Research Article

Antidiabetic Activity in Flowers of *Nymphaea rubra*

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ABSTRACT

As a prelude to discover new antidiabetic molecules from natural sources, an attempt was made to establish the antidiabetic activity in flowers of *Nymphaea rubra*. The various extracts of the dried flowers of *Nymphaea rubra* were prepared and evaluated for antihyperglycemic activity in validated animal models. The ethanolic extract and one of its fraction i.e. chloroform showed significant antihyperglycemic activity on Streptozotocin (STZ)-induced diabetic rats, neonatally-STZ treated rats and high fructose high fat fed insulin resistant rats. Beside these extracts and fractions also were found to improve glucose tolerance post sucrose load in normal rats and decline in blood glucose level of STZ-induced diabetic rats. The chloroform fraction was also observed to enhance 2-deoxy-(3H) glucose uptake by skeletal muscle cells (L-6) in concentration dependent manner. It also inhibits the process of adipogenesis and increases the expression of glucose transporter protein-4 (GLUT-4), insulin receptor substrate (IRS) and phosphatidyl inositol-3-kinase (PI-3K).

Keywords: Antidiabetic activity, Antidyslipidemic activity, Adipogenesis, Glucose uptake, High fructose high fat fed Low-dosed STZ induced rats, Neonatal STZ treated rats, Nymphaea rubra flowers, Skeletal muscle cells.

INTRODUCTION

Diabetes mellitus is a major public health problem in the developing as well as developed countries; it is ranked seventh among the leading cause of death when its total complications are taken into considerations.[1] The cardinal manifestation of diabetes mellitus is hyperglycaemia which plays a central role in the development of diabetic complications[2] which are caused either by insulin deficiency or by insulin resistance or both. Insulin resistance is defined as a complex nutritional–metabolic state characterized by reduced sensitivity of target tissues to the physiological effects of insulin. This complicated process involves an intracellular dysfunction in the molecular machinery involved in responding to the insulin signal and mobilizing glucose uptake[3] and this increased circulating glucose concentration is associated with abnormalities in carbohydrate, protein, and lipid metabolism and a variety of microvascular, macrovascular and infectious complications.[4]

Many of the oral antidiabetic agents are available for the treatment of type 2 diabetes mellitus but they have considerable side effects and fail to significantly alter the course of diabetic complications.[5] Though few of plant treatments used in traditional medicine for diabetes have received scientific scrutiny and they also provide a basis of new synthetic antidiabetic analogues with potent activity.[6-9]

N. rubra is an aquatic plant growing on the shores of lakes and rivers, commonly known as kumuda and belongs to family Nymphaeaceae. Nymphaea species have many biological activities including antitumour and antioxidant activities. *N. caerulea* species is used as a medicine for dyspepsia, diarrhea, fever and heart palpitations.[10] Kaempferol flavonol of *N. candida* posses significant antioxidant property and the another species i.e. *N. tetragona* contains highest free radical scavenging property.[11] *N. stellata* is known to posses antidiabetic as well as anti-hepatoprotective activity.[12,13] In the present study an attempt has been made to establish antidiabetic activity in the flowers of *N. rubra* in validated animal models of type 2 diabetes.

MATERIALS AND METHODS

Chemicals and Reagents

Metformin, Streptozotocin (STZ), 2-deoxy-glucose (2-DOG), insulin, cytochalasin B, IBMX, dexamethasone and DMSO were purchased from Sigma Aldrich Co., USA. One touch glucometer (Accu-Check sensor) and glucostrips was purchased from Roche Diagnostics India Ltd. HG-DMEM, FBS, horse Serum, and trizol reagent were purchased from Life Technologies (GIBCO), USA. [^{3}H]-2-DOG was procured from Amersham Pharmacia Biotech, UK whereas antimouse IgG and antirabbit IgG HRP linked antibodies were from Cell Signaling Technology (USA). PVDF membranes were procured from immobilon Millipore. All other chemicals unless otherwise mentioned were obtained from SRLs, Mumbai.

Preparation of extracts of N. rubra flowers

The flowers of *N. rubra* were shade dried, powdered well by a mechanical grinder and extracted many times with

95% ethanol by percolation method using Soxhlet apparatus. Each percolate were collected, pooled, filtered and concentrated under vacuum to dryness using rotavapor at 40-45^{0}C. This extract is termed as 95% ethanolic extract. In the same manner the crude powder was extracted with 50 % ethanol and distilled water, respectively and 50 % ethanolic and aqueous extracts prepared.

Fractionation of 95% ethanolic, 50% ethanolic and aqueous extracts of N. rubra flowers

The 95% ethanolic, 50% ethanolic and aqueous extracts were further fractioned using column chromatography with solvents of increasing polarity viz. n-hexane, chloroform, n-butanol and water to obtain the respective fractions. Each extract was dried well and triturated with hexane and then concentrated under reduced pressure at 40°C. The insoluble portion was fractionated by chloroform and concentrated under reduced pressure. Residue obtained a er tritura ng with chloroform was then suspended in dis lled water and then frac onated with n-butanol saturated with water. n-butanol soluble frac on was concentrated under vacuum at 50^{0}C. Water soluble frac on was also concentrated under vacuum at 45-50^{0}C and finally dried under vacuum to get the final fractions.

Animals

Male albino rats of Sprague Dawley (SD) strain (8 to 10 weeks of age: body weight range 160 ± 20 g) were procured from National Laboratory Animal Centre (NLAC) of the Institute. Research on 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. Rats were housed in groups of five in polypropylene cages under controlled standard environmental conditions of temperature, humidity, light, air changes and 12 hr light-dark cycles. Prior to commencement of the experiment all the rats were acclimatized to the new environmental condition at least one week.

Induction of diabetes

A solution of STZ (60 mg/kg) in 100 mM citrate buffer, pH 4.5 was prepared and calculated amount of the fresh solution was immediately injected intraperitoneally to overnight fasted rats. Two days the animals were checked for their diabetic status. Animals showing blood glucose levels above 270 mg/dl were termed as diabetic. Blood glucose level measurements were always done by glucostrips (Roche).

Activity evaluation protocols

Improvement in oral glucose tolerance

The baseline glucose level of each animal was measured by glucometer using glucostrips after an overnight starvation. Animals showing blood glucose level between 60 to 80 mg/dl were finally selected, divided into groups each consisted of six animals. Rats of experimental group were administered the suspension of the test sample orally prepared in 1.0 % gum acacia (vehicle) at preselected dose levels i.e. 250 mg/kg body weight in the case of extracts and 100 mg/kg in case of fractions. The standard antidiabetic drug i.e. Metformin was administered at 100 mg/kg dose levels. Animals of control group were given an equal amount of 1.0 % gum acacia and termed as sham treated control. An oral sucrose load of 10g/kg body weight was given to each animal exactly after 30 min post administration of the test sample/vehicle. Blood glucose of each rat was again determined from the tail vein at 30, 60, 90 and 120 min post administration of sucrose by glucostrips. Food but not water was withheld from the cages during the course of experimentation.[14]

Decline in blood glucose level on STZ-induced diabetic rats

The STZ-induced diabetic animals having their blood glucose level between 280 to 400 mg/dl were separated, randomized into groups each consisted of six animals each. Rats of experimental groups were orally administered suspension of the desired test samples and standard antidiabetic drug metformin, respectively. The sham treated control group received 1.0% gum acacia. The blood glucose levels of each rat were measured at 0, 30, 60, 90, 120, 180, 240, 300 and 1440 min post administration of test sample/vehicle/standard antidiabetic drug. Food but not water was withheld from the cages during 0 to 300 min.[15]

