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# Insulin sensitizing potential of fractions isolated from *X. molluccensis* and *X. granatum*

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## Abstract

The present study was designed to elucidate the anti-hyperglycemic action of two fractions isolated from marine plants i.e., CDR267F018 from *X. molluccensis* and CDR134F194 from *X. granatum* in insulin resistant conditions using *in vitro* and *in vivo* models.

The glucose uptake was studied using *in vitro* model of 3T3 L1 adipocyte and L-6 skeletal muscle cell line. The effect of fractions was also studied in dexamthazaone induced insulin resistant conditions in 3T3L1 adipocyte model. The in vivo model of insulin resistance was developed using a high fructose diet. Parameters assessed were weight, fasting blood glucose, insulin, cholesterol, triglycerides and liver glycogen content. Pioglitazone was used as a positive control.

CDR267F018 and CDR134F194 demonstrated stimulation of glucose uptake in L-6 skeletal muscle cells in a dose dependent manner. Both the fractions *per se* significantly stimulated the basal 2-deoxyglucose uptake in 3T3L1 model. A stimulation of 2-deoxyglucose uptake was also observed in insulin resistant cells. The effect shown by these drugs was comparable to Pioglitazone, a known insulin sensitizer. Administration of the fractions CDR267F018 and CDR134F194 demonstrated a dose dependent decrease in sugar and insulin levels with maximum effect at the highest dose tested. These fractions also decreased the total cholesterol levels although an increase in triglycerides levels was observed at the higher doses. The maximum decrease in triglycerides was seen with lower dose.

CDR267F018 and CDR134F194 have a potential role in ameliorating insulin resistant conditions as seen in the 3T3L1 adipocyte, skeletal muscle and fructose fed model.

**Keywords:** *X. molluccensis*, *X. granatum,* glucose uptake, fructose fed model, 3T3 L1 adipocytes

## Introduction

Type 2 insulin-resistant diabetes mellitus (T2DM) accounts for 90–95% of all cases of diabetes [1]. This heterogeneous disorder afflicts an estimated 6% of the adult population in Western societies [2]. Several approaches have been recommended to reduce hyperglycemia, including increasing pancreatic insulin release by sulfonylureas, decreasing hepatic glucose production by metformin, enhancing insulin action by thiazolidinediones, and suppressing gut glucose absorption by -glucosidase. However, these treatments have limited efficacy, a high likelihood of tolerability issues, and significant mechanism-based side effects.

In response to the enormity of this growing problem, efforts to identify and develop new pharmacological agents for type-2 diabetes are been taken. The ocean which hosts the richest diversity of living organisms is a relatively unexplored source of compounds that can find uses as new pharmaceuticals and tools in molecular and pharmacological studies. Marine pharmacology deals with search for models of new bioactive compounds which in combination with proper pharmacological screening can generate new drugs against human ailment. The 'Drugs from the Ocean' project, known as MoES program has targeted development of drugs from the marine sources for diabetes.

*Xylocarpus molluccensis* and *Xylocarspus granatum* are two such plants explored for its anti- diabetic effect under this program. Both the plants belong to the natural order Meliaceae. They are mangrove plants commonly known as *pussur* in the Hindi language. In India *X. molluccensis* is found in tidal forests along the eastern and western coastal areas up to Maharashtra, and in the Andaman Islands. It shows a broad range of biological activity, including insect antifeedant and growth-regulating activities [3, 4, 5].

Traditionally, the seed paste is also used for relief of breast cancer [6, 7]. *X. granatum* is a tall tree ranging upto 20m with buttressed stem base. It is found in tidal forests along the east and west coastal areas up to Maharashtra and in Andaman Island in India.

The plant *X. molluccensis* together with *X. granatum* has yielded several limonoids (xyloccensin A–Y), which show a broad range of biological activity, including antimicrobial, anticancer, insect antifeedant and growth-regulating activities etc. [8, 9, 10, 11, 12]. Although these plants are used for the treatment of several diseases the anti-diabetic property has not been reported till date and hence an attempt was made to identify its anti-diabetic

property. Two fractions isolated from this plants *i.e.,* CDR267F018 from *X. molluccensis* and CDR134F194 from *X. granatum* have been identified through the MOES program as they have demonstrated significant anti-hyperglycemic activity in the primary screening studies. In view of these findings, further studies were carried out to explore the mechanism of action of these fractions in ameliorating the insulin resistant condition which is the major contributor for the development of type 2 diabetes.

