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Original Article

ANTIHYPERGLYCEMIC AND ANTIDYSLIPIDEMIC ACTIVITY IN ETHYL ACETATE FRACTION OF THE FRUITS OF *XYLOCARPUS GRANATUM* AND *XYLOCARPUS MOLUCCENSIS*

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ABSTRACT

Objectives: Although various species of *Xylocarpus i. e. Granatum, moluccensis* are known for their medicinal properties. Yet, its anti-diabetic activity remains to be defined. So the aim of this study was to evaluate the antihyperglycemic and antidyslipidemic activity in ethyl acetate fraction of the fruits of *X. granatum* and *X. moluccensis* on validated animal models as well as *in-vitro* glucose uptake stimulatory effect and their cytotoxicity effect in L6 skeletal muscle cells.

Methods: The ethyl acetate fraction of the fruits of *X. granatum* and *X. moluccensis* were administered to diabetic groups daily up to 10 days for prolonged study. Biochemical parameters notably glucose tolerance, insulin level, lipid profile were assessed. The ethyl acetate fraction of the fruits of *X. granatum* and *X. moluccensis* were also tested for glucose uptake effect by skeletal muscle cells in the concentration dependent manner.

Results: The present study show that the ethyl acetate fraction of the fruits of *X. granatum* as well as *X. moluccensis* are effective in improving glucose tolerance, declining blood glucose as well as serum cholesterol and triglycerides levels in low dosed streptozotocin-induced diabetic rats and dyslipidemic hamsters, respectively. These fractions were also found efficient in increasing glucose uptake by L6 skeletal muscle cells but did not show any effect on cell viability of L6 skeletal muscle cells.

Conclusion: Based on the results, the present study revealed that ethyl acetate fraction of the fruits of *X. granatum* and *X. moluccensis* lowered blood glucose profile by increasing the glucose uptake by L-6 and this may be the possible mechanisms for the antidiabetic and antidyslipidemic action.

Keywords: *Xylocarpus granatum, Xylocarpus moluccensis*, Streptozotocin-induced diabetic rats, Antihyperglycemic activity, Antidyslipidemic activity, Glucose uptake assay.

INTRODUCTION

Diabetes mellitus is one of the most severe, incurable metabolic disorders characterized by hyperglycemia as a result of a relative, or an absolute, lack of insulin, or the action of insulin on its target tissues or both [1]. Currently, diabetes mellitus is recognized as the world's most common metabolic disorder and the current prevalence of type 2 diabetes is 2.4 % in the rural population and 11.6% in the urban population of India. It has been estimated that by the year 2025, India will have the largest number of diabetic subjects in the world [2].

The use of medicinal plants has flourished as an alternative for the treatment of diabetes because modern medicines have several side-effects and are expensive. A multitude of herbs and medicinal plants have been described for the treatment of diabetes throughout the world as they might provide a basis of new synthetic antidiabetic analogues with potent activity[3,4]. The genus *Xylocarpus* (Family: Meliaceae) is commonly known as Cannon ball tree and abundantly available in India, has been reported for various biological activities exerted by various chemical constituents [5]. The aqueous extract of the different parts of this plant has been reported to have significant anti-filarial activity [6]. Recently DPPH radical scavenging activity of the methanol extract of the bark has been reported [7]. Various chemical classes of compounds, including limonoids, sterols, alkaloids, tannins, with different biological activities have been reported from *X. granatum* [8-12]. Although *Xylocarpus* has been used for the treatment of several diseases, antidiabetic property of this plant has not been explored till date, hence an attempt has been made to identify its antidiabetic property and further reconfirm it in different animal models of diabetes. In the present study we reported the antihyperglycemic and antidyslipidemic activities of the ethyl acetate fraction of the *X. granatum* and *X. moluccensis* in streptozotocin-induced diabetic rats and high fructose high fat diet fed dyslipidemic Syrian golden hamsters. *In-vitro* glucose uptake stimulatory effect of ethyl acetate fraction of *X. granatum* and *X. moluccensis* and their cytotoxicity effect on L6 skeletal muscle cells were also evaluated.

