Chalcone-based aryloxypropanolamine as a potential antidiabetic and antidyslipidaemic agent

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The hybrid congener 3 derived from hydroxychalcone and pharmacophore oxypropanolamine for adrenergic receptor, along with its enantiomers 9a and 9b were selected from a series of compounds for detailed studies of their antidiabetic profile in sucrose-challenged, low-dosed, streptozotocin-induced diabetic rats and in db/db mice, and antidyslipidaemic profile in high fat diet-induced dyslipidaemic hamsters. The test compounds exhibited significant and consistent antidiabetic and antidyslipidaemic activities in the above models. The pharmacodynamic studies of two metabolites, 10 and 11, were undertaken. Metabolite 10 having greater bioavailability in plasma was synthesized and found to exhibit significant antidiabetic activity. The parent compound together with its active metabolites exhibited significant oral bioavailability, thus establishing compound 3 as a potential lead molecule for further studies.

Keywords: Antidiabetic and antidyslipidaemic activity, chalcone, diabetes mellitus, metabolites, rodents.

DIABETES mellitus is a disease of abnormal glucose metabolism. This results in hyperglycaemia due to inherent deficiency of insulin secretion and its resistance, leading to type-2 diabetes mellitus (T2DM) that afflicts ~90% of the diabetic population. T2DM is characterized by chronic hyperglycaemia, absence of insulin response to nutrient ingestion and its insensitivity in fat and muscle cells. Due to defect in insulin tolerance in T2DM, regulation of metabolic pathways is altered, including excess glycogen accumulation in the liver[1], initiating liver disease[2] and cardiac hypertrophy[3]. Among diabetic complications, hepatic fat accumulation is a well-recognized phenomenon in which fat is stored in the form of triglycerides through increased transportation to the liver, enhanced hepatic synthesis and decreased oxidation or removal from the liver leading to steatosis of micro- or macrovesicular organs, which eventually causes fibrosis and cirrhosis[4]. Current treatment regimens are unsatisfactory and less successful in managing the disease. New therapies are therefore needed targeting metabolic pathways that minimize weight gain and attenuate dyslipidaemia with control over glycaemia. The present treatment regimens are intended to restore the function of β-cells by either enhancing insulin secretion through the involvement of potassium channel or sensitization of resistant insulin by activation of nuclear receptors[5]. Hence, we designed the compounds to positively regulate β_3-adrenergic receptors for their agonistic activity. It was noted in late 1990s that among people working under extremely cold conditions, undifferentiated brown adipose tissue cells are interspersed among white adipose tissues which generate heat to maintain homeostatic energy levels, ensuring their survival[6]. The above tissues are under direct control of the sympathetic nervous system and can be stimulated by β_3-adrenergic receptor agonists. The stimulation of the receptor mediates lipolysis to initiate the thermogenic process devoid of ATP synthesis, increase oxygen consumption, decrease glucose and food intake[7]. The dual actions of antiobesity and antidiabetic propensities make this target an attractive option for the development of drugs.

Since the discovery of thermogenic properties in β_3-adrenergic agonists, sincere efforts are being made and analogs of both the arylethanolamine and aryloxypropanolamine classes have been discovered[8–10]. These agents, however, were unsuccessful in clinical settings because of their unfavourable pharmacokinetic property[11]. We have also initiated a programme to design compounds with better lipophilic character in order to enhance bioavailability based on aromatic moieties present in the fruits and vegetables, so that their metabolites prove

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much safer. Flavonoids and chalcones are the most ubiquitous polyphenolic compounds in foods of plant origin exhibiting biological activities through free-radical scavenging mechanism[12]. The above properties led us to utilize these ring systems for the synthesis of their hybrids with oxypropanolamines and screen them for their antidiabetic and antidyslipidaemic profiles. In this series we have earlier reported flavone- and chalcone-based oxypropanolamines as antidiabetic and antidyslipidaemic agents[13–15]. The encouraging biological profiles of flavone-based compounds led us to extend our study towards chalcone hybrids, earlier reported by us. Details of the biological profile of the most active compound **3** (S001-469) of earlier studies and its enantiomers (**9a** and **9b**) are reported here (Figure 1).

Materials and methods

Structural analysis

^{1}H NMR spectra were recorded at 300 MHz using Bruker Avance DRX-300 spectrometer. Chemical shifts (δ) are given in parts per million (ppm) and coupling constants (J) in hertz. Electro-spray ionized (ESI) mass spectrum (MS) was carried out using Micromass Quatro II mass spectrometer (Waters). The purity of the compounds was observed by HPLC on Chira Sphere NT (250 mm × 0.4 mm, 5 µm, Merck) using 2-propanol/hexane/*n*-butylamine (in the ratio 75 : 25 : 0.1%).

(*R*, *E*)-4′-[3-*tert*-butylamino-2-hydroxy-propoxy]-3, 4-methylenedioxy-chalcone (**9a**): Compound **6** (2.0 g, 7.4 mmol) obtained by condensation of 4-hydroxyacetophenone (**4**) with piperonal (**5**) and S-(+)-epichlorohydrin (3.0 ml, 38 mmol) in a round-bottom flask was heated at ~100–20°C for 7–8 h. On completion of the reaction (followed by TLC, 4% MeOH in $CHCl_3$), the residual epichlorohydrin was removed under high vaccum and the product purified on silica-gel using ($CHCl_3$/MeOH). The compound **7a** so obtained was cyclized on stirring with aq. NaOH and PTC (triethylbenzylammonium chloride, ~1%) in toluene for 6–7 h. On completion of the reaction, organic layer was separated and aqueous layer was again extracted with $CHCl_3$. The combined layer was dried (Na_2SO_4) and the solvent removed. The compound **8a** so obtained was treated with *tert*-butyl amine in methanol for 8–10 h. The solvent and reagent were removed and the product purified with column chromatography to get the desired product **9a**. Yield: 34%, m.p.: 100–103°C; MS (ESI): 398 (M + 1); IR (KBr): 1598, 2919 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.04 (d, J = 8.79Hz, 2H, 2′, 6′-H); 7.75 (d, J = 15.51 Hz, 1H, β-H); 7.40 (d, J = 15.48 Hz, 1H, α-H); 7.19 (s, 1H, 2-H); 7.14 (d, J = 8.07 Hz, 6-H); 7.02 (d, J = 8.82 Hz, 2H, 3′, 5′-H); 6.86 (d, J = 8.01 Hz, 1H, 5-H), 6.05 (s, 2H, CH_2); 4.09 (d, J = 3.21 Hz, 2H, OCH_2); 4.07 (m, 1H, CHOH); 2.88 (d, 1H, NCH); 2.71 (d, 1H, NCH); 2.19 (s, 1H, OH); 1.15 (s, 9H, CH_3). $[\alpha]_D$: +11.34 ($CHCl_3$, c = 0.10%).

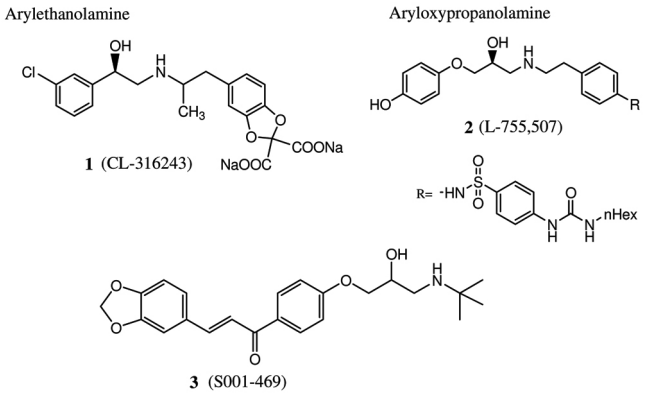


Figure 1. Some lead molecules from the literature and the present study.

*(S, E)-4′-[3-tert-butylamino-2-hydroxy-propoxy]-3, 4-methylenedioxy-chalcone (**9b**):* The above procedure was adopted for the synthesis of compound **9b** using similar amount of reagents and *R*-(–)-epichlorohydrin. The product **9b** was also purified using column chromatography. Yield: 41%; m. p.: 100–103°C; MS (ESI): 398 (M + 1); IR (KBr): 1597, 2970 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.02 (d, J = 8.85Hz, 2H, 2′, 6′-H); 7.73 (d, J = 15.48 Hz, 1H, β-H); 7.38 (d, J = 15.51 Hz, 1H, α-H); 7.17 (d, J = 1.38Hz, 1H, 2-H); 7.13 (d, J = 8.04 Hz, 6-H); 7.00 (d, J = 8.82 Hz, 2H, 3′, 5′-H); 6.84 (d, J = 7.98 Hz, 1H, 5-H); 6.03 (s, 2H, CH_2); 4.08–4.06 (m, 3H, OC$\underline{H_2}$C$\underline{H}$); 2.88 (d, 1H, NCH); 2.72 (d, 1H, NCH); 2.14 (s, 1H, OH); 1.25 (s, 9H, 3CH_3). $[\alpha]_D$: –14.08 ($CHCl_3$, c = 0.10%).

