard treated with Glimepride (Table 6 and Figure 5C). HBAO in the dose of 60 mg/kg. p.o. treated in diabetes holds the best output of drug.

Effect of HBAO on levels of serum LDL cholesterol

The analysis of serum LDL was measured and as intelligible from (Table 6 and Figure 5D) diabetic rats had maximum level of serum LDL. When HBAO in different doses was incorporated in animals, the best diminishing serum LDL outcome was noticed with HBAO 60 mg/kg. compared to all other groups, as well as to standard Glimepride.

Effect of HBAO on levels of serum VLDL cholesterol

There was an increased level of serum VLDL providing logically the affirmation that the diabetic rat had maximum level of serum VLDL and when the same diabetic rats were given HBAO at increased doses of 20, 40 60 mg/kg p.o., maximum serum VLDL decrease was noticed with HBAO 60 mg/kg. compared to all other groups as well as to standard Glimepride.

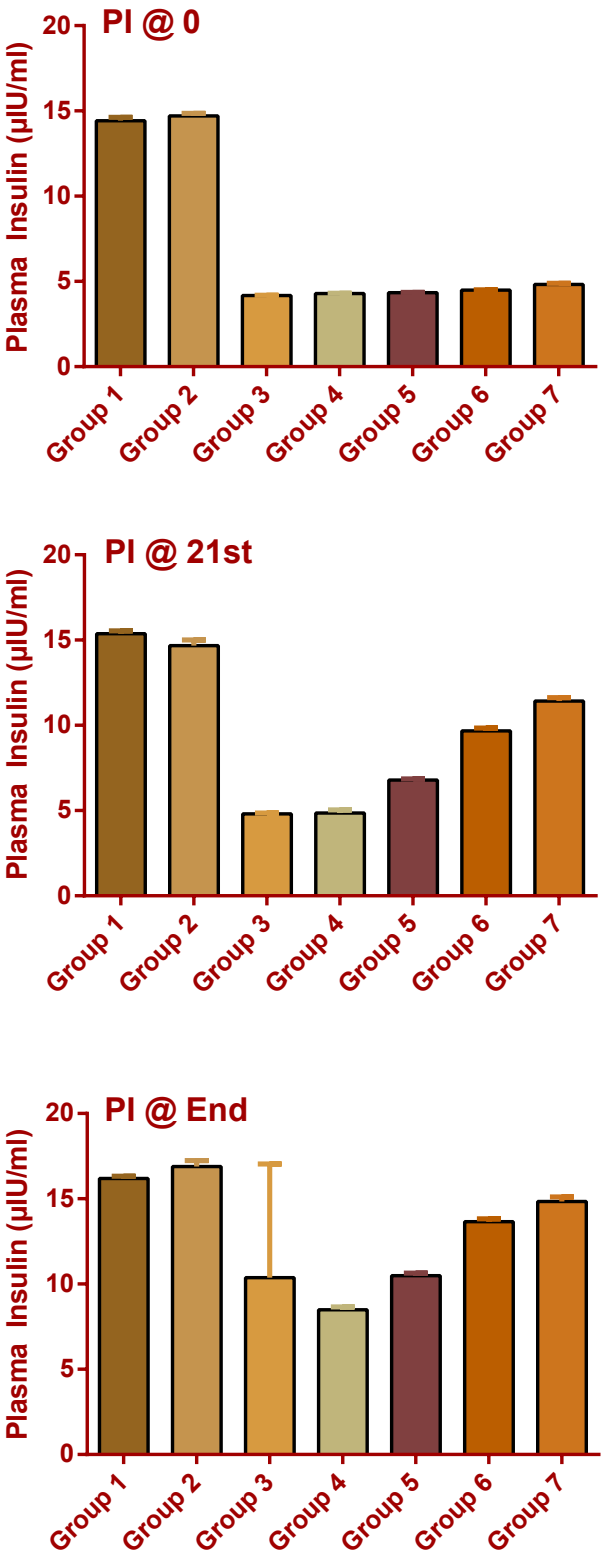

Figure 3. Effect of HBAO on Plasma Insulin at different time interval of therapy: Group 1: Normal control; Group 2: Normal + HBAO (60 mg/kg p.o.); Group 3: Diabetic Control (Infused with STZ 60 mg/kg i.p); Group 4: Diabetic + HBAO (20 mg/kg p.o.) & continue till End; Group5: Diabetic + HBAO (40 mg/kg p.o.) & continue till End; Group6: Diabetic + HBAO (60 mg/kg p.o) & continue till End; Group 7: Diabetic + Glimepiride (10mg/kg p.o.) & continue till End. END = 45 Days.

