RESEARCH PAPER

Pomiferin As A Dual-Action Bioactive For Therapeutic Use: In Vitro Antioxidant And Anti-Inflammatory Insights

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Abstract

Background: The naturally occurring prenylated isoflavone, pomiferin, extracted from maclura pomifera, is receiving growing interest as a naturally occurring bioactive molecule with potential application in the management of diseases caused by oxidative stress and inflammatory processes.

Objective: In this study, the antioxidant and anti-inflammatory activities of pomiferin were investigated in vitro through biochemical and molecular methods.

Methods: The evaluation of antioxidant activity included nitric oxide (no*) scavenging, dpph radical scavenging, and xanthine oxidase inhibitory activities. Modulation of oxidative stress was determined by assessing the levels of malondialdehyde (mda) and hydrogen peroxide (h_2o_2) in hepg2 and l6 myotubes. To clarify certain facets of natural defenses, we assayed the activities of sod, cat, and gsh. Inhibitory effects on inflammation were characterized by interleukin inhibitory activity and gene expression levels of nf-κb, tnf-α, and pkc as determined by q-rt-pcr.

Results: The results indicated that pomiferin is a strong radical scavenger and xanthine oxidase inhibitor. In addition to the decrease of mda and h_2o_2 contents and the increase of the activities of sod, cat, and gsh, this evidence supported the effectiveness of antioxidants. Pomiferin inhibited the degradation of cellular proteins and consequently caused the reduction in the levels of nf-κb, tnf-α, and pkc, hence establishing the inhibitory effect of pomiferin on inflammatory actions.

Conclusion: On the whole, our data support pomiferin as a bifunctional bioactive molecule with very strong antioxidant and anti-inflammatory properties, thus perhaps serving as the basis of its use as a natural remedy for diseases triggered by oxidative stress and inflammation.

Keywords: Antioxidant, Anti-Inflammatory, Gene Expression, Invitro, Nf-Kb, Pomiferin.

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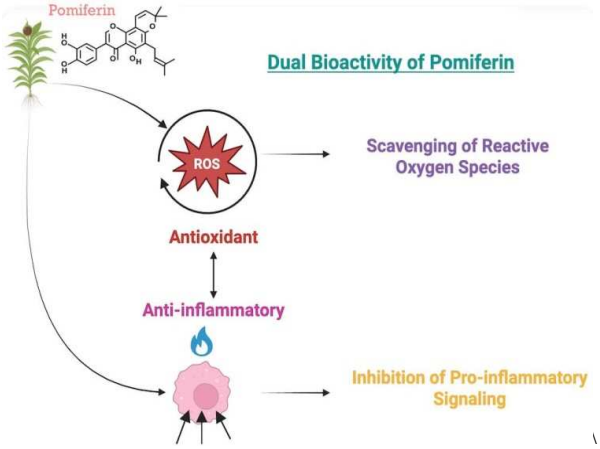
INTRODUCTION

Oxidative stress and inflammation are two biological processes that are related. When the body produces excess reactive oxygen species (ROS), oxidative stress is established, and this damages the cells. Redox homeostasis imbalance may perpetuate inflammatory mechanisms further to create a vicious cycle that encourages pathologies like cardiovascular disease, diabetes mellitus, cancer, and neurodegenerative disorders (1). Overproduction of the ROS may cause damage in proteins, lipids, and DNA molecules and can also trigger inflammatory pathways by activating the inflammatory regulators, including NF-κB, inflammasomes, and other transcription factors, and stimulates the synthesis of pro-inflammatory cytokines and chemokines (2). Direct activation of innate immune receptors like Toll-like receptors to detect the damage-associated molecular pattern can also be produced by oxidative stress in a manner that may trigger the development of inflammation (3). Chronic inflammation, on the other hand, facilitates the production of excess ROS, thereby sustaining a cycle of cellular damage and dysfunction within tissues, which can be sustained and even worsen (4). The increased incidence of chronic conditions, which are closely associated with oxidative stress and inflammatory conditions, presupposes the need to investigate new therapeutic agents that can control such disease-causing mechanisms (5). Since oxidative stress and inflammation are closely connected, the use of treatment that aims at the two simultaneously could be the most promising way to prevent or at least delay the course of the chronic diseases. Although most traditional interventions often center on one pathway, unifocal intervention approaches might be inadequate to address the multifactorial nature of chronic conditions. Bioactive compounds that have antioxidant and anti-inflammatory properties have a special interest because they can exert synergistic effects to restore redox homeostasis and prevent pro-inflammatory signaling (6).

Flavonoids have received much attention as a single category of dietary polyphenols that are common in fruits, vegetables, tea, and medicinal plants. They are direct scavengers of reactive oxygen species (ROS) by virtue of their hydroxyl structures and chelating transition metal ions, and they destroy oxidative damage at its origin. Other pathways that are regulated by flavonoids include NF-κB, which regulates inflammation; MAPKs (ERK, JNK, and p38), which mediate the stress response; and the Nrf2 pathway that participates in cellular antioxidant responses. Flavonoids appear to be an attractive long-term treatment option because they scavenge free radicals and simultaneously alter protective signaling. Most of the experimental research has already proven that flavonoids typically have low levels of toxicity when they are cultured in cells and administered to animal models at physiological and supplemental doses and still have significant bioactivity. The benefits of flavonoids include a safe and versatile response to oxidative stress and inflammation (7).