*3-Benzo-(1, 3)-dioxol-5-yl-1-[4-(3-tert-butylamino-2-hydroxy-propoxy)-phenyl]-propan-1-one (**10**):* To a solution of compound **9** (1.0 g, 2.51 mmol) in methanol was added 10% Pd-C (0.1 g) and hydrogenated in Parr-hydrogenation assembly at 40 lb hydrogen pressure. On completion of the reaction, catalyst was filtered and solvent removed under vacuum to get compound **10**, which was purified over silica-gel column. Yield: 34%; m.p.: 105°C; MS (ESI): 400 (M + 1); IR (KBr): 1618, 2919 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.93 (d, J = 8.80 Hz, 2H, 2′, 6′-H); 6.95 (d, J = 8.75 Hz, 2H, 3′, 5′-H); 6.75–6.71 (m, 2, 5, 6-H), 5.93 (s, 2H, CH_2); 4.22 (m, 1H, C$\underline{H}$OH); 4.10–4.04 (m, 2H, OCH_2), 3.47 (s, 1H, OH), 3.20 (t, 2H, CH_2); 3.06–2.96 (m, 3H, C$\underline{H_2}$, NC$\underline{H}$); 2.85 (d, 1H, NC$\underline{H}$); 1.28 (s, 9H, CH_3).

In vivo antihyperglycaemic and antidyslipidaemic activity evaluation

Inhibitory effect on the rise of postprandial hyperglycaemia post sucrose load in normal rats: Male albino Sprague Dawley (SD) rats weighing 140 ± 20 g procured

from the National Laboratory Animal Centre (NLAC) of the Central Drug Research Institute, were grouped and acclimatized for 3–4 days in polypropylene cages. The animals were starved overnight and fasting blood glucose (FBG) level was determined using a glucometer (Roche, Germany). Animals showing blood glucose levels between 3.33 and 4.44 mM were selected and divided into groups, each consisting of six animals. Rats of experimental groups were orally treated with standard drugs, i.e. glybenclamide, metformin, acarbose, galvus or test samples at indicated doses. Animals of control group received an equal volume of 1.0% gum acacia. A sucrose load (10.0 g/kg) was given to each animal orally after 30 min post administration of the test sample/vehicle. Blood glucose profile of each rat was determined just prior to the administration of test sample (–30 min), sucrose (0 min) and thereafter at 30, 60, 90 and 120 min post administration of sucrose by the glucometer. Food but not water was withdrawn from the cages during the course of experimentation[16]. Quantitative inhibition on the rise of postprandial hyperglycaemia post sucrose load by the test sample was calculated by plotting duration and blood glucose level on the *x*- and *y*-axis respectively. The area under curve (AUC) of each group was determined using Prism software. The percentage lowering in AUC of standard drugs/test sample treated groups was compared to control group to determine the percentage inhibition on the rise in postprandial hyperglycaemia post sucrose load.

Antihyperglycaemic activity in sucrose-challenged, low dose streptozotocin-induced diabetic rats: Male albino SD rats weighing 140 ± 20 g were used in the study. Streptozotocin (STZ; Sigma, USA) was dissolved in 100 mM citrate buffer (pH 4.5) and a calculated amount of fresh solution of STZ was injected to overnight fasted rats at 45 mg/kg intraperitoneally. FBG level of each animal was checked 48 h later using the glucometer. Animals showing blood glucose levels between 8.0 and 12 mM were finally included in the experiments and named as STZ-induced diabetic animals. These diabetic animals were divided into groups, each consisting of six animals. Rats of experimental groups were orally given standard antidiabetic drugs: metformin, glybenclamide, galvus, januvia or test compounds at indicated doses. Animals of control group were given an equal amount of 1.0% gum acacia. A sucrose load of 2.5 g/kg body wt was given to each rat 30 min after administration of the test sample/standard drugs/vehicle. The blood glucose profile of each animal was monitored just before administration of the test sample/standard drug/vehicle (–30 min), sucrose load (0 min), and thereafter at 0.5, 1, 1.5, 2, 3, 4, 5 and 24 h respectively. Food but not water was withdrawn from the cages during the experimentation. The blood glucose profile was plotted against time and AUC of each group determined. The percentage lowering in AUC of experimental/standard drug-treated groups was compared to control group to determine the per cent inhibition on the rise in postprandial hyperglycaemia post sucrose load.

Blood glucose lowering effect in streptozotocin-induced diabetic rats: Diabetes was induced in 12–16 week male albino rats (SD strain) by injecting streptozotocin intraperitoneally, as described earlier. Animals showing FBS over 270 mg/dl on day-3 post streptozotocin injection were finally included in the experiment. The animals were divided into groups consisting of six animals each. Animals of the experimental groups were treated with glybenclamide, metformin, galvus, januvia or compounds at indicated doses. Animals of the control group received an equal amount of 1.0% gum acacia. The blood glucose level of each animal was determined just before administration of the standard drugs (0 min) and thereafter at 0.5, 1, 1.5, 2, 3, 4, 5 and 24 h (ref. 17). Food but not water was withdrawn from the cages during 0–5 h. The average lowering in blood glucose level between 0 and 5 h as well as 0 to 24 h was calculated by plotting blood glucose level on the *y*-axis and time on the *x*-axis, to determine AUC. Comparing the AUC of experimental group with that of control group provided the per cent lowering of blood glucose level during the above periods.

Antihyperglycaemic activity evaluation in db/db mice: C57BL/KsBom-db/db male mice (12–18 weeks old) weighing ~35–40 g bred in the NLAC were used in the present study. The mice were housed in a room controlled for temperature (23 ± 2.0°C) and 12/12 h light/dark cycle for 3–4 days before start of the experiment. The body weight and food intake of the animals were measured everyday, from day-1 to day-15 of the experiment. All animals had free access to freshwater and pellet diet, except on the desired days before the oral glucose tolerance test (OGTT) was done on day-10 and day-15 respectively. Random blood glucose profile of each animal was checked every morning till day-17. The standard antidiabetic drug pioglitazone and test samples of compounds **3**, **9a**, **9b** and **10** were given to the experimental groups at indicated doses. An equal amount of vehicle (1.0% gum acacia) was given to the control group and named as treated and sham-treated control group respectively. On day-10 and day-15, an OGTT of each animal was performed as follows. Blood glucose of each animal was measured at –30.0 min and then standard antidiabetic drug and test samples were administered to the respective groups. The blood glucose level of each animal was again measured at 0 min post-treatment, and at this juncture glucose solution was given at a dose of 3.0 g/kg per-oss to all the groups, including the control group. The blood glucose levels were again checked at 30, 60, 90 and 120 min post glucose load[18]. Finally on day-17, blood was collected from the retro-orbital plexus for serum

insulin and lipid profile (triglycerides (TG), total cholesterol (TC) and HDL-cholesterol) analysis on Cobas Integra 400 Analyzer using proper kits according to the instructions of the manufacturer.

Antidyslipidaemic activity evaluation in high fat diet-fed Syrian golden hamsters: Male Syrian golden hamsters weighing ~100 g as procured from NLAC were acclimatized for 3–4 days. Dyslipidaemia was induced in these animals by feeding a high fat diet (HFD) for 7–15 days. Dyslipidaemic hamsters were divided into five groups based on their serum lipid profile. Each group consisted of six animals. The dyslipidaemic animals had free access to HFD and water throughout the experimental period. The treated groups received a fine suspension of compounds **3**, **9a**, **9b** or **10** that were fed orally at a dose of 30 mg/kg for seven consecutive days. Control animals were given the vehicle, i.e. 1.0% gum acacia only, and served as sham-treated control. The body weight and food intake of each animal were recorded everyday to check the effect of the test samples on them[19]. At the end of the experiment, i.e. on day-8, blood from retro-orbital plexus of each animal was withdrawn into tubes and kept for 15 min to separate serum. Biochemical analysis of serum was performed on the same day for TG, TC, HDL-cholesterol, LDL-cholesterol, glycerol and non-esterified fatty acids (NEFA) content using enzymatic diagnostic kits (Roche).

Skeletal muscle cell culture and glucose uptake studies

Wild-type rat L6 skeletal muscle cells which express rat GLUT4 with a *myc* epitope (L6-GLUT4*myc*) were obtained as a kind gift from Amira Klip (Program in Cell Biology at Hospital for Sick Children, Toronto, Canada). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Foetal Bovine Serum (FBS) and 1% antibiotic/antimycotic solution in a humidified atmosphere of air and 5% CO_2 at 37°C. They were allowed to differentiate into myotubes stage in DMEM with 2% FBS and differentiated myotubes were used for experimentation.

For determination of glucose uptake, differentiated myotubes were treated with test compounds for 16 h and glucose uptake was assessed for 5 min in HEPES-buffered saline containing 10 μM 2-deoxyglucose (0.5 μCi/ml, 2-[^{3}H] deoxy-glucose) at room temperature. Subsequently, cells were washed with an ice-cold saline solution. To quantify the radioactivity incorporated, the cells were lysed with NaOH and lysates were counted with scintillation fluid in a β-counter. Nonspecific uptake was determined in the presence of cytochalasin B during the assay, and this value were subtracted from all other values. Glucose uptake measured in triplicate and normalized to total protein, was expressed as fold induction with respect to control cells.

Method used for pharmacokinetic studies

Intra-day and inter-day accuracy and precision for compounds **3, 9a** and **9b** were assessed at low, medium and high concentrations to validate the developed LCMS/MS method for their quantification. The bio-analytical method is accurate, as the bias is within the acceptance limits of ±20% of the theoretical value at the lower limit of quantification and ±15% at all other concentration levels. The precision around the mean value never exceeded 15% at any of the concentrations studied. The results showed that the bio-analytical method for analytes is accurate and precise over the concentration range 0.78–400 ng/ml. Recovery calculated from the spiked plasma samples was more than 93%. The method was applied for quantitization of analytes in different stability and pharmacokinetic samples of male SD rats.