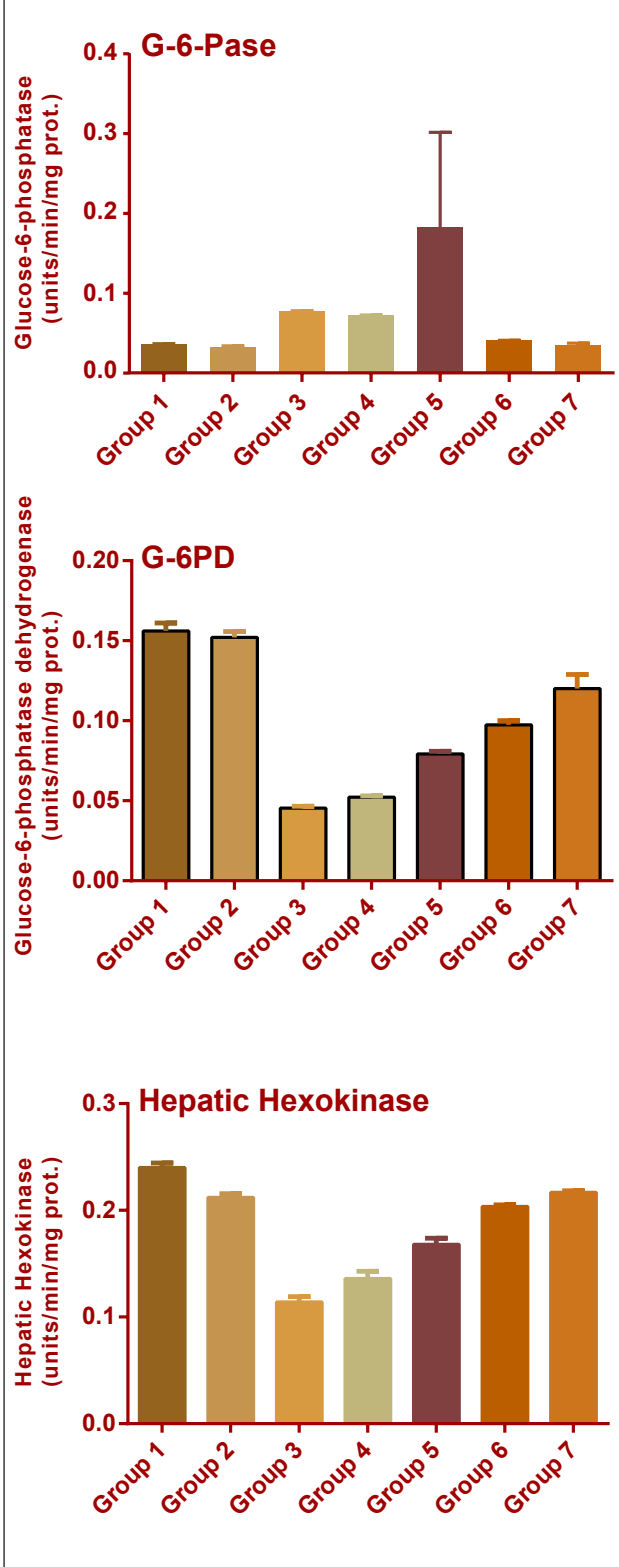

Figure 4. Effect of HBAO on Gluconeogenic hepatic enzymes at different time interval of therapy: Group 1: Normal control; Group 2: Normal + HBAO (60 mg/kg p.o.); Group 3: Diabetic Control (Infused with STZ 60 mg/kg i.p); Group 4: Diabetic + HBAO (20 mg/kg p.o.) & continue till End; Group5: Diabetic + HBAO (40 mg/kg p.o.) & continue till End; Group6: Diabetic + HBAO (60 mg/kg p.o) & continue till End; Group 7: Diabetic + Glimepiride (10mg/kg p.o.) & continue till End. END = 45 Days.

Effect of HBAO on serum triglyceride levels

With the outcome of Table 6 and Figure 5B it is evident that STZ diabetic rats showed noticeable increase in triglyceride level of serum. Similarly to other parameters, here too HBAO with 60 mg/kg. resulted in better response compared to other dosage concentrations as well as to diabetic rats given Glimepiride.

Effect of HBAO on superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activity

As expected and as clear from Table 7 and Figure 6A, 6B and 6C, there was significantly decreased activity of SOD, CAT and GPx (p<0.01) in STZ induced diabetic rats compared to normal control rats. The dose of 60 mg/kg HBAO yielded the best result with significant level of (p<0.01) increased in SOD, CAT and GPx in comparison to other given concentrations and standard Glimepride.

Effect of HBAO on body weight variation

Reduced body weight was observed to an extent compared to the normal group. HBAO showed a markedly increased body weight in the STZ induced diabetic rats with maximum weight gain in the group given 60 mg/kg HBAO (Table 8 and Figure 7).

Effect of HBAO on histopathology of pancreas, kidney & liver

Pancreas

Normal control animals displayed a typical histological design. Many rounded typical proportions of islet of Langerhans were discovered all around the pancreatic acini. Noticeable nuclei with very much systematic lobules with encompassing islet cells were found in typical control rats (Figure 8). The diabetic group showed spoiled cells of pancreatic islets and acini which consequently proved pancreatic β- cell damage and deteriorated with irregular vacuoles. HBAO treated to STZ induced group demonstrated distinct change of the cell injury, as apparent from the partial restoration of islet cells, diminished β-cell damage, more symmetrical vacuoles and an expansion in number of islet cells.

Kidney

Ascending & descending loops of Henle, collecting ducttubules and prominent glomeruli were among the major morphological features of the kidney in the normal control group. The diabetic group showed deposition of crystals upon glomeruli as well as infiltration of red blood cells (Figure 9).The groups that received HBAO demonstrated the reversibility of the destruction resulting in retention of crystal deposited and cell regeneration.

Liver

Normal control groups exhibited influential hepatocytes with central vein along with portal triad (Figure 10). On the other hand, a clearly visible damaged central vein, hepatocytes and portal triad was observed in STZ induced diabetic rats. All the HBAO groups overturned the damage to liver cells.

Discussion

At present, traditional and complementary medicine has seen an upsurge in its popularity for the treatment of different diseases. India is endowed with a rich tradition of herbal medicines as is evident from the fact that the Susruta Samhita differentiated between genetically and acquired forms of diabetes and recommended many herbal medicines in different oral formulations for treatment of the disease. Recently, search for appropriate anti-hyperglycemic agents has focused on plants used in traditional medicine because of leads provided by natural products that may provide better treatment than currently used drugs.

In the main, a wide range of secondary metabolites and substances are synthesized by plants playing a key role for their pharmacological potentials. Alkaloids, triterpenoids and steroids were reported to have anti-diabetic activity already in ancient times (Erememisoglu *et al.,* 1995).

The present research depicts the isolation of a novel pentacyclic triterpene (Figure 1) isolated from the methanolic extract of *Euryale ferox salisb.* seeds further

Table 6. Effect of HBAO on lipid profiles in normal control and STZ-induced diabetic treated rats.

