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Nitrovanillin derivative ameliorates AGE-RAGE nexus associated inflammation: A step towards the amelioration of vascular complications under diabetic environment

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ABSTRACT

Introduction: Advanced glycation endproducts (AGEs) are implicated in various pathological conditions, including diabetes, inflammation, and cardiovascular diseases. Methylglyoxal (MGO), a potent glycation agent, leads to the formation of MGO-derived AGEs, which promote structural and functional anomalies in various cellular and tissues proteins. AGEs stimulate the proliferation of monocytes, and induce a pro-inflammatory state through AGE-RAGE interactions, triggering oxidative stress, and inflammatory condition that contribute to the progression of atherosclerosis, and other diabetic complications.

Objective: The current study was aimed to explore the antioxidant and anti-inflammatory properties of a series of novel antiglycation compounds, nitrovanillin derivatives, by modulating the AGEs-stimulated intracellular signaling pathways involved in inflammation.

Methods: The preliminary safety profile of nitrovanillin derivatives was assessed by using human hepatocytes (HepG2), and monocytes (THP-1) cell lines *via* MTT, and WST-1 assays, respectively. Antioxidant activity of the compounds was determined by using DCFH-DA technique. Western blotting, immunocytochemistry, and ELISA methods were employed to assess the levels of pro-inflammatory markers (RAGE, COX-1, COX-2, NF-κB, and PGE_2) in MGO-AGEs stimulated THP-1 monocytes.

Result: Among the nitrovanillin derivatives **1**–**11**, only compound **2**, ((*E*)-2-methoxy-6-nitro-4-(((2-(trifluoromethyl)phenyl)imino)methyl)phenol), was found non-toxic to HepG2, and THP-1 cells. Compound **2** lowered the MGO-AGEs-induced reactive oxygen species (ROS) production by inhibiting the upstream signaling of NADPH oxidase and MAPK-p38, which subsequently inhibited the NF-κB activation in THP-1 monocytes. Compound **2** also reversed the AGEs-mediated COX-1 suppression, COX-2 upregulation, and PGE_2 production by blocking the AGE-RAGE ligation in THP-1 monocytes.

Conclusion: In conclusion, nitrovanillin **2** ((*E*)-2-methoxy-6-nitro-4-(((2-(trifluoromethyl)phenyl)imino)methyl) phenol) is a potential candidate for mitigating MGO-AGEs mediated vasculopathy by the inhibition of AGE-RAGE-p38/NF-κB nexus in THP-1 monocytes. It may offer a therapeutic option for the patients with diabetes and chronic inflammatory vascular complications, and thus offering new avenues for treatment development.

1. Introduction

The non-enzymatic glycation reactions between sugars and proteins, lipids or nucleic acids, give rise to the formation of advanced glycation endproducts (AGEs) *via* the Maillard reaction. In hyperglycemic conditions, excessive glucose in the blood stream promotes the formation of

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AGEs by initially forming Schiff bases, and then to a more stable Amadori products. Further modifications and non-enzymatic peptide-cross-linking lead to the formation of diverse AGEs. Moreover, AGEs are also formed through other mechanisms, including glucose autoxidation, lipid peroxidation, hexosamine and polyol pathways upsurged, and protein kinase c activation [1].

Methylglyoxal (MGO) is a byproduct of glycolysis, formed by the non-enzymatic degradation of triose phosphates (glyceraldehyde-3-phosphate (G3P), and dihydroxyacetone-phosphate (DHAP)). Though the concentration of MGO is comparatively lower (~25,000-fold) than glucose in plasma, it is 50,000-fold more reactive than glucose, and generates glycation adducts at a faster pace than glucose. MGO reacts with arginine in proteins, and deoxyguanosine in DNA, causing the formation of MGO-derived hydroimidazolone 1 (MG-H1), and N2-carboxyethyl-2- deoxyguanosine (CEdG), respectively. Deposition of MGO-AGEs within the tissues promotes structural and functional anomalies in cellular proteins [1]. Thus, AGEs are mainly responsible for the on-set of multiple diseases, including chronic inflammation, arthritis, cancers, *etc.* [2–4].