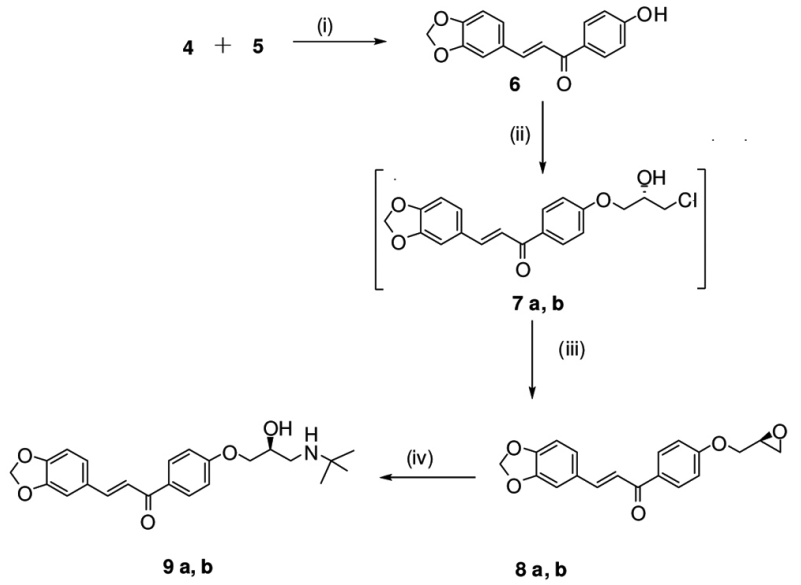
Statistical analysis

Values are given as mean ±SEM. Analysis of statistical significance of differences in measurements between samples was done by one-way ANOVA with Dunnets post hoc test (Graph Pad Prism version 3). Quantitative glucose tolerance of each animal was calculated by AUC method. $P < 0.05$ was considered statistically significant.

Results and discussion

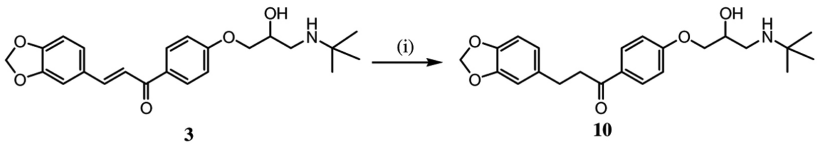
*Synthesis of compound **3** and its enantiomers*

The synthesis and antihyperglycaemic activity of a series of racemic compounds have been reported earlier by us. The most active compound **3** exhibiting consistent results in repeat tests was selected for detailed biological studies[13,14]. The biological studies of compound **3** and its enantiomers are presented here. The enantiomers of compound **3** have been obtained by asymmetric synthesis with optically pure epichlorohydrins following the protocol used earlier for atenolol, a β-blocker drug[16]. In the method, hydroxyl-chalcone (**6**) obtained by the condensation of 4-hydroxy-acetophenone (**4**) and piperonal (**5**) was heated at ~100–120°C with either *R*- or *S*-epichlorohydrin to give chlorohydrin intermediates **7a** or **7b** (Scheme 1), which were then cyclized *in situ* under basic conditions (aq. NaOH and PTC in toluene) to yield epoxy-chalcones with inversion of stereochemistry, i.e. *S*-epichlorohydrin yielded *R*-epoxy-chalcone (**8a**), while *R*-epicholorohydrin yielded *S*-epoxy-chalcone (**8b**). The optically active *R*- or *S*-isomer of epoxides were refluxed with 5–6 eq. of *tert*-butyl amine in methanol to give *tert*-butyl-amino-carbinols **9a** (*R*-isomer) and **9b** (*S*-isomer), retaining the configuration of the epoxide. The enantiomeric purity of the compounds was checked on chiral HPLC column



Reagents: (i) 20% aq. NaOH, MeOH, r.t., (ii) *S*-epichlorohydrin or *R*-epichlorohydrin, 100–120°C, (iii) aq. NaOH, PTC, toluene, (iv) t-butyl-amine, MeOH, r.t.

Scheme 1.



Reagents: 10% Pd-C, H_2.

Scheme 2.

(Chira Sphere NT 250 mm × 0.4 mm, 5 μm, Merck); optical rotation $[\alpha]_D$ of *R*-isomer (**9a**) was recorded to be (+) 11.3 and that of the *S*-isomer (**9b**) to be (–) 14.08 respectively. Further the metabolite **10** identified during pharmacodynamic studies was prepared by hydrogenation of compound **3** in Parr shaker at 40 lb pressure of hydrogen gas at room temperature (Scheme 2).

Comparative efficacy of chalcone and its enantiomers on glucose tolerance in normal rats

The sucrose-loaded rat model (SLM) is the primary model for the study of antidiabetic compounds. Here, we have studied the efficacy of a compound to improve glucose tolerance against rise in hyperglycaemia by artificial load of sucrose in normal rats. First we validated SLM with a series of known antidiabetic drugs. Table 1 presents the per cent inhibition on the rise of hyperglycaemia by standard antidiabetic drugs, i.e. glybenclamide, metformin, acarbose and galvus, and test compounds **3**, **9a** and **9b** at the indicated dose levels. The per cent inhibition on the rise of hyperglycaemia by standard drugs, i.e. glybenclamide, metformin, acarbose and galvus was calculated to be ~43.3, 20.6, 30.2 and 20.8 respectively, whereas the test compounds **3**, **9a** and **9b** showed values ~19.6, 22.4 and 17.6 respectively, on the rise of hyperglycaemia post sucrose load in rats. In this model, insulin production was triggered from β-cells of the pancreas due to excessive amount of glucose. The secreted insulin stimulates peripheral glucose utilization and controls the production of glucose through various mechanisms. However from this study (sham-treated control), it is clear that the secreted insulin requires 2 h to bring back the glucose to normal level. The groups treated with test compounds **3**, **9a** or **9b** showed significant inhibition on the rise of hyperglycaemia and brought back the glucose levels to normal much earlier, demonstrating the ability of the compounds to increase insulin secretion resembling the mechanism(s) of action of antidiabetic sulphonylureas[20,21]. Compound **9a** showed greater ability to improve glucose tolerance in sucrose-loaded rats.

Inhibition of hyperglycaemia in sucrose-loaded streptozotocin-induced diabetic rats

STZ-induced diabetic rats are a well-known model for development of drugs for diabetes mellitus. Streptozotocin, a specific cytotoxic agent, causes destruction of

Table 1. Comparative inhibitory effect of compounds **3, 9a** and **9b** and standard antidiabetic drugs on postprandial rise in hyperglycaemia post-sucrose load in normal rats

Test sample	Dose (mg/kg)	% Inhibition on the rise of postprandial hyperglycaemia post-sucrose load
Glybenclamide	50	43.3 ± 2.01**
Metformin	100	20.6 ± 1.08*
Acarbose	50	30.2 ± 1.68**
Galvus	50	20.8 ± 1.19*
Compound **3**	100	19.6 ± 1.43*
Compound **9a**	100	22.4 ± 1.57*
Compound **9b**	100	17.6 ± 1.26*

Values are mean ± SEM of three independent experiments. Number of animals in each group was six. Statistical significance: *$P < 0.05$ and **$P < 0.01$.

Table 2. Comparative inhibitory effects of standard drugs and test compounds on the elevated hyperglycaemia post-sucrose load in streptozotocin-induced diabetic rats

		% Inhibition on rise of hyperglycaemia	
Test sample	Dose (mg/kg)	0–5 h	0–24 h
Metformin	100	24.4 ± 1.68**	19.8 ± 2.01**
Glybenclamide	50	32.2 ± 2.68***	27.4 ± 2.48**
Galvus	50	25.4 ± 2.16**	27.2 ± 2.57***
Januvia	50	24.0 ± 1.98**	28.7 ± 3.01**
Compound **3**	100	32.9 ± 2.98***	22.6 ± 1.89**
Compound **9a**	100	37.3 ± 3.17***	19.9 ± 2.01**
Compound **9b**	100	32.3 ± 3.01***	21.3 ± 2.28**

Values are mean ± SEM of three independent experiments. Number of animals in each group was six. Statistical significance: **$P < 0.01$; ***$P < 0.001$.

β-cells of the pancreas by necrosis[22], leaving less functional cells and resulting in decreased insulin secretion, thereby causing hyperglycaemia[23]. It is also a reliable test for the screening of antidiabetic compounds. The possible mechanism of lowering the blood glucose level may be either directly or indirectly by increasing insulin or insulinomimetic effects, or even both.

Table 2 presents the average antihyperglycaemic profile of standard antidiabetic drugs: metformin, glybenclamide, galvus, januvia, and of compounds **3**, **9a**, and **9b** at the indicated dose levels in sucrose-loaded STZ-induced diabetic rats. It is evident from the results that all antidiabetic drugs and compound **3** inhibited the rise in postprandial hyperglycaemia post sucrose challenge on streptozotocin-induced diabetic rats between 0 and 5 h, and the effect persisted till 24 h. Metformin caused ~24.4% and 19.8% peak inhibition on the rise of postprandial hyperglycaemia post sucrose load on streptozotocin-induced diabetic rats during 0–5 h and 0–24 h post-treatment respectively, whereas the peak effect of glybenclamide was slightly better, i.e. 32.2% and 27.4% during the same periods. The peak effect of galvus and januvia was found to be ~25.4%, 27.2% and 24.0%, 28.7% respectively, during the same periods. The per cent inhibition caused by test compounds **3**, **9a** and **9b** on the rise of postprandial hyperglycaemia on streptozotocin-induced diabetic rats was found to be ~32.9, 37.3 and 32.3, and ~22.6, 19.9, 21.3, during 0–5 h and 0–24 h post treatment respectively.

