Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 1, 2014

Research Article

ANTIHYPERGLYCAEMIC AND ANTIDYSLIPIDEMIC ACTIVITIES IN ETHYL ACETATE FRACTION OF FRUITS OF MARINE MANGROVE *XYLOCARPUS MOLUCCENSIS*

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Received: 23 Oct 2013, Revised and Accepted:20 Nov 2013

ABSTRACT

Objective: To investigate the antihyperglycaemic and antidyslipidemic activities in ethyl acetate fraction of fruits of marine mangrove *Xylocarpus moluccensis* (EAXm) by measuring the status of biochemical parameters of diabetic animal models and in vitro glucose uptake effect.

Method: The ethyl acetate fraction of the epicarp from the fruits of *Xylocarpus moluccensis* (EAXm) (Family: Meliaceae) was tested for their glucose tolerance, declining blood glucose, lipid, renal and hepatic function markers, enzymes of carbohydrate metabolism of low dosed streptozotocin-induced diabetic rats, high fructose/high sucrose high fat fed streptozotocin induced rats and db/db mice, respectively for 10 consecutive days and in vitro glucose uptake effect by L-6 skeletal muscle cells.

Results: The EAXm was found effective in improving glucose tolerance, declining blood glucose, serum fructosamine levels of low dosed streptozotocin-induced diabetic rats, high fructose/high sucrose high fat fed streptozotocin induced rats , and db/db mice, respectively. HFD/HSD-STZ rats and dyslipidemic hamsters, when treated with EAXm for 10 consecutive days displayed decline in their serum cholesterol, triglycerides, LDL-cholesterol and elevation in their HDL-cholesterol levels and improvement in the hepatic as well as renal functions of HFD/HSD-STZ rats as evidenced by decline in their serum AST, ALT, ALP, urea, uric acid, creatinine levels. Treatment with EAXm also restored the altered activities of few key regulatory enzymes like glucokinase, phosphofructokinase, pyruvate kinase, glucose-6- phosphatase, and fructose 1-6 bisphosphatase in liver, muscle and renal tissues and glycogen degrading enzyme i.e. glycogen phosphorylase in liver and muscle of STZ-induced diabetic rats and db/db mice, respectively. EAXm also increased glucose uptake by L-6 skeletal muscle cells and inhibits the intestinal brush border enzyme alpha-glucosidase in vitro with IC_{50} around 28.4 µg /ml. The inhibition was found mixed type with respect to the substrate i.e. p-nitrophenyl-beta-D-glucopyranoside.

Conclusion: These results provide enough evidence regarding the antidiabetic and antidyslipidemic nature of epicarp of *X. moluccensis* fruits and needs further exploration.

Keywords: *Xylocarpus moluccensis*, Ethyl acetate fraction; Streptozotocin induced diabetic rats, db/db mice, Glucose uptake, Regulatory enzymes of Carbohydrate metabolism.

INTRODUCTION

Diabetes mellitus is a serious chronic metabolic disorder that has a significant impact on the health, quality of life, and life expectancy of patients, as well as on the health care system. The World Health Organization (WHO), has projected that the global prevalence of type 2 DM will more than double from 135 million in 1995 to 300 million by the year 2025 [1] With the present population of 19.4 million diabetics and a projected increase of 300% and thereby leading to approximately 60 million by the year 2025, India would rank first in sharing global burden of diabetes [2]. Recently there has been a shift in universal trend from synthetic to herbal medicine which we can say "Return to Nature". Herbal drugs are generally more prescribed widely because of their effectiveness, less side effects and relatively low cost [3]. Therefore, investigation on natural antidiabetic substances from traditional medicinal plants may become extremely useful [4].

Xylocarpus moluccensis is found in abundance in the coastal region of India, Bangladesh, Burma, Ceylon and Malaya. It has earlier been reported that *X. moluccensis* have been used in the treatment of cholera and fever whereas the fruits are aphrodisiac. The bark as well as pnematophore of *X. moluccensis* have been reported for neuro pharmacological properties [5] whereas its fruits husk has been reported as bactericidal. The kernels are used in tonics and in relieving colic. The seeds or peels of the fruits are utilized to poultice swellings and ash of the seeds is applied to itch. The fruits are used as a cure for swellings of the breast and in elephantiasis. The bark pressings are used to treat fevers including those caused by malaria. A recent review reveals the various biological activity, chemical constituents and other properties in the *Xylocarpus* genus [6]. The most characteristic of the genus Xylocarpus are xyloccensins, a class of limonoids. Perhaps even most limonoids are active as insect antifeedants, but most of these are not directly insecticidal. Some limonoids have also been found to be active against some types of cancer. Isolation and identification of two limonoids, gedunin (1) and 1α-hydroxy-1,2-dihydrogedunin (2) have been recently reported [7]. An earlier report from our laboratory revealed the antihyperglycaemic and antidyslipidemic activity in the ethanolic extracts of *Xylocarpus granatum* fruits in the validated animal models [8]. The present paper adds antihyperglycaemic as well as antidyslipidemic activity in ethyl acetate fraction of the epicarp portion of the fruits of *X. moluccensis* (EAXm) on streptozotocin-induced diabetic rats, HFD/HSD-STZ rats, db/db mice and high fructose high fat fed Syrian golden hamsters.

MATERIALS AND METHODS

Animals

8 to 10 weeks old male albino rats (Sprague Dawley strain), 10 to 12 weeks old db/db mice and 6 to 8 weeks old male Syrian golden hamsters were procured from the animal colony of CSIR-Central Drug Research Institute, Lucknow, India. Animals were always placed in groups of three to five in polypropylene cages. The following norms were always maintained in animal room environment: temperature 23 ± 2°C; humidity 50-60%; light 300 Lux at floor level with regular 12 h light cycle; noise level 50 decibel; ventilation 10-15 air changes per hour. The animals were fed ad libitum standard pellet diet and had free access to water unless stated otherwise. Research on these animals was conducted in accordance with the guidelines of the Committee for the Purpose of

Control and Supervision of Experiments on Animals (CPCSEA) formed by the Government of India in 1964.

Chemicals

Tissue culture medium DMEM, antibiotics, insulin, 2-deoxyglucose, cytochalasin B, Streptozotocin, metformin, and fenofibrate were purchased from Sigma Chemical Company, St. Louis, USA. Fetal bovine serum was purchased from Gibco BRL, USA and 2-^{3}H-deoxy glucose was purchased from GE Healthcare, UK. Fructose, sucrose, casein and cholesterol and all other biochemicals used in this study were obtained from Sisco Research Laboratory (India). Assay kits for the measurement of AST, ALT, ALP, urea, uric acid, creatinine, cholesterol, triglycerides, HDL and LDL-cholesterol in serum were purchased from Roche Diagnostics and used according to the instructions of the manufacturer on Cobas Integra 400 automated analyzer.

Cell culture

L6 rat skeletal muscle cell lines, procured from National Center of Cell Sciences (NCCS), Pune, India were available in animal facility division of the institute. Cells were maintained following previously established method [9, 10] in Dulbecco's modified Eagle medium (DMEM) with 10% FBS supplemented with penicillin (100 units/mL), streptomycin (200 µg/mL) and gentamycin (50µg/mL) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. For differentiation, cells were transferred to DMEM with 2% FBS and allowed to reach confluence, align and fuse into myotubes before being used for experimentation. The extent of differentiation was established by observing multinucleation of cells and ~90% fusion of myoblasts into myotubes was observed after 4-6 days post confluence and considered for experimentation

Extraction and fractionation of fruits

The whole fruits of *X. moluccensis* were always collected during January to March from coastal regions of India. Epicarp portion of approximate 10.0 kg of fruits were removed, shade dried, and powdered in mechanical disintegrator. The fine powder as obtained was submerged in 50% ethyl alcohol and this process was repeated several times. The combined extract was filtered and the solvent was removed in rotavapor. The dried ethanolic extract was macerated with hexane and hexane insoluble fraction was further macerated with ethyl acetate. The ethyl acetate soluble fraction was dried in rotavapour. The dried ethyl acetate fraction stored in airtight containers and termed as EAXm. Fig 1 shows the typical HPLC profile of EAXm. The major peaks were identified as xyloccensis.

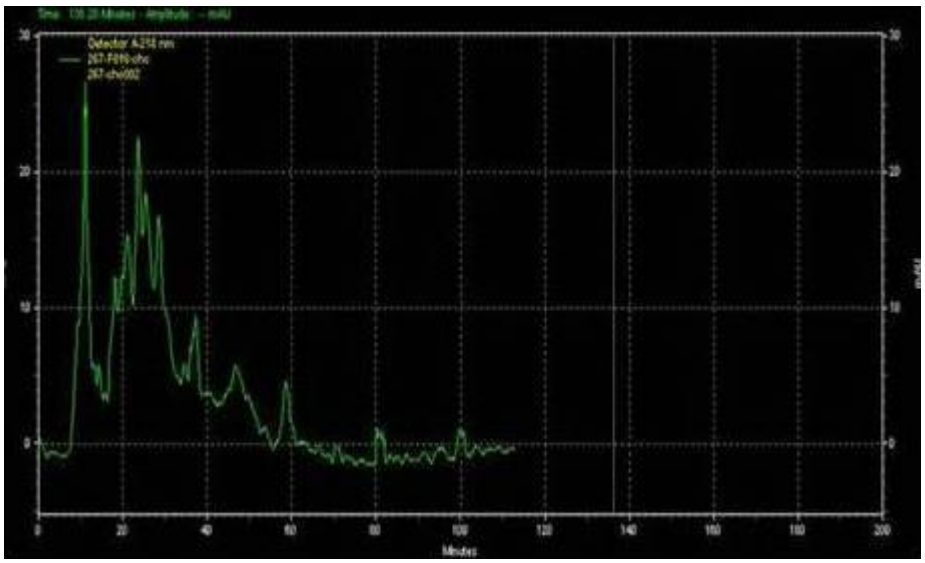

Fig. 1: Typical HPLC profile of ethyl acetate fraction of epicarp of Xylocarpus moluccensis

Effect on ^{3}H-2-deoxyglucose uptake by L-6 skeletal muscle cells

L6 cells grown in 24 well plates (6x10^{4}cells/well) were subjected to 2-^{3}H-deoxyglucose uptake as reported by Tamrakar et al. [11]. Briefly L6 myotubes were incubated with different concentrations of EAXm for 24 h with final 3 h in serum-deprived medium and a sub-set of cells were stimulated with 100 nM insulin for 20 min. Glucose uptake was assessed for 5 min in HEPES-buffered saline [140 mM NaCl, 20 mM HEPES, 5 mM KCl, 2.5 mM MgSO4, 1 mM CaCl2 (pH 7.4)] containing 10 µM 2-DG (0.5 µCi/ml 2-[^{3}H] DG) at room temperature. After uptake period, radioactive solution was rapidly aspired, and the cell mono layers were washed three times with ice-cold HEPES (0.9% Nacl and 25mM D-glucose) buffered saline solution. Cell associated radioactivity was determined by cell lysis in 0.05N NaOH, followed by scintillation counting (Beckman Coulter, USA). All assays were performed in triplicates and normalized to total protein, was expressed as fold change with respect to control.

