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Evaluation of 4-Amino-5-benzofuran-2-oyl-2-(4-methoxyphenyl)aminothiazole for antidiabetic applications: an *in vitro* and *in vivo* study

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ABSTRACT:

The aim of the present study is to investigate the *in vitro* and *in vivo* antidiabetic activity of 4-amino-5-benzofuran-2-oyl-2-(4-methoxyphenyl)aminothiazole. Toxicity and *in vitro* antidiabetic study of the compound was carried out in L6 cell lines. *In vivo* antidiabetic studies were carried out in alloxan induced diabetic Wistar albino rats. In toxicity study, the compound showed an IC_{50} value of 973.62 µM. Significant changes in glucose uptake and Glut 4 translocation by 4-amino-5-benzofuran-2-oyl-2-(4-methoxyphenyl)aminothiazole suggested that the molecular mechanism of action is similar to that of insulin. *In vivo* treatment with 4-amino-5-benzofuran-2-oyl-2-(4-methoxyphenyl)aminothiazole at a concentration of 60 mg/kg bodyweight significantly decreased blood glucose and glycosylated haemoglobin levels in diabetic rats than in non-

diabetic control rats. Oral administration of 4-amino-5-benzofuran-2-oyl-2-(4-methoxyphenyl)aminothiazole to the experimental groups also showed a highly significant reduction in total cholesterol level when compared with diabetic control rats These findings revealed that 4-amino-5-benzofuran-2-oyl-2-(4-methoxyphenyl)aminothiazole can be considered as a lead molecule for the control of type II diabetes.

Keywords: 4-Amino-5-benzofuran-2-oyl-2-(4-methoxyphenyl)aminothiazole, Glucose uptake, Glut 4, SGOT, SGPT, liver marker enzymes

INTRODUCTION:

Diabetes mellitus, a serious metabolic disease, arises from an acquired deficiency in the production of insulin by pancreas or by the desensitization of insulin receptors for insulin. Secondary complications of diabetes mellitus such as neuropathy, nephropathy and retinopathy further aggravate the outcome of the disease[1].

The screening studies for the identification of insulin sensitizing agents to counteract insulin resistance for the treatment of diabetes mellitus continue to be an active research area. Glucose uptake from blood in muscles and adipocytes are stimulated by inducing glucose transporter 4 to the plasma membrane by insulin[2]. Any compound that is capable of inducing Glut 4 expression and translocation to plasma membrane will be able to enhance glucose uptake.

Diaminothiazoles[3] have been shown to be cytotoxic towards cancer cell lines and to exhibit antiangiogenic activity at low concentrations[4]. A recent work from our group reports the mechanochemical synthesis of derivatives of diaminothiazoles and a study of their *in vitro* alpha-glucosidase and alpha-amylase inhibition activities[5]. Among the few diaminothiazoles studied thus, 4-amino-5-benzofuran-2-oyl-2-(4-methoxyphenyl)aminothiazole was found to be the potent alpha-glucosidase and alpha-amylase inhibitor. During the above study, we became aware that there exists no detailed report on the antidiabetic potential of 2,4-diaminothiazoles.

Consequently, the present study reports an investigation on the glucose uptake and Glut 4 translocation in L6 cell lines and the results of an *in vivo* screening of the antidiabetic effect of 4-amino-5-benzofuran-2-oyl-2-(4-methoxyphenyl)aminothiazole using Wistar albino rat models.

MATERIALS AND METHODS:

Chemicals and reagents

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum, antibiotic antimycotic solution, phosphate buffered saline etc. were obtained from Himedia (Pennsylvania, USA). MTT (3-(4,5–dimethyl thiazol-2-yl)-5-diphenyl tetrazolium bromide) was purchased from VWR International LLC, Solon. 2-NBDG (2-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino-2-deoxy-D-glucose) was purchased from Molecular Probe (Invitrogen Life Technologies, Carlsbad, CA, USA) and FITC Goat Anti-Rabbit IgG was purchased from BD Biosciences (San Jose, California). Rosiglitazone, alloxan, glibenclamide, trypsin etc. were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). All other chemicals used were of standard analytical grade.