Inhibition of hyperglycaemia in streptozotocin-induced diabetic rats

Table 3 presents the per cent lowering of blood glucose levels of streptozotocin-induced diabetic rats during 0–5 h and 0–24 h by glybenclamide, metformin, galvus, januvia, and test compounds **3**, **9a** and **9b** respectively. Test compounds **3**, **9a**, and **9b** showed significant blood glucose lowering effect comparable to that of metformin, glybenclamide, galvus and januvia at 100, 50, 50 and 50 mg/kg dose levels. Metformin caused ~26.4% and 21.6% peak lowering on blood glucose level during 0–5 h and 0–24 h post-treatment, whereas the peak effect of glybenclamide was slightly more pronounced, i.e. 26.6% and 30.6% during the period. The peak effect of galvus and januvia was 24.9% and 25.8% at 0–5 h, and 24.2% and 25.5% at 0–24 h respectively. The per cent peak glucose lowering caused by compounds **3**, **9a**, **9b** and **10** in streptozotocin-induced diabetic rats was found to be ~30.8, 29.6, 28.3 and 21.2 respectively, during 0–5 h and ~26.5, 27.2, 25.7 and 17.6 respectively, during 0–24 h post-treatment.

Table 3. Blood glucose lowering effect of standard antidiabetic drugs and compounds **3**, **9a**, **9b** and **10** on streptozotocin-induced diabetic rats

Test sample	Dose (mg/kg)	% Lowering in blood glucose levels	
		0–5 h	0–24 h
Metformin	100	26.4 ± 1.87**	21.6 ± 1.76*
Glybenclamide	50	26.6 ± 1.79**	30.6 ± 2.48**
Galvus	50	24.9 ± 2.57*	24.2 ± 2.29*
Januvia	50	25.8 ± 1.68**	25.5 ± 2.98**
Compound **3**	100	30.8 ± 2.98**	26.5 ± 2.57**
Compound **9a**	100	29.6 ± 2.17**	27.2 ± 2.45**
Compound **9b**	100	28.3 ± 2.78**	25.7 ± 3.01**
Compound **10**	100	21.2 ± 1.87**	17.6 ± 1.46**

Values are mean ± SEM of three independent experiments. Number of animals in each group was six. Statistical significance: *$P < 0.05$, **$P < 0.01$.

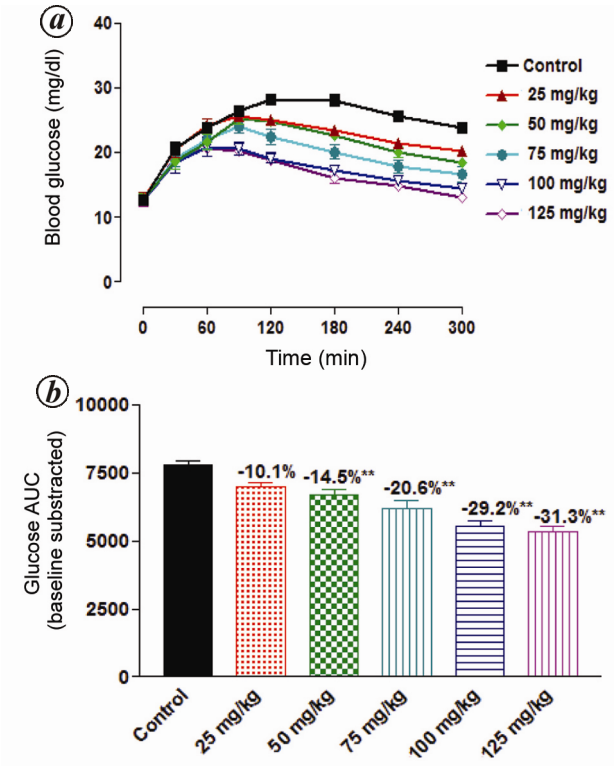


Figure 2. Comparative effects of varying doses of compound **3** on the rise in hyperglycaemia post-sucrose challenge on streptozotacin (STZ)-induced diabetic rats ($n = 6$). Statistical significance: **$P < 0.01$.

Figure 2 *a* and *b* presents the blood glucose profile of control and compound **3**-treated groups at various time intervals and dose levels, and the average AUC of blood glucose profile verses time respectively. At 25 mg/kg dose, the blood glucose level did not decline significantly during 0.5–5 h post-administration; where an average inhibition in the rise of postprandial hyperglycaemia post sucrose load was calculated to be ~10.1%. However, at 50, 75, 100 and 125 mg/kg dose, a considerable and significant inhibition on the rise of postprandial hyperglycaemia was observed and the average inhibition at these doses was calculated to be ~14.5%, 20.6%, 29.2% and 31.3% respectively.

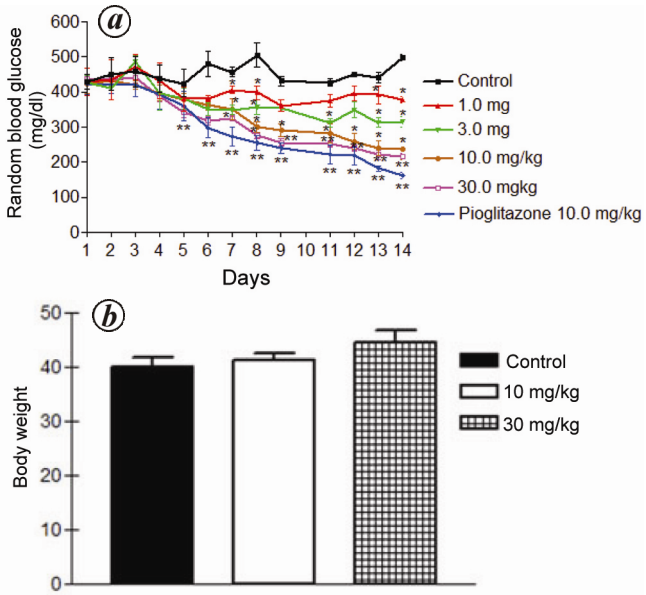


Figure 3. (***a***) Effect of test compound **3** and pioglitazone on random blood glucose levels and (***b***) effect of compound **3** on body weight of db/db mice. Statistical significance: *$P < 0.05$, **$P < 0.01$ ($n = 6$).

*Antihyperglycaemic activity of compound **3** and pioglitazone in db/db mice*

The collected data revealed that compound **3** exhibited significant and consistent antihyperglycaemic activity on normal as well as streptozotocin-induced diabetic rats. Therefore, it was further evaluated for its effect in a more appropriate model of type-2 diabetes mellitus, i.e. db/db mice. In db/db mice we tested compounds against glucose tolerance defects and diabetic complications as well. First, we studied the dose-dependent effect of compound **3** on random blood glucose profile of db/db mice (Figure 3 *a*). It is evident from the results that test compound **3** at doses 1.0, 3.0, 10.0, 30.0 mg/kg and standard drug pioglitazone at 10.0 mg/kg body weight dose lowered the blood glucose profile of db/db mice compared with the vehicle-treated control group. The blood glucose lowering effect by test compound **3** was more evident at 10.0 and

Table 4. Blood glucose profile of db/db mice during an oral glucose tolerance test (OGTT) in sham control, compound **3** and pioglitazone-treated groups on day-10 post treatment

Group	Blood glucose profile (mg/dl) at varying time intervals (min) post-glucose load					OGTT (AUC; 0–120 min)
	0	30	60	90	120	
Sham-treated control	406.0 ± 51.7	557.4 ± 34.9	597.4 ± 2.6	557.2 ± 15.9	514.1 ± 22.7	65,150 ± 2096
Compound **3** (1.0 mg/kg)	339.4 ± 30.5	416.8 ± 62.6	597.8 ± 2.2	550.1 ± 30.1	510.1 ± 38.4	59,680 ± 3217 (8.40%)^{ns}
Compound **3** (3.0 mg/kg)	315.1 ± 32.7	576.1 ± 14.7	539.2 ± 28.5	458.1 ± 14.1	388.6 ± 11.9	57,750 ± 1050 (11.4%)^{ns}
Compound **3** (10.0 mg/kg)	280.2 ± 8.98	519.8 ± 39.9	511.2 ± 4.18	422.1 ± 20.7	373.3 ± 34.6	53,380 ± 2337 (18.1%)*
Compound **3** (30.0 mg/kg)	223.2 ± 13.6	446.2 ± 44.6	455.2 ± 27.2	403.0 ± 41.4	322.0 ± 18.3	47,320 ± 3612 (27.3%)**
Pioglitazone (10.0 mg/kg)	184.3 ± 8.49	422.2 ± 46.4	444.5 ± 32.4	365.5 ± 17.1	284.5 ± 8.74	44,000 ± 2766 (32.4%)**

Values are mean ± SEM of three independent experiments. Number of animals in each group was five. Statistical significance: *$P < 0.05$, **$P < 0.01$ and ns, Not significant.