Monocytes are largely responsible for innate immunity, thus play a pivotal role in inflammation [5]. Under the influence of hyperglycemia and AGEs, monocytes contribute to the progression of vasculopathies [6]. AGEs exacerbate the proliferation of monocytes by stimulating granulocyte-macrophage colony-stimulating factor (GM-CSF), modifies extracellular matrix proteins and circulating lipoproteins, and initiate the signaling of pro-inflammatory molecules through AGE-RAGE ligation. These events consequently contribute to the formation of atherosclerotic plaques in diabetic patients [7,8].

AGEs exert their action primarily by interacting with its receptor, RAGE, which expresses in response to various pathological stimuli on different cell types, such as monocytes, macrophages, endothelial cells, and vascular smooth muscle cells [9]. In monocytes, AGE-RAGE ligation instigate redox-sensitive pathways, including upregulation of NOX proteins, and mitochondrial dysfunction, tilting the balance towards oxidative stress [10]. This in turn, promotes the activation of nuclear transcription factor (NF-κB), and multiple signaling cascades, including mitogen activated protein kinase (MAPK-p38), extracellular regulated kinase (ERK1/2), protein kinase-C (PKC), and JNK pathways [11,12]. Activation of NF-κB also modulates gene expression, and upregulates the expression of pro-inflammatory cytokines and chemokines, such as IL-1 (interleukin-1), TNFα (tumor necrosis factor-alpha), and COX-2 (cyclo-oxygenase-2) [13,14]. AGE-RAGE signaling also reported to down-regulate the COX-1 protein, suggesting their plausible role in the development of various diseases [15]. Thus, AGE-RAGE mediated signaling increases the oxidative stress, promote inflammation, insulin resistance, β-cell dysfunction/apoptosis, and diabetes-associated nephropathy, retinopathy, neuropathy, and cardiomyopathy [16,17]. Therefore, preventing the deleterious effects of AGEs at various stages is a validated therapeutic approach.

Various compounds have been reported as inhibitors of AGEs in *in vitro* and *in vivo* models. The inhibitors of AGE-RAGE pathway may target different aspects of glycation, and associated downstream effects. These inhibitors collectively represent a multi-pronged approach to attenuate the pathological consequences of AGEs accumulation, and RAGE activation. However, their therapeutic uses have been limited due to lower efficacy, and safety concerns. Literature survey also indicates the challenges and complexities in the development of safe and effective AGEs inhibitors. For instance, benfotiamine, a lipid-soluble derivative of thiamine (vitamin B1), has demonstrated efficacy in reducing the AGEs formation and oxidative stress, with beneficial effects on diabetic neuropathy and nephropathy, but it was not developed further due to insufficient clinical evidences [18]. Similarly, ALT-711 (alagebrium) is specific inhibitor of MGO, inhibits the synthesis of MGO by increasing the metabolism of triose phosphate, and reducing the production of highly reactive AGEs. Moreover, it is a metal chelator, AGE-cross-link breaker, and antioxidant. It has demonstrated the potential to cleave the AGE-induced protein-protein cross-links, and reduce arterial stiffness in clinical studies. However, it was discontinued due to its insignificant positive effect on cardiovascular health [19–21]. Inhibitors of the glycation reaction, such as aminoguanidine (prototype AGE inhibitor), and pyridoxamine (AGE inhibitors), act by trapping reactive carbonyl species, such as methylglyoxal (MGO) and glyoxal, preventing the early formation of AGEs. RAGE blockers, including soluble RAGE (sRAGE) and pharmacological agents like azeliragon, function by interfering with the binding of AGEs to their receptor (RAGE antagonist), thereby mitigating RAGE-mediated inflammatory and oxidative stress signaling. Azeliragon have undergone clinical trials, but none of the above reached the level of drug development due to their potential adverse effects, and regulatory and safety challenges [19].

To this notion, synthetic compounds, specifically designed to interfere with the AGEs-RAGE nexus, could provide targeted and effective therapeutic options with potentially fewer side effects. Vanillin, a phenolic aromatic aldehyde, naturally found in plants like *Vanilla planifolia*, *V. pompon*, and *V. tahitensis* [22], and its synthetic derivatives possess a range of biological activities, including antioxidant, anti-glycation, anti-inflammatory, anticancer, antimicrobial, wound healing, and antiviral properties [23–25]. Recently, we reported the anti-glycation activity of novel derivatives of nitrovanillin using MGO-AGEs model [26]. Considering their potential as AGE-inhibitors, the nitrovanillin derivatives were further investigated for their anti-inflammatory effects, and the ability to modulate expression of cell signaling molecules in THP-1 monocytes. To asses the safety and efficacy of lead inhibitors at the discovery phase, metabolism-associated toxicity was assessed using human hepatocytes (HepG2), which contain the drug-metabolizing enzymatic system cytochrome P450 [27]. Further investigation on these compounds can provide valuable insights into the molecular mechanisms of AGEs-induced inflammation, potentially leading to the identification of new drug candidates against vascular complications.