Groups	Lipid Profiles			
	Total cholesterol (TC) (mg/dl)	LDL cholesterol (LDL-c) (mg/dl)	Triglycerides (mg/dl)	HDL cholesterol (HDL-c) (mg/dl)
Group 1	118.9±0.39	24.40±0.44	71.97±0.29	55.79±0.31
Group 2	115.4±2.24	23.76±0.61	72.10±0.49	54.25±0.27
Group 3	231.8±0.31[a]	144.6±0.54[a]	281.7±0.42[a]	19.83±0.26[a]
Group 4	218.1±0.47	134.0±0.58	211.6±0.57	27.07±2.3
Group 5	142.8±0.31	103.2±0.62	140.9±0.29	37.09±0.37
Group 6	121.4±0.48***	37.10±0.54**	84.17±1.16***	49.14±0.18***
Group 7	135.7±0.52**	43.51±0.65**	91.99±0.53**	46.62±0.39***

The data are expressed as mean ± SEM. The comparisons were made by one way ANOVA followed by Dunnett's test. *p< 0.05 is considered significant when compared to the control group (0 h); **p< 0.001 is considered very significant when compared to the control group (0 h); ***p< 0.001 is considered extremely significant when compared to the control group (0 h). [a] – compared to normal; * – compared to diabetic control

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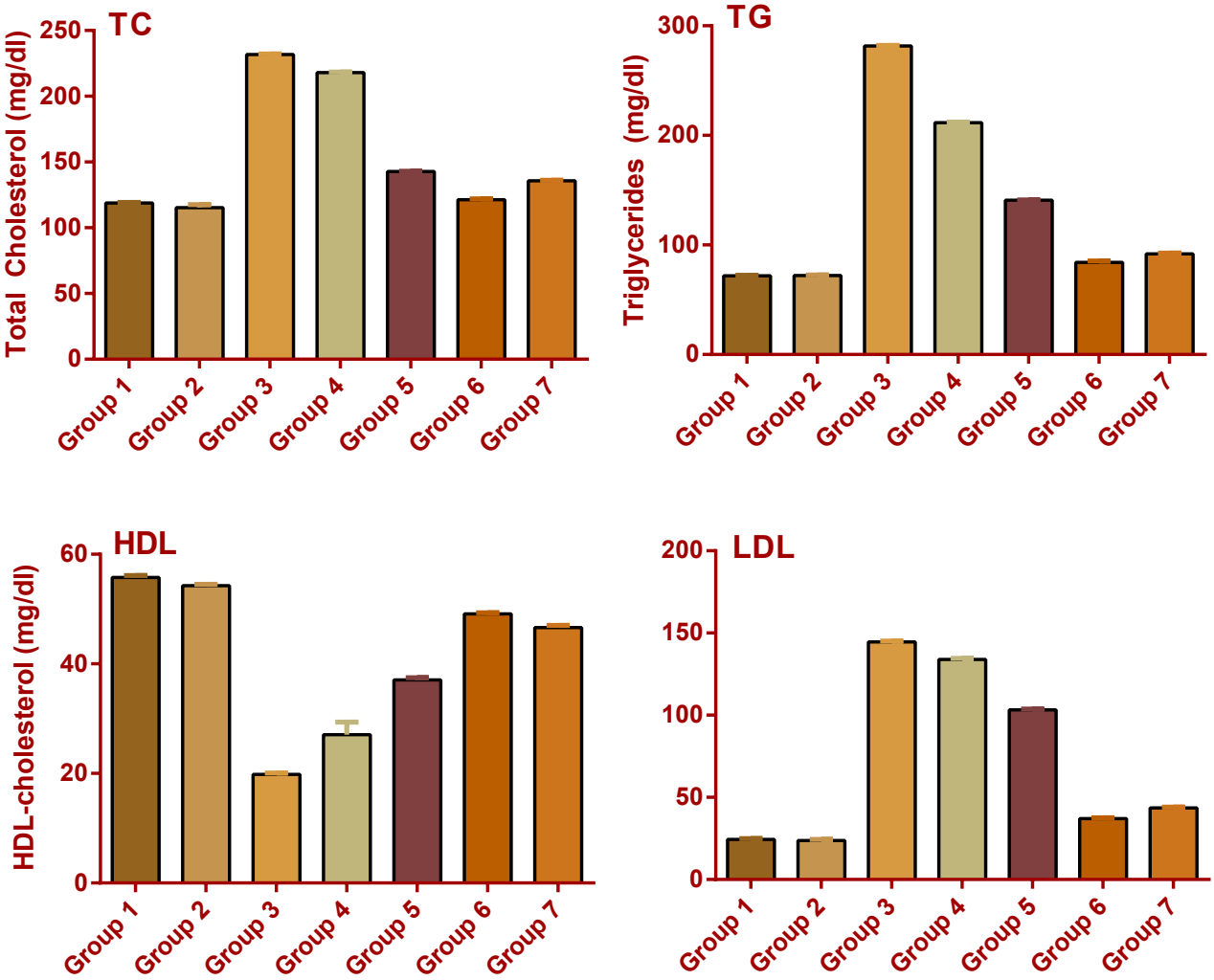

Figure 5. Effect of HBAO on Lipid Profile at different time interval of therapy: Group 1: Normal control; Group 2: Normal + HBAO (60 mg/kg p.o.); Group 3: Diabetic Control (Infused with STZ 60 mg/kg i.p); Group 4: Diabetic + HBAO (20 mg/kg p.o.) & continue till End; Group5: Diabetic + HBAO (40 mg/kg p.o.) & continue till End; Group6: Diabetic + HBAO (60 mg/kg p.o) & continue till End; Group 7: Diabetic + Glimepiride (10mg/kg p.o.) & continue till End. END = 45 Days.