Table 5. Blood glucose profile of db/db mice during an OGTT in sham control, compound **3** and pioglitazone-treated groups on day-15, post-treatment

Group	Blood glucose profile (mg/dl) at varying time intervals (min) post-glucose load					OGTT (AUC; 0–120 min)
	0	30	60	90	120	
Sham-treated control	297.3 ± 14.3	592.3 ± 4.09	588.0 ± 7.2	582.3 ± 3.3	573.6 ± 4.63	65,930 ± 1103
Compound **3** (1.0 mg/kg)	210.1 ± 21.1	497.3 ± 36.1	589.3 ± 6.06	530.1 ± 27.5	388.6 ± 48.4	57,480 ± 614.3 (12.8%)*
Compound **3** (3.0 mg/kg)	199.1 ± 23.4	495.3 ± 73.3	521.3 ± 11.6	462.3 ± 16.5	298.4 ± 23.1	51,320 ± 2505 (22.1%)**
Compound **3** (10.0 mg/kg)	184.8 ± 18.6	418.4 ± 52.5	479.1 ± 16.2	394.8 ± 18.2	289.2 ± 24.9	46,320 ± 1784 (30.6%)*
Compound **3** (30.0 mg/kg)	169.3 ± 17.1	353.0 ± 60.2	442.3 ± 15.1	356.6 ± 9.83	288.6 ± 35.2	41,430 ± 3053 (35.2%)*
Pioglitazone (10.0 mg/kg)	148.7 ± 21.1	409.5 ± 87.5	391.2 ± 45.2	270.7 ± 43.7	244.2 ± 38.5	38,490 ± 1261 (42.3%)**

Values are the mean ± SEM of three identical experiments. Number of animals in each group was five. Statistical significance:*$P < 0.05$ and **$P < 0.01$.

30.0 mg/kg dose, where the lowering in blood glucose profile was significant from day-6 onwards, which persisted till the last day of the experiment. The blood glucose lowering effect by pioglitazone was evident from day-5 onwards and an overall average fall in blood glucose was calculated to be ~45.9% during the period from day-5 to day-14 post-treatment. Treatments with compound **3** (at 10.0 and 30.0 mg/kg doses) did not cause significant effect on body weight of the animals compared to control animals (Figure 3 *b*).

*Effect of compound **3** on oral glucose tolerance in db/db mice*

We further studied the effect of compound **3** against glucose tolerance defect on day-10 and day-15 in db/db mice. Table 4 presents the blood glucose profile of db/db mice during an OGTT on day-10 post-treatment with compound **3** at various dose levels and pioglitazone at 10.0 mg/kg. It is evident from the results that treatment with compound **3** or standard antidiabetic drug pioglitazone showed marked improvement in the oral glucose tolerance test (OGTT) in db/db mice. Pioglitazone improved OGTT by ~32.4% ($P < 0.01$) at 10.0 mg/kg dose on day-10 post-treatment. Test compound **3** showed significant improvement in OGTT at 10.0 and 30.0 mg/kg dose level, even on day-10, where the percentage improvement in OGTT was calculated to be ~18.1 and 27.3 respectively. Whereas compound **3** did not show any significant improvement in OGTT on day-10 at either 1.0 or 3.0 mg/kg dose level, but showed significant improvement on day-15 where the improvement in OGTT was calculated to be ~12.8% ($P < 0.05$) and 22.1% ($P < 0.01$), respectively, at the above doses.

Table 5 presents the blood glucose profile of db/db mice during an OGTT of sham, compound **3** and pioglitazone-treated groups on day-15 post-treatment. It is evident from the results that compound **3** improved the oral glucose tolerance of db/db mice when orally given for 15 consecutive days. At 1.0, 3.0, 10.0 and 30.0 mg/kg dose levels, the per cent improvement in OGT was calculated to be ~12.8, 22.1, 30.6 and 35.2 respectively. The pioglitazone treatment to db/db mice improved their OGT by ~42.3% at 10.0 mg/kg dose levels.

Effect of compound 3 on serum lipid profile of db/db mice

Figure 4 shows the effect of compound **3** and standard drug pioglitazone on serum lipid profiles of db/db mice at 1.0, 3.0, 10.0 and 30.0 mg/kg doses. Compound **3** at 10.0 and 30.0 mg/kg dose level effectively lowered the serum triglyceride level and this decline was ~31.3% and 33.3% ($P < 0.05$) respectively. Whereas a decline of ~16.2%

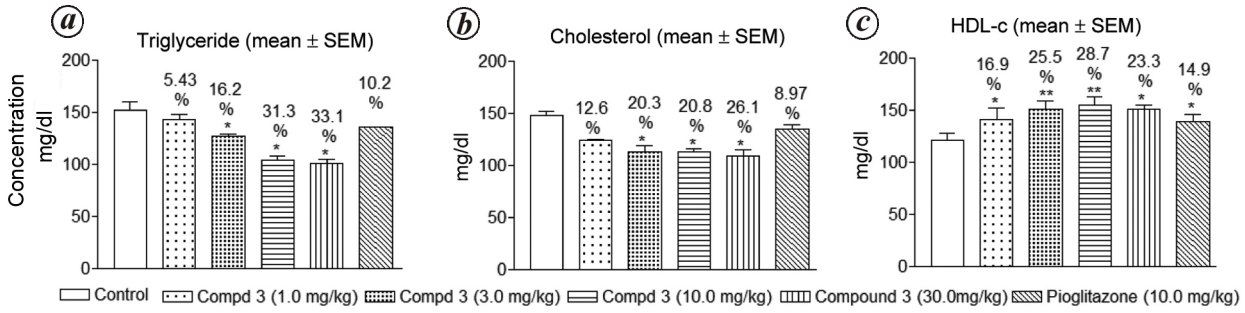


Figure 4. Effect of the compound **3** and standard drug pioglitazone on (***a***) serum triglycerides (mg/dl), (***b***) cholesterol (mg/dl) and (***c***) HDL-cholesterol (mg/dl) levels of db/db mice. Statistical significance: $*P < 0.05$, $**P < 0.01$.

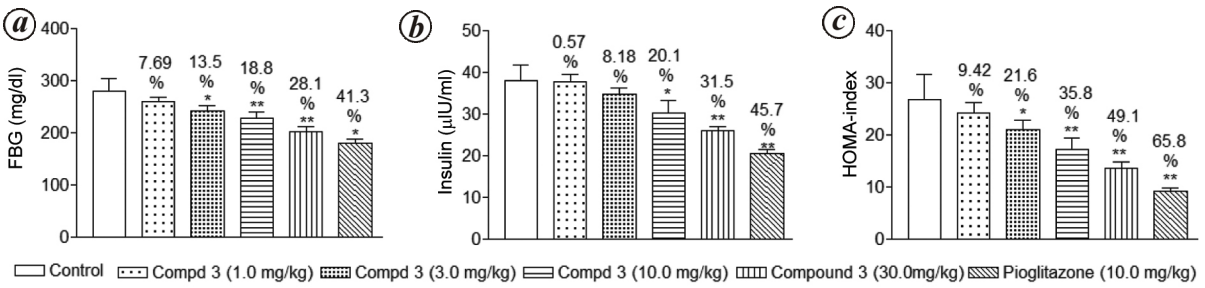


Figure 5. Effect of compound **3** and standard drug pioglitazone on (***a***) fasting blood glucose (FBG) profile (mg/dl), (***b***) serum insulin levels (μIU/ml) and (***c***) HOMA-index of db/db mice. Statistical significance: $*P < 0.05$, $**P < 0.01$.

($P < 0.05$) was observed when the test compound **3** was given at 3.0 mg/kg dose level. Test compound **3** also lowered the serum cholesterol levels of db/db mice at 3.0, 10.0 and 30.0 mg/kg dose levels by ~20.3%, 20.8% and 26.1% ($P < 0.05$) respectively. The increase in serum HDL-cholesterol levels was observed with test compound **3**-treated groups: ~16.9 ($P < 0.05$), 25.5% ($P < 0.01$), 28.7 ($P < 0.01$) and 23.3% ($P < 0.05$) observed at 1.0, 3.0, 10.0 and 30.0 mg/kg dose level respectively. The standard antidiabetic drug pioglitazone increased the serum HDL-cholesterol level in db/db mice to ~14.9% ($P < 0.05$) only at 10.0 mg/kg dose level.

*Effect of compound **3** on fasting blood glucose, serum insulin and HOMA-index of db/db mice*

Figure 5 shows the dose-dependent effect of compound **3** on fasting blood glucose, serum insulin and homeostatic model of assessment (HOMA)-index of db/db mice. It is evident from the figure that compound **3** when given to db/db mice for 15 consecutive days at 1.0, 3.0, 10.0 and 30.0 mg/kg dose levels effectively lowered their fasting blood glucose by ~7.09%, 13.5%, 18.8% ($P < 0.05$) and 28.1% ($P < 0.01$) respectively, and also lowered their serum insulin levels by 8.18%, 20.1% ($P < 0.05$) and 31.5% ($P < 0.01$) respectively. To quantify insulin resistance on the basis of fasting blood glucose and fasting serum insulin levels, the HOMA was adopted. Figure 5 shows that compound **3** remarkably improved the HOMA-index at 3.0, 10.0 and 30.0 mg/kg, and improvement was calculated to be ~21.6%, 35.8% and 49.1% respectively. The standard antidiabetic drug pioglitazone lowered the fasting glucose level by ~41.3%, serum insulin level by ~45.7% and improved insulin sensitivity of db/db mice by ~65.8%, when orally given at 10.0 mg/kg dose for 15 consecutive days.

*Blood glucose lowering effect of enantiomers **9a** and **9b**, and the metabolite **10** on random blood glucose profile of db/db mice*

Figure 6 shows the effect of compounds **9a**, **9b** and **10** on random blood glucose profile of db/db mice at various time intervals. It is evident from the results that all the test compounds **9a**, **9b** and **10** effectively lowered the blood glucose levels of db/db mice and this decline was observed from day-7, day-5 and day-4 for test compounds **9a**, **9b** and **10** respectively. This effect on blood glucose persisted till the end of the experiment, i.e. day-14. An average fall in random blood glucose profile was observed as ~30.6%, 26.3% and 37.5% for test compounds **9a**, **9b** and **10** respectively.