Antidiabetic effects on streptozotocin-induced diabetic rats

Diabetes was induced in rats by intraperitoneally injecting STZ (solution prepared in 100 mM citrate buffer (pH 4.5) at 60 mg/kg body weight dose and animals showing blood glucose level between 270-450 mg/dl 48 hours later were considered as diabetic. The diabetic rats were divided into two sets consisted of three groups of six animals in each. In one set of the experiment the experimental groups were given suspension of metformin (100 mg/kg) and EAXm (100mg/kg) prepared in 1% gum acacia, respectively. Rats of control group were given an equal amount of 1% gum acacia (vehicle) and this group was termed as diabetic control. Blood glucose levels of all animals were again monitored at 30, 60, 90, 120, 180, 240, 300 min and thereafter at 24 hours post administration of test substances. Food but not water was withheld from the cage during 0-300 min. The blood glucose level of each animal was plotted against time (min post treatment) and the area under curve (AUC) was calculated using Prism Software. The percent decline in AUC of experimental group compared to diabetic control group determined the percentage blood glucose lowering activity.

In another set treatment of metformin, EAXm and 1.0 % gum acacia was continued for 10 consecutive days at the desired dose levels. The fasting blood glucose level of each animal was determined at the time of start and on day 10th when an oral glucose tolerance test of each rat was also performed post 3g/kg post glucose load. On day 11th the blood was withdrawn from the retro-orbital plexus of each rat for the estimation of glucose, insulin, triglycerides, cholesterol, HDL-C and LDL-C levels. The insulin target tissues like muscle and adipose as well as non target tissues like liver and kidney were excised and the activities of few rate limiting enzymes of glucose metabolism in these tissues were determined [12]. The serum

insulin content was determined using the Elisa kit for rat serum insulin as provided by Linco Research Inc, USA.

Antidiabetic effect on high fructose high fat diet fed and high sucrose high fat diet fed -low dosed streptozotocin treated diabetic rats

Rats of one group were kept on homemade high fructose high fat (HFHFD) and the other high sucrose high fat diet fed (HSHFD) diets for two weeks. Half of the animals showing high serum triglycerides and cholesterol levels in these two groups were given streptozotocin by intraperitoneal injection at a dose of 45 mg/kg, whereas the other half served as control. The animals of the HSHFD and HFHFD groups injected with STZ showing impaired oral glucose tolerance two weeks later were separated in each and termed diabetic. At this stage the control as well as the diabetic animals were sub grouped into four consisted of six animals in each. First sub group is non diabetic control group; second group is diabetic control group while the other two groups were experimental group. Rats of experimental groups were treated with metformin and EAXm at 100 mg/kg respectively for 10 consecutive days whereas the rats of control group received only 1.0 % gum acacia during this period. The oral glucose tolerance test of each animal in each sub group was determined on both day 0 and day 10^{th}, respectively. At the end of experiment the animals were bled; serum was separated and analyzed for insulin, triglycerides, cholesterol, HDL and LDL-cholesterol, AST, ALT, ALP, urea, uric acid, creatinine as described earlier.

Antidiabetic effect on db/db mice

Prior to start of the test sample feeding on db/db mice, a vehicle training period was followed from day -3 to day 0 during which all the animals were given vehicle (1% gum acacia) at a dose volume of 10 ml/kg body weight. At day 0 the animals having their random blood glucose levels between 180 to 300 mg/dl were finally selected and divided into three groups consisted of 6 animals in each. One group was considered as control group while the other two groups as experimental groups. The experimental groups were given metformin and EAXm (at the dose 50 and 100 mg/kg body weight dose), respectively. The control group always received an equal amount of vehicle. The blood glucose of each animal was determined everyday and on day 10th and day 15th glucose tolerance (OGTT) of each animal post 3 g/kg glucose load. On day 16th blood was withdrawn from the retro-orbital plexus of each animal for the estimations of serum triglycerides, cholesterol, high density lipoprotein cholesterol (HDL-C) and insulin levels as described earlier. At the end of the experiment the insulin target tissues like muscle and adipose and non insulin target tissues like liver and kidney were taken out of each mice under light ether anesthesia and approximate 100 mg of each tissue was frozen at -70^0C and the rest was stored at -20^0C and used for the estimation of enzyme activities as described earlier.

Antidyslipidemic activity effects on dyslipidemic Syrian golden hamsters

Dyslipidemia in male Syrian golden hamsters was produced by keeping the animals on high fructose high fat diet (HFD) for 40 days. The dyslipidemic hamsters as evidenced by high cholesterol and triglycerides levels in their sera were divided into five groups consisted of six animals in each, The experimental groups were administered the standard antidyslipidemic drug fenofibrate at 100 mg/kg dose and the test substance EAXm at the doses of 50 and 100 mg/kg body weight, respectively, for 10 consecutive days. The control animals were always given an equal amount of vehicle only and served. At the end of the experiment period i.e. on day 11^{th}, the blood of each animal was withdrawn from retro-orbital plexus of each, serum separated and analyzed for total cholesterol, triglycerides, HDL and LDL-cholesterol using assay kits from Roche on Cobas Integra 400 automated analyzer.

Preparation of tissue homogenates

After the completion of treatment, animals were killed after an overnight starvation, and their liver, muscle and kidney were quickly excised for activity determination of key enzymes of carbohydrate metabolism. A 10 % homogenate of each was prepared in 150mM KCl (w/V) using Potter Elvejhem glass homogenizer fitted with Teflon pestle. The homogenates were centrifuged at 1000 r/min for 15 min at 4^0C; the supernatants were stored and used as enzyme source. Protein content of the supernatant was determined by the method of Lowry et al. [13].

Activity of Glucokinase (GK; EC 2.7.1.2)

It was assayed according to the Porter and Chassy (14), which was based on the formation of G6Pase. The 1.0 ml assay mixture contained 4-(2-hydro-xyethyl)-1-piperazineethanesulfonic acid (HEPES) of 1 M (pH 7.5), Adenosine-5'-triphosphate (ATP) of 0.1 M (pH 7.0), $MgCl_2$ of 0.1 M, Niotinamide adenine dinucleotide phosphate (NADP) of 0.01 M (pH 7.0), D-glucose of 0.5 M, G-6-phosphatase dehydrogenase of 5 IU, and enzyme protein. Change in optical density was measured at 340 nm at 30 s interval for 3 min.

Activity of Pyruvate kinase (PK; EC 2.7.1.40)

PK was assayed according to the method of Buchner and Pfleiderer [15]. The reaction mixture contained 0.2 mM Tris-HCl (pH 7.4), 0.1 mM KCl, 10 mM $MgCl_2$, 5 mM Adenosine diphosphate (ADP), 5 mM Phosphoenolpyruvate (PEP).4 units LDH, 0.24 mM $NADH_2$, and enzyme protein. Change in optical density was measured at 340 nm at 30 s intervals for 3 min.

Activity of Glycogen phosphorylase (GP; EC 2.4.1.1)

The activity of GP was measured according to the method of Berthet et al., 1956 [16]. The 1.0 ml assay mixture contained 0.2 ml nixture A (57 mg of glycogen, 188 mg of glucose-1-phosphate, 42 mg of NaF, and 5' Adenosine monophosphate (AMP) (4 mM) in 10 ml distilled water) and 0.1 ml mixture B, enzyme protein. Thr tubes were incubated at 37^oC for 30 min after which the reaction was terminated by the addition of 0.1 ml of 10% Tri-chloroacetic acid (TCA) and then 0.4 ml sodium acetate (100 mM) was added to prevent the spontaneous hydrolysis of glucose-1-phosphate present in the reaction mixture. The estimation of inorganic phosphate in the protein free supernatant was done according to the method of Taussky and Shorr [17].

Activity of Glucose-6-phosphatase (G-6-Pase; EC 3.1.3.9)

The enzyme activity of G6Pase was measured according to the method of Hubscher et al [18]. The 1.0 ml assay system contained 0.3 ml of citrate buffer 0.01 M (pH 6.0), 28 mM of ethylenediaminetetraacetic acid,14 mM of NaF, 200 mM of glucose – phosphate, and appropriate amount of enzyme protein. The tubes were incubated at 37^oC for 30 min after which the reaction was terminated by the addition of 1.0 ml of 10% TCA. Estimation of inorganic phosphate in the protein-free supernatant was done according to the method of Taussky and Shorr [17].

Activity of Fructose bis phosphatase (FBPase; EC 3.1.3.11)

The enzyme activity of FBPase was measured according to the method of Ulm et al [19].The 1.0 ml assay mixture contained 20 mM triethanolamine (pH 7.5), 2 mM $MgCl_2$, 40 mM $(NH_4)_2SO_4$, 0.15 mM fructose-1,6-bisphosphatase, 0.5 mM NADP, 0.1 mM EDTA, 1 U/ml of glucose-6-phosphatase dehydrogenase, and 1 U/ml of glucose phosphate isomerase. Change in the optical density was measured at 340 nm at 30 s interval for 3 min.

Activity of Phosphoenol pyruvate carboxykinase (PEPCK; EC 1.1.3.2)

It was assayed according to the method of Ward et al [20]. The 1.0 ml assay mixture contained Tris-HCl buffer of 200 mM (pH 7.4), MnCl2 of 19 mM, $NaHCO_3$ of 20 mM, Guanidine phosphate (GDP) of 1 mM, 9 units of malate dehydrogenase, PEP of 5mM, $NADH_2$ of 0.24 mM, and enzyme protein. Optical density was measured at 340 nm at 30 s interaval for 30 min.

***In vitro* effect on purified enzyme**

α-glucosidase (EC 3.2.1.20)

This was done according to a slight modification of the procedure reported by Pistia-Brueggeman and Hollingsworth [21]. A reaction

mixture containing 500 µl of phosphate buffer (50 mM; pH 6.8), 100 µl of intestinal α- glucosidase (1 U/ml), 190 µl of TDW and 10 µl of fraction of was pre-incubated for 10 min at 37°C and then 100 µl of glutathione (1 mg/ml) and 100 µl of 1 mM PNPG was added to the mixture as a substrate. After further incubation at 37°C for 20 min, the reaction was stopped by adding 500 µl of Na_2CO_3 (0.1 M). All the enzyme, inhibitor and substrate solutions were made using the same buffer. α-glucosidase activity was determined spectrophotometrically at 405nm by measuring the quantity of para-nitrophenol released from pNPG. The concentration of EAXm required to inhibit 50% α -glucosidase activity under the assay conditions was defined as the IC50 value. Experiments were done in triplicates. The inhibition percentage (%) was calculated by the equation:

% Inhibition = $(A_{sample}/A_{control})$* 100-100

Kinetics of Inhibition: α- glucosidase activity was measured with increasing concentration of p-NPG as a substrate in the absence or presence of EAXm. Enzyme kinetics data was analyzed by Lineweaver-Burk plot to point out the type of inhibition.