2. Material and methods

2.1. Assessment of safety profile through MTT and WST-1 assays

The cytotoxicity evaluation of nitrovanillin derivatives was performed on HepG2 and THP-1 cells [28]. HepG2 (human hepatocytes), and THP-1 cells (human monocytes) were obtained from (ATTC, USA). HepG2 cells were cultured in a 75 cm^2 tissue-culture treated flask, in a MEM (minimal essential medium), containing FBS (10 %) (Thermo Fischer Scientific, USA). The cells were maintained in a sterile humidified environment. After reaching 80 % confluence, cells were detached by using trypsin-EDTA (0.25 %) (Thermo Fisher Scientific, USA), and centrifuged at 1000 rpm for 10 min. The supernatant was disposed of, while cell pellet was dissolved in a fresh medium. Trypan blue assay was performed for cell counting.

HepG2 cells (8×10^4/mL) were seeded in a 96-well tissue culture plate (Nest Biotech Co. Ltd., China), containing 2 % FBS. The cells were incubated overnight at 37 °C and 5 % CO_2. The cells were treated with different concentrations of nitrovanillin derivatives (100, 50, 30, and 10 μM), and incubated for 24 h. After incubation, the test medium was discarded, 100 μL MTT (0.5 mg/mL) was added, and kept for 3 h. The optical density was recorded at 540 nm *via* Varioskan LUX multimode microplate reader (Thermo Fischer Scientific, USA).

THP-1 Cells were cultured in a 75 cm^2 flask using RPMI-1640 medium (Gibco, Thermo Fisher Scientific, USA), containing FBS (10 %), L-glutamate, and sodium pyruvate (1 mM) (Gibco, Thermo Fisher Scientific, USA), and glucose anhydrous (1 g/100 mL). Upon confluence, cells (20×10^4/mL) were seeded in a 96-well tissue culture plate containing FBS (2 %). After overnight incubation, cells were exposed to different concentrations (100, 50, 30, and 10 μM) of nitrovanillin derivatives for 24 h. Then, WST-1 dye (20 μL) was added in the cells, and incubated for 3 h at 37 °C. The plate was carefully shaken for 5 min, and the optical

density of cells was recorded at 450 nm through Varioskan LUX multimode microplate reader (Thermo Fischer Scientific, USA).

The percentage of inhibition was calculated to assess the cytotoxicity of test derivatives on HepG2 (hepatocytes) and THP-1(monocytes) cells by using the formula given below:

Cell inhibition percentage = 100–[(Absorbance of test compound–Absorbance of blank)/(Absorbance of control–Absorbance of blank) x 100]

2.2. Preparation of MGO-AGEs

MGO-AGEs were prepared employing the method outlined by Jahan *et al.* [15]. Sodium hydrogen phosphate and sodium dihydrogen phosphate were dissolved in distilled water to prepare sodium phosphate buffer (0.1 mM), and the pH was adjusted to 7.2. The glycation agent MGO (500 mM) was mixed with fatty acid-free bovine serum albumin (BSA, Sigma, USA) (10 mg/mL), the mixture was filtered through a 0.22 μm filter, and incubated at 37 °C for 24 h in a sterile environment. Similarly, BSA (10 mg/mL) without glycation agent, dissolved in sodium phosphate buffer, was used as a control. The mixture was dialyzed in a sterile PBS to remove unbound MGO at 4 °C for 2 h. The AGEs-specific fluorescence was measured at 355/460 nm by using a Varioskan Lux Microplate reader (Thermo Fischer, USA). The endotoxin levels were assessed using a LAL assay kit (Thermo Fisher Scientific, USA), and the AGEs were stored in aliquots at −80 °C for future use. The experiments were carried out on a non-physiological highly-modified MGO-albumin, as prepared with a very high concentration of non-purified MGO (500 mM).