Muscle and adipose tissue are insulin-responsive tissues and express the insulin-sensitive glucose transporter GLUT-4. GLUT-4 translocates from intracellular vesicles to the plasma membrane in response to insulin and causes increased glucose transport into muscle and fat cells. In insulin resistance conditions, there is decreased GLUT-4 translocation to plasma membrane in response to insulin. As this plays an important causative role in the pathogenesis of type 2 diabetes, we evaluated the role of CDR 267 F018 and CDR 134 F194 on glucose transport using skeletal muscle (L-6) and adipocyte (3T3L-1) cell lines.

The effect on the insulin resistance condition was also evaluated using a fructose fed rat model. This model is considered as a fine model for type-2 diabetes as it displays many of the characteristics of the diabetes including hyperglycemia, insulin resistance and progressive obesity [13]. In animal models, the potential danger of fructose consumption and its links to various metabolic disorders have been widely documented [14]. Deleterious effects of high fructose intake on body weight, insulin sensitivity/glucose homeostasis, dyslipidemia, and atherosclerotic disease has been identified, and potential mechanisms have been proposed.

With this background the present study was designed to elucidate the anti-hyperglycemic action of the select two fractions in insulin resistant conditions by using the *in vitro* and *in vivo* models.

# Materials

## Study Material

## Xylocarpus molluccensis

## Collection of Medicinal Plant

The fruit of the mangrove plant *X. molluccensis* was collected from the South Andaman Coast, India, and the authentification was done by Botany Division of Central Drug Research Institute, Lucknow.

## Extraction

Powdered *X. molluccensis*'s fruit was placed in glass percolator with 95% ethanol and allowed to stand for 24hours at room temperature. The percolate was collected and these processes were repeated for four times. The combined percolate was evaporated under reduced pressure at $50^0$ C to a brown viscous mass, which was further dried under high vacuum to remove the last traces of solvent to afford ethanol extract. The weight of extract was found to be 10-12% of fruits (CDR-267).

## Fractionation

The ethanol extract was macerated with hexane. The hexane soluble fraction was separated and evaporated under reduced pressure to afford hexane fraction (9-10% of ethanol extract). Distilled water was added to hexane insoluble portion, which was fractionated with ethyl acetate and the resultant solution was evaporated under reduced pressure to afford ethyl acetate fraction (20-25% of ethanol extract) known as CDR-267-F018.

## Xylocarpus granatum

## Collection of Medicinal Plant

Fruits of the *X. granatum* (CDR-134) mangrove were collected from South Andaman Coast, India in the month of January have been preserved in the herbarium of the Botany Division at Central Drug Research Institute, Lucknow, India.

## Extraction and Fractionation

Powdered epicarp of the fruits was placed in glass percolator with 50% aqueous-ethanol and allowed to stand for 24hours at room temperature. The percolate was collected and these processes were repeated for four times. The combined percolate was evaporated under reduced pressure at $50^0$ C to a dark brown powder (10-15% of the epicarp: CDR-134-D123). The dark brown powder was macerated with chloroform to give chloroform fraction (8-9% of 50% aqueous-ethanol) known as CDR-134-F194.

## *In vitro* studies

## Reagents

DMEM, NBCS and Fetal Bovine Serum (FBS) were obtained from Invitrogen Corporation, USA. Trypsin, Dexamethazone (DEX), Iso-butyl methyl xanthine (IBMX) and Insulin were obtained from Sigma-Aldrich Chemicals Co., St. Louis Mi, USA. 2-deoxy-D-[ H] glucose was obtained from Board of Radiation and Isotope Technology (BRIT), Unit of Department of Atomic Energy, the Govt. of India from Deonar, Mumbai. All other chemicals were purchased from SD Fine Chemicals, Mumbai.

## Cells

The mouse fibroblast 3T3-L1 preadipocytes, and rat skeletal myoblast L6, cell lines were obtained from National Centre for Cell Sciences (NCCS), Pune and maintained in DMEM culture medium supplemented with 10% FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin in an atmosphere of 5% CO2 at 37 C. For adipogenesis, 3T3-L1 pre-adipocytes were grown into confluence

in a 1 X $10^4$ cells/well and then treated with the adipogenic cocktail (1 mg/L insulin, 120 mg/L isobutylmethylxanthine, and 0.1mg/L dexamethasone) for 48 hrs. This was followed by incubation in insulin-supplemented medium for an additional 48 hrs. The normal medium was used on day 5 to maintain the adipocytes up to day 12. For differentiation of myotubes, L6 cells were placed into 24 well plates in DMEM with 10% FBS for 24 h. The cells were then maintained in 2% FBS medium (DMEM) for 6 days to induce differentiation. The medium was changed every 48 h .