Statistical analysis

Each parameter was expressed as mean±SE of three independent experiments. Statistical analysis of each parameter between the groups was done by student's t test. The p value of less than 0.05, 0.01 and 0.001 were considered as significant, more significant and most significant, respectively.

RESULTS

Effect on 2-^{3}H-deoxy-glucose uptake

Differentiated myotubes were incubated with different concentrations of EAXm for 16 h under culture conditions and then 10 µM 2-^{3}H-deoxy-glucose (0.5 µCi/ml) in HEPES buffer, pH was added. The uptake was assessed for 5 min at room temperature. It was found that EAXm stimulated glucose uptake in a concentration-dependent manner from 1 to 10 µg. The maximum effect was seen at 10 µg/ml concentration which was calculated around 5.84 fold compared to control cells. On comparison the standard antidiabetic drug metformin caused nearly 1.78 fold stimulation at 500µg/ml concentration (Fig 2).

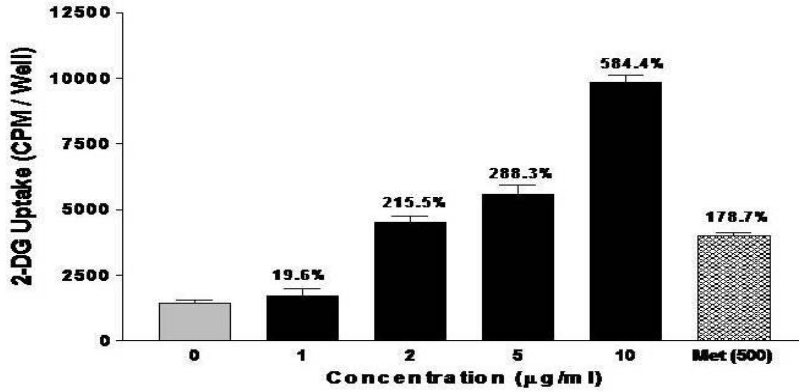

Fig. 2: Effect of EAXm and metformin on 2-^{3}H-deoxyglucose uptake by differentiated myotubes (L-6)

Single Dose Effect

Effect on streptozotocin-induced diabetic rats

Table 1 shows the effect of EAXm and metformin on blood glucose profile on streptozotocin-induced diabetic rats. Single dose treatment of EAXm at 100 mg/kg dose to streptozotocin-induced caused lowering on their blood glucose profile to the average of 22.8 % during 0 to 5 hours and 34.1 % during 0 to 24 hours. The effect of EAXm was comparable to the blood glucose lowering effect of standard antidiabetic drug metformin which showed nearly 24.2 and 33.8 % lowering in blood glucose level during this period on streptozotocin treated diabetic rats.

Table 1: Effect of EAXm and metformin on blood glucose profile of streptozotocin-induced diabetic rats

Treatment	Blood Glucose profile (mg/dl) min post treatment									AUC		% glucose lowering	Blood
	0´	30´	60´	90´	120´	180´	240´	300´	1440´	0–5h	0–24h	0–5h	0–24h
Sham treated Control (1.0% gum–acacia)	410.6 ±34.4	415.6 ±47.3	418.4 ±30.4	416.6± 22.9	378.8± 24.9	405.2 ±15.8	419.0 ±15.7	446.8 ±26.1	432.0± 14.6	123600	624500	-	-
EAXm treated (100 mg/kg)	439.8 ±39.4	405.2 ±45.0	391.6 ±37.6	347.8± 33.8	340.4± 22.2	288.0 ±29.9	243.4 ±30.6	241.4 ±31.5	313.0± 16.1	95380	411400	22.8*	34.1**
Metformin treated (100 mg/kg)	404.8 ±7.46	395.4 ±26.5	350.0 ±27.7	347.6± 35.7	296.4± 13.3	279.2 ±15.3	281.0 ±25.3	263.8 ±29.3	297.4± 26.7	93720	413600	24.2**	33.8**

Values are mean± S.E. of 5 rats.Statistical significance p*<0.05, p**<0.01, p***<0.001

Multiple Dose Effect

Antidiabetic effect of EAXm on streptozotocin-induced diabetic rat

Effect on fasting blood glucose and oral glucose tolerance (OGTT)

Fig 3a and 3b depict the effect of EAXm on OGTT post glucose load on day 0 and 10 post treatment. It is evident from results that EAXm lowered the fasting blood glucose level to around 25.7 % and improved OGTT to around 21.7 % when given at 100 mg/kg for 10 consecutive days. The metformin treated group showed nearly 35.6 % lowering on fasting blood glucose profile and 26.2 % improvement on OGTT on day 10 (Table 2).

Effect on serum insulin, fructosamine and lipid profile

Table 2 also showed the serum insulin, fructosamine and lipid profiles of sham treated control, EAXm treated and metformin treated groups on day 11. EAXm and metformin treated groups showed increased serum insulin profiles compared to sham treated control group and the respective increase were calculated to be around 36.6 % and 25.5 %. EAXm also caused nearly 24.9 % decline in the serum fructosamine level whereas the metformin treated group showed though nearly 19.0 % decline on serum fructosamine level but the effect was insignificant. Around 31.5% lowering in serum triglyceride, and around 9.96 % in serum LDL-C level was seen respectively in the EAXm treated group. There was found no significant effect on serum cholesterol level either by EAXm or metformin, however, EAXm treatment increased serum HDL-C level to around 24.7 %. Metformin treatment did not cause significant effect on serum HDL-C level on streptozotocin-induced diabetic rats.

Effect of EAXm on regulatory enzyme of carbohydrate metabolism of muscle liver and kidney of streptozotocin-induced diabetic rats

The activity profile of few key regulatory enzymes of glycolysis, gluconeogenesis and glycogenolysis in sham treated non diabetic, sham treated streptozotocin-induced diabetic control and streptozotocin-induced diabetic animals treated with EAXm for 10 consecutive days are presented in Tables 3 to 5. The activities of pyruvate kinase and phosphofructokinase were found elevated in liver and kidney of streptozotocin-induced diabetic rats whereas, the activity of glucokinase was found elevated in liver of these rats only. Although in muscle tissues the activities of said enzymes were found decreased, however, glucose-6-phosphatase, fructose-1, 6 bisphosphatase and phosphoenolpyruvate carboxykinase were likewise elevated in these three tissues i.e. liver and kidney and but glucose-6-phosphatase was not detected in the muscle of streptozotocin-induced diabetic rats. Glycogen phosphorylase was found to be significantly elevated especially in muscle tissue of streptozotocin-induced diabetic rats compared to normal rats. Administration of EAXm to the streptozotocin-induced diabetic rats for 10 consecutive days induced significant decrease in glucose-6-phosphatase and fructose-1, 6-bisphosphatase activity mainly in the liver. EAXm treatment resulted in greater decrease of glycogen phosphorylase enzyme activity in all three studied tissues.

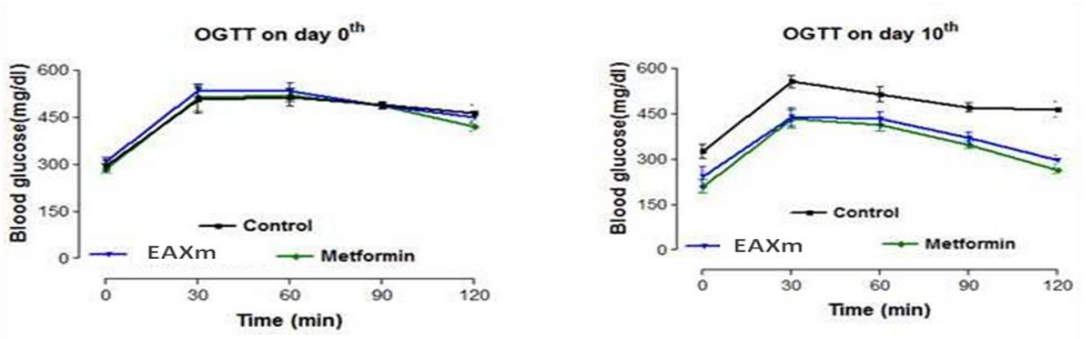

Fig. 3a and 3b depicts the oral glucose tolerance on day 0 and day 10th post treatment in streptozotocin induced diabetic rats

Table 2: Effect of EAXm and metformin on blood glucose, serum insulin, fructosamine and lipid profiles of streptozotocin treated diabetic rats

Groups	Biochemical profiles (Blood/Serum)						
	FBG (mg/dl)	Insulin (µU/ml)	Fructosamine (µmol/l)	TG (mg/dl)	TC (mg/dl)	HDL-C (mg/dl)	LDL-C- (mg/dl)
Non- Diabetic Control (1.0% gum-acacia)	82.0±3.20	41.5±1.32	67.8±4.62	68.4±4.20	45.9±2.34	34.2±2.21	26.3±2.21
Sham treated Diabetic control (1.0% gum-acacia)	325.0±22.1	14.5±2.6	327.3 ±18.2	42.2±6.8	26.8±3.4	21.6±3.5	21.1±2.4
EAXm treated (100 mg/kg)	241.4±22.6 (25.7)*	19.8±1.5 (+36.6)*	245.4±15.8 (24.9)*	28.9±5.2 (31.5)**	33.4±4.0 (+24.6)ns	26.8±1.8 (+24.7)*	19.0±1.4 (9.96)ns
Metformin treated (100 mg/kg)	209.4±20.3 (35.6)*	18.2±1.4 (+25.5)ns	265.0±20.4 (19.0)ns	39.2±7.4 (7.10)ns	30.9±3.5 (+15.2)ns	22.4±2.1 (+3.70)ns	22.2±2.3 (+5.21)ns

Values are mean ±S.E. of six rats, Statistical significance *<.05, **<.01, ***<.001 and ns not significant

Table 3: Effect of EAXm on key enzymes of glycolysis, glycogenolysis and gluconeogenesis in liver of streptozotocin induced diabetic rats