2.3. ROS Assessment by DCFH-DA technique

For the assessment of intracellular ROS production, DCFH-DA technique was employed [15]. THP-1 monocytes (1×10^6/mL) were labelled with DCFH-DA probe (2,7-Dichlorodihydrofluorescein diacetate) (Sigma-Aldrich, USA) (10 μM) for 45 min at 37 °C in a dark sterile environment. Cells were washed with PBS twice, and treated with different concentrations (100, 50, 30, and 10 μM) of test compounds for 1 h. Apocynin (100 μM), an NADPH oxidase inhibitor, and rutin (100, 50, 30, and 10 μM), as a reference antiglycation agent, were used as standards. Subsequently, cells were treated with MGO-AGEs (200 μg/mL) [15], and incubated at 37 °C for 24 h. Untreated, and BSA-treated cells were used as negative controls, while hydrogen peroxide (H_2O_2) (100 μM) was taken as a positive control against MGO-AGEs only. The fluorescence generated by reactive oxygen species (ROS) was measured at 490/520 nm (excitation/emission) through the Varioskan Lux microplate reader (Thermo Fisher Scientific, USA). The inhibition of AGEs-induced ROS by test compounds was determined by using the following formula:

% Inhibition = 100–[(Fluorescence of test compound
– Fluorescence of blank/Fluorescence of control
– Fluorescence of blank) x 100]

2.4. Determination of NF-κB activation through immunocytochemical studies

THP-1 cells (1×10^6 cells/mL) were pre-exposed to reference compounds (pyrrolidine dithiocarbamate (PDTC), NF-κB inhibitor, and rutin), and test compounds (100 μM) for 1 h at 37 °C. Later, cells were treated with AGEs (200 μg/mL) for 1 h. After incubation, media was removed, and cells were fixed using 4 % formaldehyde, followed by permeabilization with 0.2 % triton X-100 for 10 min. Cells were further exposed to blocking solution (1 % BSA dissolved in PBS and 0.1 % Tween) for 1 h at room temperature in order to prevent the non-specific protein binding. Later, cells were incubated overnight with NF-κB p65 (Thermo Fischer Scientific, USA) primary antibody at 4 °C. This step is followed by incubation with secondary antibody (FITC conjugated anti-rabbit IgG polyclonal) (Abcam, UK) for 1 h, while DAPI (Thermo Fischer Scientific, USA) was used to stain nuclei for 2 min. At the end of each step, cells were washed with chilled PBS thrice for 5 min. The images were recorded through a TE2000 fluorescence microscope (Nikon, USA), attached with NIE software. The DAPI-positive cells (10 different fields for each treatment) were used to measure the fluorescence intensity by using Image J software (NIH, USA) [29].

2.5. Assessment of inflammatory markers and cell signaling proteins via western blotting

Protein expression was determined using the western blot technique, as explained previously [30]. Initially, THP-1 cells (1×10^7 cells/mL) were treated with test compounds (100 μM), and standard reference (PDTC, and rutin, 100 μM) for 1 h. Later, cells were incubated with MGO-AGEs (200 μg/mL) for 6 h at 37 °C. Cells were centrifuged at 1200 rpm for 10 min, followed by cell lysis, using RIPA lysis buffer, on ice for 30 min. The cell content was sonicated with three pulses, each lasting 10 s, with 30 s rest on ice. The cell content was centrifuged at 12,000 rpm at 4 °C for 30 min, and stored at −20 °C for further experimentation.

The proteins samples (50 μg/mL) were separated through SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), and the protein bands were transferred to a nitrocellulose membrane through the Power Blot XL system (Invitrogen, USA). The blot was blocked with 3 % BSA for 1 h, followed by overnight incubation with COX-1, COX-2, RAGE, p38 (ThermoFisher Scientific, USA), and β-actin (Cloud Clone Corp., China) primary antibodies at 4 °C. Afterward, the membrane was washed three times with TBST (Tris-buffered saline with Tween 20), with continuously shaking, and then incubated with secondary antibody (HRP-conjugated mouse monoclonal anti-rabbit IgG) (Abcam, UK) for 1 h. Chemiluminescence (ECL) detection kit (Sangon Biotech, China) was used to detect the bands and visualized through a gel doc system (Azure Biosystems, USA). Densitometric analysis was performed using Image J software (NIH, USA).