MATERIAL AND METHODS

Materials

Streptozotocin, metformin, and fenofibrate were obtained from Sigma Chemical Company (St. Louis, USA). Biochemical kits used in the study were obtained from the Roche diagnostics. DMEM, fetal bovine serum, trypsin, antibiotic/antimycotic solutions were procured from Gibco, USA. 2-deoxy-D-[3H]-glucose (2-DG) was from GE Healthcare, UK. All other chemicals used were of the highest purity grade.

Preparation of plant fraction

The whole plant of *Xylocarpus granatum* and *Xylocarpus moluccensis* were collected from the coastal region of India, and was identified in the Botany Division of the Institute. The collection details and representative voucher specimens of the plants have been documented in the herbarium of the Botany Department, Central Drug Research Institute, Lucknow for future reference. Each plant materials (1.0 kg) was dried in shade and grounded in mechanical disintegrator. The fine powder was extracted four times with 95 % ethanol. The pooled extract was filtered and concentrated under high vacuum in a rotavapour. The ethanolic extract thus obtained was macerated with hexane at room temperature and the hexane insoluble fraction was further macerated with ethyl acetate. The

ethyl acetate fraction of each plant was concentrated under reduced pressure below 50ºC to a viscous mass which was finally dried under high vacuum to greenish powders. Ethyl acetate fraction of both plants were used for antidiabetic and antidyslipidemic studies and coded as CDR-134-F194 (from *X. granatum*) and CDR-267-F018 (from *X. moluccensis*). HPLC profiles of CDR-134-F194 and CDR-267-F018 are given in fig. 1a and 1b, respectively.

***In vivo* assays**

Animals

Male albino rats of Sprague Dawley strain (8 to 10 weeks of age; body weight, 120 ± 20 g) and Syrian golden hamsters (6 to 8 weeks old, body weight 160 ± 20 g) were available in the animal facility of the institute. The work with these animals was approved by an institutional ethics committee for animal study and 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. The animals were housed in groups of four to five in a polypropylene cage in the animal house, with temperature 23±2ºC; humidity 50–60%; light 300 Lx at floor level with regular 12 h light cycles. The animals were provided food and drinking water *ad libitum*.

Induction of diabetes

Diabetes was induced in rats by injecting streptozotocin (60 mg/kg in 0.1M citrate buffer pH 4.5) to overnight-fasted rats, intraperitoneally. Fasting blood glucose was checked after 48 hours of injection by glucometer (Boehringer Mannheim) and animals showing blood glucose value between 270 to 450 mg/dl were considered as diabetic.

Antidiabetic activity evaluation on Streptozotocin-induced diabetic rats

Streptozotocin-induced diabetic rats were selected and divided into four groups containing six rats in each. Rats of experimental groups were given suspension of CDR-134-F194, CDR-267- F018 and standard drug metformin prepared in 1% gum acacia orally at a dose 100 mg/kg body weight, respectively. Rats of the control group were given an equal amount of 1% gum acacia (vehicle) and this group was considered as diabetic control. Blood glucose level of all animals was monitored at 1h, 2h, 3h, 4h, 5h, and 24 h post-administration of test samples. For multiple dose experiment diabetic rats were divided into four groups of six rats, each. One group was considered as diabetic control while the other three groups as experimental groups. Animals of experimental groups were administered suspensions of CDR-134-F194, CDR-267-F018 and standard drug metformin orally for 10 days at a dose of 100 mg/kg body weight. Fasting blood glucose profiles of each rat was determined at the day of start of the treatment (day 0) and thereafter on day 10. The oral glucose tolerance was performed on day 7 and 10 post treatments. At the end of the experiment i. e. On day 11, blood was withdrawn from the retro-orbital plexus for the estimation of insulin, triglyceride, cholesterol, HDL-C and LDL-C content in the serum.