*Antidiabetic effect of enantiomers **9a, 9b**, and the metabolite **10** on oral glucose tolerance of db/db mice*

To observe the effect of compounds **9a**, **9b** and **10** on improvement of glucose tolerance, oral glucose tolerance

Table 6. Effect of the test compounds on OGTT in db/db mice on day-10

Group	Blood glucose profile (mg/dl) at varying time intervals (min) post glucose load					OGTT (AUC; 0–120 min)
	0	30	60	90	120	
Sham control	296.4 ± 8.68	518.6 ± 39.8	577.8 ± 17.1	528.1 ± 16.4	515.1 ± 20.2	60,910 ± 2611
Compound **9a** (30 mg/kg)	190.1 ± 14.4	469.3 ± 12.1	396.6 ± 28.9	372.6 ± 18.4	331.2 ± 17.3	44,980 ± 2248 (26.1)*
Compound **9b** (30 mg/kg)	280.2 ± 32.1	424.6 ± 30.3	411.3 ± 25.9	485.1 ± 23.4	420.3 ± 26.2	50,140 ± 1964 (17.6)*
Compound **10** (30 mg/kg)	242.6 ± 22.8	411.9 ± 23.1	390.1 ± 7.35	357.6 ± 18.9	300.5 ± 7.49	42,940 ± 384 (29.5)*

Values are mean ± SEM of three identical experiments. Number of animals in each group was five. Statistical significance: *$P < 0.05$.

Table 7. Effect of the test compounds on OGTT of db/db mice on day-15

Group	Blood glucose profile (mg/dl) at varying time intervals (min) post-glucose load					OGTT (AUC; 0–120 min)
	0	30	60	90	120	
Sham control	364.2 ± 21.4	597.8 ± 2.2	589.4 ± 10.6	579.8 ± 12.9	587.4 ± 6.6	67,350 ± 725.4
Compound **9a** (30 mg/kg)	212.6 ± 27.6	372.8 ± 92.8	403.4 ± 81.5	394.8 ± 83.8	314.6 ± 55.3	43,040 ± 894 (36.1)*
Compound **9b** (30 mg/kg)	182.6 ± 38.1	503.1 ± 72.2	466.4 ± 57.2	44.2 ± 53.3	333.6 ± 38.3	50,150 ± 585 (25.5)*
Compound **10** (30 mg/kg)	183.3 ± 32.6	320.6 ± 38.9	357.6 ± 24.5	317.1 ± 22.1	248.6 ± 15.3	36,340 ± 1882 (46.1)*

Values are mean ± SEM of three identical experiments. Number of animals in each group was five. Statistical significance: *$P < 0.05$.

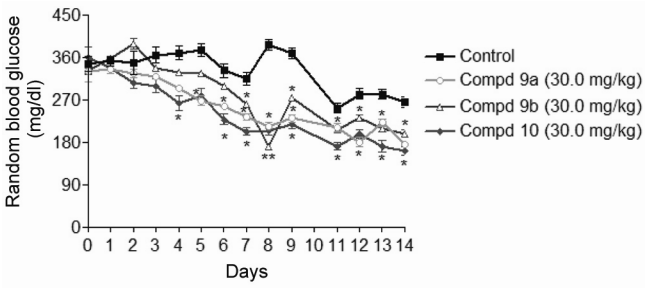


Figure 6. Effect of the test compounds **9a**, **9b** and **10** on random blood glucose profile of db/db mice. Statistical significance: *$P < 0.05$, **$P < 0.01$.

was carried out on day-10 and day-15 of the experiment. The fasting baseline value of blood glucose at 0 time-interval significantly declined on day-10 and day-15 of the experiment. Compounds **9a**, **9b** and **10** effectively lowered the rise in postprandial blood glucose post-glucose administration at 30.0 mg/kg body weight. The overall improvement in glucose AUC (0–120 min) on day-10 was calculated to be ~26.1%, 17.6% and 29.5% ($P < 0.05$) by compounds **9a**, **9b** and **10** respectively (Table 6). A similar galvanizing result was observed on day-15 for OGTT data (Table 7). Compounds **9a**, **9b** and **10** effectively resisted rise in postprandial hyperglycaemia post glucose administration and overall improvement of ~36.1%, 25.5% and 46.1% ($P < 0.01$) in glucose AUC was found during 0–120 min by compounds **9a**, **9b** and **10** respectively.

To evaluate the additional beneficial effects of compounds **9a**, **9b** and **10** on db/db mice, their serum lipid profile was analysed after 15 days of consecutive dosing. The results suggest that all these compounds have marked antidyslipidaemic effect. Test compounds **9a**, **9b** and **10** lowered the serum triglycerides levels of db/db mice by ~17.7%, 10.5% and 29.9% ($P < 0.05$) respectively, whereas the decline in serum cholesterol levels of db/db mice was recorded only in the case of **9a** and **9b**-treated groups (~17.6% and 13.3% respectively). These two compounds **9a** and **9b** also raised the HDL-cholesterol level by ~12.9% and 16.1% respectively (Figure 7).

Figure 8 presents the effect of compounds **9a**, **9b** and **10** on fasting blood glucose, serum insulin levels and HOMA-index of db/db mice after 15 days of consecutive dosing. It is evident from figure that compound **10** most effectively lowered the fasting blood glucose, serum insulin level and HOMA-index to the tune of 39.1% ($P < 0.01$), 35.9% ($P < 0.05$) and 55.8% ($P < 0.01$) respectively. Whereas compounds **9a** and **9b** lowered the fasting blood glucose by 29.1% and 30.5% ($P < 0.05$), serum insulin level by ~17.1% and 19.4% ($P < 0.05$) and HOMA-index by 41.1% ($P < 0.05$) and 44.5% ($P < 0.01$) respectively, compared to vehicle treated control group.

The db/db mice are a well-characterized model of type-2 diabetes mellitus; however, their major deficiency is lack of a functional leptin receptor, which leads to defective leptin signalling and a complete lack of feedback from leptin resulting in hyperphagia, decreased energy expenditure, obesity, insulin resistance, hyperinsulinaemia and dyslipidaemia. The test compounds **3**, **9a** and **9b** significantly lowered blood glucose levels, improved postprandial glucose tolerance and lowered the serum insulin levels of db/db mice. Interestingly, the treated mice also showed a decrease in total cholesterol, triglycerides and LDL-cholesterol levels and attenuated lipid accumulation in the plasma like those of antidiabetic drugs, i.e. thiazolidinedione.

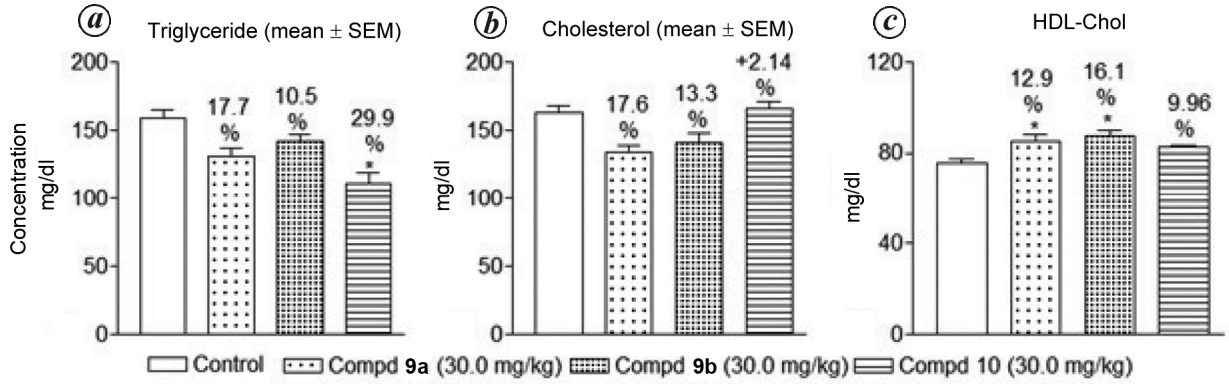


Figure 7. Effect of compounds **9a**, **9b** and **10** on serum lipid profile of db/db mice. Statistical significance: $*P < 0.05$.

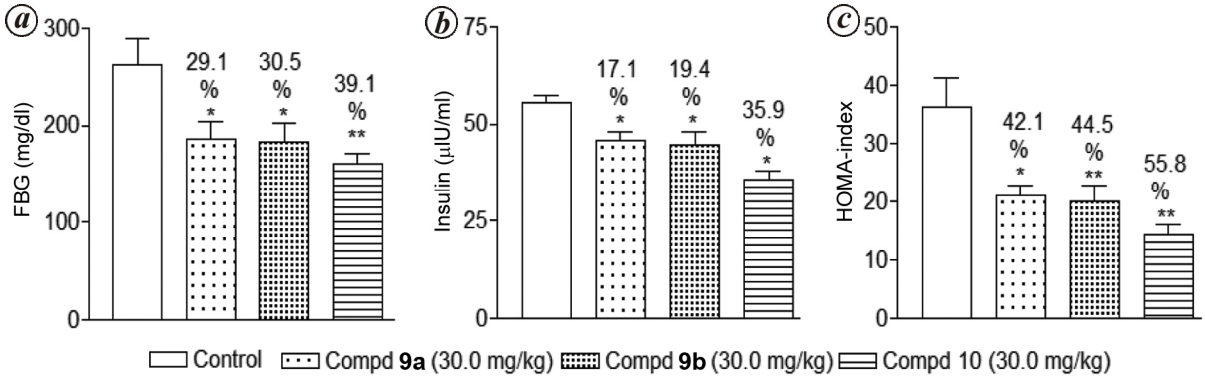


Figure 8. Effect of compounds **9a**, **9b** and **10** on (***a***) fasting blood glucose, (***b***) serum insulin (***c***) and HOMA-index of db/db mice. Statistical significance: $*P < 0.05$, $**P < 0.01$.