2.6. Assessment of PGE_2 production through ELISA

The THP-1 cells (5×10^6 cells/mL) were pre-treated with test compounds (100 μM), and standard reference (PDTC, and rutin) for 1 h at 37 °C, followed by stimulation with AGEs (200 μg/mL) for 6 h. The cells were centrifuged for 10 min at 1000 rpm. The supernatant was collected, and used to quantify the PGE_2 concentration by using an ELISA (enzyme-linked immunosorbent assay) kit (Cloud-Clone Corp., China) [15].

2.7. Statistical analysis

The results were derived from three independent experiments, and presented as mean ± SD. A one-way ANOVA, followed by Tukey's *post hoc* test, was performed to analyze the significant difference between compounds' activities. Statistical significance was considered at $p \leq 0.05$ for each analysis.

3. Results

The analogs of nitrovanillin **1**–**11** were synthesized by the modification of (4-hydroy-3-ethoxyphenyl)-nitromethane through the substitution of different moieties on phenylhydrazine ring. Our group reported the antiglycation activity of nitrovanillin analogs using *in vitro* MGO-AGEs model [26]. Considering their promising antiglycation activity (STable 1), the current study was aimed to assess the impact of nitrovanillin analogs on the ligation of AGEs with their receptor at the cellular level, and on the signaling mechanism involved in the AGEs-induced inflammatory response in THP-1 monocytes.

3.1. Cytotoxicity assessment of nitrovanilin derivatives on HepG2 and THP-1 monocytes

The nitrovanillin analogs **1**–**11** that showed antiglycation potential (STable 1) [26] were initially assessed for the safety profile by using HepG2, and THP-1 cell lines. The cells were treated with various concentrations (10, 30, 50, and 100 μM) of test compounds, while doxorubicin and rutin were used as reference compounds. Compounds **3**, and **7** were found highly cytotoxic at 100 μM concentration, showing cell inhibition (75.5 %, and 91.09 %, respectively) greater than the standard doxorubicin (72.47 %). However, compounds **1**, **4**, **5**, and **8**–**11** exhibited a moderate toxicity (Table 1) at 100 μM concentration, as compared to the doxorubicin (Fig. 1). Whereas, compound **2** was found to be non-toxic, exhibiting 18 % cell inhibition at 100 μM concentration. Similarly, rutin (standard antiglycation compound) showed 24.2 % cell inhibition at 100 μM. Considering the preliminary safety profile and antiglycation potential of compound **2**, it was further evaluated for cytotoxicity on THP-1 monocytes. Where it was found completely non-toxic at all the tested concentrations (10, 30, 50, and 100 μM), as compared to the doxorubicin which exhibited 70.3 % inhibition at 100 μM concentration (Table 1, Fig. 2). Whereas, rutin showed a minimum inhibition (22.2 %) at 100 μM concentration. Considering the non-cytotoxic profile of compound **2** in HepG2, and THP-1 cells, it was further studied for anti-oxidant and anti-inflammatory properties under *in vitro* diabetic environment, using AGEs on THP-1 monocytes.

3.2. Compound **2** prevents AGEs-mediated ROS formation in THP-1 monocytes

AGEs causes the activation of NADPH oxidases, tilting the balance towards excessive ROS formation, which in turn cause oxidative stress and cellular toxicity [15]. In this study, we evaluated the role of compound **2** in the inhibition of AGEs-mediated ROS production in THP-1 monocytes through DCFH-DA technique. Our results showed that MGO-AGEs (200 μg/mL) significantly ($p < 0.001$) increased the intracellular ROS (87 %) production in THP-1 cells, as compared to positive (H_2O_2) (58 %), and negative controls (untreated, and BSA-treated cells) (Fig. 3). The pre-treatment of cells with NADPH oxidase inhibitor (apocynin; 100 μM) blocked the AGEs-induced ROS formation ($p < 0.001$) by 65 %. Similarly, compound **2**, and rutin (reference compound) inhibited the production of ROS in a concentration dependent manner (10–100 μM) (55–71 %, and 51–71 %, respectively) (Fig. 3). Both compounds were found equally effective in suppressing AGEs-induced oxidative stress in THP-1 monocytes.