Antidyslipidemic activity evaluation in Syrian golden hamsters

Dyslipidemia in male Syrian golden hamsters was induced by feeding the animals with high fructose high fat diet (HFHFD) for 30 to 40 days. Dyslipidemic hamsters were divided into four groups of six animals each, based on their serum lipid profile and termed as control and experimental groups. The experimental groups were administered respectively the suspensions of CDR-134-F194, CDR-267-F018 and standard drug Fenofibrate orally at a dose of 100 mg/kg for 10 days. Control animals were given the drug vehicle only. At the end of the experimental period i. e. On day 11, the blood of each animal was withdrawn from the retro-orbital plexus for determining their blood glucose. Insulin and lipid profiles.

***In vitro* assays**

Cell culture

L6 rat skeletal muscle cell lines were maintained following previously established method [13] in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS with penicillin (100 units /ml), streptomycin (200µg/ml), and gentamycin (50µg/ml) in a humidified atmosphere of 5% CO2 and 95% air at 37ºC. Differentiation was induced by switching confluent cells to medium supplemented with 2% FBS. The extent of differentiation was established by observing multi nucleation of cells and 90% fusion of myoblasts into myotubes was observed after 4–6 days post confluence and considered for experimentation.

Measurement of 3 H-2-deoxyglucose uptakes

Differentiated myotubes (L6) were used for 2-deoxyglucose uptake measurement as described previously [14]. Briefly, myotubes were incubated with myotubes were incubated with increasing concentrations of CDR-134-F194, CDR-267-F018 for 24 hr. After incubation, cells were washed in glucose free HEPES-buffered saline solution (140 mM NaCl, 1 mM CaCl2, 5 mM KCl, 2.5 mM MgSO4 and 20 mM HEPES, pH 7.4). The glucose uptake was determined by adding 10µM 3H-2-deoxyglucose (0.5µCi/well) and after incubation for 10 min at room temperature. Nonspecific uptake was determined in parallel well in the presence of 10 µM cytochalasin B and was subtracted from the total uptake. After uptake period, radioactive solution was rapidly aspired, and the monolayer was washed three times with ice-cold (0.9% Nacl and 25 mM D-glucose) solution. Cell associated radioactivity was determined by cell lysis in 0.05 N NaOH, followed by scintillation counting (Beckman Coulter, USA). All assays were performed in triplicates and results are expressed as fold stimulation over control. Results were expressed as % glucose uptake with respect to control.

Cytotoxicity analysis

Cytotoxicity effect of plant fractions on L6 muscle cells was evaluated by MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay [15].

Statistical analysis

Quantitative glucose tolerance of each animal was calculated by the area under the curve (AUC) method using Prism software. The AUC of the control group and the experimental group was compared to determine the percent antihyperglycemic activity. Statistical comparisons between groups were performed by one-way analysis of variance (ANOVA), followed by Dunnett's multiple range test. Results were expressed as mean ± S. E. Statistically significance difference was set at following levels * p< 0.05, ** p< 0.01, *** p< 0.001.

RESULTS

Extraction and fractionation of fruits

Fig. 1a shows the typical HPLC profile of CDR-134-F194 and the major peaks were identified as gedunin peak whereas fig 1b shows the typical HPLC profile of CDR-267-F018.