STRUCTURAL FEATURES AND BIOACTIVITY OF POMIFERIN

Pomiferin is an isoflavonoid precursor that is extracted primarily from the Osage orange, also known as *Maclura pomifera*. Recently, it has been highlighted that it possesses a lot of bioactivities, hence becoming a candidate for therapeutic use in most medical conditions, such as oncology, neurology, and organ protection. The weird molecular structures enable pomiferin to serve in different biological roles, which include but are not limited to antioxidants, anticancer, and enzyme inhibitors.



One of the classes of isoflavonoids, where a prenyl group (3-methylbut-2-enyl) is attached to an isoflavone backbone, is pomiferin. Its chemical formula is 3-(3,4-dihydroxyphenyl)-5-hydroxy-8,8-dimethyl-6-(3-methylbut-2-enyl) pyrano [2,3-h] chromen-4-one (8). Pomiferin contains quite a number of hydroxyl groups, rendering it very reactive and capable of forming hydrogen bonds. These hydroxyl groups do not only determine the solubility of the molecule but also can be the possible location of electron transfer and cellular

enzyme interactions or receptors. Considering the nucleus of the isoflavone as a basic structure, it is highly likely to penetrate any biological target upon taking this core as a main core. The prenyl group is hydrophobic and helps to increase the recognition of the protein and the permeability of the membrane. Aromatic rings are sites of π-π stacking and also hydrophobic interactions with enzymes and receptors (9,10). Polyhydroxylated aromatic groups in combination with a lipophilic prenyl side chain make pomiferin a molecule with rather a diverse morphology. The amphipathic nature mediates interaction with system domains that may be either hydrophilic or hydrophobic, thus making it highly probable to disrupt a wide range of cellular pathways, further expanding the utility of an attractive multifunctional drug. Figure-1 represents the structure of pomiferin

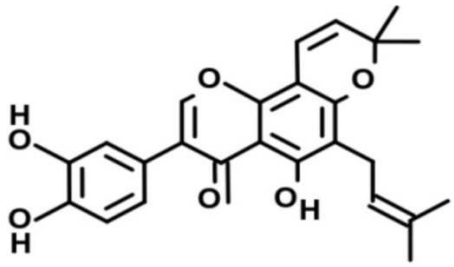


Figure 1. Structure of Pomiferin

The Pomiferin structure facilitates the inhibition of the action of histone deacetylase (HDAC), mTOR, and carbonic anhydrase enzymes. The active sites of these enzymes are helped to bind by the isoflavone core in combination with the prenyl group, and the hydroxyl groups are able to form hydrogen bonds, and this could enhance the effectiveness of the inhibition (11). The prenyl group and aromatic framework of pomiferin aid its interaction with cellular proteins, which comprise cell death processes (apoptosis, autophagy, and ferroptosis). Also, its structure allows it to prevent drug resistance proteins, such as P-glycoprotein (P-gp) (12). Pomiferin makes efforts on alternative methods to cause cell death in apoptotic resistance cancer cells through autophagy. It has suppressed the activity of SERCA and triggered the CaMKKb-AMPK-mTOR pathway that has a very critical role in escaping through drug resistance. It also prevents P-gp efflux that causes chemotherapy drugs such as cisplatin to have a better effect on cancers that are resistant to them (13). Pomiferin induces cell death in high-risk neuroblastoma, which occurs via the mitochondria. This involves a series of events, which are augmented caspase 3/7 activation and cleavage of PARP, each of which is an emblem of mitochondrial apoptosis (intrinsic). It involves the implication of permeabilization of the outer membrane of mitochondria, allowing the release of pro-apoptotic factors (14). This discovery indicates the level of bioactivity by pomiferin, which goes far beyond free radical scavenging, in which it has been found in processes that are essential in the life of cells, including survival, adaptation, and recovery.

MATERIALS AND METHODS

Reagents and Chemicals

Pomiferin, sodium nitroprusside, sulfanilic acid, naphthyl ethylenediamine dihydrochloride, bovine serum albumin (BSA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), thiobarbituric acid (TBA), trichloroacetic acid, hydrochloric acid, xanthine oxidase, DTNB (5,5'-dithiobis-2), Ellman reagent (nitrobenzoic acid), diclofenac sodium, ascorbic acid and hydrogen peroxide (H_2O_2) were acquired at Sigma-Aldrich (USA). Dulbecco Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Penicillin, and the antimicrobial agent streptomycin were acquired from Himedia (India). Cipla was the source of Metformin (India). Commercial assay kits of superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and hydrogen peroxide were bought at Cayman Chemical (USA). HepG2 cells and L6 myotubes were bought in the National Centre of Cell Sciences (NCCS), Pune. TRIzol reagent, a reverse transcription kit purchased from Thermo Fisher Scientific, and SYBR green master mix (USA) were also included. All the chemicals and reagents used in this study were of analytical grade.

