Pharmaceutical Sciences Asia

Pharm Sci Asia 2025; 52(3), 417-426
DOI:10.29090/psa.2025.03.25.4795

Research Article

Piroxicam attenuates oxidative stress in glucolipotoxic C2C12 myotubes and protein glycation damage: Models relevant to T2DM pathophysiology

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ABSTRACT

Type 2 diabetes mellitus (T2DM) is characterized by insulin resistance and associated oxidative and glycative stress, contributing to disease progression and complications. Non-steroidal anti-inflammatory drugs (NSAIDs) such as piroxicam (PIR) and naproxen (NAP) have shown potential repurposable antidiabetic effects, partly through inhibition of dipeptidyl peptidase-4 (DPP-4), an enzyme regulating glucose metabolism. This study evaluated PIR and NAP (10 μM) in a glucolipotoxic (GLT) cellular model of insulin resistance. C2C12 mouse myotubes were incubated in standard tissue culture media, or media supplemented with 28 mM glucose, 200 μM palmitic acid, and 200 μM oleic acid as a cellular model of diabetic glucolipotoxicity. GLT conditions elevated intracellular reactive oxygen species (ROS) by 73.5% ± 9.4% ($p < 0.05$). Both NAP and PIR significantly reduced ROS levels by 87.0% ± 14.6% and 74.1% ± 9.9%, respectively ($p < 0.05$). PIR (10 μM) was prioritized for further assessment for effects on glucose uptake in the same model where it enhanced glucose uptake by 191.7% ± 98.6% ($p > 0.05$). Subsequently, PIR's biochemical activities (40 ppm) were evaluated in cell-free assays. PIR significantly inhibited AGE formation in the BSA-methylglyoxal model, reducing fluorescence by 245.4% ± 36.3% at day 7 and 145.2% ± 29.2% at day 14 ($p < 0.001$). It showed a protective trend against amyloid fibril formation, decreasing Congo red absorbance by 55.0% ± 3.8%, although this effect was not statistically significant ($p > 0.005$). Pancreatic lipase inhibition was limited (~12%, $p > 0.05$), indicating a lack of anti-obesity potential at tested concentrations. While PIR shows promising antioxidative and antiglycation effects, further studies are needed to fully elucidate its clinical relevance in T2DM management.

Keywords:
Type 2 diabetes; Non-steroidal anti-inflammatory drugs; Glucolipotoxicity; Reactive species scavenging; Anti-glycation

1. INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a global public health crisis, affecting an estimated 588.7 million adults as of 2024, with projections reaching 852.5 million by 2050[1]. In the Philippines, over 4.3 million individuals were diagnosed in 2021, making diabetes the fifth leading cause of mortality[2]. T2DM is strongly associated with obesity, sedentary lifestyles, and diets high in refined sugars and fats, which contribute to insulin resistance, pancreatic β-cell dysfunction, and chronic metabolic imbalance [3–5].

Given the high attrition rates, extended timelines, and financial costs of novel drug development, drug repurposing (DR) has gained attention as a viable strategy for discovering new therapies using existing compounds. Repurposed drugs benefit from established pharmacokinetic profiles and known safety margins, enabling faster clinical translation. DR successes include the use of calcium channel blockers in gestational diabetes,

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and amlexanox, an anti-asthma drug, for glycemic control[6,7]. This strategy may provide insights into disease mechanisms beyond the drugs' original indications.

Nonsteroidal anti-inflammatory drugs (NSAIDs) have recently attracted attention for their potential roles in metabolic disorders, including T2DM. Among these, piroxicam (PIR) and naproxen (NAP) have emerged as candidates due to their distinct mechanisms affecting glucose metabolism and insulin resistance. PIR was identified through computational and in vitro assays as a candidate dipeptidyl peptidase-4 (DPP-4) inhibitor, exhibiting strong binding affinity to the DPP-4 active site and concentration-dependent enzymatic inhibition (IC_{50} of 9.9 μM)[8]. DPP-4 is a clinically validated therapeutic target in T2DM; it is a serine protease that degrades incretin hormones such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP), which enhance insulin secretion and suppress glucagon release in a glucose-dependent manner[9,10]. Beyond its glycemic control, DPP-4 is also implicated in oxidative stress, chronic inflammation, and β-cell dysfunction all of which contribute to the progression of T2DM and its complications[11]. Thus, PIR's DPP-4 inhibition may not only enhance incretin signaling but also mitigate oxidative damage, reduce advanced glycation end product (AGE) formation, and suppress protein aggregation–processes central to diabetic pathophysiology.

NAP, another FDA-approved NSAID, was identified as an inhibitor of glycogen synthase kinase-3β (GSK-3β), a kinase involved in insulin resistance, impaired glycogen synthesis and metabolic inflammation[12]. The drug demonstrated potent in vitro inhibition (IC_{50} = 1.5 μM) and improved metabolic parameters in diabetic and obese mouse models, including reductions in serum glucose and body weight, and increases in insulin, C-peptide, adiponectin, and hepatic glycogen levels[13–16]. While GSK-3β inhibition is a promising therapeutic strategy, it remains less clinically advanced and mechanistically validated in T2DM compared to DPP-4 inhibition. While these mechanisms provide strong scientific bases for repurposing PIR and NAP, this study was designed as an initial screening of therapeutic potential, focusing on measurable bioactivities rather than investigating specific molecular mechanisms. Mechanistic analysis may be pursued in future studies if strong activity is confirmed.

Although both compounds were initially screened for reactive oxygen species (ROS) scavenging activity, PIR was prioritized for further investigation based on the strength of evidence supporting DPP-4 as a druggable, clinically exploited target in T2DM. The translational potential of DPP-4 inhibition, already realized in approved therapies, provided a clearer path for repurposing PIR within a validated therapeutic framework. Subsequent in vitro biochemical assays further evaluated PIR's ability to enhance glucose uptake, protect against protein glycation, inhibit amyloid formation, and reduce obesity-related processes.