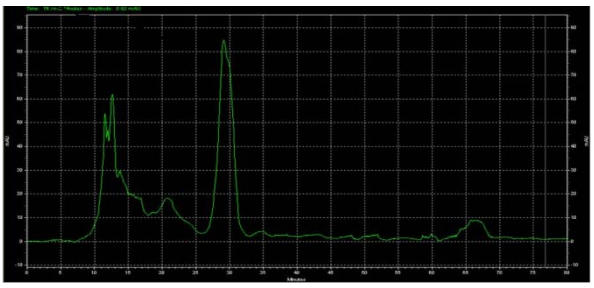

Fig. 1a: HPLC profile of CDR-134 F194 showing gedunin peak

Antihyperglycemic effect of CDR-134-F194 and CDR-267-F018 in streptozotocin-induced diabetic rat

Table 1 shows the blood glucose lowering activity of CDR-134-F194, CDR-267-F018 and standard drug metformin on STZ-induced diabetic rats. Treatment with CDR-134-F194 and CDR-267-F018

resulted in lowering of blood glucose level by 19.8 and 18.0 %, respectively, after 5h and, by 23.5 and 22.8 %, respectively after 24h of treatment at 100 mg/kg dose. The activity of these fractions was comparable to the blood glucose lowering effect of standard antidiabetic drug metformin (100 mg/kg), which showed around 17.2 and 20.8 % lowering in blood glucose level after 5h and 24h of treatment, respectively on streptozotocin induced diabetic rats.

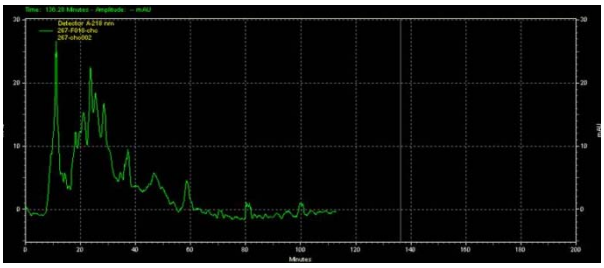

Fig. 1b: HPLC profile of CDR-267 F018

Table 2 represents the effect of repeated oral administration of CDR-134-F194 and CDR-267-F018 on glucose tolerance, serum insulin level and serum lipid profile of STZ-induced diabetic rats. When the area under the curve was compared between groups, CDR-134-F194 treated group showed 25.5% improvement whereas CDR-267-F018 treated group showed 21.4% and metformin treated group showed 26.6% improvement in glucose tolerance on 10 day respectively as compared to that in the control group whereas treatment of streptozotocin-induced diabetic rats with CDR-134-F194 resulted in the significant lowering of serum triglycerides levels to around 41.4 %. The serum HDL-C levels and serum insulin levels were found to increased around 17.5 % and 32.4 %, respectively (Table 2). Similarly treatment with CDR-267-F018 resulted in the lowering of serum triglycerides levels to around 31.5 % and caused increase in serum insulin levels to around 36.5% and serum HDL-C levels around 24.0% compared to control at this period. Treatment with CDR-134-F194 or CDR-267-F018 had no significant effect on either total cholesterol or LDL-C levels on streptozotocin induced diabetic rats (Table 2). Metformin treatment to these rats showed increase in their serum insulin level to around 25.5%. The metformin treatment had no significant effect on lipid profile of these animals.

Table 1: Single dose effect of CDR-134-F194, CDR-267-F018 and standard drug metformin on streptozotocin-induced diabetic rats

Groups	Dose (mg/kg)	% Antihyperglycemic activity	
		5h	24h
CDR-134-F194	100	19.8*	23.5**
CDR-267-F018	100	18.0*	22.80*
Metformin	100	17.2*	20.8*

Values are mean ± SE. of 6 rats, significance: * p <0.05, ** p<0.01, compared to control group

Table 2: Multiple dose effect of CDR-134-F194, CDR-267-F018 and standard drug metformin on body weight, improvement in OGTT, serum insulin level and serum lipid profile of streptozotocin treated rats

