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Research Article

Novel Piperine Derivatives with Antidiabetic Effect as PPAR-γ Agonists

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Piperine is an alkaloid responsible for the pungency of black pepper. In this study, piperine isolated from *Piper nigrum* L. was hydrolyzed under basic condition to obtain piperic acid and was used as precursor to carry out the synthesis of twenty piperine derivatives containing benzothiazole moiety. All the benzothiazole derivatives were evaluated for their antidiabetic potential by OGT test followed by assessment of active derivatives on STZ-induced diabetic model. It was observed that nine of twenty novel piperine analogues (5b, 6a-h), showed significantly higher antidiabetic activity in comparison with rosiglitazone (standard). Furthermore, these active derivatives were evaluated for their action as PPAR-γ agonists demonstrating their mechanism of action. The effects on body weight, lipid peroxidation, and hepatotoxicity after administration with active derivatives were also studied to further establish these derivatives as lead molecules for treatment of diabetes with lesser side-effects.

Key words: amide linkage, antidiabetic, gene expression, piperine, PPAR-γ

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Benzothiazoles have proved their biological importance in bioorganic and medicinal chemistry, their unique bicyclic structure allows diversified pharmacological activities, and benzothiazole finds its utility as starting material for the synthesis of several bioactive molecules reported as antidiabetic (1), antimicrobial (2), antiviral (3), antibacterial (4), anticancer (5), anti-allergic (6), antifungal (7), anti-inflammatory (8), anthelmatic, and anti-HIV agents (9,10). Plant-derived products whether pure or in crude form received immense attention in recent years. Several herbs and their formulations have been listed in ancient literature for the treatment of numerous diseases including diabetes mellitus (11). Characterized by high blood sugar, diabetes mellitus occurs due to impaired insulin sensitivity or secretion. The statistical data according to WHO revealed that the prevalence of diabetes which was estimated to be 2.8% in 2000 will rise to 4.4% in 2030 (12). Therefore, the regulation of fast growing diabetes mellitus has been still a challenge for research scientists. Furthermore, the drugs derived from natural sources are being emphasized more than those derived from synthetic routes because of their lesser side-effects. Piperine is one such plant-derived alkaloid which is well known for its wide spectra of biological activities. It is found as major constituent in the fruits of *Piper nigrum* L., commonly known as black pepper. The clinical and pharmacological studies have proved the utility of piperine as antidepressant (13), antipyretic, analgesic, anti-inflammatory (14), antioxidant (15), hepatoprotective (16), and antidiabetic (17,18) agent. It has also been accounted that piperine enhances the bioavailability of several synthetic and natural drugs like sulfadiazine, streptomycin, rifampicin (19), pyrazinamide, isoniazid, nateglinide (20), propranolol (21), curcumin, and Boswellic acid (22). Moreover, it has recently been reported that piperine exerts anti-adipogenesis effect by activating PPAR-γ receptors (23,24). As PPAR-γ also plays a crucial role in insulin sensitization (25), the present study aims at the synthesis of new piperine derivatives containing benzothiazole moiety and their evaluation for antidiabetic activity as PPAR-γ agonists.

Experimental Protocols

Isolation of piperine

Grounded black pepper (5 kg) was placed in cold percolator and 15 L of chloroform was added to it. The extract was filtered through Buchner funnel to remove plant material, and the filtrate was concentrated on rotaevaporator. Petroleum ether was slowly added to the concentrated paste. Precipitates so obtained were filtered and crystallized from methanol. Yellow-colored piperine (**I**) (30 g) was obtained. ^{1}H-NMR and melting point of the compound were recorded and were found consistent with the reported one (26).