Table 7. Effect of HBAO on oxidative stress estimation in normal control and STZ-induced diabetic treated rats.

Groups	Oxidative stress parameters		
	Superoxide dismutase (SOD) (U/mg protein)	Catalase (CAT) (nM H2O2 decomposed/min/g)	Glutathione peroxidase (GPx) (U/mg protein)
Group 1	11.10±0.28	83.24±0.87	13.11±0.41
Group 2	11.07±0.23	84.68±0.84	12.47±0.18
Group 3	3.15±0.04 a	26.89±0.31 a	5.62±0.082 a
Group 4	4.36±0.01	28.08±0.4	7.154±0.03
Group 5	7.32±0.05*	52.81±0.62*	9.69±0.12*
Group 6	9.214±0.04167**	71.88±0.2619**	10.64±0.04104**
Group 7	10.15±0.01428***	75.70±1.075***	11.73±0.1861**

The data are expressed as mean ± SEM. The comparisons were made by one way ANOVA followed by Dunnett's test. *p< 0.05 is considered significant when compared to the control group (0 h); **p< 0.001 is considered very significant when compared to the control group (0 h); ***p< 0.001 is considered extremely significant when compared to the control group (0 h). [a] – compared to normal; * – compared to diabetic control

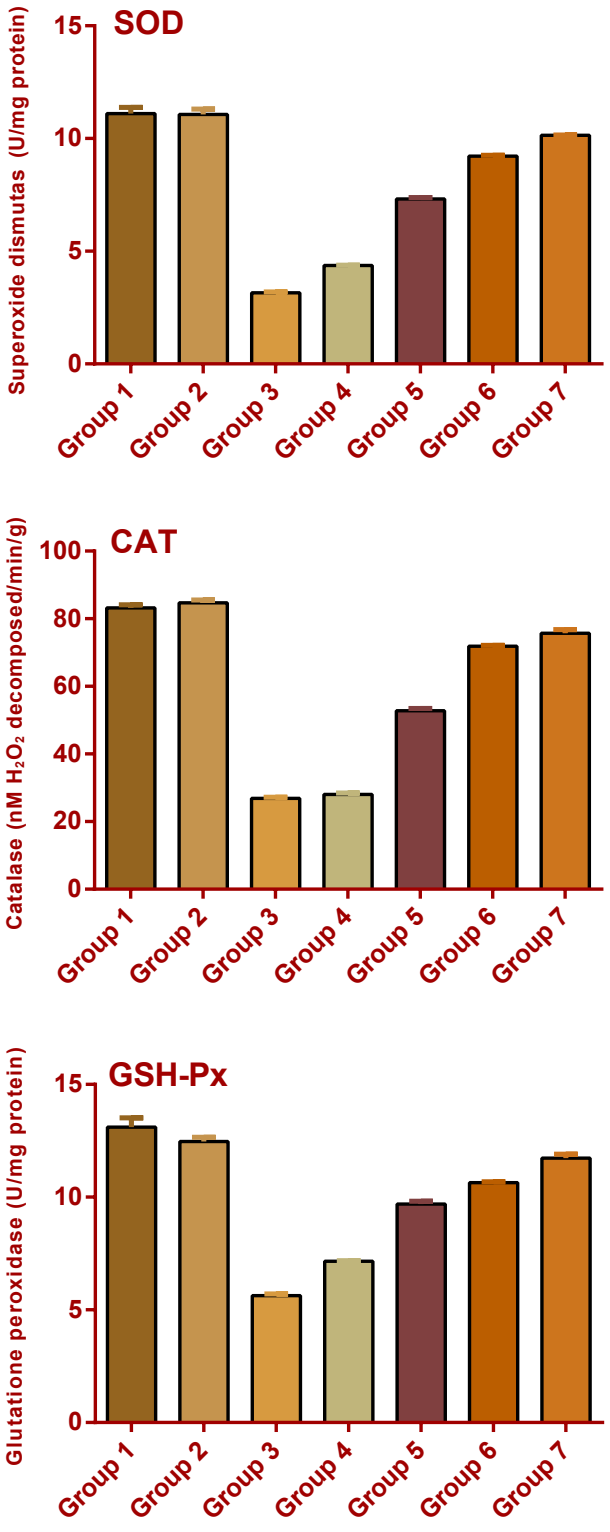

Figure 6. Effect of HBAO on Antioxidant marker enzymes at different time interval of therapy: Group 1: Normal control; Group 2: Normal + HBAO (60 mg/kg p.o.); Group 3: Diabetic Control (Infused with STZ 60 mg/kg i.p); Group 4: Diabetic + HBAO (20 mg/kg p.o.) & continue till End; Group5: Diabetic + HBAO (40 mg/kg p.o.) & continue till End; Group6: Diabetic + HBAO (60 mg/kg p.o) & continue till End; Group 7: Diabetic + Glimepiride (10mg/kg p.o.) & continue till End. END = 45 Days.