Antidyslipidaemic activity of test compounds in high fat diet-fed Syrian golden hamsters

Table 8 represents the antidyslipidaemic activity profile of test compounds **3**, **9a**, **9b** and **10** on HFD fed male Syrian golden hamsters. It is evident from the results that these test compounds at 30 mg/kg caused significant decrease in serum triglyceride levels that was found to be ~25.9%, 51.5%, 37.4% and 34.9% respectively. The serum cholesterol level was lowered by ~19.0%, 35.4%, 33.4% and 41.4% by compounds **3**, **10, 9a**, and **9b** respectively. The decline in serum LDL-cholesterol levels was recorded to be ~22.2%, 40.9%, 22.6% and 39.6% respectively. The serum glycerol (Gly) level was lowered by ~18.2%, 23.9%, 21.6% and 14.3% respectively. A mild decline in NEFA was also seen in compounds **3**, **10, 9a** and **9b**-treated groups and this was ~7.50%, 15.2%, 19.4% and 8.60% respectively; but it was not significant. Decrease in body weight of the compounds **3**, **10**, **9a**, and **9b**-treated groups was observed but it was also not significant.

*Glucose uptake stimulatory effect of compounds **3**, **9a** and **9b** in skeletal muscle cells*

Glucose uptake stimulatory effect of test compound **3** and its isomers **9a** and **9b** was determined in L6 skeletal muscle cells. L6 myotubes were incubated with increasing concentrations of test compound **3** or its isomers **9a** and **9b** for 16 h, followed by determination of glucose uptake. Results suggest that incubation of L6 myotubes with test compound **3** increased glucose uptake in a concentration-dependent manner (Figure 9 *a*). Compound **3** stimulated glucose uptake at significant level with a minimal concentration of 5 μM ($P < 0.05$). No significant difference in biological activity was observed among the racemic mixture **3** and isomers **9a** and **9b** (Figure 9 *b*). Compound **3**-induced glucose uptake was completely blocked in the presence of cytochalasin-B (50 μM), added to the transport solution, suggesting the involvement of glucose transporter-mediated uptake in response to test compound **3**.

Simulation of glucose uptake in skeletal muscle is mainly accredited to enhanced translocation and redistribution of GLUT4 to the cell membrane. In order to evaluate the potential activity of compound **3** to stimulate glucose uptake, the effect was examined on the translocation of GLUT4 to the plasma membrane in L6 myotubes that stably express GLUT4 with a myc epitope inserted in the first exofacial loop (L6-GLUT4myc). L6-GLUT4myc myotubes were treated with compound **3** and its isomers **9a** and **9b** (10 μM) for 16 h and cell surface GLUT4 level was measured via an antibody-coupled colorimetric assay, as described previously[24]. Under control condition,

Table 8. Effect of the test compounds on high fat-induced dyslipidaemia in male Syrian golden hamsters

Group	Body wt	Effect on lipid profile					
		TG (mg/dl)	Chol (mg/dl)	HDL-Chol (mg/dl)	LDL-Chol (mg/dl)	Gly (µmol)	NEFA (mmol/l)
Sham control	118.2 ± 4.10	747.1 ± 64.9	334.4 ± 37.5	151.1 ± 2.17	108.9 ± 23.6	1630 ± 55.1	7.39 ± 0.16
Compound **3**	112.2 ± 4.01	553.0 ± 46.4* (–25.9)	270.6 ± 22.5* (–19.0)	139.6 ± 3.44 (–7.60)	84.7 ± 6.17* (–22.2)	1334 ± 87.3 (–18.2)	6.84 ± 0.28 (–7.50)
Compound **10**	103.1 ± 3.35	362.0 ± 37.9** (–51.5)	216.1 ± 18.1** (–35.4)	140.7 ± 6.42 (–6.90)	64.4 ± 10.7* (–40.9)	1240 ± 78.0* (–23.9)	6.27 ± 0.23 (–15.2)
Compound **9a**	114.5 ± 6.78	467.7 ± 39.8* (–37.4)	222.8 ± 19.3* (–33.4)	148.5 ± 5.83 (–1.70)	84.3 ± 4.94 (–22.6)	1277 ± 49.4 (–21.6)	5.95 ± 0.48 (–19.4)
Compound **9b**	110.4 ± 3.58	485.7 ± 56.8* (–34.9)	196.1 ± 15.8** (–41.4)	151.6 ± 8.08 (+0.30)	66.3 ± 8.14* (–39.6)	1397 ± 66.1 (–14.3)	6.77 ± 0.40 (–8.60)

Values are average ± SEM of three identical experiments. Number of animals in each group was six. Statistical significance: *$P < 0.01$, **$P < 0.001$ and ns, Not significant.

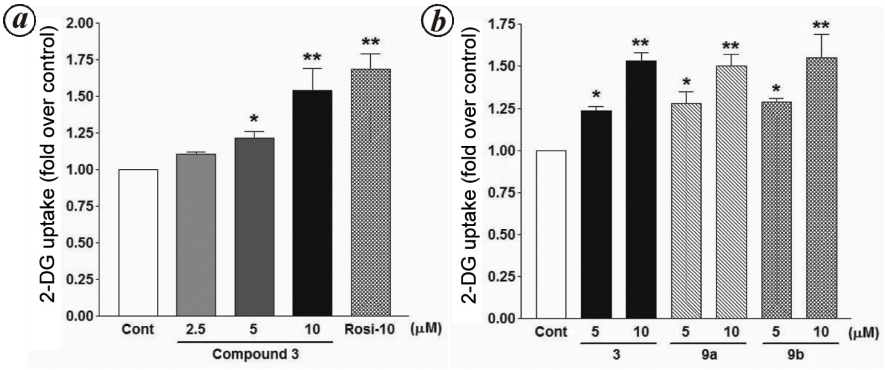


Figure 9. Effect of compounds **3**, **9a** and **9b** on glucose uptake by L6 skeletal muscle cells. Statistical significance: *$P < 0.05$, **$P < 0.01$, relative to control condition ($n = 3$).

insulin treatment (100 nM, 20 min) caused 2.5-fold ($P < 0.001$) increase in cell surface GLUT4myc level. Treatment with test compound **3** or its isomers **9a** and **9b** significantly increased GLUT4myc levels under basal condition, without affecting insulin-stimulated glucose uptake in L6-GLUT4myc myotubes (Figure 10). These observations suggest that insulin and compound **3** or its isomers might exert their effect through activation of identical sub-cellular pathways.

Further, to study the mechanism of stimulation of glucose uptake by compound **3**, the effect was examined in the presence of a specific inhibitor for PI-3-kinase wortmannin (50 nM) that blocks the insulin-signalling pathway. Presence of wortmannin completely attenuated the insulin-induced glucose uptake. Similarly, the stimulatory effect by compound **3** on glucose uptake was inhibited in presence of wortmannin in L6 myotubes (Figure 11). The results clearly suggest the activation of PI-3-kinase-dependent signalling pathway by compound **3** for stimulation of glucose uptake in skeletal muscle cells.

Preclinical pharmacokinetic studies

After establishing the antidiabetic and antidyslipidaemic activities, the test compounds were subjected to pharmacokinetic studies. The issue of chirality to avoid unnecessary drug burden on the body has also emerged as a factor in decision making for the selection of lead compound for further studies in drug discovery process, as enantiomers often differ in their pharmacological activity, toxicity and pharmacokinetic characteristics[25–27]. This prompted us to undertake pharmacokinetic study of racemic compound along with resolved individual optical isomers in laboratory animals. The comparative single intravenous and oral dose pharmacokinetic parameters for compounds **3**, **9a** and **9b** were generated in male SD rats. The intravenous pharmacokinetic study was performed at 10 mg/kg, while oral dose administration at 40 mg/kg in male rats.

Among the ADME properties, metabolic characterization is a key issue and nowadays, it is integrated into the early discovery phase. The information generated in the early discovery phase can be used to identify new chemical entities (NCEs) with undesirable metabolic behaviour, and to optimize pharmacokinetic and safety profiles by means of formulation or conversion to pro-drug by chemical transformations[28]. Metabolite profiling reduces the costs of drug development by eliminating the progression of compounds that would eventually fail due to toxicity. The advantages suggested studying the pharmacokinetic behaviour through liquid chromatography electro-spray

ionization mass spectrometry (LC-MS/MS), which is now routinely used in metabolite-profiling studies[29].

In vitro gastrointestinal (GI) stability studies: The stability of compound **3** and isomers **9a** and **9b** in a plasma was studied. It was found to be stable during freeze–thaw cycle, bench top, dry residue and long-term conditions. The compounds were also found to be highly stable in simulated gastric fluid (SGF, pH 1.2) as well as in simulated intestinal fluid (SIF, pH 6.8).

In situ absorption study: An *in situ* absorption study of compound **3** and isomers **9a** and **9b** in male rats was done using closed loop technique. It followed apparent first-order kinetics, and the absorption rate constant was calculated to be 1.51 ± 0.46/h and absorption half-life ($t_{1/2}$ α) 0.49 ± 0.13 h.