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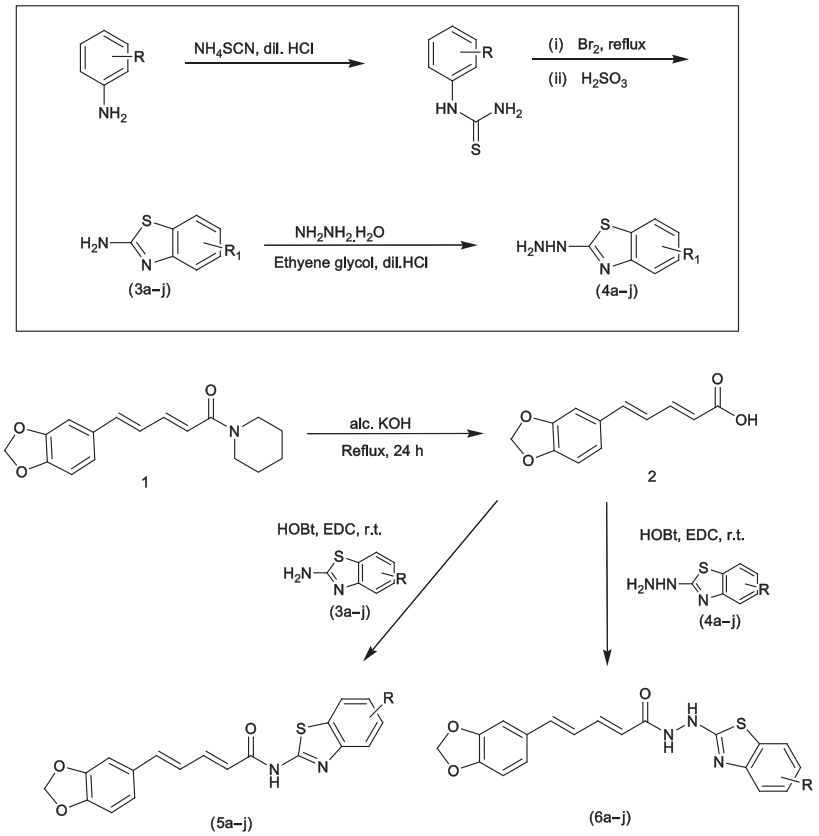
General synthesis of 5-Benzo[1,3]dioxol-5-yl-penta-2,4-dienoic acid N′(substituted-benzothiazol-2-yl) amide (5a-j)/hydrazide (6a-j)

Piperine **(1)** isolated from *Piper nigrum* was added to 20% alcoholic solution of potassium hydroxide and was refluxed for 24 h. Reaction mixture was concentrated and poured on ice. The reaction mass gradually neutralized with dil. HCl was filtered to obtain solid. Solid product was recrystallized from ethanol and identified as piperic acid **(2)**. Substituted benzothiazole reactants **(3a-j, 4a-j)** were synthesized using known method (27). The weighed amount of piperic acid (1 mol) was dissolved in dry tetrahydrofuran. A pinch of HOBt (10 mg) was added to reaction mixture. After half an hour, 1.2 mole of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was weighed and quickly added to reaction mass under nitrogen atmosphere. The reaction mass was kept on stirring for another half hour and then one equivalent of other reactant **(3 or 4)** was added to it. Reaction mixture was kept on stirring until the consumption of reactants. The reaction mass was diluted with water, and extraction was carried out using ethyl acetate. Corresponding desired products **(5a-j, 6a-j)** were obtained from organic layer. The organic layer was dried on anhydrous sodium sulfate and concentrated to give the final product as solid. The solid product was purified from methanol to get pure compound (Scheme 1).

In silico PPAR-γ docking

Protein with Accession Number 3CS8 was selected and downloaded from Protein Data Bank. This protein is reported to bind with drug rosiglitazone. The protein was imported, optimized, and minimized by removing unwanted molecules and other defects reported by the software. PPAR-γ receptor is a dimer which has two monomers chains (A & B). For the purpose of studies, chain B was deleted and water molecules near the ligands were retained. A low-energy minimized protein structure was obtained which was used for grid generation involving selected ligand as the reference as it signifies the binding sites of drug with respect to the target. The generated grid was used for docking of new molecules. Molecules drawn in 3D form were refined by LigPrep module. The molecules were subjected to OPLS-2005 force field to generate single



Compound	R	Compound	R
3a,4a,5a,6a	4-Cl	3f,4f,5f,6f	4-OCH_3
3b,4b,5b,6b	5-Cl	3g,4g,5g,6g	6-OCH_3
3c,4c,5c,6c	6-Cl	3h,4h,5h,6h	6-OCH_2CH_3
3d,4d,5d,6d	6-Br	3i,4i,5i,6i	4-CH_3
3e,4e,5e,6e	6-F	3j,4j,5j,6j	6-NO_2

Scheme 1: Synthesis of piperine derivatives.

low-energy 3D structure while maintaining their chiralities. Docking studies were carried using GLIDE software (Schrödinger, NY, USA) with extra precision. This generates favorable ligand poses which are further screened through filters to examine spatial fit of the ligand in the active site. Ligand poses which pass through initial screening are subjected to evaluation and minimization of grid approximation to generate Glide score.