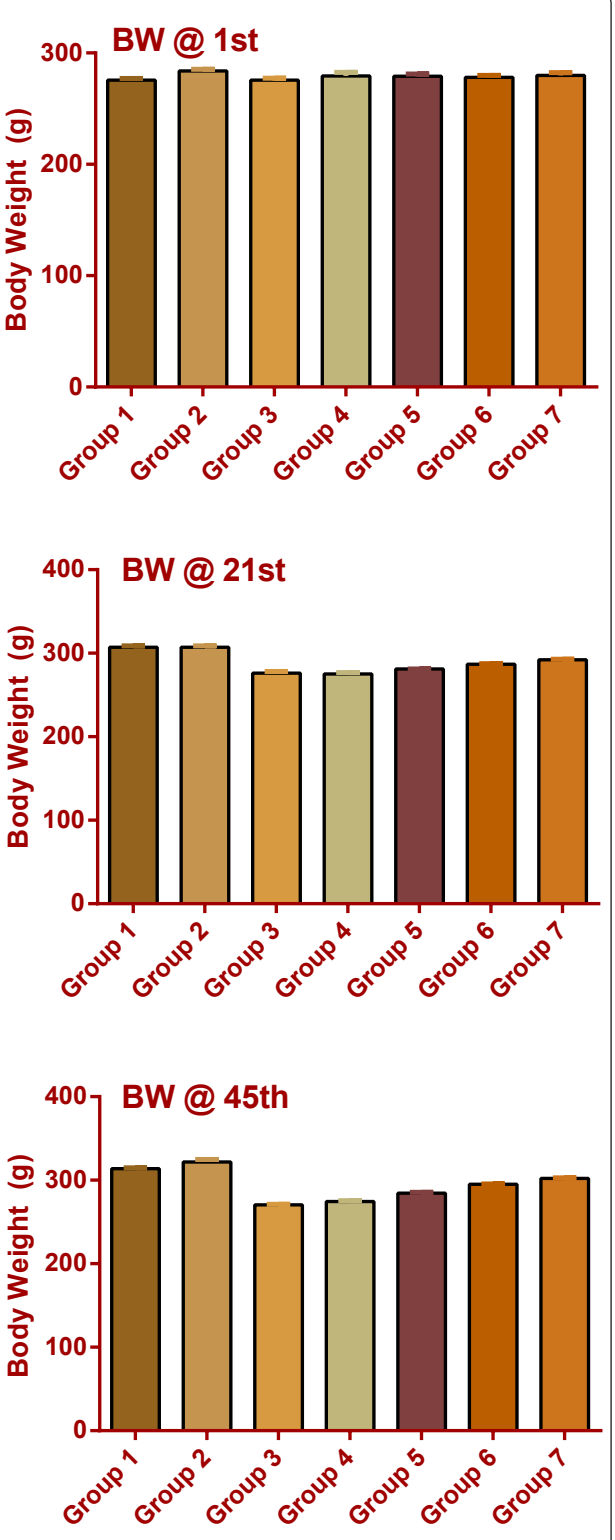

Figure 7. Effect of HBAO on Body Weight at different time interval of therapy: Group 1: Normal control; Group 2: Normal + HBAO (60 mg/kg p.o.); Group 3: Diabetic Control (Infused with STZ 60 mg/kg i.p); Group 4: Diabetic + HBAO (20 mg/kg p.o.) & continue till End; Group5: Diabetic + HBAO (40 mg/kg p.o.) & continue till End; Group6: Diabetic + HBAO (60 mg/kg p.o) & continue till End; Group 7: Diabetic + Glimepiride (10mg/kg p.o.) & continue till End. END = 45 Days.

Table 8. Effect of HBAO on body weight (grams) in normal control and STZ-induced diabetic treated rats.

Groups	Body weight (g) at different time interval of experimentation		
	At start of therapy	At 22nd day of therapy	At end of therapy (45th Day)
Group 1	275.6±1.44	307±1.95	313.8±1.07
Group 2	283.8±1.59	307.2±1.56	321.8±3.18
Group 3	275.6±1.69[a]	276±2.07[a]	270.6±0.51[a]
Group 4	279.4±3.20	275.2±1.74	274.6±0.87*
Group 5	279.2±1.83	281±0.32**	284.6±1.03**
Group 6	278.2±1.56	286.8±0.58**	295.2±0.374***
Group 7	279.8±2.65	292±0.71**	302±0.71***

The data are expressed as mean ± SEM. The comparisons were made by one way ANOVA followed by Dunnett's test. *p< 0.05 is considered significant when compared to the control group (0 h); **p< 0.001 is considered very significant when compared to the control group (0 h); ***p< 0.001 is considered extremely significant when compared to the control group (0 h). [a] – compared to normal; * – compared to diabetic control

assessed for its anti-diabetic, antihyperlipidemic, antioxidant and hepatic and pancreas protective activity against STZ-induced diabetic rats. STZ causes partial apoptosis of pancreatic cells and induces an experimental model for type-2 diabetes mellitus and it also releases free radicals which distort the mitochondrial function of β-cells (Szkudelski, 2001). This made us to take on this model for the induction of diabetes. STZ interferes with the cellular metabolic oxidative mechanisms and produces severe and irreversible hyperglycemia, if given at higher doses (Mitra *et al.,* 1996). Results of acute toxicity studies clearly demonstrate no mortality in the isolated compound treated rats and the behavior of the compound treated rats also appeared to be normal. Until the end of the research work, none of the rat showed any toxic reaction or lethality. This confirms that the isolated novel compound is non-toxic nature.

Augmented levels of blood glucose and insulin confirmed that the STZ-induced diabetic model was well founded. Plasma glucose level was measured at regular intervals (0, 5, 12, 20, 28, 35 and 45th day) in normal, diabetic and the novel compound treated Wistar rats. Administration of the novel compound HBAO clearly revealed the hypoglycemic, antihyperlipidemic, antioxidant and hepato-pancreas protective effect. There is a gradual reduction of plasma glucose in almost all the doses when compared to diabetic control rats. Maximum reduction of plasma glucose was obtained with 60 mg/kg p.o dose of HBAO. Simultaneously, it was also observed that the plasma glucose level of normal rats was unaltered upon administration of HBAO. This corroborates the substantial normoglycemic effect of the isolated compound. The decrement of plasma glucose of the HBAO treated diabetic rats advocates that the novel isolated compound acts to reduce plasma glucose either by promoting uptake metabolism by inhibiting hepatic gluconeogenesis or by advancing the entry of glucose into muscle and adipose tissues through plausible mechanism of stimulation of regenerating and revitalizing the spared pancreatic β-cells (Bolkent *et al.,* 2000).