Antidiabetic, anti dyslipidemic activities and protective effect on renal functions on STZ-induced diabetic rats

STZ-induced diabetic rats were grouped and animals showing stable or increasing blood glucose level (between 12-20mM) were finally selected and randomized into groups of 6 animals in each. One group was considered as sham treated control group while the others as experimental groups. The test sample i.e. chloroform fraction of ethanolic extract of *N. rubra* and standard drug metformin was administered to experimental groups, respectively at a dose of 100 mg/kg body weight daily for 14 consecutive days. Body weight, blood glucose levels, lipid profile renal, renal profile and OGTT were followed at preselected time intervals. At the end of experiment, the blood was withdrawn from jugular vein, serum separated and estimated for total lipid profiles and renal function markers.[16]

Antidiabetic and anti dyslipidemic activity on high fructose high fat fed STZ-induced diabetic rats

Male albino rats of SD strain were kept on high fructose high fat diet (composition: 60 % fructose, 21 % casein, 13 % saturated fat, 1.0 % salt mixture, and trace amount vital minerals and vitamins) for 6 weeks. After this period blood was withdrawn from the retro-orbital plexus of each rat for the estimation of the lipid profiles. The rats showing their serum triglyceride ≥ 300 mg/dl and total-cholesterol ≥ 150 mg/dl were considered as

hyperlipidemic. These hyperlipidemic rats were injected STZ intraperitoneally at the dose of 45mg/kg. The STZ-treated hyperlipidemic rats with the fasting blood glucose ≥280 mg/dl after 48 hours were considered diabetic and finally selected. These were divided into three groups. The rats of Group I were treated with 1.0 % gum acacia, Group II were treated with chloroform fractions of ethanolic extract of *N. rubra* at 100 mg/kg b.w, Group III was treated with standard drug metformin at 100mg/kg b.w. The treatments were continued for 14 consecutive days. The oral glucose tolerance, the serum lipid profiles were followed on day 7 and 14 post treatment.[17]

Assessment of oral glucose tolerance test on Neonatally-streptozotocin treated diabetic rats

Two-day-old pups weighing around 5 to 8g of SD strain of rats were given fresh solution of STZ prepared in citrate buffer 0.1M, pH 4.5 subcutaneously at the dose of 90 mg/kg. The pups were separated from their mothers after 4 weeks. Symptoms of type 2 diabetes mellitus in these animals were identified as polydypsia, polyurea, and abnormal glucose tolerance post glucose load. These rats were divided into three groups each consisted of 6 animals. The animals of Group I received vehicle i.e.1% gum acacia every day, whereas animals of Group II and III were treated with test sample i.e. chloroform fractions of ethanolic extract of *N. rubra* and standard antidiabetic drug metformin at 100 mg/kg b.w, respectively for 14 days. The fasting blood glucose level, serum insulin level and OGTT post glucose load of each rat was performed at selected time intervals i.e. on day 0, 7 and 14 post treatment.[18]

Measurement of serum lipids and insulin levels

Total cholesterol, Total triglycerides, LDL-cholesterol, and HDL-cholesterol in serum of each animal were measured on an automated analyzer (Cobas-Integra 400) employing the respective assay kits and instructions as provided by the manufacturer. Serum insulin level was measured employing the kit and instructions as provided by Mercodia (Uppsala Sweden).

Cell culture and differentiation

The rat skeletal muscle cells (L6 myoblasts, obtained from ATCC, USA) were cultured in DMEM supplemented with 10% FBS with penicillin (120 units/ml), streptomycin (75 µg/ml), gentamycin (160 µg/ml) and amphotericin B (3.0 µg/ml) in a 5 % CO_2 environment. For differentiation, the L6 cells were transferred to DMEM with 2% FBS up to the confluency. 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.

The mouse pre adipocyte cells (3T3L1) were grown in 24 well plates until 2 days post-confluence and the cells were induced by the differentiation medium (combination of 0.5 mM/l of IBMX, 0.25 µM/l of dexamethasone and 5 µg/l of insulin in DMEM medium with 10% FBS) to differentiate into adipocyte. Three days after induction, the differentiation medium was replaced with medium containing 5 µg/ml insulin alone. The medium was subsequently replaced again with fresh culture medium (DMEM with 10% FBS) after 2 days the extent of differentiation was measured by monitoring the formation of multinucleation in cells.

2-^{3}H-deoxy-glucose uptake by skeletal muscle cells (L6)

L6 myotubes grown in 24-well plate were subjected to glucose uptake assay. After differentiation for the stipulated period, the cells were incubated with different concentrations of chloroform fractions of ethanolic extract of *N. rubra* for 24h. After incubation, the wells were treated with 100 nM insulin and subsequently for 5 mins of incubation, followed by rinsing with HEPES-buffered saline containing [140 mM NaCl, 20 mM HEPES, 5 mM KCl, 2.5 mM $MgSO_4$, 1 mM $CaCl_2$ (pH 7.4)] containing 10 µM 2DG (0.5 µCi/ml 2[^{3}H] DG) at room temperature. The uptake of 2-^{3}H-DOG was terminated rapidly by aspirating off the radioactive incubation medium and washing the cells three times in ice-cold (0.9% Nacl and 25mM D-glucose) solution and Cell associated radioactivity was determined by cell lysis in 0.05N NaOH, followed by liquid scintillation counting (Beckman Coulter, USA). Nonspecific uptake was determined in the presence of cytochalasin B (25 µM) during the assay. The uptake measurement was made in triplicate for concordance. Results were expressed as % glucose uptake with respect to control.[19]

Adipogenesis assay (Oil Red O Staining)

Differentiated 3T3-L1 were rinsed in phosphate buffered saline (pH 7.4), and stained with Oil Red O (0.36% in 60% Isopropanol) for 30 min, finally cells were rinsed twice with phosphate buffer saline and observed under microscope. Lipid and Oil Red O were extracted using isopropanol, and absorbance was measured using a spectrophotometer at wavelength of 490 nm.[20]

RNA Extraction, Quantitative Real Time PCR and gene expression analysis

Total RNA was extracted from L6 myotubes, following the exposure to chloroform fraction of *N. rubra* by TRIZOL methods (in vitrogen Life Technologies, USA). RNA quality was determined by running a sample with RNA loading dye (Ambion, USA) on a 1% agarose gel and inspecting for distinct 18S and 28S bands, indicating lack of degradation. Quantity was determined by A260 and A280 measurement. All samples had A260/A280 ratios of 1.8–2.1.An aliquot of 2µg total RNA from each sample was reverse transcribed to synthesize cDNA using the High Capacity cDNA Reverse Transcription Kit, Applied Biosystems (ABI- 4368814) according to the manufacturer's instructions. Gene expression was analyzed by relative quantitation with the 2-ΛΛCT method using real-time PCR Light Cycler 480 System (Roche, Indianapolis, IN). Real-time PCR was performed in 96-well plates using a SYBR Premix Ex Taq (ABI- Applied Biosystems, USA) according to the supplier's instructions.

All samples were be normalized to values of GAPDH and results expressed as fold changes of threshold cycle (Ct) value relative to controls. Cycling parameters were 95^{0}C for 10 sec and then 45 cycles of 95^0 C for 5 sec and 60^{0}C for 31 sec. Quantifications was performed in triplicate, and the experiments were repeated independently two times.