Enzymes	Enzyme Activity (µmol/min/mg protein)		
	Non-diabetic Control (1.0% gum-acacia)	Diabetic Control (1.0% gum-acacia)	EAXm treated (100mg/kg)
Glucokinase	2.74±1.31	2.12±0.76 (22.6)	2.42±1.14 (+14.1)
Phosphofructokinase	5.13±1.08	4.32±0.79 (15.7)	4.93±1.01 (+14.1)
Pyruvate kinase	42.6±4.92	28.7±5.62 (32.6)	34.8±3.64 (+21.2)
Glycogen phosphorylase	9.13±2.14	19.5±2.84 (+113.5)	12.2±3.10 (37.4)
Phosphoenolpyruvate carboxykinase	36.3±7.41	127±6.40 (+251.5)	65.3±8.40 (48.5)
Fructose1,6 bisphosphatase	6.31±1.21	18.9±3.73 (+199.5)	12.1±2.15 (35.9)
Glucose-6-phosphatase	9.43±2.22	30.8±4.62 (+226.6)	24.2±2.14 (21.4)

Table 4: Effect of EAXm on key enzymes of glycolysis, glycogenolysis and gluconeogenesis in kidney of streptozotocin induced diabetic rats

Enzymes	Enzyme Activity (µmol/min/mg protein)		
	Non-diabetic Control (1.0% gum-acacia)	Diabetic Control (1.0% gum-acacia)	EAXm treated (100mg/kg)
Phosphofructokinase	5.43±0.62	6.83±0.67 (+25.7)	5.90±0.46 (13.6)
Pyruvate kinase	15.2±1.73	17.8±2.64 (+17.1)	16.3±2.04 (+8.42)
Glycogen phosphorylase	8.41±2.12	17.6±3.24 (+109.2)	10.8±1.47 (23.2)
Phosphoenolpyruvate carboxykinase	39.9±4.24	65.7±3.14 (+64.6)	58.1±6.50 (11.5)
Fructose1,6 bisphosphatase	5.43±3.47	13.8±4.24 (+154.1)	8.36±2.58 (39.4)
Glucose-6-phosphatase	10.4±2.31	20.1±3.41 (+93.2)	16.4±2.45 (18.4)

Table 5: Effect of EAXm on key enzymes of glycolysis, glycogenolysis and gluconeogenesis in muscle of streptozotocin induced diabetic rats

Enzymes	Enzyme Activity (µmol/min/mg protein)		
	Non-diabetic Control (1.0% gum-acacia)	Diabetic Control (1.0% gum-acacia)	EAXm treated (100mg/kg)
Phosphofructokinase	6.41±1.41	5.06±0.76 (21.0)	5.94±0.62 (+17.3)
Pyruvate kinase	17.3±3.29	12.3±1.02 (28.9)	16.2±1.64 (+31.7)
Glycogen phosphorylase	15.4±3.74	98.7±5.48 (+540.9)	46.1±5.67 (53.2)
Phosphoenolpyruvate carboxykinase	32.4±2.42	49.3±3.30 (+251.5)	41.8±3.41 (15.2)
Fructose1,6 bisphosphatase	4.12±0.71	12.1±0.53 (+193.6)	7.21±1.41 (40.4)

Antihyperglycaemic activity EAXm on High fructose high fat diet fed streptozotocin treated diabetic rats

Effect on body weight

Table 6 presents the effect of EAXm, metformin and fenofibrate on body weight of high fructose high fat diet fed streptozotocin-induced diabetic rats. Nearly 11.4 % decrease in body weight was observed in the EAXm treated group whereas metformin did not cause any marked effect on the body weight. Fenofibrate treated group showed nearly 14.5% decline in body weight compared to sham treated control.

Effect on fasting blood glucose and oral glucose tolerance (OGTT)

Fig 4 a and 4b depict the effect of EAXm, metformin and fenofibrate on OGTT of high fructose high fat diet fed streptozotocin treated rats on day 0 and 10 post treatment. It was evident from the results that EAXm treatment lowered the fasting blood glucose level and improved OGTT after 10 days of consecutive treatment. The respective lowering on fasting blood glucose profile was calculated to around 32.7%, whereas improvement on OGTT was calculated around 26.9% ($p<0.05$) by EAXm at 100 mg/kg dose. The metformin treated group showed around 24.3% lowering on fasting blood glucose profile and 29.6% improvement on OGTT respectively. The effect of fenofibrate on either fasting blood glucose profile or OGTT on streptozotocin-induced diabetic rats was not significant.

Effect on serum insulin level, fructosamine and lipid profile

Table 6 also showed the serum insulin, fructosamine and lipid profiles of sham treated control, EAXm, metformin and fenofibrate treated groups. The EAXm, metformin or fenofibrate treatment did not cause any effect on serum insulin levels but EAXm treated group showed decline in their serum fructosamine level to the tune of 26.1 %. EAXm treated group showed 38.4% decline in their serum triglycerides and 17.8 % decline in their serum cholesterol level and 28.3 % decline in their serum LDL-C levels compared to sham treated control. EAXm treated group also showed around 30.2 % increase in their serum HDL-C level. Fenofibrate treatment caused nearly 33.3% decline in their serum triglycerides, 13.5 % decline in their serum cholesterol and nearly 30.4 % decline in their serum LDL-C levels compared to sham treated control, Fenofibrate treatment also caused 33.2% increase in their HDL-C level. Metformin treatment did not show any significant effect on the level of either serum triglycerides, cholesterol, LDL-cholesterol or HDL-cholesterol levels.

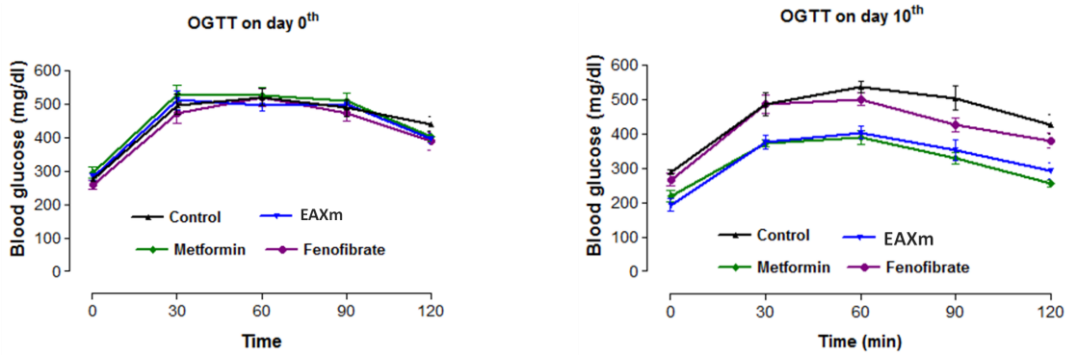

Fig. 4a and 4b: Depicts the oral glucose tolerance on day 0 and day 10th post treatment in high fructose high fat fed streptozotocin induced diabetic rats

Table 6: Effect of EAXm on blood glucose lowering and serum lipid profile in high fructose high fat fed streptozotocin treated diabetic rats

Groups	Body weight (g)	Biochemical profiles						
		FBG (mg/dl)	Insulin (µU/ml)	Fructosamine (µmol/l)	TG (mg/dl)	Cholesterol (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)
Normal control (1.0% gum-acacia)	152.8±8.27	80.6±3.21	42.4±3.42	65.8±4.21	69.6±4.27	49.5±3.21	35.7±2.32	32.2±2.31
Sham treated HFD fed control (1.0% gum-acacia)	219.2±9.30	104.6±6.20	36.8±4.42	149.8±7.32	256.4±16.7	189.6±11.8	59.5±4.19	72.4±4.25
Sham treated diabetic control (1.0% gum-acacia)	193±8.80	288.0±7.40	24.5±2.6	326.4 ±19.2	391.0±52.5	275.2±28.7	67.2±9.50	89.2±12.4
EAXm treated (100 mg/kg)	171±7.2 (11.4)	193.6±14.2 (32.7)*	23.5±1.5 (4.10)^{ns}	241.1±11.4 (26.1)*	241.2±23.4 (38.4)**	226.4±17.2 (17.8)^{ns}	87.6±7.08 (+30.4)*	64.0±5.40 (28.3)*
Metformin treated (100 mg/kg)	185±9.2 (4.14)	218.0±16.1 (24.3)*	22.2±1.4 (9.40)^{ns}	275.2.0±18.4 (15.9)^{ns}	359.1±37.4 (8.20)^{ns}	260.4± 22.6 (5.45)^{ns}	75.8±12.1 (+12.8)^{ns}	82.2±11.3 (7.95)^{ns}
Fenofibrate treated (100mg/kg)	165.5±6.6 (14.5)	267.2±10.2 (7.22)	25.2±3.2 (+2.85)^{ns}	289.5±22.5 (11.3)ns	261.2±22.6 (33.3)**	238.6±20.2 (13.5)^{ns}	89.5±8.20 (+33.2)*	62.1±5.50 (30.4)**

Values are mean± S.E. of six rats. Statistical significance *p<.05, **p<.01, ***p<.001 and ns= Not significant.

Effect on liver and kidney function tests

Table 7 showed the effects of EAXm, metformin, on liver and kidney functions on high fructose high fat diet fed streptozotocin-treated rats. EAXm treatment resulted in 25.5 % lowering in serum ALT and 16.7 % lowering in serum AST and 9.40 % reduction in the serum ALP levels whereas metformin resulted around 18.4 lowering in serum ALT, 15.5% lowering in serum AST and 21.4% lowering in serum ALP, respectively. EAXm treatment also caused decline in the serum uric acid by around 21.6 %, serum creatinine level by around 28.3% and serum urea level by around 37.9 %. Metformin did not cause any significant effect on either serum uric acid or creatinine level but it caused significant decline in the serum urea level by around 25.3 %.

Table 7: Effect of EAXm on the liver function and renal function profile of high fructose high fat diet fed streptozotocin treated diabetic rats

Groups	Liver function profile			Renal function profile		
	ALT (U/L)	AST (U/L)	ALP (U/L)	Uric acid (mg/dl)	Creatinine (mg/dl)	Urea (mg/dl)
Normal control (1.0% gum-acacia)	36.7±2.43	46.3±2.32	57.6±4.32	0.76±0.06	0.66±0.04	30.5±5.41
Sham treated HFD fed control (1.0% gum-acacia)	79.8±4.30	123.3±6.30	134.3±4.21	1.76±0.31	1.54±0.24	39.1±1.10
Sham treated diabetic control (1.0% gum-acacia)	252.5±25.6	241.3±14.6	277.5±37.4	3.65±0.64	3.36±0.18	46.2±4.05
EAXm treated (100 mg/kg)	188.6±10.9 (25.5)*	201.0±11. (16.7)*	251.4±26. (9.40)	2.86±0.18 (21.6)*	2.41±0.12 (28.3)*	28.7±1.85 (37.9)*
Metformin treated (100 mg/kg)	206.0±14.5 (18.4)*	204.3±12. (15.5)	18.2±20.2 (21.4)*	2.99±0.28 (10.9)	2.78±0.20 (17.3)	34.5±2.60 (25.3)*

Values are mean±SE. Statistical significance *p<.05, **p<.01, ***p<.001

Antihyperglycaemic activity of EAXm on high sucrose high fat diet fed streptozotocin treated diabetic rats

Effect on body weight

Table 8 presents the body weight of sham treated, EAXm, metformin and fenofibrate treated groups. EAXm caused nearly 11.4 % declines in the body weight. Metformin did not cause any significant effect on body weight whereas, fenofibrate treated group showed nearly 14.5 % decline in body weight.