Increased glycation of protein has been discovered to be a consequence of diabetic complications. Hemoglobin and various proteins are glycated to a greater extent in diabetes (Keen & Jarrett, 1982). Among the glycated proteins, HbA_{1c} is widely recognized as marker of glycation control and is considered a biological intermediate criterion for measurement of efficacy of diabetic therapy. In our research work, HBAO clearly prevents a significant increase in HbA_{1c} with a simultaneous control of plasma glucose.

The two primary complementary events that equilibrate the glucose load are glycolysis and gluconeogenesis, which is exemplified by the partial or total deficiency of insulin. Suppression of hepatic gluconeogenesis and glycogenolysis, while enhancing hepatic glycogen synthesis, was carried out by insulin. A major site of endogenous glucose production is the liver, where production of glucose was accomplished by gluconeogensis and glycogenolysis. Hydrolysis of glucose-6-phosphate to glucose was done by glucose-6-phosphatase (G-6 Pase), which is the final step of both hepatic gluconeogenesis and glycogenolysis. G-6 Pase is mainly expressed in liver, kidney and small intestine. Treatment with HBAO (60 mg/kg p.o.), causes no significant alterations. This suggests that HBAO may modulate glycogen biosynthesis by suppressing the activity of glucose-6-phosphatase and as a result decreases hepatic glycogen synthesis and storage. Insulin regulates the activity of glucose-6-phosphate dehydrogenase (G6PD). Levels of G6PD were found to be decreased in diabetic rats suggesting a lowered glucose metabolism through phosphogluconate oxidation pathway. Oral administration of HBAO (60 mg/kg p.o.) significantly restored the level of G6PD, indicating improved glucose utilization by the hepatic tissues. Fructose-bi-phosphatase (FBPase) regulates the hepatic gluocose hemostasis and is mainly expressed in liver and kidney (Nordlie *et al.,* 1999; Mithieux, 2009). In diabetic rats the hepatic glucose production was found to be increased and is directly associated with impaired function and increased level of FBPase in the liver. Enhanced

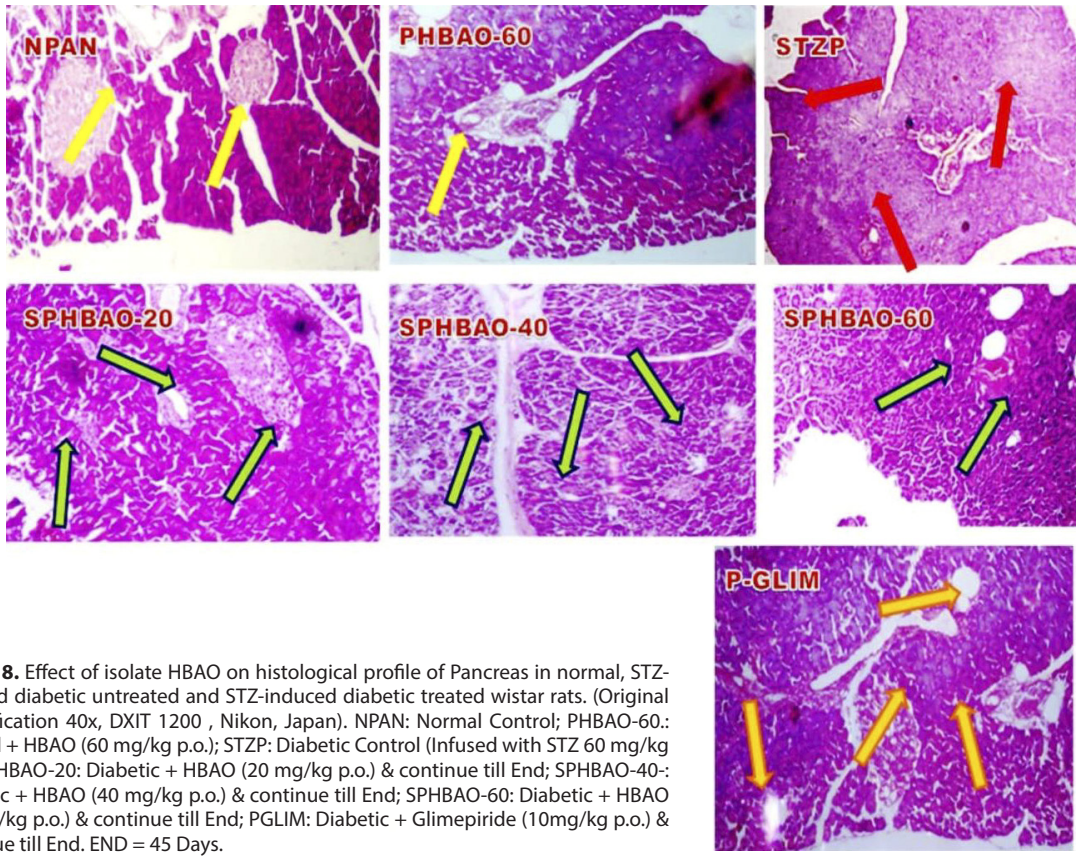

Figure 8. Effect of isolate HBAO on histological profile of Pancreas in normal, STZ-induced diabetic untreated and STZ-induced diabetic treated wistar rats. (Original magnification 40x, DXIT 1200 , Nikon, Japan). NPAN: Normal Control; PHBAO-60.: Normal + HBAO (60 mg/kg p.o.); STZP: Diabetic Control (Infused with STZ 60 mg/kg i.p); SPHBAO-20: Diabetic + HBAO (20 mg/kg p.o.) & continue till End; SPHBAO-40-: Diabetic + HBAO (40 mg/kg p.o.) & continue till End; SPHBAO-60: Diabetic + HBAO (60 mg/kg p.o.) & continue till End; PGLIM: Diabetic + Glimepiride (10mg/kg p.o.) & continue till End. END = 45 Days.