Groups	Body weight (g)	% change					
		Glucose (AUC)	Insulin (µU/ml)	TG (mg/dl)	Cholesterol (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)
Control	133±4.80	58200±2803	14.5±2.6	42.2±6.8	26.8±3.4	21.6±3.5	21.1±2.4
CDR-134-F194 (100 mg/kg)	143±3.5	43200±1450** (25.5)	19.2±1.3 * (32.4)	24.7±4.6 ** (41.4)	32.2±3.2 (20.1)	25.4±2.1* (17.5)	18.2±1.3 (13.7)
CDR-267-F018 (100 mg/kg)	141±4.2	45730±1660* (21.4)	19.8±1.5 * (36.5)	28.9±5.2 ** (31.5)	33.4±4.0 (24.6)	26.8±1.8* (24.0)	19.0±1.4 (9.90)
Metformin (100 mg/kg)	137±5.2	42750±1380** (26.6)	18.2±1.4 * (25.5)	39.2±7.4 (7.10)	30.9±3.5 (15.2)	22.4±2.1 (3.7)	22.2±2.3 (5.21)

Values are mean ± SE of 6 rats, Significance: *p<0.05, **p<0.01, compared to control group

Antidyslipidemic activity of CDR-134-F194 and CDR-267-F018 on male Syrian golden hamsters

Table 3 represents the serum lipid profile of dyslipidemic Syrian golden hamsters after 10 days treatments with CDR-134-F194, CDR-267-F018 and standard drug fenofibrate, respectively. Treatment with CDR-134-F194 resulted in significant lowering of serum triglyceride to around 55.6%, total cholesterol levels to around 24.9%, serum LDL-C levels to around 35.1 %, blood glucose levels to around 34.4%, and increased the serum insulin levels to around 37.2% and serum HDL-C levels around 27.9% of dyslipidemic golden hamsters.

Similarly CDR-267-F018 treatment showed the lowering in serum triglycerides level to around 68.4%, total serum cholesterol levels to around 22.8 %, serum LDL-C levels to around 40.7 %, and increased serum insulin level around 40.7%, and serum HDL-C levels around 24.3%. Fenofibrate was used as standard that resulted in the lowering of serum triglycerides to around 41.1%, total serum cholesterol levels to around 40.6% and serum LDL-C levels around 57.6 %, and caused an increase in serum HDL-C levels to around 20.9%. Treatment with either of these fractions or standard drug fenofibrate had no significant effect on body weight of animals.

Table 3: Effect of CDR-134-F194, CDR-267-F018 and standard drug fenofibrate on body weight, blood glucose, serum insulin and serum lipid profiles of male Syrian golden hamsters

Groups	Body weight (g)	% change					
		Glucose (mg/dl)	Insulin (µU/ml)	TG (mg/dl)	CHOL (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)
Control	170.0±3.7	87.3±6.2	34.6±10.4	308±45.9	347.0±66.3	130.6±11.1	203.9±48.3
CDR-134-F194 (100 mg/kg)	147.3±8.0	57.2±9.6* (34.4)	21.7±4.3* (37.2)	136.6±18.9** (55.6)	260.4±29.9* (24.9)	167.1±13.3* (27.9)	132.2±14.5** (35.1)
CDR-267-F018 (100 mg/kg)	140.3±8.0	74.8±10.2 (14.3)	20.5±2.9* (40.7)	97.1±16.2*** (68.4)	267.6±33.7* (22.8)	162.4±15.3* (24.3)	120.8±24.7** (40.7)
Fenofibrate (100 mg/kg)	156.0±7.5	102.3±12.4 (17.1)	37.4±7.1 (8.09)	181.4±12.16** (41.1)	205.8±28.3* (40.6)	158.0±14.8* (20.9)	86.4±15.8*** (57.6)

Values are mean ± SE. of 6 rats, significance: * p <0.05, ** p<0.01, *** p<0.001compared with control group

Glucose uptake stimulatory effect of CDR-134-F194 and CDR-267-F018 in L6 skeletal muscle cell lines