Detection of insulin signaling and other related proteins

To identify the signaling pathway and its potential protein targets for glucose transport activity of chloroform fractions of *N. rubra*, chloroform fraction of ethanolic extract of *N. rubra* were individually added to the L6 myotubes along with insulin at predetermined concentrations during glucose uptake assays, and the inhibition was measured by the glucose taken in by the cell samples treated with or without inhibitors. Cells were homogenized into PBS containing 1% NP40, 5 mM EDTA, phosphatase inhibitors and protease inhibitors cocktail (Ripalysis buffer). Samples were homogenized and incubated on ice for 15 min and centrifuged at 16000 rpm at 4°C. Then supernatant was taken and quantified by Bradford assay. For the analysis of phosphorylated and nonphosphorylated IRS-1, Akt, and PI3K, the cell lysate was loaded and separated on SDS– polyacrylamide gels. Protein was transfer red to a polyvinylidenefluoride membrane and detected using the appropriate antibodies. Immunoreactive bands will be visualized by Enhanced Chemiluminescence according to manufacturer's instructions (GE Healthcare, UK).

Statistical Analysis

Each biochemical parameter was expressed as Mean+SD of three independent experiments. Quantitative glucose tolerance of each animal was calculated by Area under curve (AUC) method (Prism Software). The average fall in AUC of experimental group compared to control group was always termed as % antihyperglycemic activity. Analysis of statistical significance of differences in measurements between samples was done by using Dunnet's test following ANOVA software. It is denoted by p values. Statistically Significance difference was set at following levels *represents p< 0.05, **represents p< 0.01 and ***represents p< 0.001.

RESULTS

Improvement in glucose tolerance on normal rats

Table 1 presents the effect of crude powder, 95% ethanolic, aqueous and 50% ethanolic extracts on the improvement of oral glucose tolerance post sucrose load in normal rats. It is evident from the results that 95% ethanolic and aqueous extracts prepared from the crude powder of *N.rubra* flowers inhibited the rise in postprandial hyperglycaemia or improved glucose tolerance (GTT) significantly post sucrose load to tune of 16.9 and 16.3 % at 250 mg/kg dose. However, the effect of the crude powder and 50 % ethanolic extract on oral glucose tolerance though was in the order of 9.60 and 14.8 % at the preselected dose i.e. 250 mg/kg but was not statistically significant. Whereas, the standard drug metformin showed around 33.7% improvement, on oral glucose tolerance post sucrose load in normal rats at 100 mg/kg dose which was highly significant. The crude powder and 95 % ethanolic extract of *N. rubra* flowers were found capable of improving glucose tolerance post sucrose load on streptozotocin-diabetic rats where their activity profiles at 250 mg/kg were calculated to be around 20.2 and 20.6 % equivalent to that of metformin at 100 mg/kg dose (Table 1).

Table 1: Effect of crude powder, 95% ethanolic, 50% ethanolic and aqueous extracts of crude powder of *N. rubra* and standard drug metformin on the improvement of glucose tolerance (GTT) post sucrose load in normal rats and decline in blood glucose level of STZ treated rats.

Test Samples	Dose (mg/kg)	% Activity	
		Normoglycaemic rats 0-120 min AUC	STZ induced diabetic rats 0-300 min AUC
Control		15150 ± 91.3	125790 ± 3361
Crude Powder	250	13689 ± 254 **(9.60)**	100350 ± 1983 **(20.2)****
95%Ethanolic extract	250	12693 ± 220 **(16.9)***	99840 ± 2122 **(20.6)****
50% Ethanolic extract	250	12909 ± 615 **(14.8)**	111021 ± 1780 **(11.7)**
Aqueous extract	250	12654 ± 309 **(16.3)***	119472 ± 1456 **(5.02)**
Metformin	100	10035 ± 155 **(33.7)*****	99336 ± 2560 **(21.0)*****

Values are mean ±S.E. of three independent experiments; p values *<0.05, **<0.01, ***<0.001

Decline in blood glucose level on STZ-induced diabetic rats

Table 2 presents the blood glucose profile shows the diagrammatic presentation of the decline in blood glucose levels of the STZ-induced diabetic rats treated respectively with crude powder, 95% ethanolic extract, 50% ethanolic extract, aqueous extract of *N. rubra* flowers and the standard antidiabetic drug metformin. It is evident from the result that crude powder showed significant decline in blood glucose level of STZ-induced diabetic rats at 250 mg/kg dose. The effect was evident even from 30 min which persisted till the end of the experiment i.e. 1440 min. The average antihyperglycemic activity was calculated to be around 20.2 and 15.3 %

Table 2: Blood glucose level of STZ-induced diabetic rats treated with crude powder, 95% ethanolic, 50% ethanolic and aqueous extracts of crude powder of *N.rubra* flowers and standard drug metformin.

Test samples	Dose (mg/kg)	Blood Glucose levels (mg/dl) post treatment									AUC	
		0 min	30 min	60 min	90 min	120 min	180 min	240 min	300 min	1440min	0-300 min	0-1440 min
Control		345.8 ± 17.1	486.4 ± 16.4	560.8 ± 15.4	503.8 ± 9.0	401.0 ± 19.0	380.0 ± 16.8	373.4 ± 13.8	360.8 ± 14.5	460.2 ± 17.7	125790 ± 3361	593760 ± 13260
Crude Powder	**250**	351.6 ± 11.4	404.0 ± 4.7 ***	382.2 ± 12.3***	366.0 ± 10.3***	344.8 ± 7.1**	321.4 ± 9.9 **	303.2 ± 12.0**	250.6 ± 4.7 ***	455.6 ± 11.7	100350 ± 1983 **(20.2**)**	502884 ± 4286 **(15.3*)**
95% Ethanolic Extract	**250**	335.6 ± 17.6	389.4 ± 10.7***	378.6 ± 20.0***	368.8 ± 14.3***	357.6 ± 9.4**	336.6 ± 5.3 *	281.4 ± 2.7 ***	251.0 ± 7.4 ***	511.8 ± 17.4	99840 ± 2122 **(20.6**)**	534636 ± 7074 **(9.95)**
50% Ethanolic Extract	**250**	350.0 ± 14.2	370.2 ± 20.3 ***	358.0 ± 20.9***	361.8 ± 11.2***	363.0 ± 14.8	363.0 ± 13.2	389.6 ± 12.7	386.0 ± 8.8	451.2 ± 13.2	111021 ± 1780 **(11.7)**	588225 ± 9611 **(0.93)**
Aqueous Extract	**250**	335.0 ± 15.4	468.8 ± 11.5	442.2 ± 9.5 **	417.0 ± 6.2***	398.2 ± 5.7	388.0 ± 3.2	374.8 ± 7.0	364.0 ± 6.6	360.6 ± 11.0***	119472 ± 1456 **(5.02)**	532494 ± 9627 **(10.3)**
Metformin	**100**	341.4 ± 3.5	450.6 ± 10.1	448.8 ± 16.8***	395.6 ± 16.5***	355.0 ± 10.8 ***	298.0 ± 14.6 ***	252.0 ± 9.4 ***	213.0 ± 3.0 ***	396.6 ± 16.7**	99336 ± 2560 **(21.0***)**	446808 ± 10317 **(24.7***)**

Table 3: Blood glucose level of STZ-induced diabetic rats treated with active fractions of extracts of crude powder of *N.rubra* flowers and standard drug metformin.