Synthesis of 4-amino-5-benzofuran-2-oyl-2-(4-methoxyphenyl)aminothiazole

Cyanamide (0.5 mmol, 22 mg) and powdered potassium hydroxide (0.6 mmol, 30 mg) were stirred in DMF (2 ml) for 5 min at room temperature and to this mixture, 4-methoxyphenyl isothiocyanate (0.5 mmol, 0.07 ml) in DMF was added dropwise with stirring for 30 min. The reaction mixture was further stirred for another 90 minutes. To this, cyanamide (0.5 mmol, 22 mg) was added again and after further stirred at room temperature for an additional 90 min. The reaction mixture containing 1-cyano-3-(4-methoxyphenyl)thiourea formed thus was treated *in situ* with 2-bromoacetylbenzofuran (0.5 mmol, 119 mg) and stirred for another 30 min. Triethylamine (1 mmol, 0.12 ml) was then added, followed by stirring for 30 min. The golden brown reaction mixture so obtained was stirred into ice cold water and the 4-amino-5-

benzofuran-2-oyl-2-(4-methoxyphenylamino)thiazole (PMG) was collected, washed with water and dried. The crude product was crystallized from DMF-water (1:3) mixture as fluorescent yellow microcrystals.

Animal Cell culture

L6 cell line (mouse myoblasts) was purchased from National Centre for Cell Science (NCCS, Pune, India). Cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% antibiotic–antimycotic solution. Cells were sub-cultured as per American Type Culture Collection (ATCC) instructions and maintained at 37˚C in a humidified atmosphere with 5% CO_2 inside incubator to attain 70-80% confluency.

MTT Assay

MTT assay was used to determine the sub-toxic concentration of 4-amino-5-(benzofuran-2-oyl)-2-(4-methoxyphenylamino)thiazole against L6 cell lines. Prior to the assay, good cell viability was confirmed by staining the cells with 0.4% trypan blue stain and counted the cells with Hemocytometer (Neubauer, Marinfield Laboratory). Cells were seeded at log phase in 96-well plates with a count of 20,000 cells per well, without the test agent and allowed the cells to grow for 24h. Appropriate concentrations of PMG (62.5, 125, 250, 500 and 1000 µM) were added in duplicates and incubated for 24h at 37˚C in a 5% CO_2 atmosphere. After the incubation period, 10 µl of MTT reagent (5 mg/mL) was added and incubated for 3h at 37˚C. After the incubation period, the formed purple formazan crystals were dissolved with 100 µl of DMSO and the absorbance was measured at 570 nm using a multi plate reader (ELX 800, BIOTEK).

The cell viability of the cells was determined by the following formula,

% of cell viability

= ((OD from control wells - OD from treated wells) / OD from control wells) x 100

***In vitro* glucose uptake assay using differentiated L6 cells**

Glucose uptake activity of the compound was determined in differentiated L6 cells. Cells were cultured in a 6 well plate at a density of 2 x 10^5 cells/2 ml and incubated at 37˚C for 24h. After incubation, cells were treated with different concentrations of PMG (2.73, 27.3, 273, 410 and 547 µM) along with control, 2 ml glucose free DMEM containing 100 µM 2-NBDG was then added and the cells were incubated for 2 hours. The uptake of 2-NBDG was stopped by removing the incubation medium and the cells were next washed with phosphate buffered saline. Cells in each well were treated with 200 µl trypsin at 37˚C for 3-4 min, followed by 2 ml culture medium. The cells were subsequently transferred directly into 12x75 mm tubes and centrifuged for 5 min at 300 x g at 25˚C (REMI R-8C, REMI, India) followed by resuspension of the cells in 1 ml of phosphate buffered saline. Wells treated with rosiglitazone served as the positive control. The cells were analyzed immediately with a Flow cytometer (BD FACS Calibur, BD Biosciences). Cells taking up 2-NBDG displayed high fluorescence with excitation and emission at 465 and 540 nm respectively and was measured in the FL1 channel which is used to detect FITC.