To model insulin resistance, a glucolipotoxicity (GLT) model was established using C2C12 mouse skeletal muscle cells exposed to high glucose, palmitic, and oleic acids. Skeletal muscle, accounting for the majority of postprandial glucose disposal, is a critical tissue in metabolic homeostasis and a valuable target for anti-diabetic intervention[17]. The GLT model mimics key features of the disease, including ROS generation, impaired insulin signaling, and reduced glucose uptake[18–20]. While this study focused on PIR, NAP's promising profile suggests it may warrant further exploration in future work.

2. MATERIALS AND METHODS

All chemicals and reagents were purchased from Sigma Aldrich (Science Park Drive, Singapore) through Chemline Scientific Corporation and Krypton Philippines; C2C12 cells were acquired from ATCC (Washington, USA) through ChemoLife Science; and cell culture reagents and plasticwares were purchased from ThermoFisher Scientific through Noveulab and Medtest, Philippines. The luminescence-based glucose uptake kit was purchased from Promega (Madison, Wisconsin).

2.1 Sample preparation

Samples were individually dissolved in sterile distilled water, with minimal drops of sterile DMSO added to aid in the dissolution of clumps, resulting in a negligible final DMSO concentration (< 1%). The solutions were then diluted to final concentrations of 10 μM for cell-based assays and 20-100 ppm for cell-free assays. The 10 μM concentration for cell-based assays was selected based on prior literature using C2C12 myocytes, in order to maintain cell viability and biological relevance. Higher concentrations were used in cell-free assays to compensate for the lack of cellular uptake and to ensure sufficient compound availability for biochemical activity.

2.2 Glucolipotoxicity (GLT) cellular model of T2DM

Mouse C2C12 skeletal myoblasts were maintained in a growth media (GM) comprised of Dulbecco's Modified Eagle Medium (DMEM)-high glucose, supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin-streptomycin, and 1%

(v/v) L-glutamine in a humidified atmosphere with 5% CO_2 at 37°C. At desired confluency, the medium was then switched to DMEM-supplemented with 2% (v/v) heat-inactivated horse serum for 7 days in order to facilitate myocytic differentiation. Cells were then incubated for a further 3 days (± drug treatment) with glucolipotoxicity (GLT) media (28 mM glucose supplemented with 200 µM oleic acid and 200 µM palmitic acid in combination) and the drug sample.

2.3 Reactive species detection assay

Muscle cells were cultured for 5 days in standard tissue culture media, or media supplemented with high glucose and high fatty acids (GLT media). Drug samples were then added and incubated for 1 h. Cells were washed 3 times in phosphate buffer solution (PBS), then 20 µM of freshly prepared and light-protected 2'7'-dichlorofluorescein diacetate (DCFDA) was loaded for 1 h at 37°C under 5% CO_2. Radical species detection was measured via fluorescence (λ_{ex} = 495 nm, λ_{em} = 530 nm) in a Victor X3 Multimode Plate Reader (PerkinElmer, Waltham, Massachusetts, USA). The intensity of 2',7'-dichlorofluorescin (DCF) fluorescence corresponds to the level of intracellular ROS formation. In all cases, reactive species are expressed as percentage change relative to the control.

2.4 Glucose uptake

A non-radioactive bioluminescent Glucose Uptake-Glo™ Assay (Promega) was used. Following the desired treatment (cell ± GLT or GLT± sample), C2C12 cells were serum-starved overnight in DMEM supplemented with 5 mM glucose, then incubated for 1h in glucose-free DMEM ($^{+}/_{-}$) 1 µM insulin. The medium was then replaced with PBS + 2-deoxy glucose (2-DG). Glucose uptake reactions were conducted for 30 min and then terminated by addition of stop buffer (0.4 M HCl + 2% dodecyl trimethyl ammonium bromide). 2DG6P detection reagent was added, and after a 1 h incubation, luminescence reading was done using a CLARIOStar luminometer (BMG Labtech, Germany).

2.5 Anti-glycation assays: BSA-Glucose and BSA-methylglyoxal (MGO) model systems

The method was adapted from Wu et al. (2013) with slight modifications[21]. Bovine serum albumin (BSA) (10 mg/mL) and glucose (90 mg/mL) were dissolved separately in PBS (pH 7.4). A non-glycated (NG) set-up serving as blank was prepared with 1 mL of phosphate buffer and 0.5 mL of BSA solution. Then, 0.5 mL of the drug sample (40 ppm) was mixed with 0.5 mL of bovine serum albumin (BSA) and 0.5 mL of glucose solution in a 5 mL polypropylene test tube. A positive control was prepared using 0.5 mL of AG solution (1 mM) mixed with 0.5 mL of BSA and 0.5 mL of glucose. The tested solution also contained 0.3 mL of NaN_3 (0.01%) to prevent microbe development. The tubes were capped and incubated for 7 days at 37 °C in the dark in a temperature-controlled incubator.

BSA (20 mg/mL) and methylglyoxal (MGO) (60 mM) were dissolved separately in PBS (pH 7.4). The rest of the procedure was the same as that for the BSA-glucose model. The incubation time was extended to 14 days at 37 °C in darkness. The fluorescence of AGEs was measured after an appropriate incubation procedure was conducted at λ_{ex} = 360 nm, λ_{em} = 420 nm for BSA-glucose and λ_{ex} = 370 nm, λ_{em} = 420 nm for BSA-MGO (CLARIOstar Plus, BMB Labtech, Germany).