Pharmacological evaluation

Animals

Healthy male Wistar rats were procured from Central Animal House, Hamdard University, New Delhi. The experiments were performed in accordance with the rules of Institutional Animals Ethics Committee (registration number 757-CPCSEA). Prior to the experiment, the animals were kept on fasting for 14 h allowing access to water *ad libitum* only.

In vivo antidiabetic evaluation by OGTT

All the synthesized derivatives were primarily evaluated for oral glucose tolerance by earlier reported method (28). Overnight fasted animals (130–180 g) were randomly divided into 24 groups containing six rats per group. A group of healthy rats was administered orally with vehicle (10% carboxymethylcellulose) and was labeled as control. Two groups treated with 36 mg/kg b.w. piperine and rosiglitazone were taken as standard groups. Remaining groups were orally administered with 36 mg/kg b.w. of synthesized derivatives suspended in 10% CMC. All the groups were orally given 3 g/kg b.w. glucose solution after 30 min of dosing. Plasma glucose level was tested just prior to glucose load and after 30 and 90 min with the help of GOD-POD kit. Working solution of each sample was prepared as per the manufacturer's instructions. The wavelength of each solution and standard solution available in kit was recorded at 505 nm. Glucose concentration in the sample can be calculated using the following formula:

$$\text{Glucose (mg/dL)} = (\text{Absorbance of Sample}/\text{Absorbance of Standard}) \times \text{Concentration of Standard.}$$

In vivo antidiabetic activity on STZ-induced diabetic model

Diabetes was induced by intraperitoneal administration of fresh solution of streptozotocin (45 mg/kg b.w.) prepared in citrate buffer (pH 4.5, 0.1 M) (29). The animals having blood glucose level more than 250 mg/dL were divided into fourteen groups of six animals each. Control group comprised of healthy animals and diabetic control containing diabetic animals were administered with 1% Tween-80 only. Other groups were treated with oral dose of 36 mg/kg b.w. piperine, rosiglitazone, and active derivatives. Dosing was carried out in same way for 15 days. Plasma glucose level was scrutinized on 0, 7, and 15th day of study by GOD-POD method.

Body weight

Body weight of all the diabetic animals was measured before carrying out the *in vivo* antidiabetic study. Finally on last day of study, body weights were again recorded to understand the effect of treatment on weight.

In vitro PPAR transactivation assay

Human embryonic kidney (HEK 293) cells were cultured in DMEM containing 10% FBS at 37 °C in 5% CO_2 humidified atmosphere until 70–80% cell confluency was obtained. Cells were inoculated in 96-well plate containing approximately 70 000 cells per well and transfected with 2.5 μL PPRE-Luc, 6.67 μL PPAR-γ, 1.0 μL Renilla, and 20 μL Lipofectamine. After 5 h of transfection, 10 μM of test derivatives was added to cells for 24 h. Cells were collected with the help of lysis buffer, and luciferase activity was monitored on luminometer using luciferase assay kit according to the manufacturer's instructions. Piperine and rosiglitazone were taken as reference drugs.

Gene expression

3T3-L1 cells (American Type Culture Collection) were cultured to attain 70% confluency and transferred to 24-well plate. Cells were incubated for 24 h at 37 °C and 5% CO_2 and treated with 10 μM of most active compound, rosiglitazone as positive control and DMSO as negative control. Treated cells were then transferred to micro-centrifuge tubes, and total RNA was isolated by TRI Reagent® (Molecular Research Centre, Cincinnati, OH, USA). The quantity and quality of RNA were determined on a NanoDrop ND-2000c spectrophotometer, and integrity was verified on a 1.5% agarose gel. The total RNA (1 μg) so obtained was used to generate cDNA using an EZ-first strand cDNA synthesis kit for RT (reverse transcription)–PCR (Biological Industries, Cromwell, CT, USA). PEARL PRIMER software was used to design primers of PPAR-γ and β-actin for real-time PCR. Reactions were run at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 second and 60 °C for 1 min. Real-time PCR was performed in triplicate on an ABI Prism 7300 Sequence Detection System (Applied Biosystems, CA, USA) using the SYBR Green PCR Master Mix (Applied Biosystems). PCR was repeated two times for each gene and each sample. Relative transcript quantities were calculated using the C_t method with β-actin as the endogenous reference gene.