Glut 4 translocation assay using differentiated L6 cells

Glut 4 translocation study using PMG was done in differentiated L6 cells. Cells were cultured in a 6-well plate at a density of 2 x 10^5 cells/2 ml and incubated overnight in a CO_2 incubator at 37˚C. After 24 hours, the spent media was aspirated and the cells were treated with 3 different concentrations of PMG (2.5, 25 and 250 µM) and control in glucose-free culture medium (2 ml) and further incubated for 2 hours. At the end of the treatment, the media was removed from all the wells and the cells were washed with phosphate buffered saline. After washing, the saline was removed, cells were detached with trypsin followed by the addition of culture medium (2 ml) to the wells and the cells were harvested directly into 12 x 75 mm tubes. The tubes were next centrifuged for 5 min at 300 x g at 25˚C followed by aspiration of the supernatant and

resuspension of the cells in phosphate buffered saline (PBS) (0.5-1 ml). The cells were incubated with primary antibody (Mouse Anti-Human Glut 4 monoclonal antibody) for 30 min on ice and then PBS was added (2 ml). The cells were washed again, resuspended in FACS buffer (100 µl) and incubated for 30 min in the dark and on ice with FITC labeled Goat Anti-mouse IgG. Samples were analyzed using a BD FACS Calibur Flow Cytometer (BD Biosciences, San Jose, CA, USA) equipped with an argon laser (wavelength 488 nm) using BD Cell Quest Pro™ Software (Version 6.0). Gates were set on M1 and M2. No permeabilization step was done as the study was focused on the GLUTs located to the surface of the cell membrane of the investigated cells. The mean fluorescence intensity was used for the analysis and interpreted as the expression of investigated GLUT isoforms.

Experimental animals

Healthy Wistar albino rats of either sex, weighing about 150-200 g were used for the study. The entire study was approved by the Institutional Animal Ethical Committee (IAEC), certified by the Committee for the Purpose of Control and Supervision of Experiments on Animals (IAEC/PDF/KMCP/23/2018) with institutional guide lines for the care of laboratory animals at KM college of Pharmacy, Madurai, Tamil Nadu. The animals were kept in clean and dry polycarbonate cages and maintained in a well-ventilated animal house in standard laboratory conditions (temperature 22±2˚C and humidity 45±5˚C with 12h day: 12h night cycle). The animals were fed with standard pellet diet and water was given *ad libitum*. For experimental purpose, the animals were kept fasting overnight but were allowed free access to water.

***In vivo* evaluation of antidiabetic potential**

Evaluation of acute toxicity

Acute toxicity was determined according to OECD guidelines 423 (Organization of Economic Co-operation and Development)[6]. Different defined doses of PMG (5, 50, 300,

2000 mg/kg body weight) were used in the method. Three healthy, wistar albino rats weighing 150-200 g were selected for the present study. The rats were fasted over-night and provided with water *ad libitum*. Following the period of fasting, the animals were treated with PMG at the dose of 300 mg/kg body weight, orally.

After oral administration, the rats were observed on hourly basis for 24 hours to assess mortality and to detect any changes in the autonomic or behavioral responses viz. alertness, spontaneous activity, salivation, respiration, urination, aggressiveness, irritability, convulsion, corneal reflex etc. The rats were observed regularly for 14 days to note the mortality or toxic symptoms. Since there was no death as per the guidelines, the study was repeated with the same dose to confirm the results.

Study design and animal grouping to assess antidiabetic potential *in vivo*

The alloxan induced diabetic rat is one of the animal models of human insulin dependent diabetes mellitus (IDDM) or type I diabetes mellitus. The rats were fasted for 12 hrs. Diabetes mellitus was induced in experimental animals by single dose by intra peritoneal administration of alloxan (150 mg/kg body weight). The injection volume was prepared to contain 1 ml/kg body weight[7]. Another six rats served as the normal control group.

Alloxan induced diabetes within 3 days by destroying the β-cells[8]. The animals were then kept for next 24 hrs in cages containing 5% glucose solution bottles to prevent the initial hypoglycemia. The control rats were injected with saline only. After 72h of alloxan administration, the blood glucose content was measured by using glucometer, a blood sample from the tail vein. The animals with blood glucose levels ≥ 180 mg/dL were considered to be diabetic and used for the experiment[9].

In the present investigation, a total of 30 rats (24 diabetic surviving and 6 non-diabetic control) were taken and divided into five groups of 6 rats each to determine the antidiabetic

activity of PMG. The actions of the extracts were compared with that of the standard oral hypoglycemic agent, glibenclamide.

Group I : Normal rats with *ad libitum* water and food

Group II : Diabetic rats with *ad libitum* water and food

Group III: Diabetic rats treated with standard drug glibenclamide at the dose of 0.5 mg /kg bw, orally for 28 days.

Group IV: Diabetic rats treated with PMG 30 mg/kg bw, orally for 28 days.