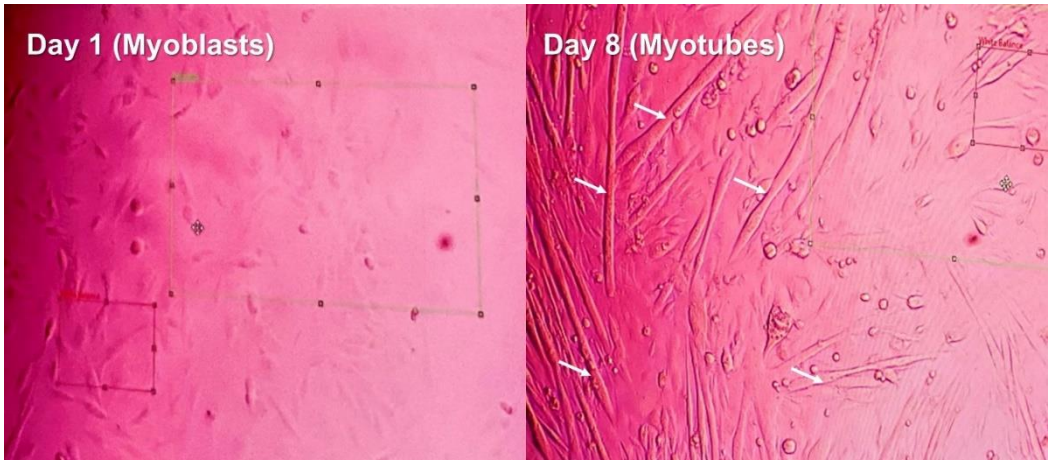

Figure 1. Representative microscopy images of C2C12 myoblast differentiation. Left: Undifferentiated C2C12 myoblasts. Right: Differentiated myotubes after 7 days in 2% heat-inactivated horse serum, showing characteristic elongation and alignment (white arrows). Images were captured using an AmScope inverted microscope with a digital camera at 10× magnification. Scale bars were not included due to lack of calibration data at the time of image capture.

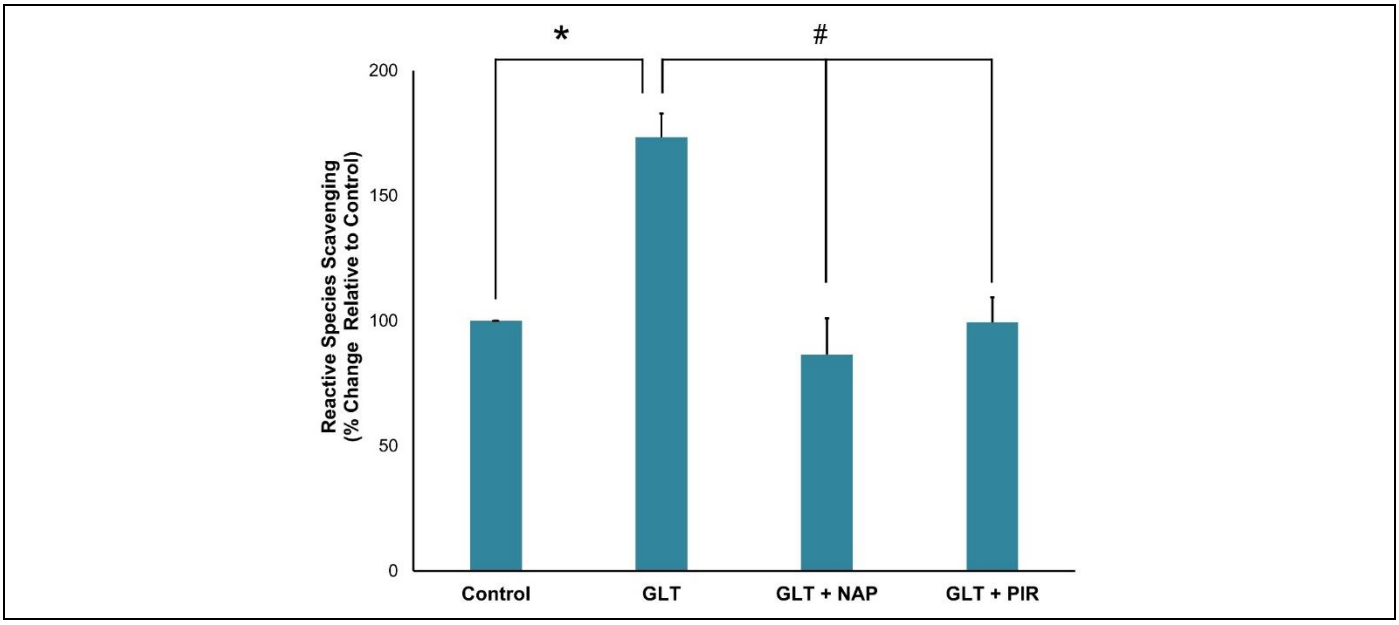

Figure 2. Naproxen (NAP, 10 µM) and piroxicam (PIR, 10 µM) attenuate intracellular reactive species formation in a glucolipotoxic (GLT) C2C12 skeletal muscle cell model C2C12 myotubes were incubated in GLT media (28 mM glucose, 200 µM palmitic acid, 200 µM oleic acid) with or without co-treatment of NAP or PIR. Reactive oxygen species (ROS) were quantified using the DCFDA assay and are expressed as percent change relative to control. Bars represent mean ± SEM from three independent experiments. *p<0.05 vs. Control, #p<0.05 vs. GLT (Tukey's post hoc test).