Acute hepatotoxicity studies

Two derivatives which showed significant plasma glucose-lowering activity were further evaluated for hepatotoxicity

risk. For this purpose, three times higher dose i.e. 108 mg/kg b.w of selected derivatives was orally administered to two separate groups of STZ-induced diabetic rats. A group of three untreated diabetic rats was taken as control. Group administered orally with 108 mg/kg b.w. piperine was taken as reference group. The livers of all rats were excised after 5 h of dosing and preserved in 10% formalin.

Lipid peroxidation assay

Lipid peroxidation effect of selected derivatives was evaluated by modified thiobarbituric acid reactive species (TBARS) assay. The liver specimen excised for hepatotoxicity risk evaluation was sliced and used for preparing homogenate. The solution was prepared by adding 0.2 mL of sodiumdodecyl sulfate (SDS; 8.1%), 1.5 mL of acetic acid (20%), and 1.5 mL of 0.9% thiobarbituric acid to 200 mL of homogenate. This solution was diluted with distilled water to make the final volume of 4 mL. The mixture was placed in boiling water to start reaction and then quickly cooled. Gradually, 1 mL of distilled water and 5.0 mL of butanol/pyridine (15:1, v/v) were added followed by centrifugation at 10 000 × *g* for 10 min. The absorbance of upper layer was recorded at 532 nm. To prevent exogenous TBARS formation, 28 mL of butylated hydroxytoluene (1 mmol/L) was added. The quantification was performed using a molar absorption coefficient of 1.56×10^5 cm^{-1} mol^{-1}, and the result was expressed as nmol malondialdehyde (MDA) per 100 mg tissue (30).

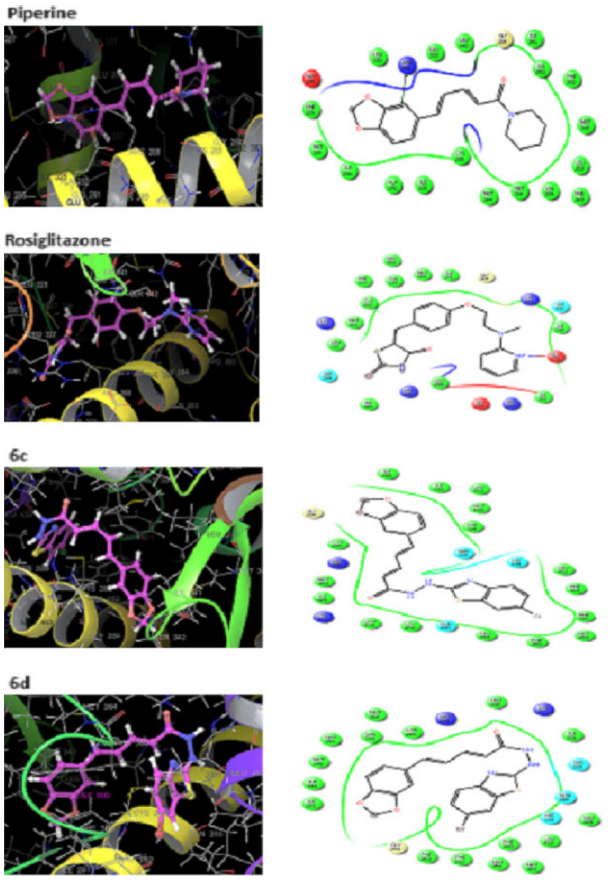


Figure 1: Docking images of piperine, rosiglitazone, and active compounds **6c** and **6d.**

Results and Discussion

Docking studies

In 2012, Coman *et al.* (31) reported interaction of piperine with ligand binding domain of PPAR-γ and related it with antidiabetic potential of piperine (32). Therefore, structures of all the synthesized piperine derivatives were docked against ligand binding domain of PPAR-γ before performing *in vivo* antidiabetic assay. Piperine and rosiglitazone were taken as reference ligands. It was found that piperine (−6.5) showed higher dock score than rosiglitazone (−5.72). Captivatingly, all the derivatives except for **6i** exhibited appreciably higher docking score than rosiglitazone and piperine. The rigid 3D structure of compound **6i** could not be aligned into the receptor site of PPAR-γ to carry out the docking study. Rest of the derivatives showed dock scores more than −7.00, and their docking images exhibited that these small ligands accommodated themselves well inside the large pocket of PPAR-γ domain resulting in high dock score (Figure 1, Table S1, supporting information).