Effect on fasting blood glucose and oral glucose tolerance (OGTT)

Fig 5a and 5b depict the effect of EAXm, Metformin and fenofibrate on fasting blood glucose and improvement on OGTT before start and after treatment. EAXm caused lowering in fasting blood glucose level and improved oral glucose tolerance of high sucrose high fat diet fed streptozotocin-treated rats. The respective lowering in fasting blood glucose profile by EAXm was calculated to be around 31.3%, whereas the respective improvement on OGTT was calculated to be around 29.1 % by EAXm at 100 mg/kg dose. Metformin caused around 32.4 % lowering on fasting blood glucose profile and 27.2 % improvement on OGTT respectively. Fenofibrate treatment did not show any effect on either fasting blood glucose level or any improvement on OGTT of high sucrose high fat diet fed streptozotocin-induced diabetic rats.

Effect on serum insulin level, fructosamine and lipid profile

Table 8 also shows the serum insulin, fructosamine and lipid profiles of sham treated control, EAXm, metformin and fenofibrate treated groups. EAXm treatment significantly decreased the serum insulin level of high sucrose high fat diet fed streptozotocin treated rats and this effect was calculated around 15.1%. Metformin and fenofibrate treatment did not cause any significant effect on serum insulin level or fructosamine level whereas EAXm treatment caused nearly 24.3% decline in serum fructosamine level. EAXm treated group showed nearly 56.5% decline in their serum triglycerides, 23.5 % decline in their serum cholesterol level and 24.5 % decline in serum LDL-C level. EAXm treated group showed increased serum HDL-C level and which was calculated to be around 32.7 %. Fenofibrate treatment also caused 44.8 % decline in serum triglycerides, 20.3 % decline in serum LDL-C, and 7.99 % decline in serum cholesterol level and nearly 17.8 % increase in serum HDL-C level. Metformin treatment resulted only 18.2 % lowering in serum triglyceride whereas no marked effect of metformin was observed on serum cholesterol, LDL-C or HDL-C levels.

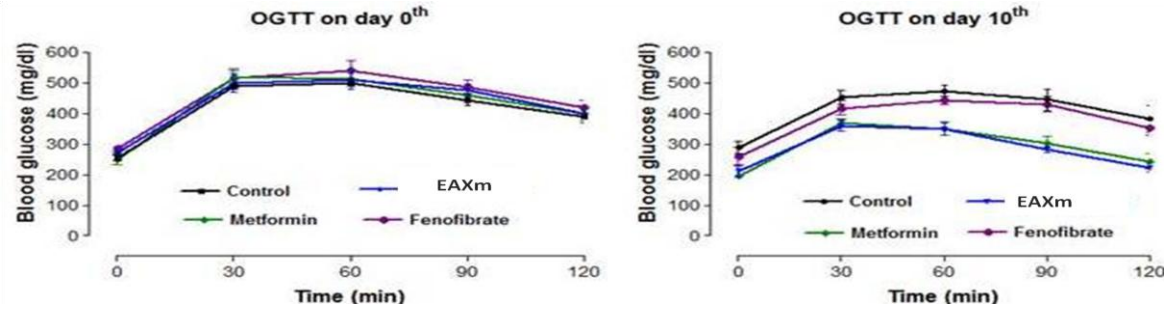

Fig. 5a and 5b: Depicts the oral glucose tolerance on day 0 and day 10th post treatment in high fructose high fat fed streptozotocin induced diabetic rats

Table 8: Effect of EAXm on blood glucose lowering and serum lipid profile in high sucrose high fat fed streptozotocin treated diabetic rats

Groups	Bodyweight (g)	Biochemical profiles (Blood/Serum)						
		FBG (mg/dl)	Insulin (µU/ml)	Fructosamine (µmol/l)	TG (mg/dl)	TC (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)
Normal control (1.0% gum-acacia)	156.8±7.21	77.8±3.29	42.4±3.42	56.8±5.36	63.3±4.58	45.4±3.89	34.9±2.78	30.4±3.39
Sham treated HFD fed control (1.0% gum-acacia)	207.2±6.82	105.6±8.12	33.4±4.48	136.4±6.92	246.4±13.4	192.6±13.8	63.5±5.30	77.8±4.95
Sham treated diabetic control (1.0% gum-acacia)	222.0±10.4	287.6±9.50	21.9±2.3	342.5±24.4	488.4±82.6	275.4±29.4	106.4±9.60	123.5±12.5
EAXm treated (100 mg/kg)	177±7.60 (20.3)*	197.4±10.2 (31.3)**	18.6±1.5 (15.1)*	259.2±12.4 (24.3)*	212.8±39.4 (56.4)**	210.6±15.2 (23.5)*	141.2±8.8 (+32.7)*	93.3±8.20 (24.5)*
Metformin treated (100 mg/kg)	192.4±9.2 (13.3)	194.4±5.99 (32.4)**	20.1±1.4 (8.20)	301.0±23.4 (12.1)	399.5±37.4 (18.2)	270.2±29.7 (1.89)	109.8±10.5 (+3.20)	115.7±13.5 (6.31)
Fenofibrate treated (100mg/kg)	188.5±6.6 (15.1)	260.4±18.9 (9.45)	21.4±3.2 (2.30)	188.5±6.6 (15.1)	269.7±30.2 (44.8)**	253.4±22.4 (7.99)	125.4±7.40 (+17.8)	98.4±6.60 (20.3)*

Values are mean± S.E. Statistical significance *p<.05, **p<.01, ***p<.001 and ns= not significant

Table 9: Effect of EAXm on the liver function and renal function profile of high sucrose high fat diet fed streptozotocin treated diabetic rats

Groups	Liver function profile			Renal function profile		
	ALT (U/L)	AST (U/L)	ALP (U/L)	Uric acid (mg/dl)	Creatinine (mg/dl)	Urea (mg/dl)
Normal control (1.0% gum-acacia)	33.2±3.41	42.1±3.20	52.1±3.30	0.86±0.05	0.61±0.03	31.2±3.15
Sham treated HFD fed control(1.0% gum-acacia)	74.6±4.80	111.3±5.20	120.8±4.61	1.65±0.35	1.82±0.28	34.6±1.21
Sham treated diabetic control (1.0% gum-acacia)	212.2±21.6	236.0±16.5	287.0±32.4	2.99±0.56	4.77±0.36	33.4±3.70
EAXm treated (100 mg/kg)	142.5±10.2 (32.8)*	189.8±10.3 (19.6)*	240.4±24.8 (16.2)	2.38±0.24 (20.4)*	3.40±0.16 (28.7)*	22.1±1.72 (33.8)**
Metformin treated (100 mg/kg)	180.0±18.4 (15.2)*	194.2±9.80 (17.7)*	232.3±21.2 (19.1)*	2.89±0.28 (3.34)	3.80±0.26 (20.3)	25.0±2.40 (25.1)*
Fenofibrate treated (100mg/kg)	190.5±20.3 (10.2)	207.5±13.2 (12.1)	254.1±22.2 (11.4)	2.73±0.26 (8.69)	4.20±0.38 (11.9)	28.4±4.15 (14.9)

Values are mean ± SE. Statistical significance *p<.05, **p<.01, ***p<.001 and ns= not significant

Table 10a: Effect of EAXm on random blood glucose profile of db/db mice

	Random Blood Glucose (mg/dl) days													
Group	0	1	2	3	4	5	6	7	8	9	11	12	13	14
Sham treated Control	267.5 ±24.8	268.5 ±29.4	272.0 ±25.1	268.3 ±21.5	279.3 ±39.3	272.8 ±36.9	281.2 ±45.3	268.7 ±22.6	240.5 ±26.4	222.3 ±29.1	217.3 ±23.2	229.7 ±26.6	239.0 ±32.9	245.0 ±24.3
EAXm treated (50.0 mg/kg)	266.8 ±25.8	259.0 ±20.4 (3.5)	255.4 ±19.1 (6.1)	256.2 ±23.3 (4.5)	255.2 ±24.3 (8.6)	249.8 ±26.4 (8.4)	236.8 ±20.2 (15.8)	215.4 ±27.1 (19.8)	222.6 ±30.3 (7.4)	193.2 ±31.6 (13.1)	176.5 ±23.9 (18.7)*	180.5 ±24.1 (21.4)*	152.5 ±22.1 (36.2)*	148.5 ±22.7 (39.4)*
EAXm treated (100 mg/kg)	268.8 ±34.8	246.0 ±29.4 (8.4)	239.4 ±25.1 (11.9)	206.2 ±21.5 (23.1)	205.2 ±29.3 (26.5)*	225.8 ±26.9 (17.2)	212.6 ±25.3 (24.4)*	203.2 ±22.6 (24.4)*	200.0 ±36.4 (16.8)	145.4 ±39.1 (34.6)**	113.5 ±23.2 (47.8)**	116.5 ±26.6 (49.3)**	114.5 ±22.9 (52.1)**	104.5 ±20.4 (57.3)**
Pioglitazone treated (10.0 mg/kg)	269.0 ±33.1	240.0 ±27.9 (10.6)	234.0 ±24.3 (14.0)	200.0 ±32.9 (25.5)*	212.0 ±43.5 (24.1)*	200.0 ±30.3 (26.7)*	192.0 ±22.1 (31.7)**	187.0 ±27.1 (30.4)**	174.0 ±18.6 (27.7)*	138.0 ±13.1 (37.9)**	100.0 ±15.6 (53.9)**	104.0 ±11.3 (54.7)**	109.0 ±18.2 (54.4)**	108.0 ±14.9 (55.9)**

Values are mean± S.E.; Statistical significance *p<.05, **p<.01, ***p<.001

Effect on liver and kidney function tests

Table 9 showed the effect of EAXm, metformin and fenofibrate on liver and kidney function tests of high sucrose high fat diet fed streptozotocin-induced diabetic rats. EAXm treatment to these rats resulted nearly 32.8 % lowering in serum ALT, 19.6 % lowering in serum AST and 16.2 % lowering in serum ALP. Metformin and fenofibrate caused nearly 15.2 and 10.2 % lowering in serum ALT, 17.7 and 12.1 % lowering in serum AST and 19.1 and 11.4 % decline in serum ALP, respectively. ETXm caused nearly 20.4 % reduction in serum uric acid level, 28.7 % lowering in serum creatinine level and 33.8 % lowering in serum urea level was observed. Metformin and fenofibrate treatment did not cause any marked effect on serum uric acid levels, whereas, these caused nearly 20.3 and 11.9 % decline in serum creatinine and nearly 25.1 and 14.9 % decline in the serum urea levels of high sucrose high fat diet fed streptozotocin-induced diabetic rats.