2.6 Congo red spectrophotometric assay

To preliminarily assess the ability of PIR to prevent modifications in protein tertiary structure, a dye-binding assay using Congo red was performed. BSA (50 mg/mL) and glucose (36 mg/mL) were each dissolved in 0.2 M PBS (pH 7.4) containing 0.01% sodium azide (3 mM NaN_3). For the non-glycated control, 2 mL of PBS was mixed with 1 mL of BSA solution. The treatment setup consisted of 1 mL of PIR sample (40 ppm), 1 mL of BSA solution, and 1 mL of glucose solution in a 5 mL polypropylene tube. A positive control was prepared by mixing 1 mL of aminoguanidine (AG, 2mM), 1 mL of BSA solution, and 1 mL of glucose solution.

All glycated mixtures were incubated at 60 ºC for 1 day to accelerate glycation. After incubation, each setup was divided into two portions: one (1.5 mL) was mixed with 0.7 mL of Congo red solution (75 µM Congo red in PBS 10% v/v ethanol), and the other half was left untreated to serve as background control. Absorbance was measured at 530 nm using a CLARIOstar Plate Reader (BMG Labtech, Germany).

2.7 Pancreatic lipase inhibition assay

Pancreatic lipase (PPL) solution was freshly prepared by suspending crude porcine pancreatic lipase powder in 0.1 M PBS (pH 8.0) to a final concentration of 2.5 mg/mL. The suspension was centrifuged to obtain a clear supernatant. For the assay, 40 µL of the test sample was pre-incubated with 40 µL of the enzyme solution at 37 °C for 10 minutes. Subsequently, 20 µL of the para-nitrophenyl butyrate (PNPB, 10 mM in absolute ethanol) was added, and the reaction mixture was incubated for an additional 20 minutes at 37 °C. Lipase activity was quantified by measuring the release of p-nitrophenol from PNPB at 405 nm using a multimode spectrophotometer (CLARIOstar Plus, BMG Labtech, Germany). Orlistat (100 ppm) was used as a positive control.

2.8 Statistical analysis

All values are expressed as mean ± standard error of the mean (SEM) from three independent experiments. One-way analysis of variance (ANOVA) was used to assess significant differences among groups. Where applicable, Tukey's post hoc test was performed. Differences were considered statistically significant at $p < 0.05$. Analyses were conducted using GraphPad Prism version 10.2.1.

3. RESULTS

3.1 Effect of PIR and NAP on reactive species formation in a glucolipotoxic skeletal muscle cell model.

Several NSAIDs have recently been reported to scavenge reactive species in oxidative stress models; however, this antioxidative activity is not a direct or universal consequence of NSAID pharmacology, as it

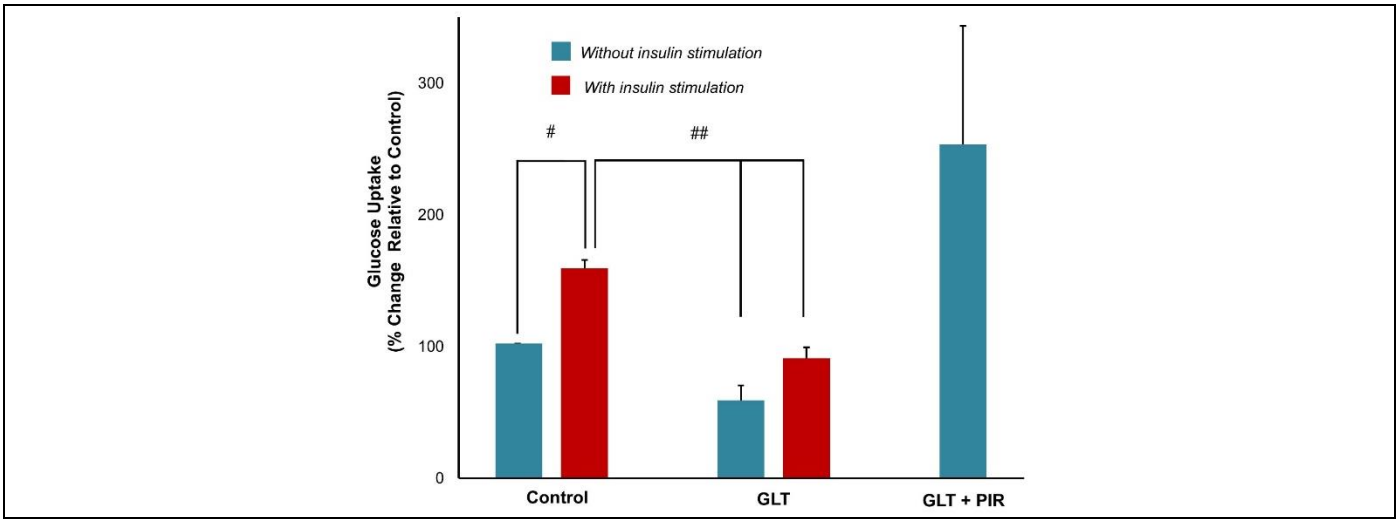

Figure 3. Piroxicam (PIR, 10 µM) enhances glucose uptake in GLT-exposed C2C12 muscle cells. Glucose uptake was measured under basal and insulin-stimulated conditions following glucolipotoxic (GLT) exposure with or without PIR treatment (10 µM). Data are presented as mean ± SEM from three independent experiments. ($^{\#}p < 0.05$ vs. Control, $^{\#\#}p < 0.05$ vs. Control with insulin simulation; Tukey's post hoc test)

varies among compounds and must be experimentally validated for each drug[22–24]. Here, we assessed the potential of PIR and NAP to reduce oxidative stress in differentiated C2C12 skeletal muscle cells exposed to GLT conditions mimicking T2DM.