In vivo *antidiabetic activity*

The antidiabetic potential of twenty derivatives which demonstrated high dock score was initially assessed by loading glucose on normal rats. The results showed that unlike docking score, piperine did not assuage plasma glucose level as significantly as rosiglitazone. However, of twenty synthesized derivatives, nine derivatives **(5b, 6a-h)** exhibited better alleviation in plasma glucose level than rosiglitazone, while seven derivatives **(5b, 5c, 5d, 5f-i)** improved plasma glucose level concentration as considerably as rosiglitazone (Figure 2). Comparison of antidiabetic activity of synthesized derivatives with that of piperine also revealed that nineteen derivatives (except **6i**) were more potent antidiabetic agents than piperine.

To avoid the superfluous use of animals, only nine active derivatives **(5b, 6a-h)** that showed more antidiabetic potential than rosiglitazone were assessed for their effect on plasma glucose concentration in streptozotocin (STZ)-induced diabetic model. It was found that all these derivatives possessed significantly higher antidiabetic activity in comparison with the standards, piperine and rosiglitazone. Additionally, the results were consistent with docking studies as well as with the results of oral glucose tolerance test. All the derivatives restored plasma glucose level near to normal level on 15th day of study (Figure 3). Therefore, the *in vivo* study clearly illustrated that the coupling of

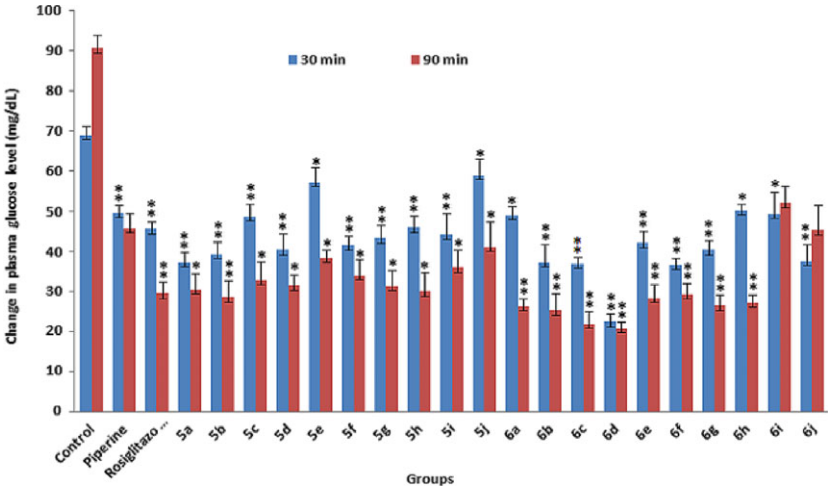


Figure 2: Antidiabetic effect of treating Wistar rats with synthesized compounds by oral glucose tolerance test. Data is analyzed by one way ANOVA followed by Dunnett test and expressed as mean ± SEM from six observations; * represents change as compared to control; ** indicate $p < 0.01$ & * indicates $p < 0.05$.

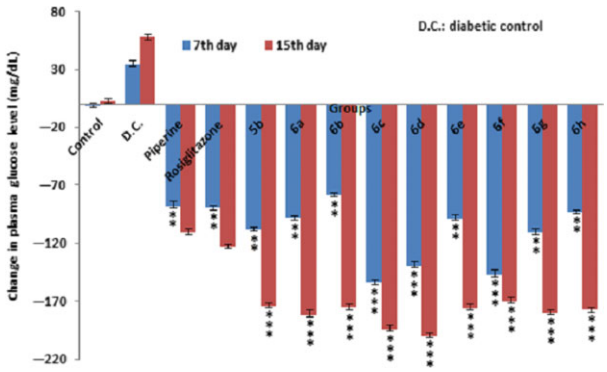


Figure 3: Antidiabetic effect of active synthesized compounds on STZ-induced diabetic animals. Data is analyzed by one way ANOVA followed by Dunnett test and expressed as mean ± SEM from six observations; * represents change as compared to diabetic control; ** indicates $p < 0.01$ & *** indicates $p < 0.001$.

piperine with benzothiazole elevated antidiabetic potential of piperine which is the main objective of study.