Antihyperglycemic and antidyslipidemic effect of EAXm and pioglitazone on db/db mice

Table 10a depicts the dose and time dependent effect of EAXm and pioglitazone on random blood glucose profile of db/db mice. The results reveals that the EAXm at 100 mg/kg dose produces more significant decline in random blood glucose of db/db mice from day 4th post daily treatment till the end of the experiment, whereas at 50.0 mg/kg dose, EAXm lowered the random blood glucose from day 11th that persisted till the end of the experiment. The standard drug pioglitazone at dose 10.0 mg/kg caused significant decline in random blood glucose from day 3rd which persisted till the end of the experiment (Fig 6a).

Table 10 b and 10c depicts the effect of EAXm and pioglitazone on improvement of OGTT post treatment. The fasting baseline blood glucose value at 0 min was found lowered in both EAXm or pioglitazone treated groups on 10 and 15 days post treatment, as compared to vehicle treated control group. Treatment of EAXm at 100 mg/kg body weight dose and pioglitazone effectively inhibited the rise in postprandial blood glucose post glucose load of 3.0 gm/kg body weight. The percent improvement on OGTT by EAXm at 50 and 100 mg/kg doses was calculated around 17.1 and 35.6 on day 10 and 24.1 and 39.8% respectively. Pioglitazone treatment to db/db at 10.0 mg/kg dose caused an improvement on OGTT by around 18.4 % on day 10 and 37.1 % on day 15, respectively (Fig 6b and 6c).

Table 10b: Effect of EAXm on oral glucose tolerance test (OGTT) on 10 day post reatment

Groups	Blood Glucose profile (mg/dl) min post glucose load					AUC (0-120 min)
	0	30	60	90	120	
Sham treated normal control (1.0% gum-acacia)	89.7±4.5	135.6±10.2	123.3±11.3	111.3±8.90	98.3±7.32	13930±854
Sham treated diabetic Control (1.0% gum-acacia)	195.0±37.5	398.8±62.6	380.6±72.2	333.6±77.4	313.8±70.2	37430±4475
EAXm treated (50.0 mg/kg)	151.6±6.8	357.4±41.5	291.2±27.1	226.0±26.8	192.2±22.2	31040±2797 (17.1)*
EAXm treated (100.0 mg/kg)	130.7±10.1	279.0±39.5	216.7±18.8	171.5±14.1	141.0±11.0	24100±2363 (35.6)*
Pioglitazone (10.0 mg/kg)	145.6±2.5	345.4±36.9	287.2±25.0	218.0±18.2	188.2±13.4	30530±2471 (18.4%)*

Values are mean±SE. Statistical significance *P<.05, **P<.01, ***P<.001

Table 10c: Effect of EAXm on oral glucose tolerance test (OGTT) on day 15th post treatment

Groups	Blood Glucose profile (mg/dl) min post glucose load					AUC (0-120 min)
	0	30	60	90	120	
Sham treated normal control (1.0% gum-acacia)	92.3±6.54	139.4±10.2	117.3±8.76	108.7±8.70	96.5±7.21	13790±766
Sham treated Diabetic Control (1.0% gum-acacia)	190.8±6.17	494.2±54.6	387.8±56.7	376.8±49.3	308.2±40.2	39550±3517
EAXm treated (50.0 mg/kg)	141.6±5.63	343.8±12.5	282.4±15.1	205.0±4.33	176.6±6.21	29981±1337 (24.1) *
EAXm treated (100.0 mg/kg)	113.6±6.01	255.8±58.3	207.2±30.3	167.8±27.9	122.2±26.3	23790±2097 (39.8) **
Pioglitazone treated (10.0 mg/kg)	119.6±16.2	325.8±36.4	211.2±18.2	179.8±12.7	160.2±22.1	24860±3552 (37.1%) **

Values are mean ± SE. Statistical significance *p<.05, **p<.01, ***p<.001

To see the other beneficial effect of EAXm and pioglitazone, their lipid lowering as well as insulin resistance reversal activity were also assessed on these mice. Table 10d presents the results of the same. Repeated oral gavages of EAXm and pioglitazone to db/db mice for 15 consecutive days caused significant decline in their fasting blood glucose and serum insulin levels. EAXm treated mice showed around 23.2 and 38.8 % decline in their fasting blood glucose on day 16th at 50 and 100 mg/kg doses. Pioglitazone treated db/db mice showed around 37.4 % decline in their fasting blood glucose level compared to sham treated control. Both EAXm as well as pioglitazone improved the insulin sensitivity by declining the hyperinsulinic conditions as compared to vehicle treated group. EAXm declined the insulin level by 14.8 and 22.2% at 50 and 100 mg/kg doses (fig 6d). Pioglitazone treatment group caused declined serum insulin level by 51.5% at 10.0 mg/kg dose. However, EAXm displayed better antidyslipidemic activity as compared to pioglitazone. EAXm declined the serum triglyceride level by 16.1 and 25.3 % at 50 and 100 mg/kg dose and enhanced serum HDL-C level by 12.4 and 27.1% these doses whereas pioglitazone treatment enhanced serum HDL-C by 18.4%. Treatment with either EAXm or pioglitazone did not cause any significant effect on total serum cholesterol levels of db/db mice (Fig 6e).

Table 10d: Effect of EAXm and pioglitazone on fasting blood glucose, serum insulin and serum lipid profiles of db/db mice

Groups	Serum profile on day 16th				
	Blood glucose (mg/dl)	Insulin (µg/l)	TG (mg/dl)	T-Cholesterol (mg/dl)	HDL-C (mg/dl)
Sham treated normal control (1.0% gum-acacia)	86.8±4.21	0.30±0.004	89.5±4.21	76.4±3.23	36.3±2.14
Sham treated Diabetic Control (1.0% gum-acacia)	192.8±4.27	2.70±0.15	154.6±18.3	187.3±22.8	78.3±5.80
EAXm (50.0 mg/kg)	148.1±5.23 (23.2)*	2.30±0.30 (14.8)*	129.7±14.3 (16.1)*	173.0±16.9 (7.6)	88.0±3.4 (+12.4)*
EAXm (100.0 mg/kg)	118.2±4.11 (38.8%)**	2.10±0.15 (22.2)*	115.0±15.5 (25.3)*	143.0±17.8 (-23.5)ns	99.5±2.20 (+27.1)*
Pioglitazone (10.0 mg/kg)	120.6±6.3 (37.4%)**	1.31±0.11 (51.5%)**	139.2±12.3 (10.2%)ns	179.4±17.3 (4.23%)ns	92.7±11.2 (18.4%)**

Values are mean ±S.E; Statistical significance *p<.05, **p<.01, ***p<.001

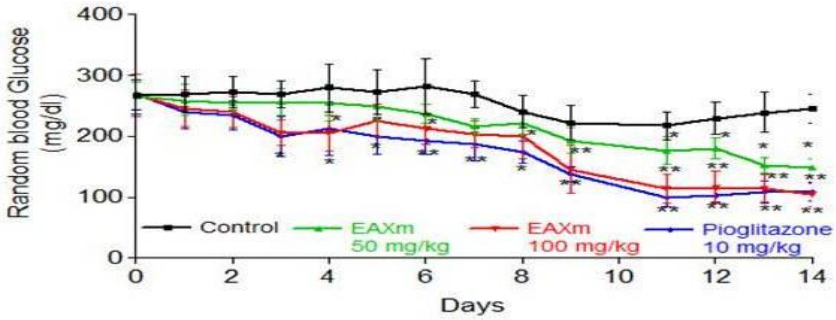

Fig. 6a: Effect of EAXm and pioglitazone on random blood glucose profile of db/db mice

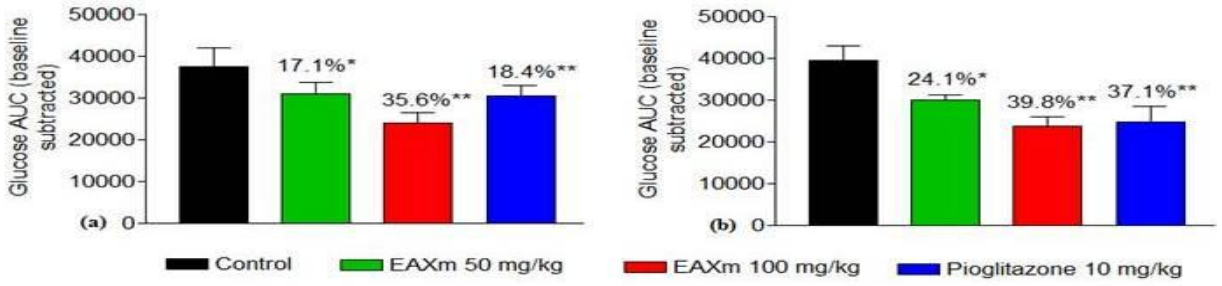

Fig. 6b and 6c: Effect of EAXm and pioglitazone on oral glucose tolerance test (OGTT) on 10 day (A) and 15 day (B) post treatment

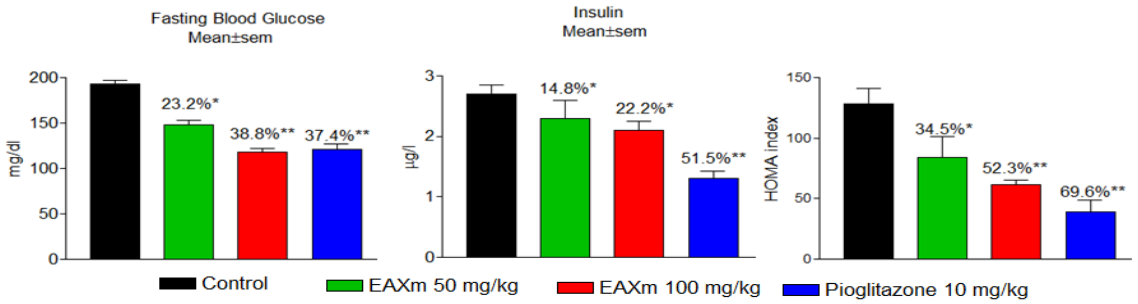

Fig. 6d: Effect of EAXm and pioglitazone on fasting blood glucose, serum insulin and HOMA-index of db/db mice