Group V: Diabetic rats treated with PMG 60 mg/kg bw, orally for 28 days.

Histopathological study

For histological examination, the liver tissues were stored in 10% formalin. Tissues were taken out and trimmed to optimal size and embedded in histology grade paraffin wax after dehydrating in gradient percentage of alcohol. These blocks were later sectioned using a microtome, and stained with haematoxylin and eosin.

STATISTICAL ANALYSIS:

In vivo experiments were done in sextuplicates (n=6). Data were reported as mean ± SD. The data were subjected to two-way analysis of variance (ANOVA), and the significance of differences between means was calculated by Duncan's multiple range test using SPSS for Windows, standard version 11.5 and the significance accepted at $P \leq 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

MTT assay

The cytotoxicity of PMG was assessed against L6 cell lines at concentrations of 62.5, 125, 250, 500 and 1000 μM. The results indicate that PMG is comparatively safe to cells and showed toxicity against L6 cells with IC_{50} value of 973.62 μM after an incubation period of 24 hours at

37˚C.

***In vitro* hypoglycaemic effect of 4-amino-5-(benzofuran-2-oyl)-2-(4-methoxyphenylamino)thiazole: Stimulates 2-NBDG uptake in L6 myotubes**

To establish whether PMG can stimulate the glucose absorption in L6 myotubes, we evaluated the effect on 2-NBDG uptake. The assay was planned in differentiated L6 myotubes and the cells were pretreated with varying concentrations of PMG (2.73-547 µM) for 1h. The results indicated a dose dependent increase in the uptake of 2-NBDG in myotubes. The test compound, PMG with different concentrations (2.73, 27.3, 273, 410 and 547 uM) showed 1.40, 1.45, 1.56, 3.01 and 4.60 fold increase in 2-NBDG-uptake in terms of Mean Fluorescence Intensity (MFI) compared to the untreated cells. The standard drug rosiglitazone showed 13.45 fold increase in 2-NBDG uptake at 100 µM concentration (Fig. 1 & 2).