Structure–activity relationship

From the results, following structure–activity relationship was drawn:

- the derivatives containing C(O)-N-N bond were found to be more active as antidiabetic agent and showed higher dock score than derivatives with amide bond.

- the derivatives substituted with nitro group were least active as antidiabetic agents.

- methyl substitution on benzothiazolyl ring imparted less significant antidiabetic activity than ethoxy followed by methoxy substitution.

- among halide substitutions, fluoro substitution on benzothiazolyl ring illustrated least antidiabetic potential.

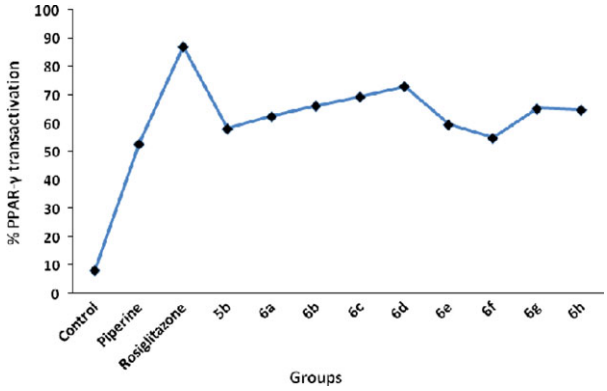


Figure 4: *In vitro* PPAR-γ transactivation assay on 3T3-L1 cell line.

In vitro *PPAR transactivation assay*

The consistency between docking results and *in vivo* studies demonstrated that the activity of synthesized piperine derivatives might be as a consequence of their action on PPAR-γ. The active derivatives were hence analyzed by *in vitro* PPAR transactivation with intent to validate the results of all the studies performed. The derivatives **5b, 6a-h** extensively transactivated PPAR-γ but to a lesser extent than rosiglitazone whereas piperine exhibited very less PPAR-γ transactivation (Figure 4). This clearly affirmed the activation of PPAR-γ by synthesized derivatives. The results of *in vitro* PPAR-γ transactivation assay, despite in agreement with *in vivo* results, were less significant. This deviation might be attributed to the synergistic effect of other genes and mediators along with PPAR-γ in *in vivo* systems.

Body weight

One of the major concerns of oral antidiabetic treatments is increase in body weight. PPAR-γ plays a vital role in management of lipids and encompasses differentiation of

pre-adipocytes into adipocytes resulting in weight gain (33). It is therefore necessary to maintain a proper balance of pleiotropic gene, PPAR-γ in order to improve glucose homeostasis and prevent adipogenesis at the same time (34). However, piperine was not found to be involved in weight gain, but the effect of synthesized derivatives on body weight of diabetic animals post-treatment was studied carefully as they behaved as PPAR-γ agonists. The data of nine active derivatives (**5b, 6a-h**) is presented in Figure 5. It was found that the treatment with active derivatives **6a, 6c, 6d,** and **6f** substantially controlled and did not allow unusual rise in body weight as observed with rosiglitazone after 15 days of study. Weight of rats treated with the derivatives **6a, 6c, 6d,** and **6f** was increasing gradually and not abruptly unlike observed in case of marketed oral treatments against diabetes. The remaining active derivatives **(5b, 6b, 6e, 6g,** and **6h)** also maintained body weight but not as appreciably as derivatives **6a, 6c, 6d,** and **6f** during the course of study.

Hepatotoxicity

Hepatic toxicity is another major drawback of oral antidiabetic drugs and remains a major reason for drug withdrawal from pharmaceutical development and clinical use. In some of the initial PPAR agonists, hepatotoxicity was a serious concern (35). Keeping these reports in view, the effect on hepatic tissue after treatment with two most active derivatives **6c** and **6d** was analyzed. The animals of diabetic control showed enormous degeneration of tissues and fatty changes in the liver lobule. The fatty change was mainly seen in the centrilobular area (Figure 6). Conversely, treating the groups with piperine, **6c,** and **6d** minimized the changes caused by the induction of diabetes and presented normal histological pattern in the hepatic tissue.