### Fractions Preparation

The fractions were evaluated over a concentration range from 5 to 40µg/ml. The fractions were dissolved in DMSO and further diluted in the cell culture medium. The concentration of DMSO in the extract and standard drug did not exceed 0.2 %, which had no effect on the obtained results. Pioglitazone, standard insulin sensitizer was evaluated at a dose of 6 µg/ml; dose extrapolated from the median therapeutic human dose.

### Viability assay

Prior to glucose uptake studies, viability tests were carried out to eliminate cytotoxic concentrations of fractions on differentiated adipocytes and myocytes using MTT assay as previously described.

### Glucose uptake

2-deoxy-D-[3H] glucose uptake was conducted according to a method described previously [15]. In brief, differentiated cells cultured in 24-well plates were treated with different concentrations (5 to 20µg/ml) of CDR 134 F 194 and CDR 267 F 018 for 24 hrs. The cells were then incubated with 2-deoxy-D-glucose (1 µCi/ml) for 5 mins and solubilized in 0.4 ml of 1% sodium dodecyl sulfate. Radioactivity of 3H-glucose was determined in the whole cell lysate using a Scintillation counter. To evaluate the effect of in insulin resistant state, differentiated adipocytes were treated with CDR 267 F018 and CDR 134 F194 for 24 hours prior to inducing to insulin resistant condition by dexamethazone. After treatment, the insulin resistant state was created by treating the adipocytes with dexamethazone (1mM) for 48 hours along with the drug fractions. Differentiated adipocytes were also treated with Pioglitazone which served as a positive control. DMSO was used as a vehicle control. After treatment, glucose uptake activity was evaluated using 2-deoxy –D- [$^3$H] glucose.

### *In vivo* studies

### Experimental animals

Wistar rats of either sex aged 12 weeks (200-230 g) were obtained from Bharat serum & Vaccines, Mumbai and housed in a group of 8 animals, for one week, in a 12:12 hour light and dark cycle, in a temperature and humidity controlled room. The animals were given free access to food and water. After the one week adaptation period, healthy animals were used for the study. The Institutional Animal Ethics Committee approved the experimental protocol.

### Treatment protocol

The animals were divided into ten groups of eight animals each and individually housed in cages. The normal control group continued to be fed a laboratory pellet chow *ad libitum* and maintained on regular normal water. Fructose fed group received laboratory pellet chow and 25% Fructose solution in water for 21 days, to create insulin resistance conditions. The standard anti-diabetic agent, Rosiglitazone, was administered to animals in the Standard Control group at a dose of 4 mg/kg body weight of rats along with 25% Fructose solution in water. Animals in the test groups were administered CDR 134 F 194 and CDR 267 F 018 along with the 25% Fructose solution in water for 21 days. 4 doses each of the test fractions were studied; *viz.* 20, 45, 90 & 180mg/kg for CDR 267 F 018 and 18, 35, 70 & 140mg/kg for CDR 134 F 194. These doses were determined based on the Effective/Therapeutic Dose ($ED_{50}$). The animals were weighed at the beginning of the experiment and then every week thereafter.

### Blood Biochemical analysis

Before and after drug administration, blood was collected by retro-orbital puncture from ether anesthetized rats and subjected to centrifugation to obtain serum at intervals of 7 days. Serum levels of glucose, triglycerides and total cholesterol were estimated using biochemical kits. Serum insulin levels were measured using Rat Insulin ELISA kit. (Mercodia, Sweden).

### Estimation of Glycogen content

The animals were sacrificed with an overdose of diethyl ether. The liver and thigh muscles were removed and weighed. Glycogen content was estimated using a previously described method [16].

### Statistical Analysis

Data was expressed as Mean ± SD. The results of the study were subjected to analysis of variance (ANOVA) using graph pad prism followed by Dunnett's t-test for multiple comparisions. A p-value <0.05 was considered statistically significant.

## Results

### *In vitro* studies

### Effect on Viability of cells

The viability was assessed over a concentration range 5µg/ml to 40µg/ml in 6 sets of experiments. There was no effect seen on the viability up to 20µg/ml as compared to the DMSO control. However viability was affected at 40µg/ml as compared to the DMSO control. Hence the concentrations selected for study were in the range of 5 to 20µg/ml.