Group	Dose (mg/kg)	Blood glucose profile (mg/dl)									AUC	
		0 min	30 min	60 min	90 min	120 min	180 min	240 min	300 min	1440 min	0-300 min	0-1440 min
Control		358.2 ± 4.7	512.0 ± 16.2	522.6 ± 13.8	503.4 ± 8.3	474.6 ± 10.8	434.6 ± 12.4	387.4 ± 6.4	405.4 ± 18.5	477.6 ± 17.7	134352 ± 1117	637662 ± 1905
Chloroform (95 % EE)	100	357.4 ± 6.3	380.2 ± 14.9 ***	378.4 ± 14.6***	375.8 ± 12.8***	364.8 ± 13.0**	373.4 ± 14.8**	366.2 ± 18.2	369.6 ± 11.8	395.4 ± 8.8 **	111273 ± 4104 **(17.1*)**	547323 ± 1832 **(14.1*)**
Aqueous (95 % EE)	100	361.8 ± 9.6	486.2 ± 18.3	457.0 ± 19.6*	390.2 ± 5.8 ***	376.6 ± 9.2 **	372.4 ± 12.2*	358.2 ± 12.6	350.0 ± 17.1*	381.0 ± 10.2*	116712 ± 1885 **(13.1)**	533382 ± 1162 **(16.3)**
Hexane (AE)	100	362.2 ± 5.8	337.0 ± 9.3 ***	362.8 ± 11.1***	324.6± 5.6 ***	328.2 ± 4.4***	321.4 ± 12.5**	293.2 ± 7.7 *	236.4 ± 7.2 ***	383.6 ± 2.7 *	94902 ± 1665 **(29.3**)**	448302 ± 4707 **(29.6**)**
Aqueous (AE)	100	357.8 ± 6.4	406.8 ± 7.7 ***	397.6 ± 13.3**	372.2 ± 14.4***	352.6 ± 9.7 **	340.0 ± 16.2**	342.6 ± 14.9**	347.6 ± 18.2*	309.0 ± 4.0 **	107916 ± 3041 **(19.3*)**	482178 ± 1277 **(24.3*)**
Aqueous (50 % EE)	100	359.8 ± 10.6	369.6 ± 5.1 ***	397.2 ± 6.8**	363.8 ± 12.7***	352.0 ± 8.9**	321.4 ± 4.9***	297.4 ± 7.4**	287.0 ± 3.4 **	386.6 ± 2.7*	100893 ± 1077 **(24.9*)**	484845 ± 3635 **(23.9*)**
Metformin	100	361.8 ± 17.9	450.6 ± 10.1 ***	448.8 ± 16.8**	395.6± 16.5***	355.0 ± 10.8**	298.0 ± 14.6**	252.6 ± 9.4 ***	213.0 ± 3.0 ***	359.4 ± 17.2**	99642 ± 2777 **(25.8***)**	425910 ± 1312 **(33.2***)**

EE-95% Ethanol Extract; AE-Aqueous Extraxt; 50% EE- 50% Ethanol Extract

Table 4: Effect of chloroform fraction of ethanolic extracts of the crude powder of *N.rubra* flowers and standard drug metformin on fasting blood glucose, improvement in OGTT and serum insulin level and serum lipid profile on STZ induced diabetic rats.

Group	Days	Fasting blood glucose (mg/dl)	OGTT 0-120 min AUC	Serum insulin (µg/l)	Triglycerides (mg/dl)	Cholesterol (mg/dl)	LDL-cholesterol (mg/dl)	HDL-cholesterol (mg/dl)
Sham treated control (1.0% Gum acacia)	0	319 ±22.3	54667.0 ±2398	0.06 ±.004	134.4 ±13.4	99.0 ± 17.7	39.4 ±3.1	28.4 ±3.8
	7	317.6 ±34.7	55674 ±1879	0.07 ±.003	133.1 ±7.5	91.6 ± 6.0	35.6 ±1.3	26.3 ±3.0
	14	339.6 ±22.0	56250.0±2218	0.04 ±.005	133.6 ±7.1	82.2 ± 5.3	69.8 ±2.2	24.1 ± 2.3
Chloroform fraction treated (100 mg/kg)	0	322 ±11.0	54779 ±1964	0.06 ±.004	134.1 ±5.3	89.8 ± 8.7	28.3 ±2.9	29.8 ±4.2
	7	261.3±18.5* **(17.7)**	40302±1495** **(27.6)**	0.07 ±.005	110.8 ±4.1* **(16.7)**	55.0 ± 0.9** **(39.9)**	34.0 ±0.7 **(4.49)**	40.3 ±5.2* **(+53.8)**
	14	242.2±14.5** (**28.6)**	35184±1939** **(37.4)**	0.09 ±.003	111.4 ±2.9* **(16.6)**	55.7 ± 7.7* **(32.2)**	59.6 ±3.7* **(14.6)**	37.8 ± 3.2* **(+56.8)**
Metformin treated (100 mg/kg)	0	327±29.9	54778 ±2987	0.05 ±.005	133.1 ±2.90	95.2 ± 2.8	22.4 ±1.4	28.8 ±0.6
	7	222.8±15.9** **(29.8)**	34086±1563** **(38.7)**	0.9 ±.003	100.0 ±2.6* **(24.8)**	60.6 ± 1.3& **(33.8)**	29.2 ±1.6 **(17.7)**	40.7 ±3.0* **(+54.7)**
	14	185.8±13.1** (**45.2)**	31560±1220*** (**43.8)**	0.12 ±.004	98.9 ±1.06** **(25.9)**	55.9 ± 1.5* **(31.9)**	49.8 ±2.0* **(28.6)**	41.1 ±1.2* **(+69.8)**

Table 5: Effect of chloroform fraction of ethanolic extracts of the crude powder of *N.rubra* flowers and standard drug metformin on renal functions profile on STZ induced diabetic rats.

Group	Day	Renal functions		
		Serum-Urea (mg/dl)	Serum-Uricacid (mg/dl)	Serum-Creatinine (mg/dl)
Sham treated control (1.0% Gum acacia)	0	57.9 ± 2.0	3.78± 0.6	0.43± 0.07
	7	66.4 ± 0.4	3.49± 0.2	0.55± 0.02
	14	71.2 ± 3.5	3.16 ± 0.3	0.58 ±0.11
Chloroform fraction treated (100 mg/kg)	0	56.9 ± 0.2	3.58 ± 0.1	0.42 ± 0.01
	7	56.1 ± 6.0* **(15.5)**	2.60± 0.2* **(25.2)**	0.46 ± 0.05* **(16.3)**
	14	59.2 ± 2.5* **(16.8)**	2.3 ± 0.2* **(26.1)**	0.48 ± 0.06* **(17.2)**
Metformin treated (100 mg/kg)	0	58.1 ± 2.4	3.65 ± 0.3	0.45± 0.02
	7	54.4 ± 3.5* **(18.0)**	2.16 ± 0.5*** **(38.1)**	0.44 ± 0.08* **(20.0)**
	14	56.1 ± 3.6** **(21.2)**	1.81 ± 0.5*** (**42.7)**	0.47 ± 0.02* **(18.9)**

Table 6: Effect of chloroform fractions of ethanolic extract of the crude powder of N. rubra flowers and standard drug metformin on fasting blood glucose, OGTT, serum insulin profile and Lipid profile of High fructose high fat fed low dosed STZ-induced diabetic rats.