Pharmacokinetics: The intravenous dosing formulation was prepared in a solution of DMF : PG : water (3 : 3 : 4 v/v) and 10 mg/kg dose was administered through tail vein in rats. After intravenous administration, measurements were made up to 48 h for compound **3** and 60 h isomers **9a** and **9b** (Figure 12). Concentration–time profile after intravenous dose shows multiple peaks for compound **3** and isomers **9a** and **9b**. The irregular concentration–time profile after intravenous dose could not be explained by any standard compartment models. Hence non-compartmental approach was used to fit the data. The elimination half-life ($t_{1/2}$) and mean residence time (MRT) values were 11.97 and 26.75 h for compound **3**, 11.49 and 20.3 h for isomer **9a**, and 9.98 and 19.3 h for isomer **9b**

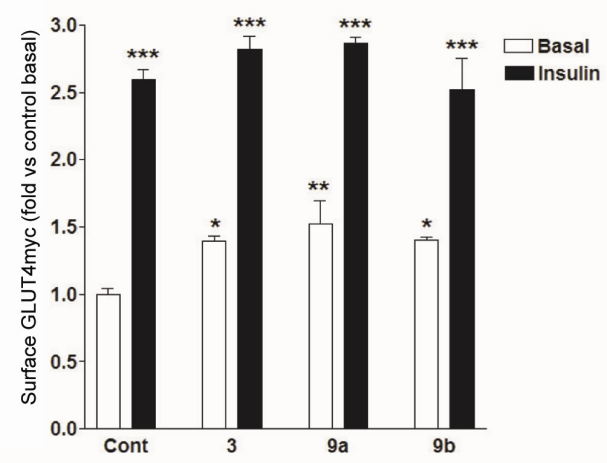


Figure 10. Effect of test compounds **3**, **9a** and **9b** on GLUT4 translocation in L6-GLUT4*myc* myotubes. Significance analysis: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ relative to control basal condition ($n = 3$).

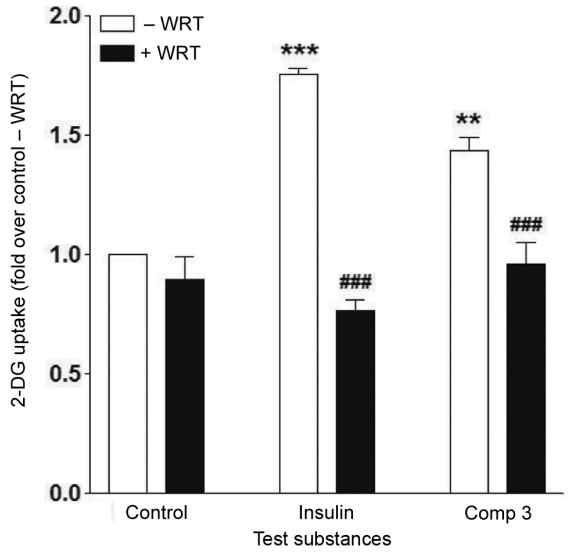


Figure 11. Effect of wortmannin on insulin- and compound **3**-induced glucose uptakes in L6 myotubes. Significance: **$P < 0.01$, ***$P < 0.001$ relative to negative control; $^{###}P < 0.001$ compared to positive control ($n = 3$).

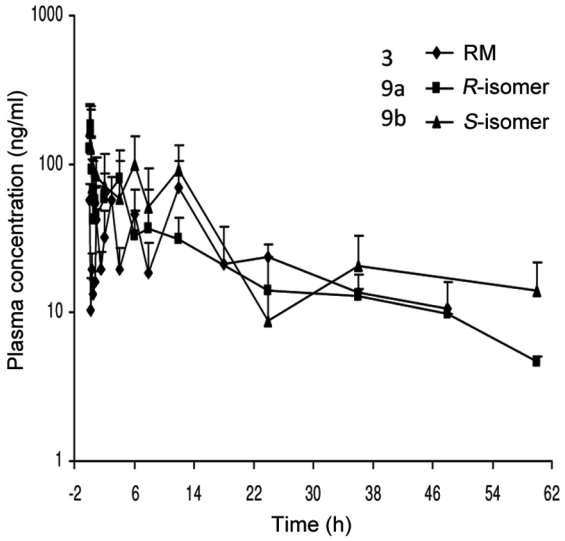


Figure 12. Plasma concentration-time profiles of compounds **3**, **9a** and **9b** in male rats after intravenous administration at 10 mg/kg dose ($N = 3$, mean ± SEM).

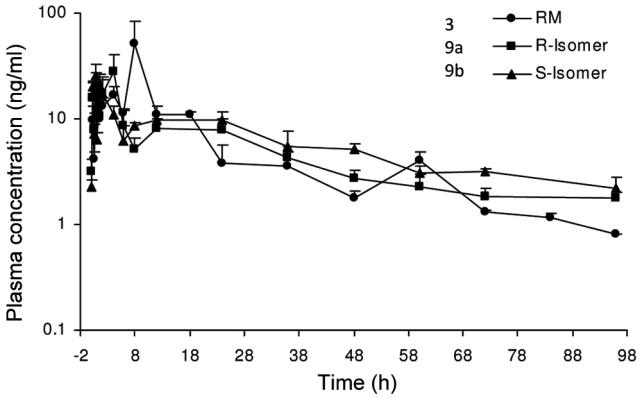


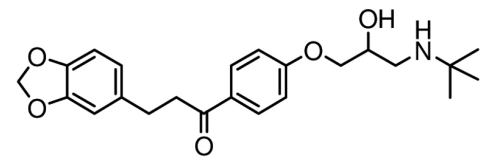
Figure 13. Plasma concentration–time profile of compound **3**, **9a** and **9b** in male rats after oral administration at 40 mg/kg dose ($N = 3$, mean ± SEM).

respectively. The volume of distribution was found to be ~112.6, 131.5 and 106.2 l/kg for compounds **3**, **9a** and **9b** respectively.

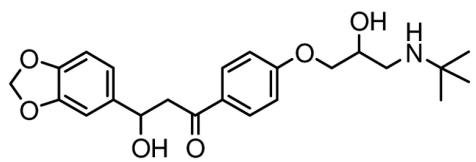
For oral pharmacokinetic study, the aqueous suspension of compounds in 0.5% methyl cellulose was administered to rats at 40 mg/kg dose. After oral administration of test compounds **3**, **9a** and **9b** these were measured in the plasma up to 96 h as shown in the Figure 13. Multiple peaks were observed in concentration-time profiles of compound **3** and isomers **9a** and **9b** after oral dose administration. Non-compartmental approach was used to fit the data to observe pharmacokinetic parameters. The half-life ($t_{1/2}$) and MRT values were 18.95 and 28.03 h for compound **3**, 17.77 and 34.6 h for **9a** and 19.48 hr and 33.41 h for **9b** respectively. The systemic exposure ($AUC_{0-\infty}$) after oral administration was 579.17 ng h/ml for compound **3**; 461.88 ng h/ml for **9a** and 557.7 ng h/ml for **9b**. The oral bioavailability was found to be ~11.62%, 8.92%, and 6.98% for **3**, **9a** and **9b** respectively, in accordance with the dose normalized AUC. A part of this work has been recently reported[30].

In vivo metabolite profiling using LC-MS/MS in rat plasma: Quantitative information of drug metabolites is of great interest during the drug discovery and development process. The generation of metabolites in *in vivo* condition and their elimination from the body is a function of time[31]. The peak area ratio (metabolite/IS) versus time profile was used to obtain time-dependent pharmacokinetic parameters of metabolite. Plasma test sample after oral and intravenous dose was further investigated for the formation of *in vivo* metabolite. Mass spectrometric parameters were optimized and used to obtain sensitive multiple reaction-monitoring chromatogram for transitions *m/z* 400 → 135.2 and *m/z*416 → 194.1 of metabolites **10** and **11** respectively (Figure 14).

The comparative area ratio–time profile for metabolites and the parent compound was found to be in decreasing order of **10** > **11** > **3** for post-oral dose administration. *In vitro* stability study also showed that compound **3** gets converted into reduced metabolite in the whole blood. The metabolites **10** and **11** are formed by the reduction and hydration of parent molecule respectively.



10



11

Figure 14. Identified metabolites.

Excretion studies: Percentage of administered dose excreted in urine was 0.048–0.095%. The hydroxylated metabolite **11** was present in higher amounts in comparison to parent and reduced metabolite **10** in urine. Percentage of administered dose excreted in faeces was found ~0.188%–0.223% (ref. 29). The reduced metabolite **10** was present in higher amounts compared to parent **3** and hydroxylated metabolite **11** in faeces.

Plasma protein binding study: Protein binding was estimated using charcoal adsorption method. The sample of test compound **3** was analysed by validated LC-MS/MS method for rat plasma at concentration of 1 μg/ml of drug and protein binding was found to be ~1.38%.

Conclusion

The results of various experiments conducted in this study establish antihyperglycaemic and antidyslipidaemic effects of the chalcone congener with aryloxypropanolamine moiety. The comparative study reveals that the enantiomers have no edge over racemic compound in terms of superior activity and hence are not useful for further studies, since they are expensive compared to racemic compound. Comparative concentration–time profile and pharmacokinetic parameters were generated in SD rats for compounds **3**, **9a** and **9b** after intravenous and oral administration. Poor oral bioavailability (<12%) was observed for compounds **3**, **9a** and **9b**, which may be due to metabolic conversion. The metabolite **10** was present in significant amounts compared to parent, which is again associated with antidiabetic activity, and metabolite **11** was identified in systemic circulation both after oral and intravenous dose administration. The presence of hydroxy radical conjugated metabolite **11** indicates the role of chalcone skeleton towards relieving oxidative stress. However, this has to be further authenticated. The study of the mechanism suggests that compound **3** follows the activation of PI-3-kinase pathway for glucose uptake in skeletal muscle. Thus, the chalcone compound offers a promising lead for development as a drug for the management of type-2 diabetes mellitus with additional antioxidant effect.

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