Cell line

The human liver cancer cells (HepG2) and mouse skeletal muscle cells (L6 myotubes) were obtained from NCCS, Pune. Passaging and maintenance of all the cell lines were carried out in a CO_2 incubator. It is supplemented with 10% fetal bovine serum (FBS), 1% antibiotics including penicillin and streptomycin, and DMEM media. For inducing oxidative stress, the cells were treated in high glucose (HG; 30 mM) for 24 hours.

DPPH radical scavenging activity

According to the given methodology (1), the potency of pomiferin to act as a DPPH radical scavenger was evaluated. DPPH powder weighing 4.3 mg was dissolved in 3.3 mL methanol and wrapped with aluminum foil to avoid sunlight. For the control, 150 µL of DPPH solution was mixed with 3 mL of methanol, with absorbance at 517 nm measured immediately on a Shimadzu UV-visible spectrophotometer, model UV-1601 (Japan). For preparing the samples, 50 µL of different concentrations (100, 200, 300, and 400 µg/mL) of pomiferin and standard antioxidant (ascorbic acid) were added to test tubes. Methanol was added to make up the volume to 3 mL. In all these tubes, 150 µL of the DPPH solution was added, and the mixtures were kept in the dark for 15 minutes at room temperature. After incubation, the absorbance of the solution is measured at 517 nm with methanol taken as a blank on the UV-Visible spectrophotometer. Based upon this value, the DPPH free radical scavenging activity is calculated by the following formula:

% scavenging = (control absorbance - sample absorbance/control absorbance) X 100

Assessment of Nitric Oxide Radical Inhibition

The inhibitory activity of pomiferin on nitric oxide was studied by the following method (15,16). The reaction mixture of 3 mL was prepared by adding sodium nitroprusside (10 mM) 2 mL, phosphate-buffered saline 0.5 mL, and pomiferin at various concentrations of 100, 200, 300, and 400 µg/mL. The mixture was maintained at 25°C for 150 minutes. Upon incubation, 0.5 mL of the mixture containing nitrite was withdrawn and was mixed with 1 mL of sulfanilic acid reagent (0.33% in 20% acetic acid). It was then left standing for 5 minutes to complete the diazotization. Thereafter, 1 mL of naphthyl ethylenediamine dihydrochloride was added, and the mixture was stirred and kept under diffused light at 25°C for 30 minutes, thus developing a pink chromophore. Ascorbic acid was used as a standard at concentrations of 100, 200, 300, 400µg/mL. The absorption of the formed chromophore was measured at 550 nm to monitor the efficacy of pomiferin in scavenging nitric oxide. The results were expressed as a percentage of scavenging by using the following formula:

Nitric Oxide radical scavenged (%) = (Control OD − Sample OD/Control OD) × 100

Xanthin*e* Oxidase Inhibitory Activity

The xanthine oxidase inhibitory effect of pomiferin was so determined (17,18). The assay mixture was prepared with pomiferin (1.0 ml) at different concentrations, 2.9 ml of phosphate buffer, and 0.1 ml of the freshly prepared solution of the xanthine oxidase enzyme, which was kept at 25°C for 15 minutes. Following this, 2 mL of xanthine substrate solution was added and incubated at 25°C for 30 minutes. The reaction was terminated by the further addition of 1 mL of 1N hydrochloric acid. With the help of the UV-visible spectrophotometer, the absorbance of the final solution was determined at 290 nm to measure the impact of pomiferin on xanthine oxidase activity.It was calculated as % inhibition = (1 - as/ac) x 100.

The absorbance in the presence of the test substance is denoted by as, while the absorbance of the control is represented by ac.

Oxidative Stress Markers

Malondialdehyde (MDA) Assay

The levels of malondialdehyde (MDA) were determined using the thiobarbituric acid reactive substances (TBARS) assay. In short, cell lysate was mixed with TBA reagent (0.67% TBA in 10% trichloroacetic acid), boiled for 15 minutes, and cooled down. The pink-colored MDA-TBA adduct was extracted and quantified spectrophotometrically at 532 nm. The levels were described in µmol/L of MDA.

Hydrogen Peroxide (H_2O_2) Assay

The concentration of H_2O_2 was quantified with a commercial kit for hydrogen peroxide detection based on the horseradish peroxidase reaction. Cell lysates were incubated for a period with the assay buffer solution containing a colorimetric probe, and the absorbance was read at 560 nm. The hydrogen peroxide concentrations were calculated from a standard curve and expressed in µmol/L.

Antioxidant Assay

Superoxide Dismutase (SOD) Activity Assay

Measurement of SOD activity was performed by a commercially available SOD assay kit. The recombinant xanthine oxidase and WST-1 reagent used for detecting

superoxide radicals were incubated with cell lysates. Absorbance was read at 450 nm. Activity was expressed as U/mg protein.