Group	Day	Fasting blood glucose (mg/dl)	OGTT 0-120 min AUC	Serum insulin (µg/l)	Triglycerides (mg/dl)	Cholesterol (mg/dl)	LDL-cholesterol (mg/dl)	HDL-cholesterol (mg/dl)
Sham treated control (1.0% Gum acacia)	0	206.9 ±9.8	51668 ±2345	2.60 ± 0.09	327 ±64.0	167 ±19.0	72.5 ± 6.3	44.1± 5.4
	7	186.0 ±17.1	51780 ± 1611	2.70 ± 0.03	332 ± 76.8	166 ±18.5	73.1 ± 3.2	40.1± 1.6
	14	189.9 ±15.0	54594 ± 1654	2.90 ± 0.02	339 ±78.6	167 ± 18.2	74.7± 3.4	38.9 ± 2.7
Chloroform fraction treated (100 mg/kg)	0	203.6 ±15.7	50923 ±2009	2.70 ± 0.05	326 ±35.0	168 ±4.4	71.0 ± 10.2	46.0 ± 6.8
	7	172.4 ±19.7 **(7.31)**	40902 ± 362.4* **(21.0)**	2.55 ±0 .09*	289 ± 36.2 **(12.9)**	146 ±5.7 **(12.0)**	66.9 ± 1.9 **(8.48)**	49.7± 3.0 **(+23.7)**
	14	168.0 ±11.6 **(11.1)**	39534 ± 836.2** **(27.5)**	2.00± 0.30	253±38.2** **(25.3)**	130 ±4.8* **(22.5)**	63.0± 0.90* **(15.6)**	49.0 ±2.3* **(+25.8)**
Metformin treated (100 mg/kg)	0	213.4 ±11.4	49887 ±1987	2.80 ± 0.10	326 ±62.1	162 ±11.3	72.4 ± 2.1	44.1 ± 0.19
	7	143.4 ±8.5* **(22.9)**	39312 ± 1300 **(24.0****)	2.08± 0.88	328 ± 73.3 **(1.20)**	161 ±13.2 **(3.01)**	72.9 ± 4.0 **(0.27)**	43.2 ± 0.17 **(+7.73)**
	14	144.2 ±9.1** **(24.0)**	32970± 1205*** **(39.6)**	1.91±0.99*	311 ± 52.1 **(8.25)**	159 ±14.2 **(4.79)**	74.3 ± 2.2 **(0.53)**	43.5 ± 0.13 **(+11.8)**

Table 7: Effect of chloroform fractions of ethanolic extract of the crude powder of *N. rubra* flowers and standard drug metformin on fasting blood glucose, OGTT and serum insulin profile of neonatally STZ treated diabetic rats.

Group	Day	Fasting blood glucose (mg/dl)	OGTT 0-120 min AUC	Serum insulin (µg /l)
Sham treated control (1.0% Gum acacia)	0	136.0 ± 14.0	31989±2123	0.03 ± 0.01
	7	135.7±11.0	34610 ±2334	0.05 ± 0.01
	14	133.2 ±7.9	35520 ±716.3	0.03 ± 0.04
Chloroform fraction treated (100 mg/kg)	0	138.0 ± 11.2	32990±1398	0.04 ±.003
	7	122.2±5.0 **(9.94)**	28571 ±2768* **(17.4)**	0.08 ± 0.01*
	14	108.2 ±4.3 **(18.7)**	25975 ±2669** **(26.8)**	0.11 ±0 .01 **
Metformin treated (100 mg/kg)	0	139.0 ± 13.5	32845±2154	0.03 ±.003
	7	103.7±6.5* **(23.5)**	27050 ±950.1** **(21.8)**	0.10 ± 0.01 **
	14	99.2 ±6.9** **(25.5)**	23865 ±2524** **(32.8)**	0.13 ± 0.03 ***

Values are mean ±S.E. of three independent experiments.

during 0-300 min and 0-1440 min, respectively. Among the three extracts i.e., 95% ethanolic, 50% ethanolic and aqueous prepared from the crude powder of *N.rubra* flowers, only 95% ethanolic extract showed decline in blood glucose level of STZ-induced diabetic rats at the selected dose 250 mg/kg till the end of the experiment whereas such effect was not evident in either aqueous extract or 50 % ethanolic extract treated group. The average antihyperglycemic activity of the 95% ethanolic extract was calculated to be around 20.6 % during the period 0-300 min and 9.95 % during the period 0-1440 min. The antihyperglycemic effect of metformin was also evident from 30 min which persisted till 300 min post treatment. The average antihyperglycemic activity of metformin was calculated to be around 21.0 and 24.7 %, respectively, during the period 0-300 min and 0-1440 min.

Table 3 presents the blood glucose levels of STZ-induced diabetic rats treated with fractions prepared from 95% ethanolic, 50% ethanolic and aqueous extracts, respectively of crude powder of *N.rubra* flowers. It is evident from the results that chloroform as well as aqueous fractions of 95% ethanolic extract showed significant decline in blood glucose levels of STZ-induced diabetic rats. The average antihyperglycemic activity of these fractions were calculated to be around 17.1 and 13.1 % during the period 0-300 min and around 14.1 and 16.3 % during the period 0-1440 min. The aqueous fraction of 50 % ethanolic extract only showed significant decline in blood glucose level of STZ-induced diabetic rats and the antihyperglycemic activity was calculated to be around 24.9 and 23.9 %, respectively during the period 0-300 min and 0-1440 min, respectively. Among the four fractions prepared from aqueous extract of the crude powder of *N.rubra* flowers i.e. hexane, butanol, chloroform and aqueous, the antihyperglycemic activity was evident in hexane and aqueous fraction only. The hexane and aqueous fraction of aqueous extract showed around 29.3 % and 19.3 % fall in blood glucose level of STZ-induced diabetic rats during the period 0-300 min and 29.6 % and 24.3 % fall in blood glucose level during the period 0-1440 min, post treatment at 100 mg/kg dose. The standard antidiabetic drug metformin showed around 25.8 and 33.2 % decline in blood glucose level of STZ-induced diabetic rats during the period 0-300 min and 0-1440 min, post treatment, respectively, at 100 mg/kg dose.

Antidiabetic, antidyslipidemic activities and protective effects of chloroform fraction of ethanolic extract of *N.rubra* flowers and metformin on renal functions of STZ-induced diabetic rats

Table 4 presents the effect of chloroform fraction of ethanolic extract of the crude powder of *N. rubra* flowers and metformin on fasting blood glucose level, improvement in OGTT, and serum insulin levels of STZ-induced diabetic rats. The chloroform fraction of ethanolic extract of the crude powder of *N. rubra* flowers as well as metformin treated showed decline in their fasting blood glucose profile on day 7 as well as 14, post treatment. The decline in fasting blood glucose level was around 17.7 and 28.6 % on day 7 and 14 in chloroform fraction treated group and was around 29.8 and 45.2 % on the said days in metformin treated group. The chloroform fraction treated group showed improvement in their i.e., 27.6% ($p<0.05$) on day 7 and around 37.4% ($p<0.01$) on day 14 whereas metformin showed improvement on OGTT to the tune of around 38.7% ($p<0.01$) on day 7 and 43.8% ($p<0.001$) on day 14th day, respectively, as compared to that of the sham treated control group. The serum insulin levels were also found increased in both chloroform fraction and metformin treated groups on day 14 post treatment where nearly 2 and 3 folds increase was noticed in the respective groups.