Skeletal muscle is the principal site responsible for the postprandial glucose utilization and accounts for more than 80% of insulin-dependent glucose disposal in humans and other animals as well. In order to investigate the observed antihyperglycemic effects in the fractions CDR-134-F194 and CDR-267-F018, their effect was also assessed on glucose uptake by rat skeletal muscle cells (L6). L6 skeletal muscle cells were allowed to differentiate into myotubes before treatment with either CDR-134-F194 or CDR-267-F018. As evident from the results in fig. 2, 24 hr incubation with CDR-134-F194 and CDR-267-F018 caused significant stimulation in glucose uptake by L-6 muscle cells in a dose-responsive manner. Incubation with 10µg/ml concentration of CDR-134-F194 and CDR-267-F018 stimulated the glucose uptake to around 5.2 and 3.5-folds, respectively, which was fairly high compared to metformin. Metformin showed stimulation in glucose uptake by L-6 muscle cells to around 2.2 fold (fig. 2).

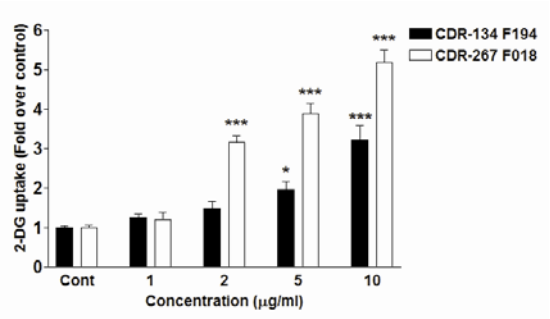

Fig. 2: Effect of CDR-134- F194 and CDR- 267-F018 at different concentration on glucose uptake by L6 cells

Cytotoxicity effect of CDR-134-F194 and CDR-267-F018 in L6 skeletal muscle cell lines

To discard the possibility that increase in glucose uptake in response to fractions CDR-134-F194 and CDR-267-F018 was not due to changes in the total amount of cells, viability of L6 myotubes were assessed after the treatment with CDR-134-F194 and CDR-267-F018. The L6 skeletal muscle cells were incubated respectively with CDR-134-F194 and CDR-267-F018 at various concentrations for 24 h and the MTT uptake by the treated cells was monitored. It is evident from the results that no significant effect in L6 myotubes viability was observed either due to treatment with CDR-134-F194 or CDR-267-F018 at concentrations ranges from 10 ng to 100 µg/ml. (fig. 3)

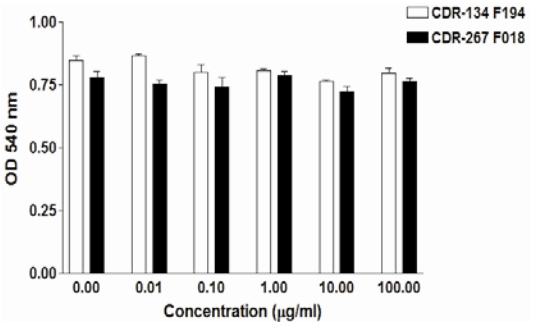

Fig. 3: Effect of CDR-134-F194 and CDR-267-F018 at different concentration on cell viability in L6 skeletal muscle cell lines

DISCUSSION

Diabetes mellitus is one of the most common endocrine disorders and is a major global health problem. Though, different types of oral hypoglycemic agents are available for the treatment of diabetes mellitus, there is a growing interest in search of herbal remedies, due to the side effects associated with the available therapeutic agents [16]. The ethnobotanical information reports about 800 plants with anti-diabetic potential but only few of these have received scientific and medical evaluation to assess their efficacy.