Catalase (CAT) Activity Assay

CAT activity was determined by the decomposition rate of H_2O_2. Cell lysates were incubated with freshly prepared 30 mM H_2O_2 in phosphate buffer (pH 7.0), and the decrease in absorbance was noted at 240 nm. 1 unit of catalase activity is defined as the activity of an enzyme that breaks down 1 µmol of H_2O_2 in a minute (19). These values were expressed after normalization with respect to the protein content and were shown to be represented as U/mg protein.

Reduced Glutathione (GSH) Assay

The assay kit for quantifying GSH is based on GSH reacting with DTNB (5,5'-dithiobis-2-nitrobenzoic acid) to form a yellow chromophore that is measured at 412 nm. The results are presented as µmol GSH/mg protein.

Gene expression by RT-PCR

Total RNA was extracted with the TRIzol reagent using the manufacturer's instructions. The RNA samples were recorded for purity and concentration by a NanoDrop spectrophotometer. A reverse transcription kit was used for synthesis of cDNA with 1 ug total RNA as a template. Gene expression levels of inflammatory markers (NF-κB, TNF-a, and PKC) were quantified by SYBR Green-based qPCR. The internal control was taken as GAPDH or β-actin. Relative gene expression was determined using 2^−ΔΔCt. Primer sequences were gene-specific and had been proved efficient. A thermal cycling profile was initiated at 95°C for 5 minutes, and then 40 cycles of two-step cycling were performed, and denaturation was done at 95°C in the time of 5 seconds. Annealing and extension were performed for 10 seconds with primer-specific temperatures of about 55-62.5°C. RT-PCR quantifications were performed on a CFX9 real-time Bio-Rad system, USA.

STATISTICAL ANALYSIS

The student's t-test, which is a parametric statistical test designed specifically to be applied for paired data in which it is utilized by three independent variables (n=3) using Graph Pad Prism 8. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test was used to find the calculation of the gene expression. Statistical significance between groups was considered at a predetermined significance level of *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$.

RESULTS

Effects of Pomiferin on DPPH Radical Scavenging Activity

A chemical assay, which is the most known method of estimating the scavenging of free radicals of natural materials in vitro, is the DPPH test. Figure 2 presents the DPPH radical scavenging activity of pomiferin, which was elevated as the concentration increased. That implies that the compound may be used as an antioxidant. As indicated in the data, pomiferin with a concentration of 100 µg/mL exhibited a weak inhibition compared to the standard antioxidant (ascorbic acid), although there was a strong inhibition at higher concentrations of 200–400 µg/mL. Pomiferin is able to scavenge the free radicals at a concentration of 400µg/ml, almost as potent as the standard, so it is a strong radical scavenger.

The polyphenolic and prenylated isoflavone structure of pomiferin provides it with the ability to contribute free radicals by taking away hydrogen atoms or electrons to neutralize them so that they would become non-reactive species. Thus, this property serves both to demonstrate the fundamental chemical reactivity's and to emphasize the biological significance of neutralizing oxidative damages within cells. Based on the trend, pomiferin, much like two known antioxidants, ascorbic acid or BHT, might be an enormous help in decreasing oxidative stress. Because pomiferin cancels free radicals, it holds therapeutic potential since oxidative stress is an important pathogenetic factor in chronic disorders like diabetes, neurodegeneration, and cancer.

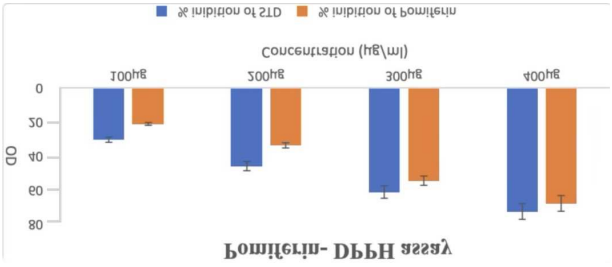


Figure 2. DPPH radical scavenging activity of pomiferin compared with standard (ascorbic acid)

Effects of Pomiferin on Nitric Oxide (NO•) Radical Scavenging Activity

Nitric oxide serves as an important physiological messenger that assists in vasodilation, neurotransmission, and immune regulation. Under oxidative stress, NO*quickly reacts with superoxide anions to form peroxynitrites (ONOO-), a hyperactive nitrogen species capable of damaging DNA, lipids, and proteins. Excessive production of nitric oxide (NO*) and resultant nitrosative stress has been closely associated with inflammation, B-cell dysfunction, and the pathophysiology of diabetic complications. Hence, the inhibition of NO* radicals is regarded as an important test for anti-inflammatory activity. Figure 3 shows that pomiferin inhibited NO* radicals in a dose-dependent manner as compared to the standard antioxidant, ascorbic acid. At a concentration of 100 µg/ml, pomiferin showed less NO* scavenging ability as compared to the standard, but the activity increased consistently as the concentration was enhanced. Pomiferin exhibited a strong inhibitory effect at 200µg/ml, and this inhibitory effect was enhanced on increasing the concentration to 300µg/ml, which considerably reduced the difference between the standard and pomiferin. The inhibition at the highest concentration of 400 µg/ml was almost equal to that of the standard indicator, which is quite good at neutralizing NO* radicals.