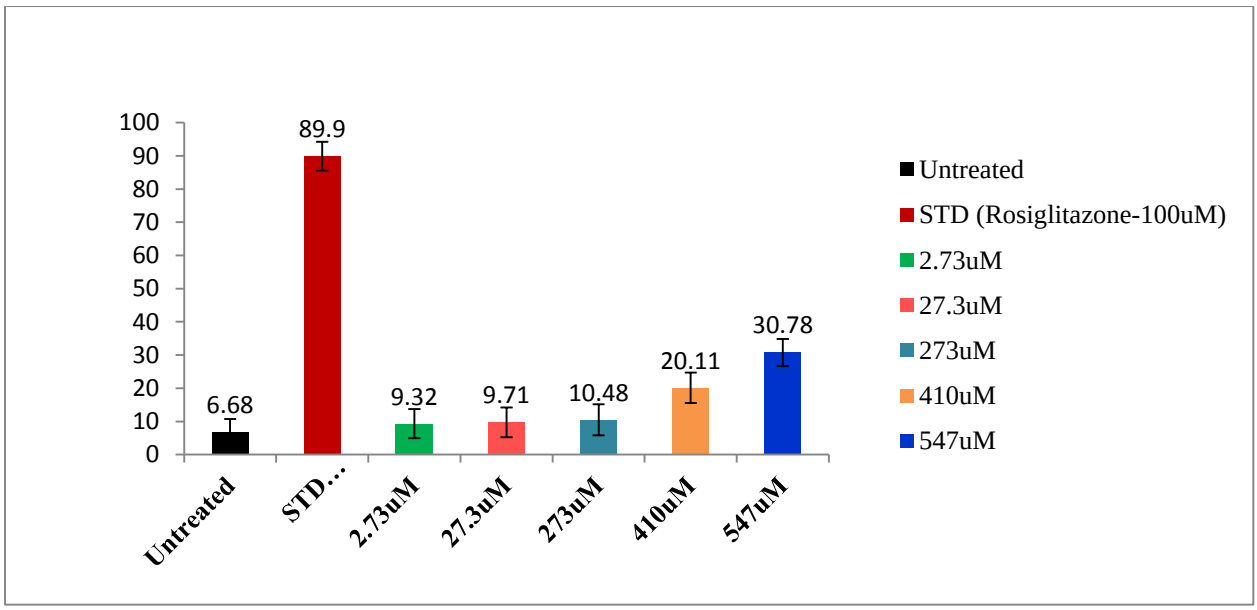

Fig. 1. NBDG Mean Fluorescence Intensity of test compounds along with control

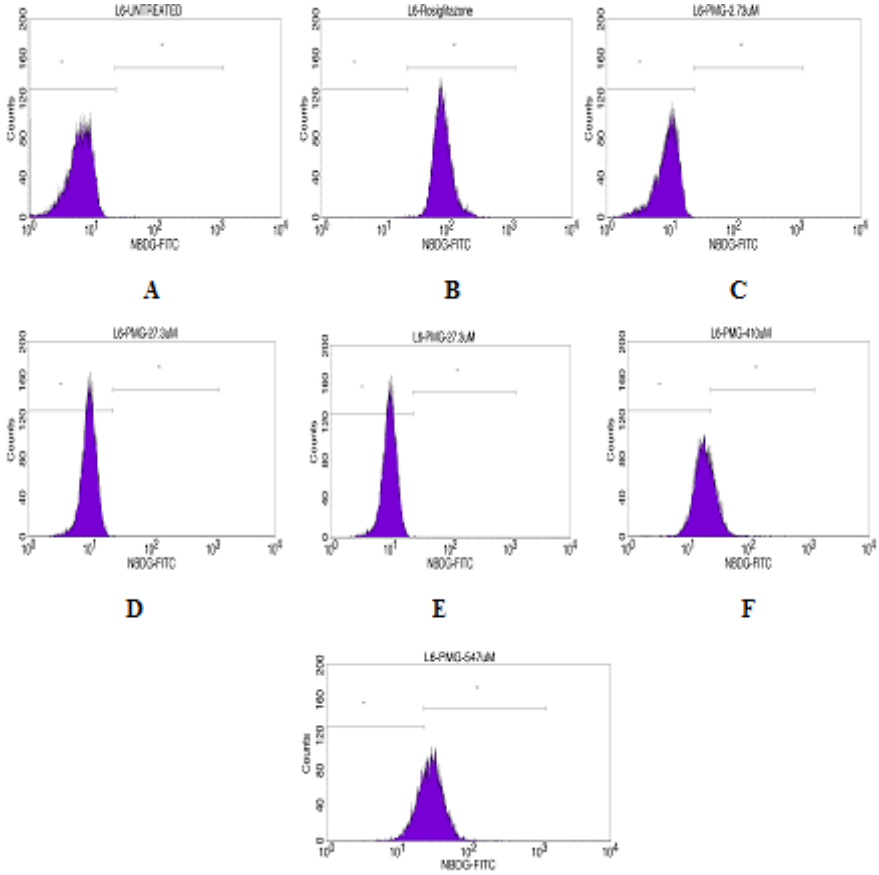

Fig. 2. Glucose Uptake study of PMG against L6 cells using BD FACS calibur

(NBDG-FITC histogram of the gated L6 singlets distinguishes cells at the M1 and M2 phases: Here, M1 refers to negative expression/region and M2 refers to the positive expression/region) **A)** Control cells **B)** Cells treated with standard rosiglitazone (100 µM) **C**) Cells treated with PMG (2.73 µM) **D)** Cells treated with PMG (27.3 µM) **E)** Cells treated with PMG (273 µM) **F)** Cells treated with PMG (410 µM) and **G)** Cells treated with PMG (547 µM)

PMG stimulates Glut 4 expression in L6 myotube cell surfaces

Glucose transporter 4 is considered as the major glucose transporter surface protein expressed in muscles. It plays an important role in the regulation of insulin stimulated glucose uptake. In the present study, different concentrations of PMG (2.5, 25 and 250 µM) showed 2.38, 3.53 and 5.40 fold increase of Glut 4 expression in terms of Mean Fluorescence Intensity compared to

untreated control cells. The standard drug rosiglitazone showed 6.62 fold increase of Glut 4 expression at 100 µM concentration (Fig. 3 & 4).