In present study ethyl acetate fraction of *X. granatum* (CDR-134-F194) and *X. moluccensis* (CDR-267-F018) were evaluated for antidiabetic and antidyslipidemic effect. The results obtained indicate that ethyl acetate fraction of CDR-134-F194 and CDR-267-F018 possess significant blood glucose lowering activity. Single dose treatment of CDR-134-F194 and CDR-267-F018 (100 mg/kg) on streptozotocin-treated diabetic rats, significantly lowered blood glucose profile. In these animals, streptozotocin causes the destruction of β-cells of pancreas leading to a hyperglycemic condition. CDR-134-F194 and CDR-267-F018 treatment led to a significant fall in the elevated blood glucose level. The antihyperglycemic effect of CDR-134-F194 and CDR-267-F018 was found to be comparable to standard drug metformin. Further, treatment of streptozotocin-induced diabetic rats with CDR-134-F194 and CDR-267-F018 (100 mg/kg), respectively for 10 consecutive days caused significant improvement in glucose tolerance but without any significant effect on body weight. The observed glucose tolerance effect of CDR-134-F194 and CDR-267-F018 was found to be associated with increase in serum insulin level, suggesting that fraction of each plant may increase the synthesis and release of insulin from pancreas. These fractions also exerted beneficial effect on lipid profile in streptozotocin-induced diabetic rats characterized by significant decline in the levels of serum triglycerides and increase in HDL-C levels.

Diabetes mellitus is often linked with abnormal lipid mobilisation. The impairment of insulin secretion results in enhanced metabolism of lipids from the adipose tissue to the serum [17]. It has been demonstrated that insulin deficiency in diabetes mellitus leads to the increased level of serum total cholesterol and triglycerides due to the enhanced mobilisation of lipids from adipose to serum in diabetic patients [18]. Hence the antidyslipidemic activity of the CDR-134-F194 and CDR-267-F018 was evaluated in high fructose high fat diet fed male Syrian golden hamster. As evident from the results that after 10 days continuous feeding of the fractions, significantly decline in total serum cholesterol, serum triglycerides, and serum LDL-C was observed while at the same time significant increase in the HDL-C level in serum of high fat fed male Syrian golden hamster was observed. These observed antidyslipidemic effect of CDR-134-F194 and CDR-267-F018 was found comparable to standard antidyslipidemic drug fenofibrate at the same dose level.

Effect of CDR-134 F194 and CDR-267 F018 were further evaluated on glucose uptake by skeletal muscle. Skeletal muscle is the major tissue responsible for the maintenance of glucose homeostasis *In vivo*. In type II diabetes the capacity of skeletal muscle to take up glucose get reduced. This diabetes-associated decrease is observed at the level of both basal and insulin stimulated uptake. Since skeletal muscle is a primary disposal site for glucose and major determinant of glycemia, it would be expected that interventions enhancing muscle glucose uptake would reduce glycemia in diabetic human and animals [19]. Incubation with CDR-134-F194 and CDR-267 F018 caused significant stimulation in glucose uptake in L6 skeletal muscle cells. Incubation with 10 µg/ml concentrations of CDR-134-F194 and CDR-267-F018 stimulated the glucose uptake by around 5.2 and 3.5-fold respectively, which is much higher than that of standard antidiabetic drug metformin which showed nearly 2.2-fold stimulation at 500 µM concentration in the medium. Since uptake of glucose is the rate limiting step in its utilization, observed antidiabetic effect of CDR-134-F194 and CDR-267-F018 may be mediated, at least in part, through increased utilization of glucose and skeletal muscle may be the major target of action. It was found that both CDR-134-F194 and CDR-267-F018 did not show any effect on cell viability of L6 cells after 24 h incubation in a concentration range of 10 ng/ml to 100 µg/m1 and thus found to be safe.

CONCLUSION

In conclusion, the present study demonstrates that ethyl acetate fraction of the *X. granatum* and *X. moluccensis* have potent antidiabetic and antidyslipidemic activity.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

ABBREVIATIONS

STZ- Streptozotocin, SEM- standard error mean, AUC- area under curve, SD strain-Sprague Dawley strain, OGTT-oral glucose tolerance test, LDL low-density lipoprotein, HDL high-density lipoprotein, TG-Triglycerides, TC-Total Cholesterol.

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