## Effect on Glucose Uptake in L6 cells

As shown in Figure 1 a, CDR 267 F018, when tested over concentrations ranging from 5 to 20 µg/ml, stimulated glucose uptake in L-6 cells in a dose dependent manner with the maximum effect seen at 20 µg/ml, which was statistically significant as compared to the DMSO control treated cells. The effect was also comparable to that obtained with Pioglitazone, the standard anti-diabetic agent used in the study. CDR 134 F194, when tested over concentrations ranging from 5 to 20µg/ml, demonstrated an increase in glucose uptake as compared to the DMSO control with maximum stimulation seen at 5µg/ml. However, with an increase in concentration, a dose dependent decrease in stimulation of glucose uptake was observed in the L6 cells. (Figure 1b)

**Figure 1: Effect on glucose uptake in L 6 cells (n=6)**

**Fig 1a: CDR 267 F018**

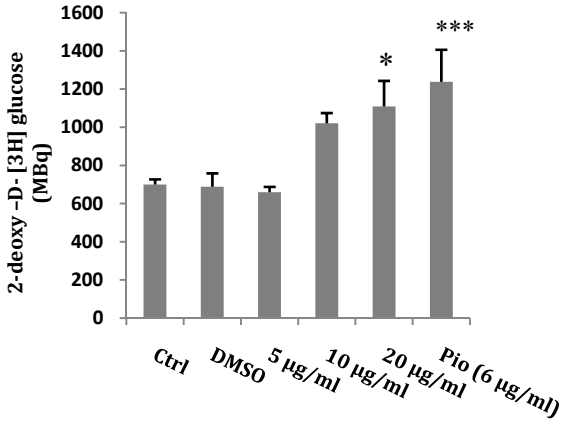
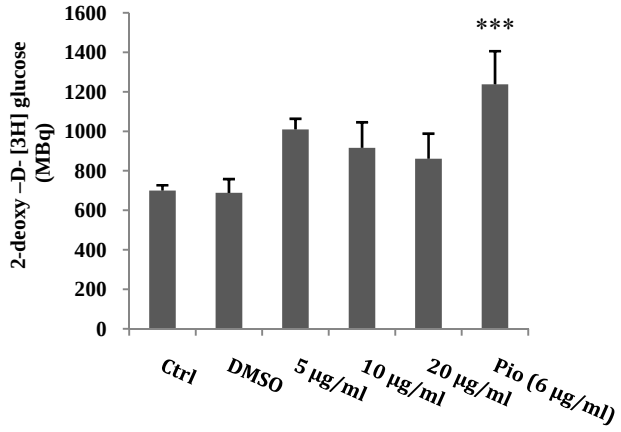



**Fig 1b: CDR134 F194**




All values are expressed as Mean ± SEM, $^{*}p<0.05$;$^{***}p<0.01$ as compared to DMSO control
(ANOVA followed by post-hoc tests)