Differentiation of C2C12 myoblasts into myotubes was confirmed by characteristic elongation and alignment, consistent with established morphological indicators (Figure 1). Following differentiation, cells were incubated for 72 hours in GLT media (28 mM glucose, 200 µM palmitic acid, and 200 µM oleic acid) with or without treatment with PIR or NAP. ROS levels were assessed using the fluorogenic probe DCFDA, which detects intracellular hydroxyl, peroxyl, and related radical species. GLT exposure significantly increased ROS formation (+73.48 ± 9.37%, $p < 0.05$ vs. control). Treatment with NAP significantly reduced ROS by 87.00 ± 14.63%, while PIR decreased ROS by 74.05 ± 9.93%, both relative to GLT ($p < 0.05$), demonstrating that both compounds possess ROS-scavenging activity under GLT conditions (Figure 2).

3.2 Potential of PIR to enhance glucose uptake.

Glucose uptake in C2C12 myotubes was significantly impaired following exposure to GLT conditions. Compared to untreated control cells, GLT exposure reduced glucose uptake by 43.44 ± 15.27% under basal conditions and by 70.35 ± 8.65% under insulin-stimulated conditions ($p < 0.05$; Figure 3). Treatment with PIR (10 µM) led to an increase in glucose uptake of 191.70 ± 98.64% relative to GLT, although this increase did not reach statistical significance ($p > 0.05$).

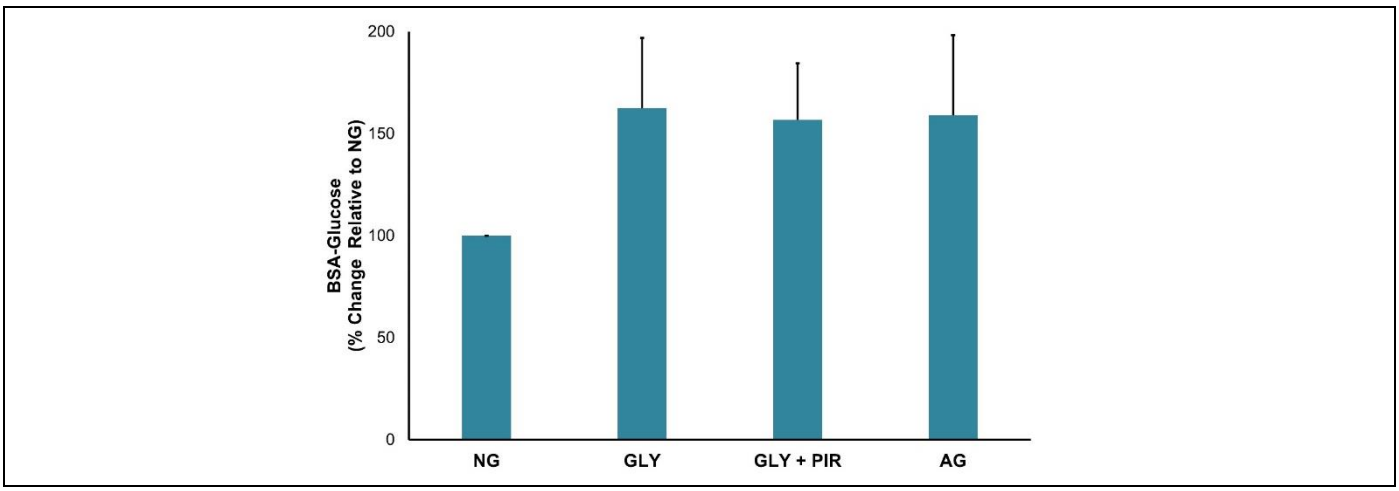

Figure 4. Antiglycation activity of piroxicam (PIR, 40 ppm) in the bovine serum albumin--glucose (BSA-glucose) model. PIR was compared to aminoguanidine (AG, 1 mM) for inhibition of advanced glycation end-product (AGE) fluorescence after 7 days. AGE formation was quantified via fluorescence and expressed as percent change relative to the non-glycated control (NG). The glycated control (GLY) consisted of BSA incubated with glucose without any test compound. AG served as a positive control. Data are presented as mean ± SEM from three independent experiments. ($p > 0.005$; one-way ANOVA).