Pomiferin decreases biosynthesis of NO*, where gradual accumulation is a sign that it can decrease nitrosative stress and worsen inflammatory responses. This role provides protection to pancreatic b-cells, blood vessels, and skeletal muscle against NO*-mediated cytotoxicity. Therefore, the fact is in favor of therapeutic opportunities on pomiferin, an antioxidant and anti-inflammatory, which is against reactive species formed out of nitrogen and oxygen.

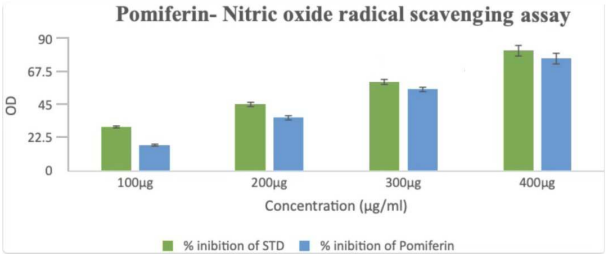


Figure 3. Nitric Oxide radical scavenging activity of pomiferin compared with standard (ascorbic acid)

Effects of Pomiferin on *Xanthine* Oxidase Inhibitory Activity

The enzyme of significance to the purine metabolism is xanthine oxidase (XO). It converts hypoxanthine into xanthine and then xanthine into uric acid. XO produces many reactive oxygen species (ROS), namely superoxide anions and hydrogen peroxide, during these conversions. Subsequently, overactive XO is linked with oxidative stress and a high level of uric acid. Natural inhibitors are needed more, as they can do the double job of lowering uric acid levels and protecting tissues from damage by ROS. As shown in Figure 4, pomiferin inhibited XO activity with respect to concentration, showing activity levels gradually increasing to that of the conventional inhibitor diclofenac sodium. Pomiferin showed a moderate inhibition at 100 µg/ml concentration, which increased with a gradual increase in concentration. At 300 µg/ml concentration, inhibition by pomiferin was almost equivalent to the standard, and at 400 µg/ml, it attained nearly similar inhibition values to those of the standard.

Contributing to this property could possibly be the isoflavone skeleton of pomiferin having hydroxyl and prenyl substituents that may be engaged in efficient binding with the active site of XO, thus barring substrate access and consequent uric acid formation. Being a potent inhibitor of xanthine oxidase activity, pomiferin halts aberrant uric acid accumulation (hyperuricemia and gout) and also curtails excessive ROS production, which protects many tissues from oxidative damage. These findings imply that pomiferin can be a natural XO inhibitor that can help in combating hyperuricemia, oxidative stress, and inflammatory disorders.

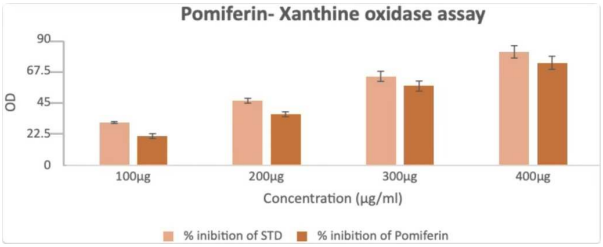


Figure 4. Xanthine oxidase inhibitory effect of pomiferin compared with standard (diclofenac sodium)

Effect of Pomiferin on Oxidative Stress Markers (MDA, H_2O_2) in HepG2 and L6 Myotubes

The bar graphs in figure-5 reflect the protein concentration of two significant indicators of oxidative stress, malondialdehyde and hydrogen peroxide. High glucose-stimulated HepG2 cells and L6 myotubes, both with and without treatment, were used in the study. Biochemical evidence suggests that exposure to HG has led to the increase of indices of oxidative stress in both cell lines, and this is indicated in an elevation of MDA and H_2O_2. In the case of HepG2 cells, the amount of MDA had risen to around 7.5 µmol/L, in comparison with the control of 3µmol/L; likewise, H_2O_2 was at a level of ~10.5 µmol/L in comparison to the control of 5.5µmol/L. In L6, MDA and H_2O_2 experienced an increase to about 9.5 and 15µmol/L, respectively, in the state of hyperglycemic stress, hence displaying potential colossal oxidative damage.

Pomiferin treatments suppressed oxidative stress significantly and dose-dependently. At a lower dose of 2 µM, Pomiferin treatment reduced hepatic MDA levels to 6.8 µmol/L and H_2O_2 levels to 9.5µmol/L, and under the higher dose of 8 µM, the oxidative stress parameters reached near-control levels, i.e., 4.5µmol/L and 6.5µmol/L, respectively. These observations pattern the results seen in L6 myotubes, with Pomiferin (8 µM) restoring the MDA and H_2O_2 levels to ~5.5 and ~8.5µmol/L, respectively. Metformin treatment reduced the oxidative stress level, similarly to Pomiferin, particularly at a concentration of 8 µM. These findings also indicate that Pomiferin exerts cytoprotection against hyperglycemia-induced oxidative damage by attenuation of lipid peroxidation and accumulation of reactive oxygen species, similar to the antidiabetic agent metformin.