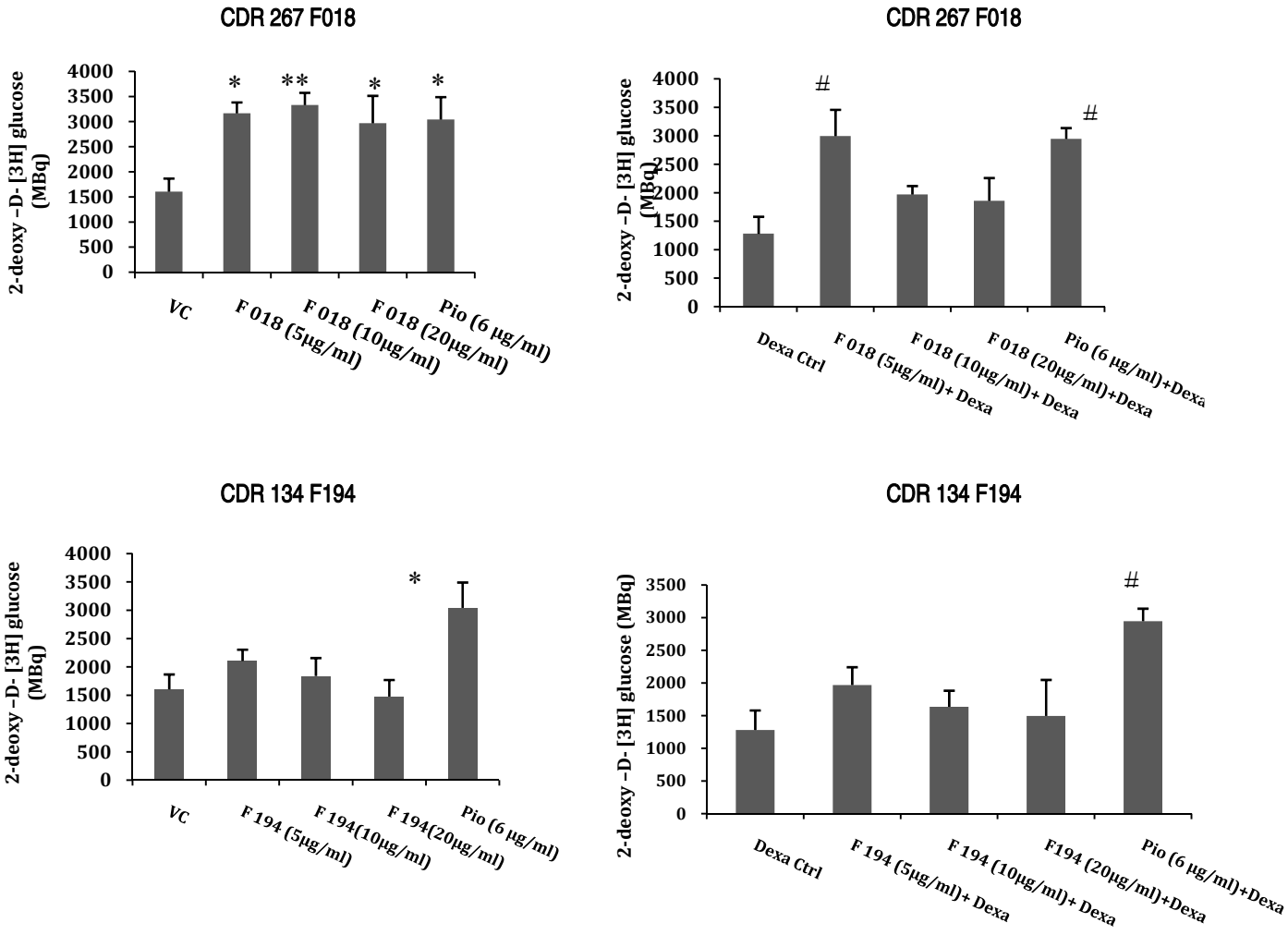
## Effect on Glucose Uptake in naïve and insulin resistant 3T3 L1 cells

As seen in Fig 2a, a decreased in the glucose uptake was observed in cells treated with dexamethazone (1000 µM) alone indicating an insulin resistant condition and an increase in cells treated with dexamethazone in presence of Insulin. Pioglitazone also demonstrated a significant increase in glucose uptake, when compared to uptake shown by the cells treated with dexamethazone alone indicating a reversal of the insulin resistant state.

CDR 267 F018 *per se* significantly stimulated the basal 2-deoxyglucose uptake with maximum effect seen at 10µg/ml concentration and this effect was comparable that of Pioglitazone. When added to the insulin resistant cells, CDR 267 F018 stimulated 2-deoxyglucose uptake, with maximum effect seen at the lowest concentration tested *i.e.* 5µg/ml which was comparable to Pioglitazone. These results indicate that these fractions have the potential to overcome the insulin resistant condition caused by dexamethazone.

CDR 134 F194 per se showed significant stimulation of basal 2-deoxyglucose uptake with maximum effect seen at 5µg/ml concentration. When added to the insulin resistant cells, CDR 134 F194 stimulated 2-deoxyglucose uptake, with maximum effect seen at the lowest concentration tested i.e. 5 µg/ml. Increase in concentration caused a decrease in effect.

Figure 2: Effect on glucose uptake in naïve and insulin resistant 3T3 L1 cells (n=6)




All values are indicative of Mean ± SEM
**p<0.01; *p<0.05 as compared to Vehicle cell control; #p<0.01 as compared to Dexa treated cells
(ANOVA followed by post-hoc tests)

## *In vivo* studies

### Effect on body weight (gms)

A decrease in body weight was observed at the end of 21 days in the disease control group as compared to normal control group. There was no significant increase observed on treatment with both CDR267F018 and CDR134F194. Rosiglitazone showed an increase in body weight when compared with disease control group. However the effect was not statistically significant.