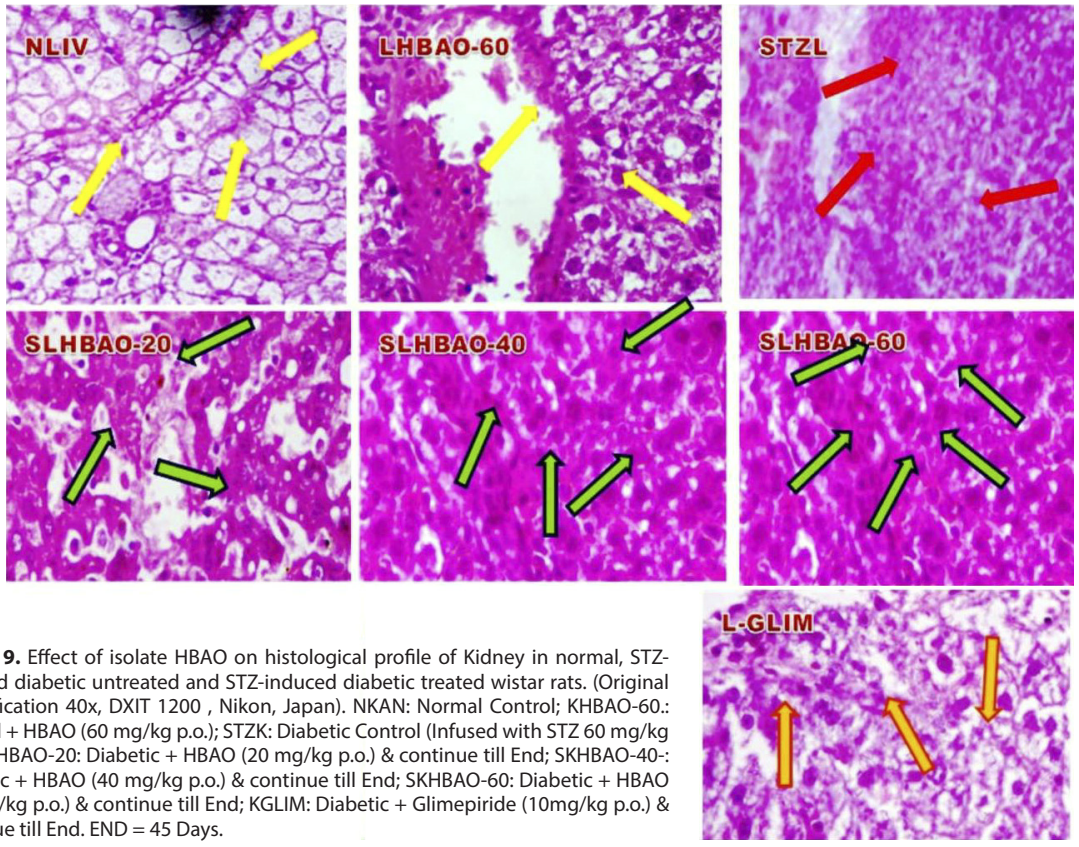

Figure 9. Effect of isolate HBAO on histological profile of Kidney in normal, STZ-induced diabetic untreated and STZ-induced diabetic treated wistar rats. (Original magnification 40x, DXIT 1200 , Nikon, Japan). NKAN: Normal Control; KHBAO-60.: Normal + HBAO (60 mg/kg p.o.); STZK: Diabetic Control (Infused with STZ 60 mg/kg i.p); SKHBAO-20: Diabetic + HBAO (20 mg/kg p.o.) & continue till End; SKHBAO-40-: Diabetic + HBAO (40 mg/kg p.o.) & continue till End; SKHBAO-60: Diabetic + HBAO (60 mg/kg p.o.) & continue till End; KGLIM: Diabetic + Glimepiride (10mg/kg p.o.) & continue till End. END = 45 Days.