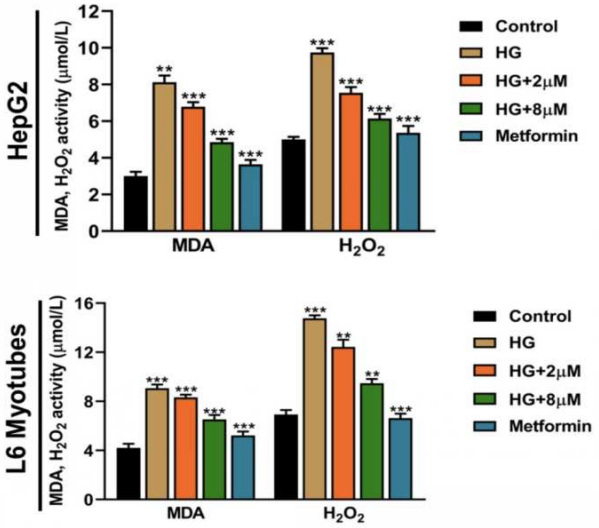


Figure 5. MDA and H_2O_2 activity by treating pomiferin by pretreating with high glucose (30mM) compared with metformin (1mM) in HepG2 cells and L6 myotubes cells.

Effect of Pomiferin on Antioxidant Activity

The bar graph in figure-6 depicts the antioxidant response of SOD, CAT, and GSH activities in HepG2 and L6 myotubes conditioned to be subjected to hyperglycemia and Pomiferin treatment. In these two cell lines, HG exposure was found to be detrimental to the antioxidant defense system. The SOD activity in HG HepG2 cells dropped to ~50 U/mg, whereas in L6 cells, it dropped to ~45 U/mg as against ~85 U/mg and ~75 U/mg in control cells, respectively. Similar results were seen in CAT and GSH activities, which reduced to 30%-40% during the HG condition, which suggests there might be oxidative stress-induced damage to the enzymatic defenses.

The activities of antioxidant enzymes are dose-dependently restored by the treatments of Pomiferin at 8 µM treatment, which elevates the amount of SOD, CAT, and GSH in the HepG2 cells to nearly the level shown by control cells (SOD ± 78 U/mg, CAT ± 68 U/mg, GSH ± 63 U/mg). The identical activities were observed in the L6 cells, where 8 µM of Pomiferin brought the level of SOD to ±70 U/mg, CAT to ±58 U/mg, and GSH to ±62 U/mg. Metformin, which also provided great protection with activity either similar to or slightly superior to pomiferin at 8 µM, is in favor of the familiar antioxidant activity of metformin. Therefore, the results indicate that

Pomiferin is effective in the context of repairing the damaged antioxidant defense mechanism from hyperglycemia. Enhancement of SOD, CAT, and GSH functions infers that Pomiferin suppresses oxidative stress by supporting the cellular redox system, which is similar to that of Metformin's therapeutic effects.

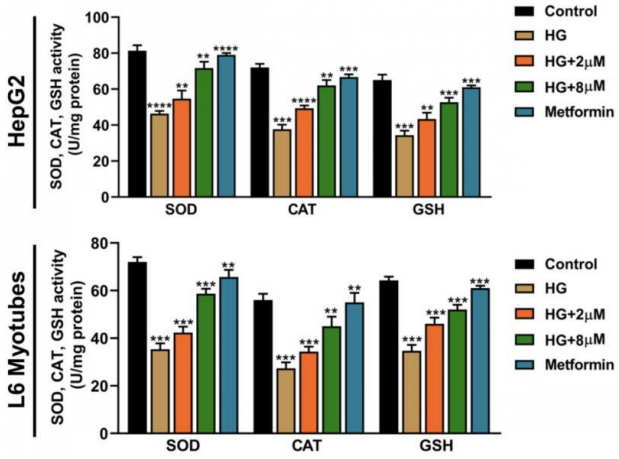


Figure 6. SOD, CAT, and GSH activity by treating pomiferin by pretreating with high glucose (30mM) compared with metformin (1mM) in HepG2 cells and L6 myotubes cells

Effects of pomiferin on NF-κB, TNF-α Protein kinase C (PKC) mRNA expression in HepG2 cells