Table 4 also presents the effect of chloroform fraction of the ethanolic extract of the crude powder of *N. rubra* flowers and metformin on serum lipid profiles of STZ-induced diabetic rats. The serum TG, cholesterol, as well as LDL-cholesterol levels were found increased and HDL-cholesterol level were found decreased in STZ treated rats at the beginning of the experiment whereas treatment of chloroform fraction and metformin resulted in lowering of serum TG, cholesterol, and LDL-cholesterol levels. The respective lowering in these parameters was observed around 16.7, 39.9 and 4.49 on day 7 and around 16.6, 32.2 and 14.6 on day 14 in chloroform fraction treated group. The metformin treated group showed lowering in these parameters to the tune of around 24.6, 33.8 and 17.7 % on day 7 and around 25.9. 31.9 and 28.6 on day 14. Both chloroform fraction as well as metformin treated group raised serum HDL-chlolesterol levels to the tune of around 53.8 and 54.7 %, respectively day 7 and this effect was not enhanced further when the treatment was continued for 14 days. Table 5 presents the effect of chloroform fraction of ethanolic extract of the crude powder of *N.rubra* flowers and metformin on urea, uric acid and creatinine levels in serum of STZ-induced diabetic rats. Both chloroform fraction as well as metformin lowered down the levels of urea, uric acid and creatinine levels in serum; the maximum effect was achieved on day 7 in each of the case. Chloroform fraction treated group showed decline in the levels of the said to around 15.5, 25.2 and 16.3, respectively on day 7 whereas the decline in the levels of these were observed nearly 16.8, 26.8 and 17.2 %, respectively on day 14, post treatment. Almost similar profile was observed in each case on day 14 post treatment.

Effect of chloroform fraction of ethanolic extract of crude powder of *N. rubra* flowers and metformin on fasting blood glucose level, improvement in oral glucose tolerance and serum lipid profiles of high-fructose high-fat fed low dosed STZ- induced diabetic rats

Table 6 depicts the effect of chloroform fraction as well as metformin on fasting blood glucose level, oral glucose tolerance and serum insulin levels of high fructose high fat fed–low dosed STZ-induced diabetic rats. There was not found any significant decline in blood glucose level of chloroform fraction treated group but this group showed

improvement in their glucose tolerance on day 7th, and 14th day, respectively. However, Metformin treated group showed both decline in fasting blood glucose level as well as improvement in oral glucose tolerance. When the area under the curve (AUC) of glucose tolerance was compared, chloroform fraction of ethanolic extract of *N. rubra* treated groups showed around 21.0% ($p<0.05$) and 27.5% ($p<0.01$) improvement on day 7th and 14th where as metformin showed around 24.0% ($p<0.01$) and 39.6 % ($p<0.001$) improvement on day 7th, and 14th, respectively. Both chloroform fraction and metformin treated groups showed decline in serum insulin level. High fructose high fat fed diet and low dosed Streptozotocin-induced diabetic rats showed elevated serum cholesterol, triglycerides and LDL-C levels and lowered HDL-cholesterol levels (Table 6). Treatment with chloroform fraction declined their serum triglycerides, LDL and cholesterol levels by around 25.3% ($p <0.01$), 15.6 ($p<.05$) and 22.5% ($p<0.05$), respectively, and increased the level of HDL- cholesterol to around 25.8% ($p<0.05$) on day 14th, post treatment respectively. Metformin treated group did not demonstrate any such effect (Table 6).

Assessment of improvement in glucose tolerance and serum insulin profiles by the effect of chloroform fraction of ethanolic extract of *N. rubra* on neonatally-STZ induced diabetic rats

Neonatally-STZ-induced diabetic rats showed abnormal glucose tolerance after 8 weeks and this was the time when these were taken for evaluating the effect of chloroform fraction of the ethanolic extract of *N. rubra* flowers. Table 7 presents the effect of administration of chloroform Fraction of ethanolic Extract of *N. rubra* and Metformin at 100mg/kg doses on n-STZ induced diabetic rats. Chloroform fraction of ethanolic extract of *N.rubra* treated rats showed significant decline in their blood glucose profile on day 7 and 14. The decline in fasting blood glucose levels were calculated to be around 9.94 ($p<0.05$) and 18.7% ($p<0.01$), respectively on 7 and 14 day while such effect was more in the metformin treated group where nearly 23.5% ($p<0.05$) and 25.5 % ($p<0.01$) lowering was observed on day 7 and 14, Both chloroform fraction and metformin treated groups also showed improvement in their oral glucose tolerance to the tune 17.4 and 21.8 and 26.8 and 32.8 %, respectively on day 7 and 14. The serum insulin levels of chloroform fraction as well as metformin treated groups were also found higher than the sham treated control group on day 7 and 14, post treatment, respectively.

Effect on 2-deoxy-3^H glucose uptake by L6 muscle cells

Chloroform fractions of the ethanolic extract of crude powder of *N. rubra* flowers increased both basal as well insulin-stimulated 2-deoxy-^{3}H -glucose uptake by L6 myotubes in a dose dependent manner. Chloroform fractions of the ethanolic extract of crude powder of *N. rubra* increase basal glucose uptake in L6 myotubes to a significant level at a minimum concentration of 2.5 µg (1.23-fold, $p<0.05$). Maximum stimulation was observed at 10 µg (1.49-fold, $p<0.01$). Effect of Chloroform fractions of the ethanolic extract of *N. rubra* on insulin-induced increase in glucose uptake was also observed. Insulin alone enhanced a significant increase in glucose uptake (1.56-fold, $P<0.01$). Pre-incubation of myotubes with different concentration of Chloroform fractions of the ethanolic extract of *N. rubra* for 24 h with insulin (100 nM) added for final 20 min, resulted in dose-dependent increase of insulin response in an additive manner (1.71-fold, p<0. 01, 1.72-fold, $p<0.01$ and 1.82-fold, $p<0.01$ at 2.5, 5.0 and 10 µg concentration, respectively vs. control, 1.56-fold) (Figure 1a).

Effect on adipocyte differentiation

The anti-adipogenic potential of chloroform fraction of the ethanolic extract of crude powders of *N. rubra* flowers was assessed by determining pre-adipocyte differentiation into adipocyte. 3T3-L1 pre-adipocytes were treated with differentiation media in the presence of chloroform fraction of the ethanolic extract of crude powders of *N. rubra* flowers and rosiglitazone (as a positive control). At 9 days after the initiation of differentiation, accumulated lipid droplets were detected by staining with Oil Red O. As shown in Fig.1b1, treatment of 3T3-L1 cells with chloroform fraction of the ethanolic extract markedly inhibited adipocyte differentiation. The OD value of the Oil Red O eluted solution significantly decrease by 17.3 % ($p<0.01$) in 3T3-L1 preadipocytes treated with 10 µg/ml concentration of chloroform fraction of the ethanolic extract of crude powders of *N. rubra* flowers (Fig.1b2). Rosiglitazone at a concentration of 10µM significantly promoted adipocyte differentiation by 26.8% ($p<0.001$) in 3T3L1 adipocyte. These results indicated that chloroform fraction of the ethanolic extract of crude powders of *N. rubra* flowers may have been efficiently blocking adipocyte differentiation and have potential of anti-obesity effects in 3T3-L1 cells.