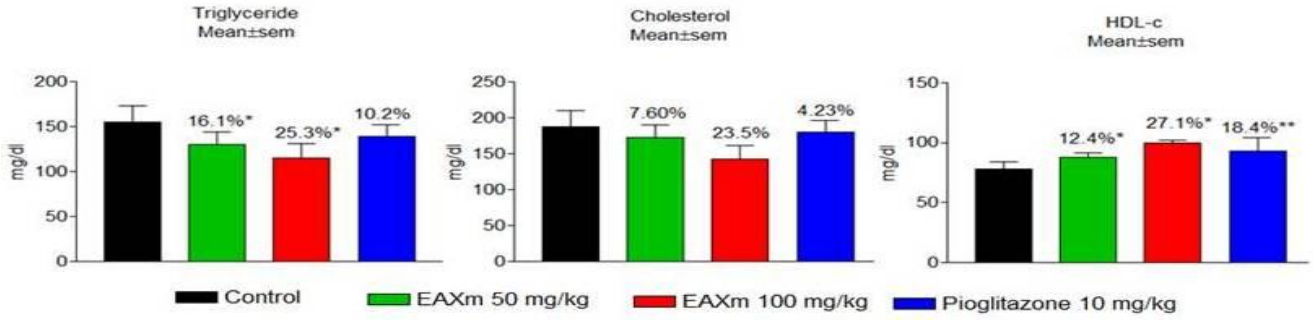

Fig. 6e: Effect of EAXm and pioglitazone on serum lipid profile of db/db mice

Effect of EAXm on regulatory enzymes of carbohydrate metabolism in muscle, liver and kidney tissues of db/db mice

Table 11-13 presents the activity profiles of few key regulatory enzymes of glycolysis, gluconeogenesis and glycogenolysis in liver, kidney and muscle tissues of sham treated non diabetic mice (db+), diabetic db/db and diabetic db/db treated with EAXm for 15 consecutive days. The activity of pyruvate kinase, and phosphofructokinase were found elevated in liver and kidney tissues of whereas the activity of glucokinase was elevated in liver tissue. In muscle tissue the activities of these enzymes were found decreased in sham treated diabetic rats compared to sham treated control. Glucose-6-phosphatase, fructose-1, 6 bisphosphatase and phosphoenolpyruvate carboxykinase were found elevated in liver and kidney tissues and fructose 1,6-bisphosphatase and Phosphoenolpyruvate carboxykinase in muscle tissue of the STZ-induced diabetic rats. Glycogen phosphorylase was found to be significantly elevated especially in muscle tissue of diabetic db/db control mice compared to db/+ lean control of the same strain. Administration of EAXm to the db/db mice for 15 consecutive days induced significant decrease in the glucose-6-phosphatase and fructose-1, 6-bisphosphatase activity mainly in the liver. EAXm treatment resulted in greater decrease of glycogen phosphorylase enzyme activity.

Table 11: Effect of EAXm on key enzymes of glycolysis, glycogenolysis and gluconeogenesis in liver of db/db mice

Enzymes	Enzyme Activity (umol/min/mg protein)		
	Sham treated non-diabetic Control (1.0% gum-acacia)	Sham treated diabetic control (1.0% gum-acacia)	EAXm treated (100 mg/kg)
Glucokinase	2.10±0.92	2.82±0.76 (+34.2)	2.53±0.10 (10.2)
Phosphofructokinase	4.12±0.71	5.30±0.57 (+28.6)	5.14±0.37 (3.01)
Pyruvate kinase	98.4±9.41	182.1±10.3 (+43.1)	151.2±11.2 (16.9)
Glycogen phosphorylase	18.7±3.26	39.4±5.29 (+110.6)	27.2±3.15 (43.9)
Phosphoenolpyruvate carboxykinase	20.4±2.61	32.9±3.28 (+61.2)	31.4±6.14 (17.3)
Fructose1,6 bisphosphatase	12.1±2.41	39.8±5.42 (+228.9)	26.6±3.41 (33.1)
Glucose-6-phosphatase	21.8±4.23	56.8±6.14 (+160.5)	41.5±7.24 (26.9)

Table 12: Effect of EAXm on key enzymes of glycolysis, glycogenolysis and gluconeogenesis in kidney tissue of db/db mice

Enzymes	Enzyme Activity (umol/min/mg protein)		
	Sham treated non-diabetic Control (1.0% gum-acacia)	Sham treated diabetic control (1.0% gum-acacia)	EAXm treated (100 mg/kg)
Phosphofructokinase	8.7±2.11	11.3±2.70 (+29.8)	10.1±1.41 (10.6)
Pyruvate kinase	84.1±7.60	120.4±12.2 (+43.1)	114.3±12.6 (5.06)
Glycogen phosphorylase	15.4±3.10	28.6±7.21 (+85.7)	22.4±3.64 (21.6)
Phosphoenolpyruvate carboxykinase	24.6±7.30	36.8±7.09 (+49.5)	31.4±6.14 (14.6)
Fructose1,6 bisphosphatase	16.4±8.12	56.5±11.4 (+244.5)	42.6±9.40 (24.6)
Glucose-6-phosphatase	23.4±6.28	62.9±6.11 (+168.8)	51.5±6.31 (18.1)

Table 13: Effect of EAXm on key enzymes of glycolysis, glycogenolysis and gluconeogenesis in muscle of db/db mice

Enzymes	Enzyme Activity (umol/min/mg protein)		
	Sham treated non-diabetic Control (1.0% gum-acacia)	Sham treated diabetic control (1.0% gum-acacia)	EAXm treated (100 mg/kg)
Phosphofructokinase	6.30±0.83	4.81±0.31 (23.6)	4.14±0.47 (+13.9)
Pyruvate kinase	68.3±4.61	52.1±6.12 (23.7)	56.7±5.00 (+8.82)
Glycogen phosphorylase	34.7±3.12	88.4±5.34 (+154.7)	46.7±6.24 (47.1)
Phosphoenolpyruvate carboxykinase	30.2±2.90	39.6±6.24 (+31.1)	34.2±3.33 (13.6)
Fructose1,6 bisphosphatase	5.44±1.40	18.6±3.15 (+241.9)	12.6±3.42 (32.2)

Effect of EAXm on α-glucosidase

Fig.7a displays the inhibition on α-glucosidase by EAXm. EAXm caused around 50% inhibition at 28.4µg/ml concentration. Fig.7b displays Lineweaver-Burk plot. The α-glucosidase was treated with various concentrations of *p*NPG (20-120 µM) in the absence or presence of EAXm. The type of inhibition by EAXm on alpha-glucosidase was analyzed using Lineweaver Burk plot. The double-reciprocal plot displayed mixed type of inhibition as both Km and Vmax were affected.

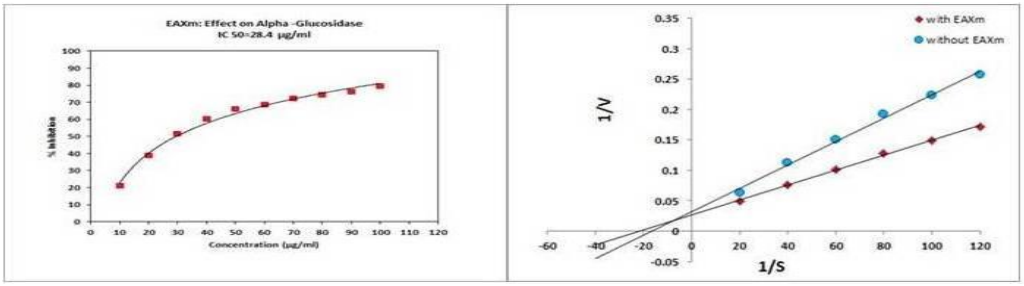

Fig. 7a and 7b: Inhibition of α-glucosidase by EAXm at various concentrations

Antidyslipidemic activity of EAXm and fenofibrate in male Syrian golden hamsters

Table 14 represents the serum lipid profile of sham treated dyslipidemic Syrian golden hamsters, and dyslipidemic hamsters treated with 50 and 100 mg/kg EAXm and 100 mg/kg fenofibrate for 10 consecutive days. EAXm caused dose dependent decline in their serum triglycerides by around 41.0, and 68.5 %, serum cholesterol by around 10.5 and 22.8 %, serum LDL-C by 25.4 and 40.7 %, glycerol level by around 19.8 and 28.4 % and increased serum HDL-C levels by around 12.7 and 24.3 %, respectively at 50 and 100 mg/kg dose respectively. EAXm also caused decline in non esterified free fatty acids (NEFA) level by around 33.0 % at 100 mg/kg dose. The serum lipoprotein lipase (LPL) activity was also found increased by 43.4 % by EAXm treatment at the dose of 100 mg/kg body weight. Fenofibrate was used as a positive standard which caused around 41.1 % lowering in serum triglyceride, 29.1 % lowering in serum cholesterol, 57.6 % in serum LDL-C, 27.3 % lowering in glycerol and around 30.5 % lowering in NEFA at 100 mg/kg dose. Fenofibrate also caused an increase in serum HDL-C level to the tune of 20.9 % and LPL activity by around 37.8 %. Treatment with either EAXm or fenofibrate did not affect the body weight of the hamsters.

Table 14: Effect of EAXm and Fenofibrate on serum lipid profiles of male golden hamsters

Groups (mg/kg dose)	Body weight	Serum lipid profiles						
		TG (mg/dl)	CHOL (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)	Glycerol (µM/L)	NEFA (mM/L)	LPL (U/L)
Sham Treated control	170±3.70	308.0±45.9	347.0±66.3	130.6±11.1	203.9±22.6	1263.0±69.7	6.60±0.61	30.4±3.45
EAXm (50 mg/kg)	147.3±8.0 (13.5)	181.6±28.9 (41.04)*	310.4±29.9 (10.5)	147.2±16.3 (+12.7)	152.2±14.8 (25.4)*	1012.5±41.2 (19.8)	ND	ND
EAXm (100 mg/kg)	140.3±8.1 (17.6)	97.1±16.2 (68.5)**	267.6±33.7 (22.8)*	162.4±15.3 (+24.3)*	120.8±14.7 (40.7)*	904.2±33.6 (28.4)*	4.42±0.31 (33.0)*	43.6±4.10 (+43.4)*
Fenofibrate (100 mg/kg)	156.0±7.5 (8.39)	181.4±12.2 (41.1)*	245.8±28.3 (29.1)*	158.0±14.8 (+20.9)	86.4±9.80 (57.6)**	917.8±30.2 (27.3)*	4.59±0.28 (30.5)*	41.9±4.40 (+37.8)*

Values are mean ± S.E. of 6 hamsters, Significance: * p <0.05, ** p<0.01, ns= not significant compared to sham treated control and ND = Not done.