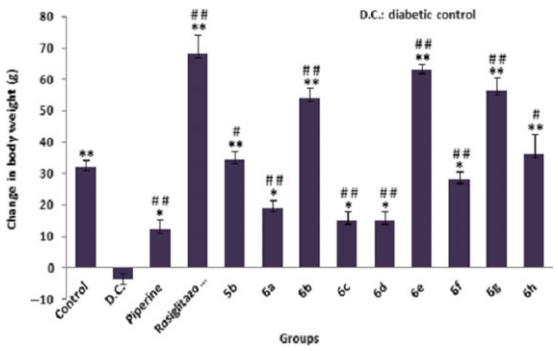


Figure 5: Change in body weight after 15 days of study on STZ-induced diabetic rats. Data is analyzed by one way ANOVA followed by Bonferroni '*t*' test and expressed as mean ± SEM from six observations; * represents significant change as compared to diabetic control; # represents significant change as compared to normal control. ** indicates $p < 0.01$ & * indicates $p < 0.05$; ## indicates $p < 0.05$ & # indicates $p < 0.01$.

Lipid peroxidation

Toxicity and degenerative processes are generally associated with lipid peroxidation (LPO). The results showed that the induction of animals with STZ (diabetic control) elevated lipid peroxide level by 2.43-fold. It was observed that the derivatives **6c** and **6d** significantly suppressed the production of LPO content. Derivatives **6c** and **6d** increased LPO level merely by 11.29% and 8.87%, respectively, whereas rosiglitazone exhibited comparatively less significant inhibition and showed 38.70% increase (Figure 7). The protective effect of these derivatives against hepatotoxicity might be attributed to the lesser production of LPO content after administration of these derivatives.

PPAR-γ gene expression

The effect of the most promising antidiabetic derivatives **6c** and **6d** on the expression of PPAR-γ target gene was also evaluated (Figure 8). The standard drug rosiglitazone enhanced PPAR-γ expression by 1.5-folds, while piperine was found to amplify the expression comparatively to much less extent (0.71 folds). However, both the active derivatives **6c** and **6d** exhibited 1.12- and 1.27-folds augmentation in PPAR-γ expression, which was found to be more than piperine but less effective than rosiglitazone.

Chemistry

A focused library of twenty piperine derivatives was synthesized. The structures **(5a-j, 6a-j)** were determined by spectral data and elemental analysis. Melting points were determined on digital instrument and are uncorrected. The amide coupling in **5a-j** derivatives was confirmed by the presence of singlet of NH proton in aromatic region at δ 7.52–7.62 p.p.m. The coupling with substituted benzothiazole hydrazine (**6a-j**) was confirmed by the appearance of two singlets due to NH protons, one singlet appearing in the aromatic region (δ 7.47–7.60 p.p.m.) and other at δ 4.80–4.90 p.p.m. The protons on conjugated double bonds also appeared downfield as a multiplet in the range of δ 6.50–6.90 p.p.m. ESI-MS of all derivatives showed $[M+H]^+$ peaks. Elemental analysis was carried out on CHNS Elementar (Vario EL III). The experimental data of each compound has been provided in supplementary information.

Conclusion

It can be concluded from the present study that the most of synthesized piperine derivatives are potential antidiabetic agents with maximum effect observed for compounds **6c** and **6d**. These compounds exhibited their effect by enhancing PPAR-γ gene expression. Moreover, the compounds **6c** and **6d** did not allow unusual gain in body weight which is generally observed in subjects suffering from diabetes. The library of these derivatives can be