### Effect on Serum Biochemical Parameters

An increase in blood glucose levels was observed in fructose fed group as compared to normal control group. Both the drugs showed a decrease in blood glucose levels as compared to the fructose fed group. Feeding fructose water caused a significant increase in serum levels of Insulin, total cholesterol and triglycerides as compared to normal control group. In contrast both the drugs inhibited the increase in the serum levels of total cholesterol and insulin that was induced by the fructose diet. Treatment with both the drugs demonstrated a decrease in triglycerides levels only at higher concentrations *i.e.,* 180 mg/kg in case of CDR267 F018 and 70 and 140 mg/kg in case of CDR134 F194.

Table 2: Effect on Serum Biochemical Parameters (n=6)

| Groups | Sugar | Insulin | Total Cholesterol | Triglycerides |
|---|---|---|---|---|
| NC | 71.18±8.31 | 0.074±0.01 | 54.51±16.74 | 106±53.18 |
| DC | 107.5±48.91 | 0.098±0.01 | 93.12±27.55** | 118.35±44.70 |
| Rosiglitazone | 82.6±10.26 | 0.077±0.033 | 77.31±15.84 | 90±61.21 |
| CDR 267 F018(20mg/kg) | 101.66±7.07 | 0.101±0.015 | 70.28±7.65 | 333.62±89.30 |
| CDR 267 F018(45mg/kg) | 86.67±3.05 | 0.091±0.024 | 83.25±15.41 | 137.34±50.21 |
| CDR 267 F018(90mg/kg) | 88.4±18.59 | 0.085±0.011 | 82.98±15.07 | 149.42±53.38 |
| CDR 267 F018(180mg/kg) | 76.88±3.61 | 0.086±0.020 | 70.63±9.27 | 102.83±21.26 |
| CDR 134 F194 (18mg/kg) | 103.2±7.68 | 0.090±0.012 | 88.35±12.23 | 175.07±71.38 |
| CDR 134 F194(35mg/kg) | 87.56±8.35 | 0.081±0.014 | 76.05±16.04 | 170.23±84.77 |
| CDR 134 F194(70mg/kg) | 90.18±16.18 | 0.078±0.016 | 75.04±13.72 | 103.75±53.21 |
| CDR 134 F194(140mg/kg) | 76.7±20.31 | 0.081±0.009 | 90.91±11.49 | 110.00±46.49 |

All values represent Mean ± SD;
**p<0.01 of Disease Control as compared to Normal Control; (ANOVA followed by post-hoc tests)

## Effect on glycogen content

Glycogen content in liver and thigh muscles was measured after sacrificing the animals. A decrease in the glycogen content in the Disease control group was observed as compared to the Normal control group. No effect in the glycogen content of the liver and thigh muscles was observed in the drug treated group as compared to the Disease Control group except for CDR 134 F 194(140mg/kg) which showed a significant increase in the glycogen content of thigh muscles as compared to the Disease Control group. Rosiglitazone showed an increase in the glycogen content as compared to the Disease Control group and statistically significant effect was observed with thigh muscles. (Table 3)

Table 3: Effect on Glycogen content (mg/gm of tissue) (n=6)

| Groups | Liver | Muscles |
|---|---|---|
| NC | 23.04±0.81 | 7.22±0.89 |
| DC | 17.73±1.49 | 3.59±1.11@@@ |
| Rosiglitazone | 21.65±0.96 | 5.93±0.81*** |
| CDR 267 F018(20mg/kg) | 18.90±0.58 | 3.83±0.65 |
| CDR 267 F018(45mg/kg) | 18.22±1.63 | 3.87±0.59 |
| CDR 267 F018(90mg/kg) | 19.21±0.80 | 4.19±0.61 |
| CDR 267 F018(180mg/kg) | 19.73±1.25 | 4.42±1.12 |
| CDR 134 F194 (18mg/kg) | 19.10±0.69 | 4.36±0.26 |
| CDR 134 F194(35mg/kg) | 18.67±1.46 | 4.63±1.20 |
| CDR 134 F194(70mg/kg) | 19.62±0.93 | 4.79±0.86 |
| CDR 134 F194(140mg/kg) | 20.59±2.75 | 4.91±0.89** |
| | | |

All values represent Mean ± SD;
@@@p<0.001 of Disease Control as compared to Normal Control;
***p<0.001; **p<0.01 of Disease Control as compared to drug treated groups
(ANOVA followed by post-hoc tests)

## Discussion

Type 2 diabetes is a common metabolic disorder characterized by chronic hyperglycemia and dyslipidemia resulting from peripheral tissue insulin resistance and impaired insulin secretion from the pancreas. It is estimated that Type 2 diabetes patients lose 15 years of an average life expectancy. Therefore, intensive treatment to control hyperglycemia is needed in diabetes. However, because clinical therapies have limited efficacy and significant mechanism-based side effects, there has been an enormous increase in the use of alternatives for diabetes management.