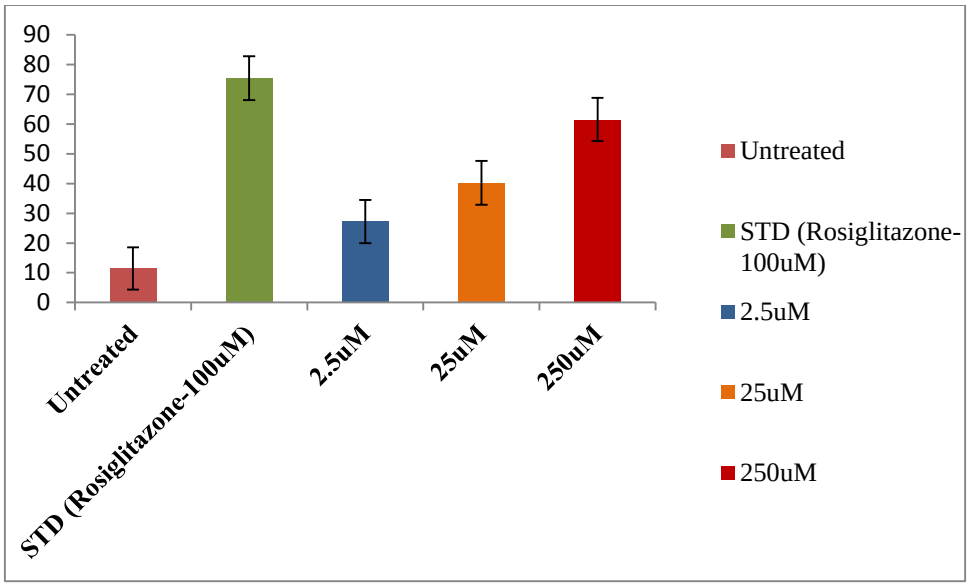

Fig. 3. Mean Fluorescence Intensity of test compounds along with control

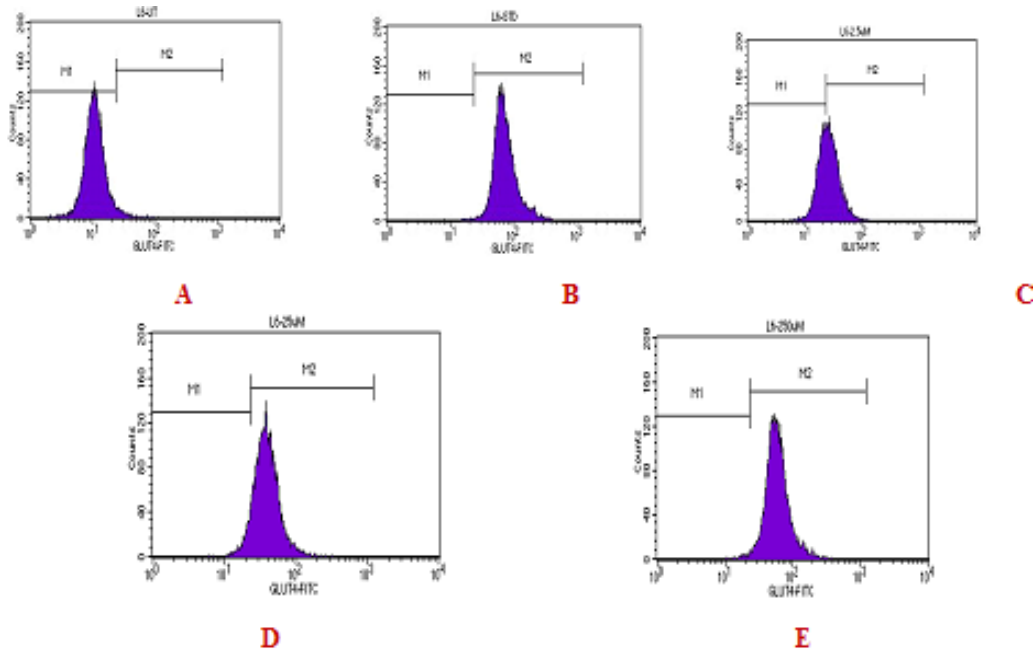

Fig. 4. Change in surface expression of GluT4 in response to PMG treatment were checked by flow cytometer. Population M1 refers to unresponsive cells and M2 regions corresponds to GluT4 expressed cells

A) Control cells **B)** Cells treated with standard rosiglitazone (100 µM) **C)** Cells treated with PMG (2.5 µM) **D)** Cells treated with PMG (25 µM) **E)** Cells treated with PMG (250 µM)

***In vivo* evaluation of antidiabetic potential**

Safety evaluation of PMG was carried out *in vivo* using Wistar albino rats as model organism. There was no mortality observed even at 300 mg/kg dosage for the PMG. All animals were found to be normal and there were no behavioral changes till the end of the observation period. Thus, PMG was found to be safe up to 300 mg/kg of body weight, known as maximum tolerated dose (MTD) by acute toxicity model study as per OECD guidelines 423. Hence in this experiment, $1/10^{th}$ and $1/5^{th}$ quantity of MTD were selected and the effective dose was fixed as 30 and 60 mg/kg body weight for further pharmacological studies.