NF-κB, TNF-α, and PKC protein expressions are essentially triggered in an inflammatory situation under hyperglycemia. Incubation of the HepG2 cell line with 30 mM glucose caused a strong activation of PKC and a more than 1.3-fold increase in the levels of NF-κB and TNF-α, supporting the role of oxidative stress in stimulating inflammatory mediators, thereby contributing to insulin resistance. Pomiferin inhibited these effects in a dose-dependent manner [Figure-8]. At a concentration of 2 μM, pomiferin was minimally effective in blocking the hyperglycemia-induced increase in NF-κB, TNF-α, and PKC, whereas at 8 μM, it reduced all three to normal levels. This study thus implies that pomiferin is capable of interrupting the whole pro-inflammatory cascade induced by hyperglycemia. It is more important to note that the anti-inflammatory effect of pomiferin at 8 μM was almost equal to that of metformin, which is medically recognized as an insulin-sensitizing agent. These findings show that pomiferin suppresses the activation of NF-κB, which causes the production of TNF-α, and blocks the PKC signaling, which is a significant connection between inflammation and insulin resistance. The combination of these data demonstrates the strong anti-inflammatory effect of pomiferin, which justifies its potential as a therapy for preventing dysfunction of the cell due to elevated levels of blood sugar.

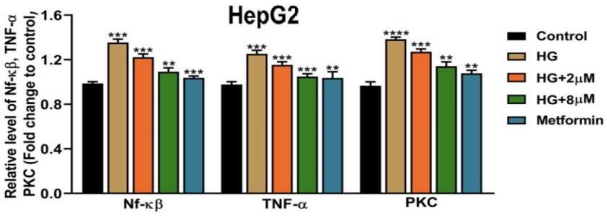
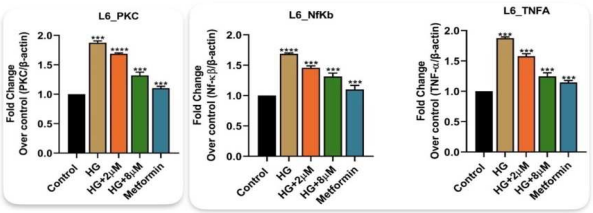


Figure 7. m-RNA expression of NF-κB, TNF-α and PKC treated by pomiferin by pretreating with high glucose (30mM) compared with metformin (1mM) in HepG2 cells.

Effects of pomiferin on NF-κB, TNF-α, Protein kinase C (PKC) mRNA expression in L6 myotubes cells

High glucose conditions in L6 myotubes have been revealed to increase gene expression of the inflammatory mediators NF-κB and TNF-α. Concomitantly, enhanced PKC expression is evident in figure 9. NF-κB levels increased by about 1.6-fold compared to the control, while TNF-α levels rose approximately 1.4-fold, and PKC expression increased nearly 2-fold. This pattern suggests that high blood sugar levels induce skeletal muscle cells to respond to inflammation. Activation of PKC is critical in promoting inflammatory signaling since PKC isoforms can induce NF-κB translocation and increase TNF-α production, thus setting up a destructive feedback loop of inflammation and signaling disruption. Increased PKC activity in the myotubes has been correlated with abnormal insulin signaling, thus suggesting the link between inflammation and metabolic dysfunction. The therapeutic use of pomiferin greatly inhibited these HG-induced changes. It acts at 2 μM to inhibit the formation of NF-κB, TNF-α, and PKC, with strong inhibition occurring at 8 μM, since almost all three indicators were restored to normal levels. Being a potent inhibitor of PKC, pomiferin is of significant interest because the blockade of this kinase prevents the signaling pathway that leads to NF-κB activation and proinflammatory cytokine generation. In comparison studies, higher levels of pomiferin had therapeutic efficacies similar to metformin; this is additional support for the clinical potential of the phytocompound. The

empirical evidence therefore supports that pomiferin triggers a significant anti-inflammatory effect in L6 myotubes by downregulating NF-κB, PKC, and TNF-α.



m-RNA expression of NF-κB, TNF-α and PKC treated by pomiferin by pretreating with high glucose (30mM) compared with metformin (1mM) in L6 myotubes cells.

DISCUSSION

The study confirms that pomiferin shows the dual bioactivity, which is free radical inhibition and anti-inflammatory activity in vitro. Pomiferin exhibited strong and concentration-dependent DPPH and nitric oxide scavenging assays, which had been demonstrated to be free radical scavenging activities, not incidental or random but dose-related and reproducible through fundamentally using a standard procedure. The strength of its scavenging capacity was nearly equal to the known reference antioxidants; therefore, it confirms the therapeutic value of pomiferin as a natural bioactive molecule. This activity is caused by the presence of a polyphenolic backbone and the prenylated isoflavone structure. Several hydroxyl groups present in the structures can release hydrogen or electron ions to the reactive species, not allowing them to react. The prenyl group also makes the compound lipophilic, hence increasing uptake by cells. Thus, such structural peculiarities might be the reason for the effectiveness of pomiferin across lipid membranes and subsequently cause an antioxidant effect intracellularly. These results are as per the previous results, indicating that pomiferin reduces oxidative stress through promoting Nrf2 and simultaneously mediating upwards and downwards inflammatory agents, thereby safeguarding cells under neuroinflammatory and metabolic stress conditions (21).