Impaired glucose uptake to peripheral tissues results in high circulating glucose levels. Therefore, the main strategy is to stimulate glucose uptake to skeletal muscle, liver, and adipocytes that consume most plasma glucose. CDR267F018 and CDR134F194 demonstrated stimulation of glucose uptake in L-6 skeletal muscle cells in a dose dependent manner. Both the fractions *per se* significantly stimulated the basal 2-deoxyglucose uptake. A stimulation of 2-deoxyglucose uptake was also observed when added to insulin resistant cells developed using dexamethazone indicating that these fractions have the potential to overcome the insulin resistant condition caused by dexamethazone. The effect shown by these drugs was comparable to Pioglitazone, a known insulin sensitizer.

High fructose consumption leads to obesity and metabolic abnormalities as observed in insulin resistance syndrome. Fructose as such doesn't stimulate insulin secretion from pancreatic-β-cells, leptin an adipose derived hormone production is regulated by insulin in response to meals, and consumption of foods and beverages containing fructose reduces circulating leptin concentration leading to insulin resistance. [17,18].The rats fed with high fructose diet provide an animal model of insulin resistance associated with weight gain, hyperinsulinemia, hyperlipidemia, hyperglycemia. The use of 25% w/v fructose in drinking water for a period of 21 days significantly raised glucose, insulin, triglycerides, cholesterol and bodyweights with a decline in liver and muscle glycogen levels.

Administration of the fractions CDR267F018 and CDR134F194 demonstrated a dose dependent decrease in sugar and insulin levels with maximum effect at the highest dose tested. These fractions also decreased the total cholesterol levels although an increase in triglycerides levels was observed at the higher doses. The maximum decrease in triglycerides was seen with lower dose. Thus we can conclude that these fractions prevented the development of hyperglycemia, hyperinsulinemia and hypertriglyceridemia induced by feeding of a fructose rich diet.

Also both the fractions demonstrated an increase in liver and muscle glycogen content as compared to the glycogen content of fructose fed rats. Insulin resistance results in accumulation of triglyceride content and reduction of glycogen content in skeletal muscle. However, very few studies have measured lipid content and glycogen content in liver associated with insulin resistance. Researchers have studied the relationship between liver lipid content, liver glycogen, and insulin resistance in high-fat-fed rats, which are animal models of insulin resistance. Thus in our study both the fractions *i.e.,* CDR267F018 and CDR134F194 might have improved insulin sensitivity in peripheral tissues, as evident from the results showing decreased glucose and insulin production and increased glycogen stores. However the increase seen in triglyceride levels at lower concentrations needs to be explored further by studying the fatty acid metabolism.

Our previous study showed the efficacy of *X. granatum* extract in declining blood glucose profile of STZ-induced diabetic rats and both glucose as well as cholesterol and triglycerides levels in low dosed streptozotocin- high fructose fed rats and dyslipidemic hamsters. Our results are in agreement with the said results.

## Conclusion

The above results prove the efficacy of CDR267F018 and CDR134F194 in ameliorating insulin resistant conditions as seen in the 3T3L1 adipocyte, skeletal muscle model and fructose fed models. This is the first study to report about the existence of insulin resistance reversal property of active fractions obtained from *X. molluccensis* and *X. granatum,* which could be of clinical interest. Further the efficacy of the fractions in management of type 2 diabetes and dyslipidemia can be proved by conducting clinical studies. Further work is in progress to identify the active constituents from the fractions CDR267F018 and CDR134F194, which are responsible for these activities.

### Author Contributions

RM and SJ have made substantial contributions in conception and design of the study, acquisition, analysis and interpretation of data. SJ drafted the manuscript and RM revised it critically for important intellectual content; TN and SPSB helped in preparation and fractionation of the extracts used in the study.

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