Effect on m-RNA level of diabetes related genes in rat skeletal muscle cells (L-6)

There was found significantly different genes expression between chloroform fraction of ethanolic extract of crude powder of *N. rubra* flowers and control group (Fig-4), Studies towards expression of genes involved in insulin signaling pathway i.e. insulin receptor substrate (IRS-1), thymoma viral proto-oncogene 2 (AKT2), phosphatidlyinositol-3 kinase catalytic subunit (PIK3CA) were markedly up regulated by 1.56, 1.27 and 2.63 folds, respectively, in the chloroform fraction treated cells. The mitogen-activated protein kinase (MAPK) which is involved in the MAPK pathway, was also found up regulated by 2.58-fold by treatment with chloroform fraction. The glucose transporter 4 (GLUT4) gene expressions, which is involved in glucose transport in membranes, was found up regulated by 1.88-fold in the chloroform fraction treated group. Furthermore, expression of hepatic nuclear factor 4α (HNF-4α) gene, a key regulator of glucose, cholesterol, and fatty acid metabolism, was found up-regulated by 1.69 fold in

chloroform fraction treated group, Gene expression of Prkaa-1, was found up regulated by 1.80-fold and Cbl was found up regulated by 2.51-fold in the chloroform fraction treated cells(Fig-1c).

Western blot analysis

Glucose uptake can be mediated by the insulin signalling pathway, which can stimulate the translocation of glucose transporter 4 (GLUT4)-containing vesicles to the plasma membrane. Subsequently, GLUT4 transports glucose across the plasma membrane into cytoplasm. To investigate the mechanistic aspects of the antidiabetic action of chloroform fraction of the ethanolic extract of the crude powder of *N. rubra* flowers the expression of genes involved in insulin signalling pathway were studied by western blot analysis. As shown in the Fig-1d chloroform fraction of ethanolic extract of the crude powder of *N. rubra* flowers, the proteins expression profile of IRS-1 and GLUT-4 was found up regulated. Insulin induces activation of Insulin Receptor Tyrosine Kinase (IRTK) through autophosphorylation and this leads to employment of Insulin Receptor Substrate (IRS) proteins, followed by activation of phosphatidyl inositol 3 kinase (PI3K) and subsequent translocation of glucose transporter-4 (GLUT-4) and glucose uptake in muscle. PI3-kinase plays a critical role in insulin-stimulated glucose uptake. In an analysis whether PI3K enzyme participates in the stimulation of glucose uptake by chloroform fraction of ethanolic extract of crude powder of *N. rubra* flowers, elevated PI3 kinase expression similar to insulin on treatment with chloroform fraction. Insulin was used as a negative control. Chloroform fraction of ethanolic extract of crude powder of *N. rubra* flowers were found to up regulate the expression of IRS-1 and GLUT-4 in the treated muscle cells at both mRNA and protein levels.

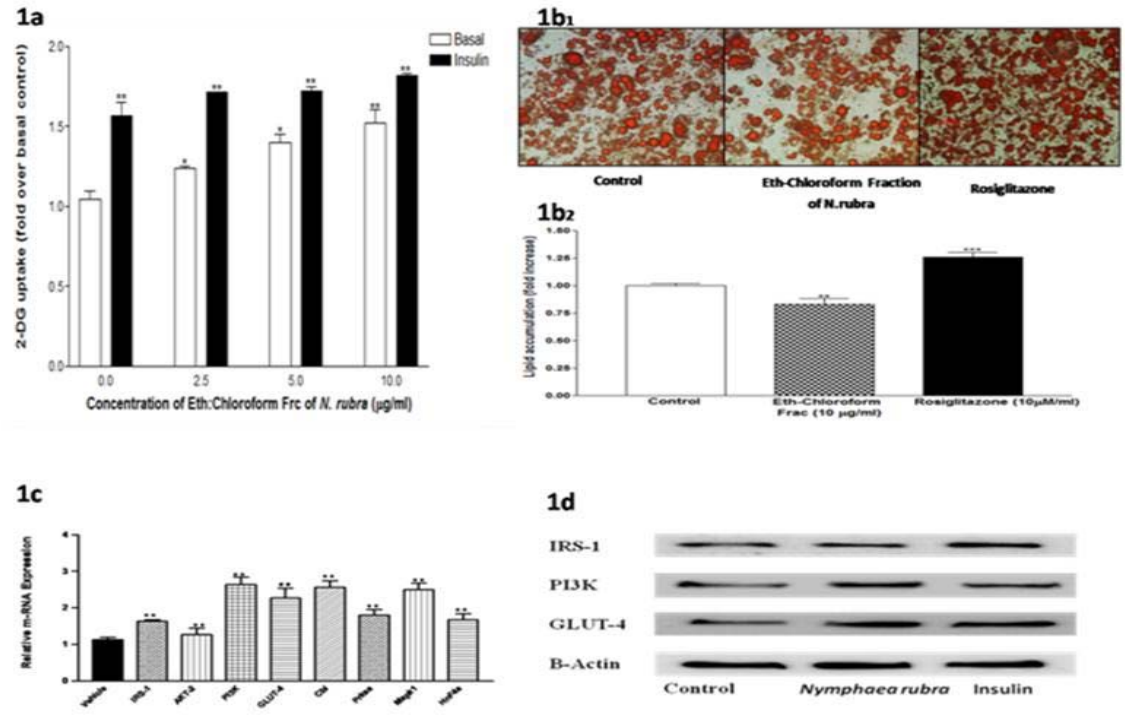

Figure 1: Effect of Chloroform fraction of Ethanolic extract of *N. rubra* on Insulin signaling pathway of glucose uptake in L6 myotubes and adipogenesis in 3T3-L1 pre-adipocytes. 1a: Glucose uptake by L6 skeletal muscle cells, 1b: Adipocytes differentiation in 3T3-L1 pre adipocytes, 1c:Expression of selected genes by treatment of chloroform fraction of N-rutra skeletal muscle cells, 1d:Western blot analysis on gene expression in L6 muscle cell lines.

DISCUSSION

The treatment goal for diabetes mellitus is to prevent or reduce the risk and severity of complications associated with it. This target is only achieved by maintaining normal or near normal blood glucose, lipid levels and renal functions.[21,22] With a disturbing rise in the prevalence of this metabolic disease and associated healthcare costs, interest in alternative or complementary therapies has grown. Plant medicines have a long history as treatment for diabetes mellitus. With this aim the present study was performed to find out the active anti-hyperglycemic and hypolipidemic fraction isolated from the active extract of flowers of *N. rubra*. Results of the present study revealed that chloroform fraction of ethanolic extract of *N. rubra* showed significant improvement in the blood glucose profile of normoglycemic rats to validated animal models of type 2 diabetes mellitus and increase in insulin-stimulated glucose uptake by skeletal muscle cells L-6 cell lines in a dose-dependent manner and inhibited adipogenesis in adipocyte cells (3T3L1) and increased GLUT-4 translocation by insulin receptor mediated tyrosine phosphorylation of insulin receptor substrate (IRS-1) proteins and subsequent activation of PI-3- kinase.[23, 24]