DISCUSSION

The results of the present study clearly indicate that ethyl acetate fraction of the fruits of *X. moluccensis* (EAXm) has both antidiabetic and antidyslipidemic effects in a variety of animal models of diabetes mellitus and dyslipidemia. In streptozotocin-induced diabetic rat model, streptozotocin causes the destruction of β-cells of pancreas leading to a hyperglycemic condition [22]. EAXm treatment led to a significant fall in the elevated blood glucose level. The antihyperglycaemic effect of the fraction was found to be comparable to standard drug metformin. However, single dose treatment of fraction did not lower the blood glucose to the normal level, multiple dose treatment of EAXm for 10 consecutive days improved glucose tolerance without any significant effect on either food uptake (data not shown) or the body weight of the animals (Fig 2a, 2b and Table 2). The observed glucose lowering effect of the fraction was found to be associated with significant increase in serum insulin level, suggesting that fraction may induce the release of insulin from pancreas. The fraction also exerted beneficial effect on lipid profile in streptozotocin-induced diabetic rats characterized by significant decline in serum triglycerides and increase in HDL-C levels. The antidiabetic and antidyslipidemic efficacy was also studied both high fructose as well as high sucrose high fat fed animal models that displayed hyperglycemia, dyslipidemia as well as

obesity. It is well documented that intake of high carbohydrate high fat diet (HCHF) are the main causative factors for the development of hyperglycemia, dyslipidemia, obesity, insulin resistance and hypertension in rats [23, 24]. In the present study the hyperglycemic and hyperlipidemic rat models were developed by feeding high carbohydrate and high fat fed (HCHF) diets for two weeks and then injecting mild dose of streptozotocin that results in the development of persistent hyperglycaemia and other secondary diabetic complications i.e. elevated levels of liver and kidney functions parameters. These results are in accordance with the published reports [25, 26, 27]. These models for screening of wide variety of plant fractions and synthetic compounds for their antidiabetic, antidyslipidemic, insulin resistance reversal activity and also for studying the effects on secondary complications like liver and kidney damage in type 2 diabetes mellitus has also been advocated by many of the investigators.

EAXm treatment for 10 consecutive days caused significant improvement on glucose tolerance to either high fructose high fat fed streptozotocin diabetic rats (HFD-STZ) or high sucrose high fat fed streptozotocin treated diabetic rats (HSD-STZ). The probable mechanism of antihyperglycaemic activity may be due to either insulin mimetic or insulin secretagogue activity of EAXm. However, the possibility of insulin resistance reversal activity in EAXm can't be ruled out. The standard drug metformin which is an insulin sensitizer, also showed antihyperglycaemic activity in these two models. The increase in serum insulin levels in these two models by the development of insulin resistance in these animals due to high fat feeding. Therefore the fraction is thought as insulin mimetetic, insulin secretaceous and insulin sensitizers. EAXm treatment also displayed promising triglyceride and LDL-C lowering activity in HFD-STZ and HSD-STZ rats. EAXm also lowered the elevated level of cholesterol in HCHF-STZ and fraction was found to have more triglyceride and cholesterol lowering activity as compare to fenofibrate treatment at the same oral dose. The interesting feature is that continuous feeding of EAXm results in the significant elevation of the cardio-protective HDL-C level in HFD- STZ and HSD-STZ rats which is a favorable effect [28].

The high carbohydrate high fat diet fed streptozotocin rats showed liver and renal damage. The treatment of both the EAXm and metformin showed improvement in the liver and renal functions. EAXm displayed enormous ability to improve the liver and renal functions as evidenced by decline in the activity of AST, ALT, urea and levels of uric acid and creatinine levels in the serum, thus improving the liver and kidney functions. Metformin also showed ability to improve liver functions in the present animal model. Metformin is well known drug in the treatment of nonalcoholic steatohepatitis [29]. EAXm and metformin also decreases the levels of serum fructosamine i.e. improving the diabetic injury and the degree of improvement in diabetic injury by fraction is greater than that to metformin.

Further antidyslipidemic activity was confirmed in high fructose high fat fed Syrian golden hamsters. As evident from the results that after 10 days continuous feeding of the EAXm, significantly reduced total cholesterol, triglyceride, and LDL-C while at the same time significantly increased the HDL-C level in serum of high fat fed male Syrian golden hamster. The observed antidyslipidemic effect of EAXm was comparable to standard antidyslipidemic drug fenofibrate at the same dose level.

Finally antihyperglycaemic and antidyslipidemic activity of the fraction EAXm was assessed in the knockout mice model i.e. db/db mice. db/db mice exhibit an initial phase of hyperinsulinemia, hyperglycemia, hyperphagia and obesity [30]. This fraction has shown better antidyslipidemic activity as compare to piogltazone at the same dose and also overcomes the limitations of piogltazone as remarkable glucose lowering activity and insulin reversal activity was seen in db/db mice. The plioglitazone treated group showed strong glucose and insulin lowering activity but unable to lower down the increased level of triglyceride and cholesterol in the serum of db/db mice. The treatment of plioglitazone did not increase the level of HDL-C besides mildly altering its level in the serum of db/db mice at same doses. EAXm displayed significant effect on diabetic hypertriglyceridemia this could be through its control of hyperglycemia. This is in agreement with the fact that (i) the level of glycemic control is the major determinant of total and very low density lipoprotein (VLDL) triglyceride concentrations (ii) improved glycemic control following sulfonyl urea therapy decreases levels of VLDL and total triglycerides [31]. Evidence from other studies suggests an important role for tissue lipid levels in insulin action [32].

In diabetes mellitus, deficiency or insensitivity of the insulin causes derangement in carbohydrate metabolism, a decrease in the enzymatic activity of glycolytic pathway and an increase in the enzymatic activity of gluconeogenesis. In the present study, the activities of the enzyme of these pathways were significantly altered in the diabetic control. The activity of the regulatory enzyme of carbohydrate metabolism was measured as the second step, after insulin measurement, to find out whether these antihyperglycaemic leads implicated as antihyperglycaemic agent modulate the key enzyme involved in the glucose metabolism during its course of action. The severity of diabetic state as indicated by the degree of hyperglycaemia bear a significant correlation with the alteration in hepatic glycolytic and gluconeogenic enzyme activities. Enzyme abnormalities in the tissues of diabetic rats [33], db/db mice [34] and NOD mice [35] have been well documented. Liver and skeletal muscles are insulin dependent tissue while kidney is insulin independent tissue for the uptake of glucose and this primarily reflects the differences in their ability to metabolize cellular glucose. As described earlier, in the diabetic condition the theory of glucose over and under utilization by the peripheral tissues plays a central role in disorder of glucose metabolism leading to elevated systemic glucose [36]. Results of the present studies indicate lower levels of the hepatic glycolytic enzymes while elevated levels of hepatic gluconeogenic enzymes in the diabetic animals. These results are consistent with earlier published results [37]. Both these actions are directly responsible for decreased uptake and utilization of glucose in the liver. In kidney, overutilization of cellular glucose occurs through elevated activities of the glycolytic enzymes. However renal gluconeogenic enzymes are elevated, similar to hepatic gluconeogenic enzymes thereby correlated to the uncontrolled glucose homeostasis at both cellular and systemic level. Activities of GK and PFK have shown to be very sensitive signs of the glycolytic pathway [38] and these are decreased in the liver and muscle in diabetic state. Administration of insulin causes normalization of the enzymatic activities [39] and therefore measurement of the activity of these enzymes represents a method to assess peripheral utilization of glucose. Administration of the fraction EAXm increased the activity of all three enzymes towards nondiabetic controls suggesting that the antihyperglycaemic action seen is the result of increased glucose utilization at the level of skeletal muscle as well as liver. However it is not possible to deduce from the present finding that this increase in glycolytic enzymatic activity occurred secondary to the treatment mediated release of insulin or whether a component of the fractions EAXm have insulinomimetic action. Since STZ-insulin diabetes is an insulin deficient model, the probably of insulinomimetic effect seems more probable.

The hepatic gluconeogenic enzyme activities in diabetic animal were found much higher as compare to non diabetic animals. Gluconeogenic enzyme activities were also significantly elevated in kidney of diabetic animals but these elevations were less pronounced than those observed for liver. These antihyperglycemic agents helped in the significant restoration of the elevated enzymatic activity of glucose-6-phosphatase in the diabetic animals post treatment. Glucose-6-phosphatase enzyme plays an important role in glucose homeostasis in liver and kidney [40]. These results are similar to others where several plant extracts decreased the activity of this enzyme in diabetic condition [41, 42]. Activity of other two enzyme of gluconeogenesis i.e. fructose 1, 6-bisphosphatse and phosphoenolpyruvate carboxykinase was also found to be suppressed after treatment with these antihyperglycaemic agents. Thus these antihyperglycaemic agents may have acted by decreasing glucose production by gluconeogenesis.

The present results support the possibility that treatment of EAXm to diabetic animals could affect regulatory enzyme of carbohydrate

metabolism. Alteration in the activities of enzymes effected by these antihyperglycaemic agents treatment as elucidated in this study suggest that a normal glucose metabolism, in peripheral tissues such as liver and kidneys, is critical in achieving normoglycemia or normal blood glucose homeostasis.

Postprandial hyperglycemia is the key problem in diabetes mellitus. The enzyme alpha-glucosidase (EC 3.2.1.20), present in the epithelial mucosa of small intestine is an important enzyme, catalyses the final step in the digestive process of carbohydrates i.e. conversion of polysaccharide to monosaccharide which is absorbed through intestine [43]. Inhibition of alpha-glucosidase can slow the uptake of dietary carbohydrates and suppress postprandial hyperglycaemia [44]. In the present study EAXm showed a dose dependent inhibition of alpha-glucosidase activity with an IC50 value of 28.4 µg/ml. These findings suggests antidiabetic effect of ethyl acetate fraction EAXm may be mediated via the inhibition of alpha-glucosidase.

On the basis of present findings, the fraction EAXm have shown enormous ability to lower down blood glucose and normalizing insulin level, strong antidyslipidemic activity in the validated animal model of diabetes mellitus, it also improves insulin resistance in db/db mice hence it is advantageous to develop insulin sensitizer with additional lipid lowering properties for clinical use. It can be assumed that EAXm from *X. moluccensis* could be a potential lead discovery for the management of type 2 diabetes mellitus.

ACKNOWLEDGEMENTS

This investigation received financial support from Ministry of Earth Sciences (MoES) in the form of research project "Development of Potential Drugs from Ocean" to CDRI, Lucknow. This paper bears CDRI communication number

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