Blood glucose changes in Type 2 diabetes rats

Administration of alloxan significantly increased the blood glucose level compared to normal control rats. Blood glucose level was determined in various groups of experimental animals at frequent intervals of 7 days for a period of 28 days. The blood glucose level decreased significantly after an oral administration of varying doses (30 and 60 mg/kg bw) of PMG and glibenclamide than that seen in diabetic control rats ($p<0.001$) (Table 1).

Table 1
Blood glucose level analysis of PMG

Treatment groups	Blood glucose level (mg/dL)				
	Days of treatment				
	0	**7**	**14**	**21**	**28**
Normal control	82.35 ± 2.25	81.62 ±1.12	83.23 ± 1.23	80.71 ± 1.21	80.58 ± 1. 54
Diabetic control	201.42 ± 3.55	222.83 ± 6. 48	234.44 ± 2.71	243.85 ± 5. 41	235.22 ± 3.26
Standard control Glibenclamide (0.5 mg/kg bw)	202. 60 ± 2.14	155. 76 ± 1. 23	142. 26 ± 2.81	123.60 ± 1.80	100.53 ± 2.81
Treatment with PMG (30 mg/kg bw)	181.23 ± 2.22*	171.71 ± 2.63	145.23 ± 2.72	141.21 ± 2.72**	127.54 ± 2.35**
Treatment with PMG (60 mg/kg bw)	181.28 ± 2.50*	164.83 ± 2.48	145.27 ± 1.56	139.41 ± 2.30**	125.66 ± 2.80**

All values are expressed as mean ± SEM for 6 animals in each group.
*p<0.05, **p<0.01 shows significance between normal control vs diabetic control and drug treated groups

Lipid profile analysis

The lipid profile of different groups of experimental animals was determined and results are presented in Table 1. The level of total cholesterol in blood of alloxan-induced diabetic rats (Group II) were found to be significantly ($p<0.01$) elevated when compared with normal control rats (Group I). Oral administration of PMG to the experimental groups (Group IV & V) showed a highly significant ($p<0.001$) reduction in total cholesterol level when compared with diabetic control rats (Group II). Administration of PMG was able to restore the triglyceride and lipoprotein levels significantly ($p<0.001$) on diabetic induced rats. In restoration of HDL, 60 mg/kg dose of PMG showed a promising effect, even though less significant ($p<0.1$). Compared to diabetic control rats, administration of 30 and 60 mg/kg dose of PMG significantly reduced the LDL-C levels ($p<0.01$), which resulted in better values compared to normal control.

Table 2
Lipid profile analysis of PMG

Treatment groups	Total cholesterol IU (mg/dL)	Triglycerides IU (mg/dL)	HDL IU (mg/dL)	LDL IU (mg/dL)
Normal control	1.52 ± 0.25	2.78 ± 0.13	1.15 ± 0.03	0.87 ± 0.05
Diabetic control	3.28 ± 0.80	3.70 ± 0.16	0.77 ± 0.05	1.05 ± 0.11
Standard control Glibenclamide (0.5 mg/kg bw)	1.66 ± 0.18	2.28 ± 0.07	1.26 ± 0.03	0.76 ± 0.07
Treatment with PMG (30 mg/kgbw)	1.70 ± 0.03***	2.75 ± 0.46***	0.51 ± 0.06	0.84 ± 0.02*
Treatment with PMG (60 mg/kg bw)	1.55 ± 0.054***	2.30 ± 0.55***	0.81 ± 0.05	0.78 ± 0.12**

All values are expressed as mean ± SEM for 6 animals in each group.
*p<0.05, **p<0.01, ***p<0.001 shows the significance between normal control vs diabetic control and drug treated groups

Analysis of liver marker enzymes

The levels of liver marker enzymes in serum were increased significantly (p<0.001) in diabetic rats (Group II) when compared to normal control group (Group I). Administration of both doses of PMG restored the SGOT highly significant (p<0.001) on diabetic induced rats. In restoration of SGPT, high dose of PMG (p<0.001) showed highly significant effect whereas low dose showed only significant (p<0.01) effect. Histopathological studies showed that (Fig. 5) there was less significant effect (p<0.05) found with low dose on reduction of ALP whereas higher dose of PMG significantly (p<0.001) reduces the ALP in diabetes induced rats (Table 3).