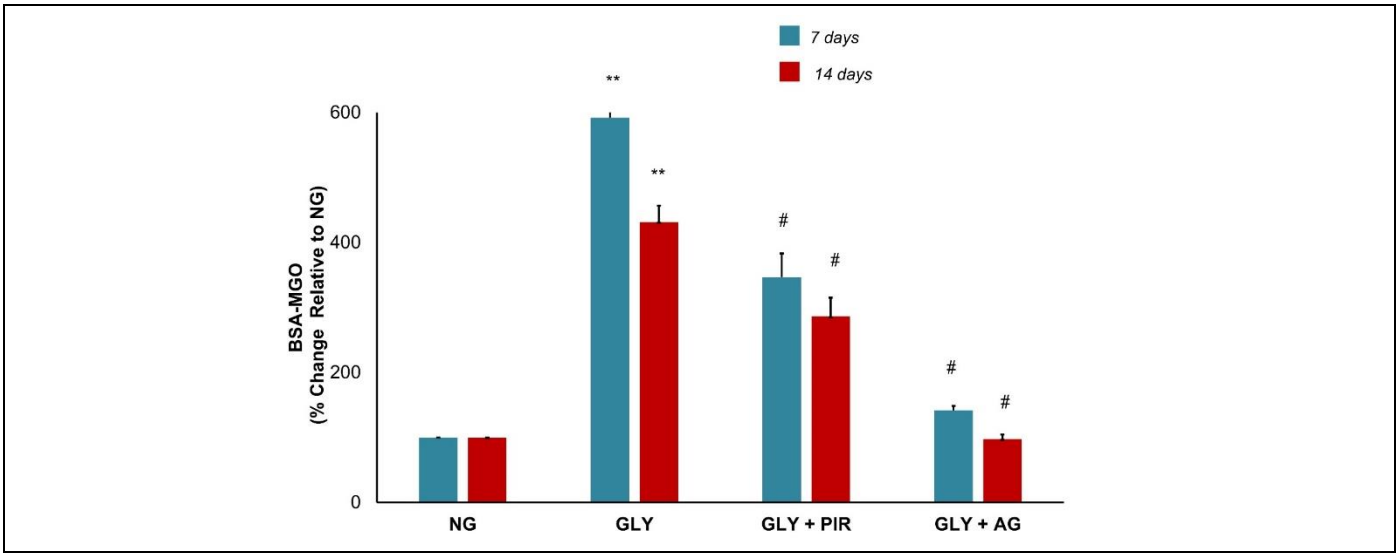

Figure 5. Antiglycation activity of piroxicam (PIR, 40 ppm) in the bovine serum albumin–methylglyoxal (BSA–MGO) model after 7 and 14 days of incubation. Formation of advanced glycation end-products (AGEs) was quantified via fluorescence and expressed as percent change relative to the non-glycated control (NG). The glycated control (GLY) consisted of BSA incubated with MGO without any test compound. Aminoguanidine (AG, 1 mM) served as a positive control. Data represent mean ± SEM from three independent experiments. (**p<0.0001 vs. NG, #p<0.05 vs. GLY; Tukey's post hoc test).

3.3 Inhibitory effect of PIR on advanced glycation end products (AGEs).

The BSA-glucose model system showed a marked increase in fluorescence intensity in the glycated (GLY) group compared to the non-glycated (NG) control, with a 62.54 ± 34.31% increase. PIR treatment (40 ppm) resulted in a slight reduction of 5.80 ± 27.72% in fluorescence intensity, while the aminoguanidine (AG) control showed a reduction of 3.56 ± 39.30% (Figure 4). In the BSA-MGO model, a significant increase in fluorescence intensity was observed in the GLY setup compared to NG controls. On day 7 and day 14, fluorescence increased by 492.10 ± 20.00% and 331.33 ± 25.50% respectively ($p < 0.0001$; Figure 5). PIR supplementation (40 ppm) reduced fluorescence by 245.40 ± 36.25 (day 7) and 145.16 ± 29.24% (day 14). In comparison, AG (1 mM) reduced fluorescence more substantially, to 42.01 ± 6.77% and 2.64 ± 7.08% on days 7 and 14, respectively.

3.4 Protective effect of PIR against change in tertiary protein structure.

The glycated BSA group exhibited a 58.03 ± 29.08% increase in absorbance compared to the NG control (Figure 6). PIR treatment (40 ppm) reduced absorbance by 54.96 ± 3.81% , while AG treatment resulted in a 39.61 ± 3.70% decrease.

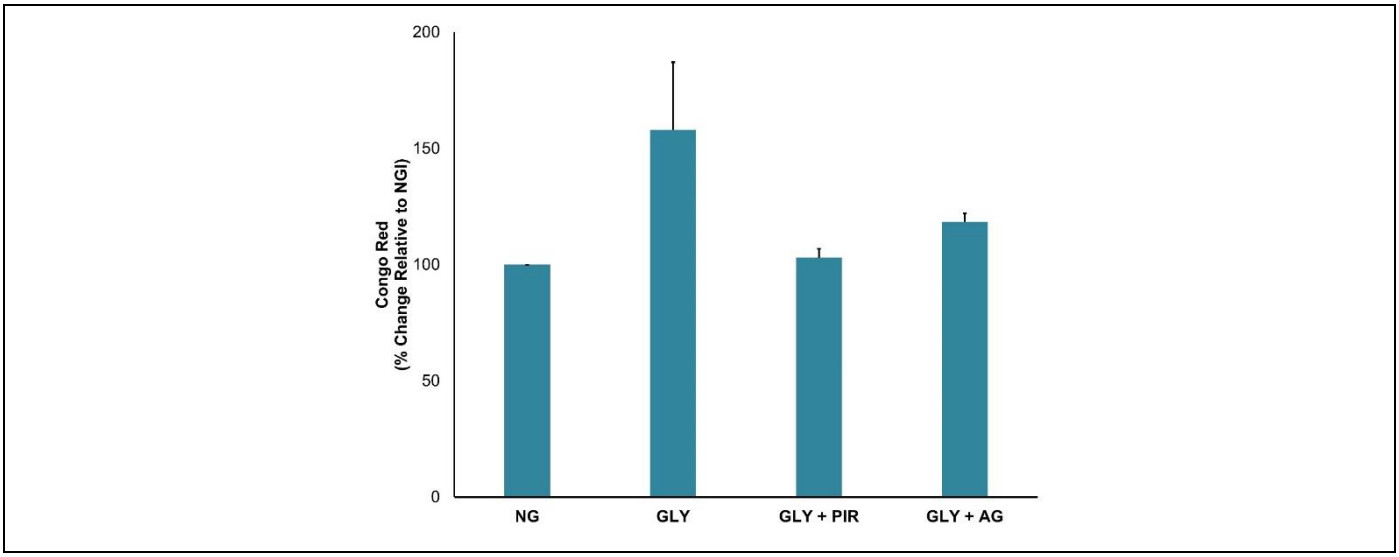

Figure 6. Protective effect of piroxicam (PIR, 40 ppm) against glycation-induced protein aggregation. Congo red absorbance was measured as an indicator of protein fibrillation. NG: non-glycated control; GLY: glycated; AG: aminoguanidine. Results are expressed as means ± SEM from three independent experiments. ($p > 0.005$; one-way ANOVA).