Significantly, these effects were not confined to cell-free systems but were also observable in cellular models. In HepG2 liver cells and L6 myotubes, pomiferin-treated cells exhibited decreased markers of oxidative stress, such as malondialdehyde (MDA) and hydrogen peroxide (H_2O_2), while increasing endogenous antioxidant defenses such as superoxide dismutase (SOD), catalase (CAT), and GSH. The above results thus indicate that pomiferin acts to scavenge free radicals and maintain cellular antioxidant systems so that redox homeostasis can be preserved in high-glucose-mediated oxidative stress. Very similar results have been observed in vivo, suggesting that pomiferin and osajin enhanced SOD and glutathione peroxidase activity, with a decrease in MDA levels from cardiac tissues undergoing ischemia-reperfusion injury (22). Further, oral treatment of pomiferin on streptozotocin induced diabetic rats improved glycemic control, inhibited lipid peroxidation, and restored antioxidant enzyme activity, thereby supporting its role in redox regulation during metabolic stress (23).

Pomiferin has a significant effect of inhibiting the activity of xanthine oxidase, an enzyme that works in the purine catabolic pathway. Hypoxanthine is first changed to xanthine and further to uric acid. This system also produces reactive oxygen species (ROS), including superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). Overproduction of XO leads to hyperuricemia and gout, as well as results in the emergence of oxidative stress and vascular endothelial dysfunction and inflammation. This current evidence indicates the justification of pomiferin inhibiting the activity of XO, hence unlocking its possibility as a naturally occurring XO inhibitor with systemic effect. This has been supported by literature that is extant; prenylated isoflavones similar to the pomiferin show strong xanthine oxidase inhibition and anti-lipid peroxidative effects, especially reducing the oxidation of low-density lipoprotein (LDL), an important initial step in atherosclerosis (24). In addition, its proven action of inhibiting xanthine oxidase highlights the therapeutic potential of the compound.

When treated with pomiferin, the transcriptional expression of pro-inflammatory cytokine genes, namely NF-κB, TNF-α, and PKC, in both the HepG2 hepatocytes and L6 skeletal muscle myotubes was significantly reduced. These observations make pomiferin an antagonist of signal cascades that combine both oxidative stress and inflammatory reactions. NFκB is a major transcription factor that is involved in the transcription of cytokines, adhesion molecules, and inflammatory mediators. Conversely, TNF-α is an effective upstream activator that reinforces the further inflammatory cascade. PKCs are also involved in stress-

activated signaling pathways with the development of insulin resistance, thereby linking oxidative imbalance and metabolic dysfunction. By suppressing the expression of these genes, pomiferin seems to have an ability to regulate cellular stress responses and maintain tissue function. Consistent with the in vitro evidence, nickel intoxication of the liver in Sprague-Dawley rats revealed that pomiferin supplementation significantly downregulated the hepatic expression of NF-κB, COX-2, TNF-α, IL-1b, and IL-6, diminishing the effect of oxidative stress, thereby corroborating the regulation of inflammatory signaling pathways at the transcriptional level, while simultaneously promoting antioxidant enzyme activities, thereby showing the effect of pomiferin at the organismal level [4].

Pomiferin is a dual bioactive compound with antioxidant and anti-inflammatory effects and can scavenge reactive oxygen and nitrogen species besides regulating important antioxidant activity enzymes- superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) to maintain cellular redox homeostasis. Simultaneously, it suppresses pro-inflammatory mediators including NF-kB, TNF- alpha and PKC in HepG2 hepatocytes and L6 myotubes, stabilizing the proteins of cells and suppressing the inflammatory cascades. Pomiferin combats the co-modulating mechanisms of chronic disease, such as metabolic syndrome, type 2 diabetes, cardiovascular disease and neuroinflammatory disorders by simultaneous control of redox homeostasis and gene transcription. These bifunctional activities highlight its therapeutic potential as the agent that would be able to restore cellular and tissue homeostasis.

CONCLUSION

The present results indicate the strong antioxidant and anti-inflammatory effects, where pomiferin transports within itself. Pomiferin was found to exhibit an activity to scavenge free radicals directly and disrupt the cellular antioxidant defense mechanism. The pomiferin inhibited oxidative markers and raised enzyme antioxidant effects on HepG2 hepatocytes and L6 myotubes. It also suppressed pro-inflammatory mediators such as NF-κB, TNF-α, and PKC. Pomiferin is working on the oxidative stresses as well as inflammatory signaling pathways, hence providing a treatment opportunity for the complex disorders of metabolism and inflammation. The current evidence identifies pomiferin as a dual-action bioactive compound that combines strong antioxidant and anti-inflammatory properties; however, more studies are still necessary to convert the evidence into a practical medicine.

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AUTHOR CONTRIBUTIONS

Arockya Stafi Arockyasamy: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft. Sridevi Gopathy: Supervision, Validation Writing – review & editing. L. Durga: Formal analysis. All authors have read and approved of the final manuscript.

CONFLICT OF INTEREST

The authors hereby declare that there is no conflict of interest.

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Data Availability Statement

All data generated or analyzed during this study are included in this published article.

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