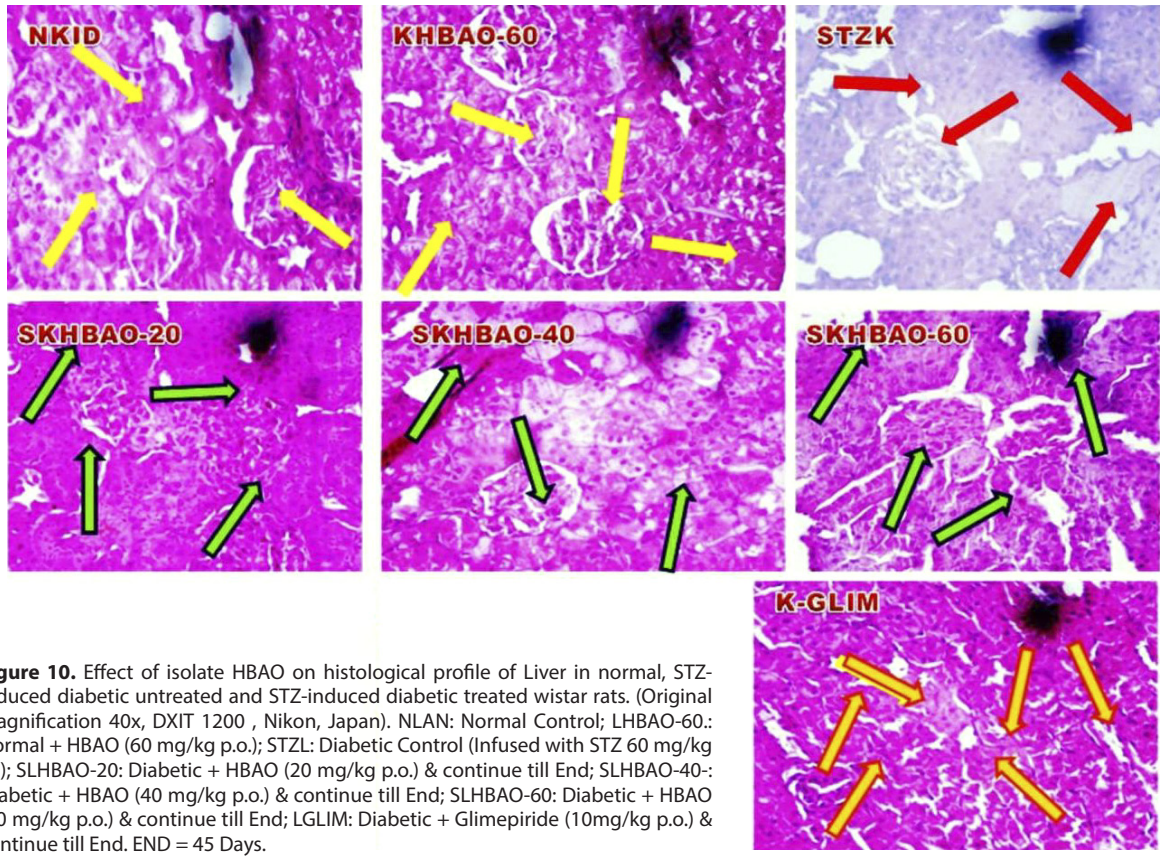

Figure 10. Effect of isolate HBAO on histological profile of Liver in normal, STZ-induced diabetic untreated and STZ-induced diabetic treated wistar rats. (Original magnification 40x, DXIT 1200 , Nikon, Japan). NLAN: Normal Control; LHBAO-60.: Normal + HBAO (60 mg/kg p.o.); STZL: Diabetic Control (Infused with STZ 60 mg/kg i.p); SLHBAO-20: Diabetic + HBAO (20 mg/kg p.o.) & continue till End; SLHBAO-40-: Diabetic + HBAO (40 mg/kg p.o.) & continue till End; SLHBAO-60: Diabetic + HBAO (60 mg/kg p.o.) & continue till End; LGLIM: Diabetic + Glimepiride (10mg/kg p.o.) & continue till End. END = 45 Days.

hepatic glucose production in STZ-induced diabetic rats is associated with dysregulation of these hepatic enzymes. Treatment of diabetic Wistar rats with HBAO (60 mg/kg p.o.) clearly depicts the significant decline in the level of FBPase, which is escorted by reduction in hepatic glucose production. Hepatic hexokinase is an insulin independent enzyme in the glyolytic pathway and fundamental for glucose homeostasis. Reduction in hexokinase activity in STZ-induced diabetic rats may be due to deficiency of insulin, as insulin simulates and activates hepatic hexokinase. Administration of HBAO (60 mg/kg p.o) to STZ-induced diabetic rats significantly improved the activity of hexokinase, which in turn results in enhanced glucose utilization for energy production.

In diabetes mellitus, increased blood glucose level is accompanied with dyslipidemia which is characterized by increase in triglyceride (TG), total cholesterol (TC), LDL cholesterol and a decrease in HDL cholesterol (Gupta *et al.,* 2009). High level of TG, TC, LDL cholesterol are significant and major risk factors for cardiovascular disorders, while improved level of HDL cholesterol is associated with improvement in cardiovascular profile. HDL plays a key role in transfer of cholesterol from blood to liver by reverse cholesterol pathway (Wang *et al.,* 2010). Disturbed lipid profile was reversed after oral administration of HBAO. Thus HBAO has the potential to prevent atherosclerosis and coronary heart disease formation in STZ-induced diabetic rats.

Some important research indicates that chronic hyperglycemia mediated oxidative stress is a major risk factor for dysfunction of liver in diabetes (Evans *et al.,* 2002; Rochette *et al.,* 2014). In oxidative stress, free radicals are generated, associated with increased plasma glucose and liver dysfunction. In STZ-induced diabetic rats, a significant decrease in hepatic antioxidants including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities were observed. The role of SOD is imperative in eliminating reactive oxygen species (ROS) originating from peroxidative process in tissues of liver and scavenging superoxide redicals by converting them to less toxic H_2O_2 and molecular oxygen (Packer *et al.,* 1978; Kaleem *et al.,* 2006). Upon administration of HBAO, the activity of SOD was significantly increased. In the present research work, a reduction in CAT activity was observed in STZ-induced diabetic rats. A reduction in CAT activity is primarily involved in the direct elevation of ROS, and might be associated with enhancement in oxidative stress in diabetes (Arulselvan & Subramanian, 2007). Our research work indicates a significant increase in CAT activity in HBAO treated STZ-induced diabetic rats. Another important enzyme, glutathione peroxidase (GPx), depicts a severe decline due to free radical induced inactivation and glycation of GPx in STZ-induced diabetic rats (Zhang & Tan, 2000). HBAO administration to STZ-induced diabetic rats increases GPx activities characteristic of insulin stimulatory activity of HBAO.

Conclusion

The novelty of this study is that HBAO, a pentacyclic triterpene, is a novel compound found in *Euryale ferox salisb* seeds. Oral administration of HBAO alleviates glycemic homeostasis and oxidative stress in STZ-induced diabetic rats, further HBAO normalized plasma glucose, glycosylated hemoglobin (HbA_{1c}), hepatic gluconeogenic enzymes, plasma insulin, ameliorated pancreatic β-cell, hepatic and renal histology and β-cell functions, improvising dyslipidemia and antioxidant enzymes in STZ-induced diabetic rats. Thus it can be concluded that HBAO derived from *Euryale ferox salisb* seeds may help prevent the important complications in diabetic rats and it might be a potential therapeutic candidate to combat diabetes.

Authors' contributions

DA and MS developed and designed the study. DA and MIK performed the experiments. DA and MFK analyzed the data. DA and MIK wrote the manuscript. All authors finalized the reading and inputs of the manuscript.

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