In the present study, the experimental conditions (normoglycemic and STZ-induced diabetes mellitus) were selected with the aim to find out the active fractions have an insulin-like rapid onset of action to the moderate to severe diabetic state. In sucrose loaded normal rat's insulin production triggered from pancreatic β cells due to excessive amount of glucose. This secreted insulin will stimulate peripheral glucose consumption and controls the production of glucose through different mechanisms. However, from the study (glucose control) it was clear that the secreted insulin requires 2-3 h to bring back the glucose level to normal. In the case of chloroform fraction of ethanolic extract of *N. rubra,* in glucose utilization were tested for anti hyperglycemic activity found effective with significant ($p<0.01$) lowering in blood glucose level as evidenced from the fact that it inhibits rise in postprandial hyperglycemia at a dose level of 100 mg/kg and this effect may be due to the increased insulin release resembling the mechanism of actions of sulphonylureas.[25,26]

Streptozotocin a cytotoxic agent caused destruction of pancreatic cell by necrosis[27] leaving less functional cells results in Type I Diabetes mellitus and also produces hyperglycemia.[28] It is the confirmatory animal model for the screening of antidiabetic compounds for type 1 and 2 diabetes mellitus. Single dose treatment of ethanolic extract and chloroform fraction of ethanolic extract of *N. rubra* to STZ induced diabetic rats significantly improve the glucose tolerance comparable to that of standard antidiabetic drug metformin. The probable mechanism behind the anti hyperglycemic activity is that the fractions could directly or indirectly act as insulin secretaceous, mimetic, sensitizer and by other mechanisms like α-glucosidase inhibition etc.

The antihyperglycemic and anti dyslipidemic activity of the chloroform fraction of the ethanolic extract was confirmed by multiple dose treatment of this fraction from *N. rubra* flowers on STZ induced diabetic rats. It was found to improve glucose tolerance significantly as these plant fractions were found insulin secreatogougs in nature i.e. markedly elevates the serum insulin level of diabetic rats. Hyperglyceamia also induces the elevation of plasma levels of urea, uric acid and creatinine, which are considered as the significant markers of renal dysfunction.[29,30] But treatment of chloroform fraction of ethanolic extract were significantly ($P<0.05$) decreased.

High fructose-high fat diet followed by low dose STZ injection was used to develop obese-diabetic rats that mimic human diabetes in terms of obesity and impaired insulin sensitivity which plays a pivotal role in the development of diabetes.[31,32] In high fructose-high fat diet, fructose is mostly responsible for induction of dyslipidemia as it bypasses the main regulatory steps causing continuous production of PGA and Acetyl CoA resulting in hyper triglyceridemia and hyper cholesterolemia.[33] On the other hand fat also plays an important role in the development of obesity and insulin resistance. The levels of serum lipids are usually elevated in diabetes mellitus. This abnormal high level of serum lipids is mainly due to the uninhibited actions of lipolytic hormones on the fat depots mainly due to the action of insulin. In diabetic state lipoprotein lipase is not activated due to insulin deficiency resulting in hypertriglyceridaemia.[34] The findings in the present article reveals that administration of chloroform fraction of ethanolic flower extract of *N.rubra* to high-fructose high fat fed rats, significantly reversed the hyperglycemia, hyperinsulinemia and it also improves the serum lipid profile declining the levels of triglycerides, total cholesterol, LDL-cholesterol along with increasing the level of HDL-cholesterol. In our study the maximum activity was found in chloroform fraction of ethanolic extract may be due to presence of higher amount of main classes of active constituents in the chloroform fraction of ethanolic extract of *N. rubra* while the metformin 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 rats. The treatment of metformin did not increase the level of HDL-C besides mildly altering its level in the serum at same dose levels.

The n-STZ treated rats are suitable model of type II diabetes as it exhibiting the various stages of Type 2 diabetes mellitus such as impaired glucose tolerance, mild, moderate and severe hyperglycemia with alteration of dose and days of STZ injection. The n-STZ rats exhibit slightly lowered plasma insulin, slightly elevated plasma glucose levels and lowered pancreatic insulin content.[35] The b cells in the n-STZ rats bear a resemblance to the insulin secretory characteristics found in Type 2 diabetic patients.[36] After 14 days of treatment with chloroform fraction of *N.rubra* improved insulin level and glycemic control.

The current study also reveals the glucose transporting efficacy of chloroform fraction of ethanolic extract of *N. rubra* and demonstrates the molecular mechanism involved in mediating glucose metabolism through insulin dependent pathway in the *in vitro* model. Insulin resistance in type 2 diabetes is manifested by decreased insulin -stimulated glucose uptake and metabolism in skeletal muscle cells.[37] Glucose transport is the rate-limiting step in glucose utilization in insulin targeted skeletal muscle. This transport is mediated by the major glucose transporter proteins in skeletal muscle.[38-40] L6 muscle cell line is a well-established in vitro model to study the regulation of glucose transport and it is the major site for primary glucose disposal and glucose utilization.[41] Chloroform fractions strongly stimulate the basal glucose uptake in L6 cells in concentration-dependent manner. In addition, the effect of chloroform fraction of ethanolic extract of *N. rubra* on adipogenesis was assessed in 3T3L1 adipocytes. Adipogenesis is the process of preadipocyte differentiation into adipocytes is controlled by various positive and negative regulators such as hormones, adipogenic genes, adipokines and growth factors.[42] 3T3L1 adipocytes incubated chloroform

fraction of ethanolic extract of *N. rubra* clearly revealed a significant decrease in lipid droplets formations. Taken together these findings suggest that chloroform fraction of ethanolic flower extract of *N.rubra* exhibit glucose-lowering efficacy without inducing adipogenesis in 3T3L1 preadipocytes.

Insulin resistance is a characteristic feature of type 2 diabetes and other pathophysiological states in humans; therefore amelioration of insulin sensitivity is an important therapeutic goal. Furthermore, insulin regulates glucose transport by activating insulin receptor substrate-1 (IRS-1)-dependent PI3K which activates AKT and the downstream proteins, such as GLUT4, a key role of the rate-limiting step in glucose metabolism. Insulin signalling is a cascade of events initiated when insulin binds to its surface receptor and it then auto phosphorylate, leading to activate tyrosine phosphorylation of IRS.[43] The binding of the regulatory subunit of (PI3K) to IRSs activates PI3K and AKT, which are also called protein kinase B (PKB). The activation of this process is necessary for insulin to regulate glucose transporter 4 (GLUT4) protein translocation from the cytosol to the membrane for glucose transportation in skeletal muscle.[44] Our study clearly indicates that chloroform fraction of the ethanolic extract of *N. rubra* flowers showed significant improvement in the expression of IRS-1, Glut-4 and PI-3K which were associated with improvements in glucose transport and insulin signalling pathways and this may explain the effects of the fraction in improving insulin resistance.[45-47] The up-regulated protein expression of IRS-1, PI-3K and GLUT-4 in skeletal muscle, by chloroform fraction suggested that this might exert its insulin sensitizing function by enhancing signal transduction of insulin pathway. The present study also disclosed that chloroform fraction of ethanolic extract of *N. rubra* upregulated the expression of MAPK (mitogen-activated protein kinase) pathway and GLUT4 at mRNA level.

In summary, our studies clearly provide evidence that chloroform fraction of ethanolic extract of N.rubra has both anti hyperglycemic as well as dyslipidemic activities and warrants further work.

CONCLUSION

The chloroform fraction of ethanolic extract of *N.rubra* flowers was found to have the enormous ability to lower down the blood glucose and normalizing insulin level, has strong anti dyslipidemic activity in the validated animal models of type 2 diabetes mellitus. It also showed insulin resistance reversal activity and therefore, may be considered as candidate plant for the identification of antidiabetic molecules for the management of type 2 diabetes mellitus.

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