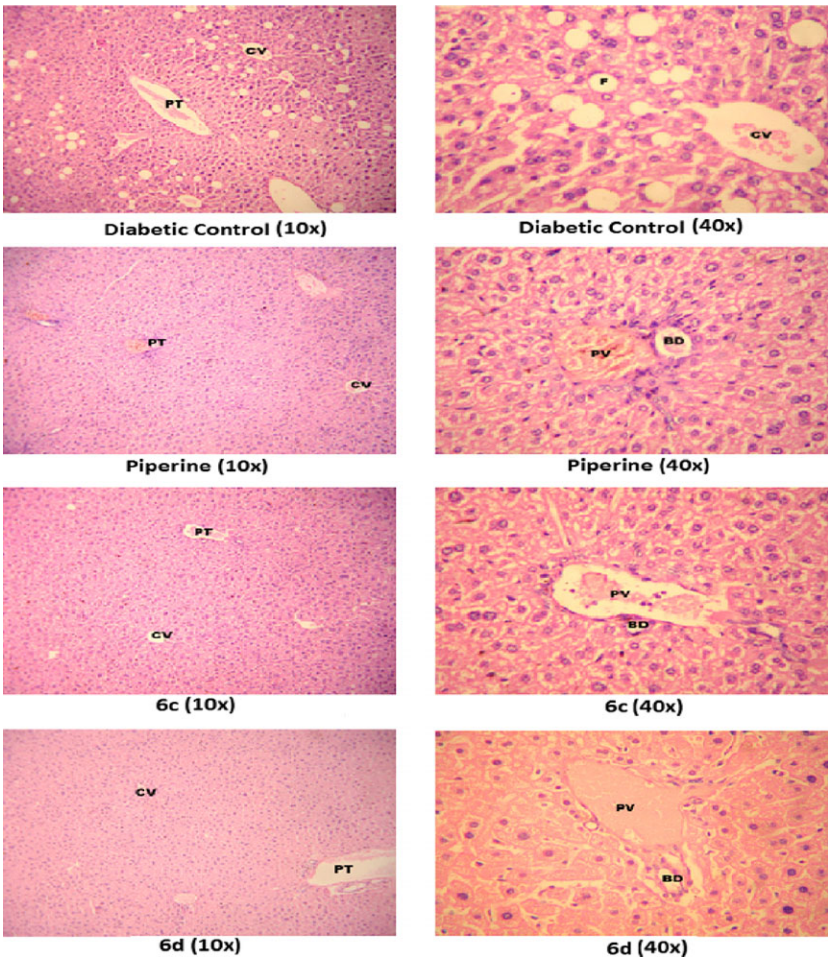


Figure 6: Histological study of STZ-induced rat liver. Where; PT, Portal triad; BD, bile duct; CV central vein; F, fatty change in Hepatocyte.

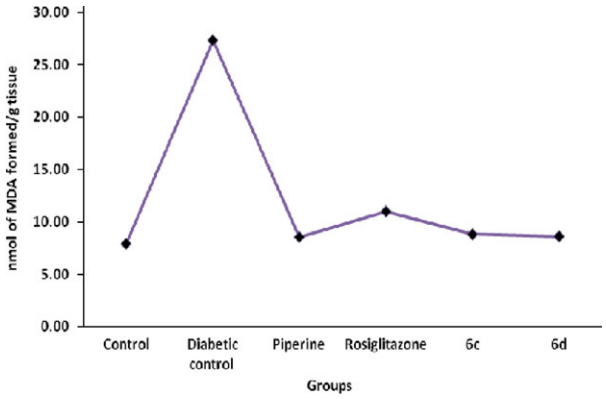


Figure 7: Lipid peroxidation assay of compounds **6c** and **6d.**

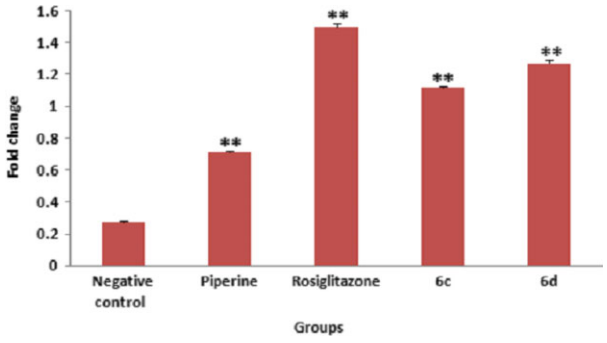


Figure 8: PPAR-γ gene expression evaluation of compounds **6c** and **6d** in comparison with standards, piperine and rosiglitazone. Data is analyzed by one way ANOVA followed by Dunnett test and expressed as mean ± SEM from three observations. * represents change as compared to control; ** indicates $p < 0.01$.

therefore taken into further consideration to develop prospective antidiabetic drug with fewer side-effects.

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Conflict of Interest

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Appendix S1. Spectral data.

Table S1. Dock score of all the synthesized compounds.