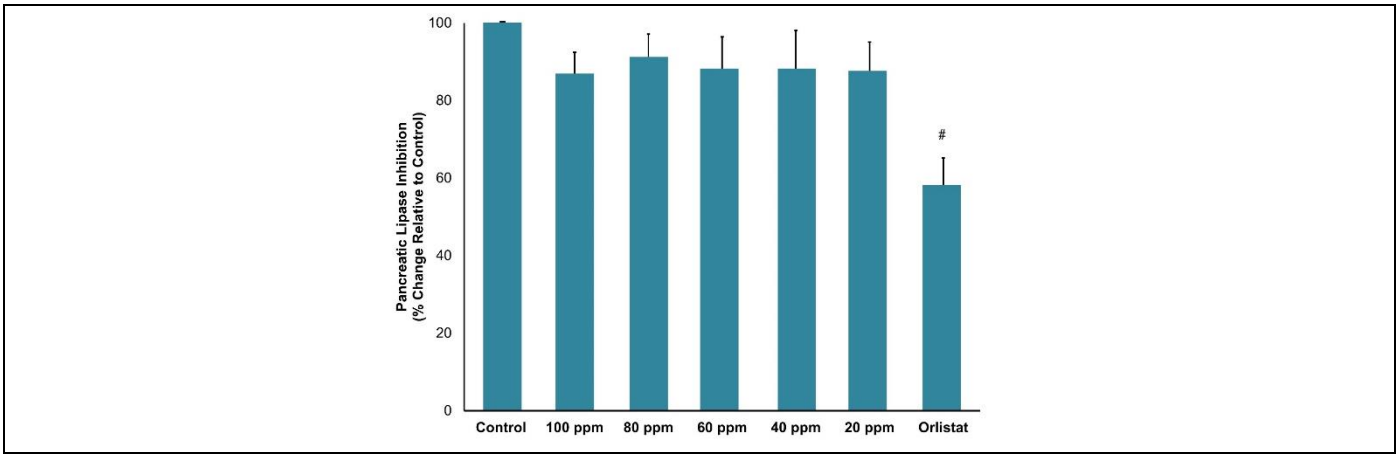

Figure 7. Pancreatic lipase inhibition by piroxicam (PIR, 20-100 ppm) compared to orlistat (100 ppm). Enzyme activity was measured using p-nitrophenyl butyrate substrate. Data are presented as mean ± SEM from three independent experiments. ($p < 0.005$; one-way ANOVA; $^{\#}p < 0.05$ vs. control; Tukey's post hoc test).

3.5 Pancreatic lipase inhibition.

PIR exhibited limited inhibitory against porcine pancreatic lipase across the tested concentrations (20, 40, 60, 80, and 100 ppm), with an average inhibition of 11.93% (Figure 7). No clear dose-dependent trend was observed. PIR did not produce a statistically significant inhibition of enzyme activity, indicating a lack of effect. The reference inhibitor orlistat (100 ppm) significantly reduced enzyme activity by 41.89 ± 7.02%.

4. DISCUSSION

The rationale for exploring NSAIDs in the context of T2DM extends beyond their established anti-inflammatory properties. Drug repurposing efforts have identified NSAIDs as potential DPP-4 inhibitors, with PIR specifically shown to reduce blood glucose levels in diabetic animal models and to alleviate neuropathic symptoms[8,25,26]. These therapeutic benefits are thought to arise from a combination of mechanisms, including DPP-4 inhibition, cyclooxygenase (COX) suppression, and AMP-activated protein kinase (AMPK) activation, which is a central regulator of cellular energy balance.

A primary finding of this study was the ability of both NSAIDs to counteract glucolipotoxicity-induced oxidative stress. Oxidative stress plays a central role in the pathogenesis of T2DM, as excessive ROS disrupt cellular redox homeostasis, impair glucose metabolism, and contribute to insulin resistance. Attenuating oxidative stress is therefore a key therapeutic target in preventing the progression of metabolic dysfunction. In C2C12 myotubes, NAP and PIR reduced intracellular ROS by approximately 87% and 74%, respectively. Although earlier reports have described NSAID-induced ROS generation, our results align with more recent studies indicating that PIR possesses antioxidant capacity comparable to that of ascorbic acid[27–29]. Since oxidative stress is a known initiator of downstream diabetic complications, its attenuation may directly contribute to the additional protective effects observed. These actions may involve the suppression of upstream ROS sources such as COX-2 or activation of AMPK, leading to metabolic reprogramming.

Glucose transport is impaired in insulin-resistant states such as T2DM, where the normal signaling pathway that facilitates glucose uptake is disrupted. In healthy individuals, insulin plays a central role in maintaining blood glucose, typically around 5 mM, by triggering the translocation of GLUT4 to the muscle cell membrane[30]. This allows efficient glucose entry into the cell. In our GLT-induced model of insulin resistance, the mechanism was compromised, as shown by the reduced response to insulin stimulation. Persistent disruption of insulin signaling contributes to the progression of T2DM and is associated with complications such as cardiovascular disease, kidney failure, and diabetic retinopathy. Therefore, compounds that improve glucose uptake, either by restoring insulin responsiveness or by enhancing glucose entry through alternative routes, may offer therapeutic value. This effect may reflect secondary benefits from PIR's oxidative activity, or, alternatively, the single concentration assessed may have been insufficient to elicit a significant response. NSAIDs such as PIR have been shown to activate AMPK and promote Glucose Transporter type 4 (GLUT4) translocation, but dose-response studies will be needed to determine the extent of its pro-glycemic effects in insulin-resistant cells[31]. The ability of PIR to reduce intracellular ROS may indirectly influence the insulin signaling pathway. Oxidative stress is known to impair insulin sensitivity by disrupting insulin receptor substrate (IRS) activation and downstream PI3K/Akt signaling, leading to reduced glucose uptake in insulin-responsive cells[32]. Although insulin signaling markers were not measured directly in this study, the observed improvement in glucose uptake

in GLT-treated myotubes, together with ROS attenuation, is consistent with previous findings where antioxidant treatment was associated with restored insulin responsiveness.