Table 3

Analysis of PMG on liver marker enzymes

Treatment groups	Hb (mg/dL)	Glycosylated hemoglobin (mg/dL)	SGOT	SGPT	ALP
Normal control	13.24 ± 1.2	4.16 ± 0.25	25.15 ± 2.35	30.70 ± 1.60	142.38 ± 4.84
Diabetic control	10.81 ± 0.35	12.15 ± 1.09	56.10 ± 4.13	50.73 ± 1.14	164.05 ± 5.17
Standard control Glibenclamide 0.5 mg/kg bw	14.26 ± 1.35	5.08 ± 1.71	25.78 ± 2.03	32.19 ± 1.01	155.09 ± 43.28
Treatment with PMG (30 mg/kg bw)	12.13 ± 0.12*	7.09 ± 1.19*	26.03 ± 1.14***	38.39 ± 5.05**	160.25 ± 5.18*
Treatment with WEBL 60 mg/kg bw)	12.11 ± 1.90*	4.29 ± 1.50**	23.52 ± 1.42***	32.32 ± 0.30**	160.84 ± 3.70*

All values are expressed as mean ± SEM for 6 animals in each group.

*p<0.05, **p<0.01, ***p<0.001 shows the significance between normal control vs diabetic control and drug treated groups

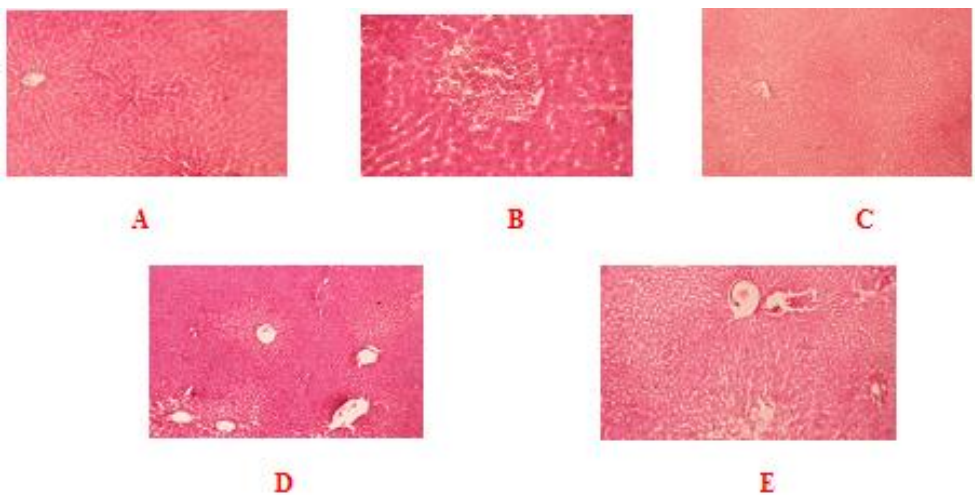

Fig. 5. Images showing histopathological results of liver from control rats treated with different concentration of PMG.

A) Normal control liver **B)** Diabetic control liver **C)** Standard control liver (Glibenclamide 0.5 mg/kg bw) **D)** Treated with low dose of PMG 30 mg/kg bw **E)** Treated with high dose of PMG (60 mg/kg bw)

CONCLUSION:

The results of the present study indicate that 4-amino-5-(benzofuran-2-oyl)-2-(4-methoxyphenylamino)thiazole is a lead molecule of a new class of antidiabetic compounds that would enhance the glucose uptake activity as it showed significant antidiabetic activity in diabetic rats. The *in vitro* results provided additional support for the results obtained in *in vivo* studies. The results of both the 2-NBDG uptake study and Glu4 expression study strongly suggest that PMG has promising anti diabetic potential.

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