The glycation of proteins is not only identified as a marker of the progression of diabetic complications but also a key driver of diabetes-associated disorders [33,34]. Binding of excess sugars to proteins in the living system modifies their structures and functions in a manner that damages various organs[35]. These cellular and molecular changes eventually become detrimental and pathogenic[36]. Several glycation inhibitors have been studied in the past decades, such as aminoguanidine, but have not been approved for clinical use, mainly because of their cytotoxicity, and other adverse effects[37,38]. On the other hand, drugs such as metformin, aspirin, and diclofenac have been approved by the FDA (USA), but they are not efficient enough to inhibit glycation under chronic hyperglycemic conditions[39]. Some common drugs, such as PIR, are under investigation for this purpose. PIR demonstrated protective effects against protein damage associated with diabetic complications. In anti-glycation assays, it showed moderate inhibition of AGE formation in the BSA-glucose model and stronger suppression in the BSA-MGO model, suggesting preferential reactivity toward carbonyl stress rather than glucose itself. These results are consistent with the findings of Rasheed et al. (2018), although PIR's antiglycation activity remains lower than that of aminoguanidine[40].

In the Congo red assay, PIR reduced β-sheet-rich amyloid fibril formation, supporting its ability to interfere with amyloidogenic protein aggregation. Proteins must fold correctly to function properly, with their amino acid sequence guiding this process. Misfolded proteins can aggregate, forming harmful polymers[41]. T2DM is a common protein misfolding disease (PMD), and its pathogenesis is considered to be tightly associated with the aggregation of amylin or islet amyloid polypeptide[42]. Previous research indicates that there is a widespread conformational change in the proteins involved in T2D that form β-sheet-like motifs[38]. This transition, typical of amyloid deposits, can lead to aggregation and the generation of toxic proteins which can catalyze the misfolding of other proteins, disrupting cellular function, and contributing to disease progression.

In the assay, the non-glycated (NG) sample served as the control, while the glycated (GLY) group showed an apparent increase in Congo red binding, indicating enhanced β-sheet content associated with glycation. Although this difference did not reach statistical significance, it is biologically consistent with early conformational changes. Treatment with PIR reduced absorbance relative to the glycated setup, suggesting a protective effect against glycation-induced structural modification. While PIR's activity might involve non-covalent interactions, such as hydrophobic binding or π–π stacking, and aligns with previous proposals of NSAIDs like PIR as scaffolds for anti-amyloidogenic drug design[43].

PIR was also studied for its anti-obesity potential. Obesity results from the disequilibrium between energy intake and expenditure, where pancreatic lipase inhibition is classified as one of the approaches to treat obesity due to the fact that 50-70% of total dietary fat hydrolysis is performed by pancreatic lipase[44]. Hence, inhibiting pancreatic lipase is an important strategy for treating obesity and other metabolic disorders. To date, orlistat is the only approved lipase inhibitor on the market, and researchers are looking for similar drugs with fewer side effects. Based on the results, PIR exhibited weak and non-dose-dependent inhibition of pancreatic lipase in a p-nitrophenyl butyrate (pNPB) substrate assay, suggesting a lack of effect on lipid digestion, relevant to obesity associated with T2DM. This is consistent with its chemical structure, which lacks features typical of lipase inhibitors, such as long hydrophobic tails or reactive electrophilic centers. an important strategy for treating obesity and other metabolic disorders. Moreover, it is worth noting that obesity is a state of low-grade chronic inflammation, which is a persistent, low-level inflammatory state in the body that can occur over an extended period. NSAIDs can effectively reduce inflammation and alleviate pain in the short term, but their long-term use for managing chronic low-grade inflammation is not typically recommended due to potential side effects and risks.

This study has several limitations. All experiments were performed in vitro, and the systemic relevance of the findings remains to be validated in vivo. All of the assays, except the pancreatic lipase inhibition, used only a single concentration of PIR, limiting conclusions regarding dose-dependency. Myotube formation was assessed qualitatively through morphology rather than molecular markers, and mechanisms were not confirmed through protein-level assays.

5. CONCLUSION

This in vitro study demonstrates that PIR can attenuate multiple cellular stress responses associated with T2DM, including oxidative stress, protein glycation, and amyloidogenesis. While its effect on glucose uptake and lipid metabolism were limited, PIR's activity profile supports its potential as a multi-target candidate for further investigation in the context of metabolic disease.

Given the scope, these findings should be interpreted as preliminary. The cellular responses observed provide a functional basis for future in vivo

validation and mechanistic studies. Additionally, NAP, which demonstrated comparable antioxidative effects, highlights its potential and warrants further exploration.

6. AKCNOWLEDGMENTS

We thank the Cell Culture and Cell-based Assay Laboratory at the Center for Natural Products and Drug Discovery (NPDD) of the Premier Research Institute of Science and Mathematics (PRISM), for their invaluable support.

Author Contribution

LML conducted experiments, collected and analyzed data, and prepared the manuscript. HJE conducted experiments and analyzed data. QJL conducted experiments and analyzed data. CL directed the overall study design, analyzed data, and reviewed the manuscript.

Funding

This work was supported by the Mindanao State University - Iligan Institute of Technology (MSU-IIT) Research Funds and the Department of Science and Technology - Science Education Institute (DOST-SEI), Philippines.

Conflict of Interest

The authors declare no conflict of interest.

Ethics Approval

No human participants or samples were involved in this study. All cell lines used were established and acquired from the American Type Culture Collection (ATCC).

Article info:

Received April 30, 2025
Received in revised form July 04, 2025
Accepted July 12, 2025

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