3.3. Compound **2** inhibits AGEs-mediated activation of NF-κB in THP-1 monocytes

Activation of NF-κB (p65) is crucial for the expression of pro-inflammatory genes, and production of cytokines and chemokines in inflammatory cells, which are associated with various pathophysiological conditions [31]. Our previous studies also showed that AGEs-induced ROS formation activate the NF-κB signaling in THP-1 monocytes, resulting in the activation of inflammatory signaling molecules [15,28]. As compound **2** exhibited a greater ROS inhibition at 100 μM concentration. Its effect was further examined in blocking the NF-κB (p65) signaling in AGEs-treated (200 μg/mL) THP-1 monocytes (SFig. 2). Our findings exhibit that pre-treatment with compound **2** (100 μM) significantly halt the p65 activation ($p < 0.001$, RFU: 16) in AGEs-stimulated THP-1 monocytes (Fig. 4A, and B, SFig. 3), which is comparable to the signals (RFU: 52) generated by AGEs- treated cells. Furthermore, reference compounds: rutin, and PDTC (100 μM), substantially reduced p65 signaling ($p < 0.001$, RFU: 32, and 9, respectively), as compared to AGEs-treated cells (Fig. 4A, and B, SFig. 3).

3.4. Compound **2** modulates the AGEs-induced expressions of RAGE, p38, COX-1, and COX-2 in THP-1 monocytes

The interaction between AGEs and RAGE triggers the activation of various signaling molecules, including MAPK-p38 pathway, which subsequently activates NF-κB, and hence promotes the synthesis of pro-inflammatory genes and mediators [13,14,17,32]. To elucidate the mechanism by which compound **2** inhibits the AGEs-NF-κB -mediated inflammatory responses, the expression of various signaling proteins, including MAPK-p38, RAGE, and COX-1 and -2 were assessed using Western blotting. The expression of protein bands was quantified as fold change, with beta-actin (a housekeeping protein) was used to normalize the signals.

We observed that compound **2** suppressed ($p < 0.001$) RAGE expression by 2.62-fold, as compared to AGEs-treated cells, which increased the RAGE levels by 8.39-fold than BSA and untreated cells (control). Rutin, and PDTC (100 μM) also suppressed ($p < 0.001$) RAGE levels by 4.79-, and 2.60-fold, respectively (Fig. 5A, and C). Furthermore, compound **2** (100 μM) significantly ($p < 0.001$) inhibited the AGE-RAGE mediated activation of MAPK-p38 in THP-1 monocytes. The level of p38 was decreased by 1.22-fold, as compared to the AGEs-treated cells. PDTC treatment suppressed p38 levels by 1.12-fold, which is equivalent to the effect of compound **2**. Whereas, rutin was ineffective in reducing p38 levels, exhibiting the p38 protein levels equivalent to AGEs treated cells (Fig. 5B, and C).

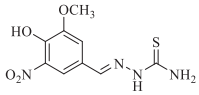
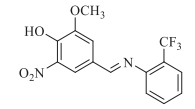
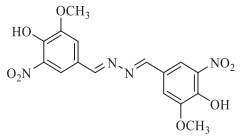
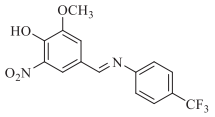
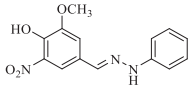
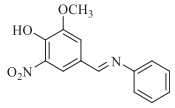
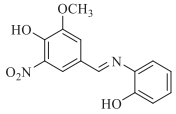
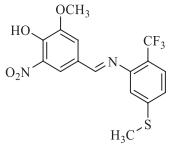
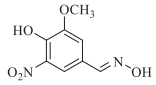
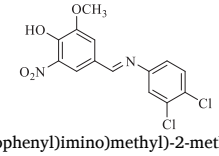
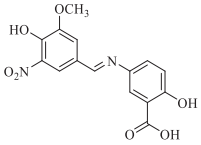
Activation of MAPK-p38, and NF-κB p65 leads to the induction of COX-2 gene, which expressed as inflammatory mediator, and associated with the production of prostaglandin E_2 (PGE_2) [33]. Since compound **2** inhibited the AGE-RAGE-mediated inflammatory signaling (p38, and p65), it was further assessed for its ability to mitigate COX-2 expression in THP-1 monocytes. AGEs increased the COX-2 levels by 3.68-fold, which was significantly ($p < 0.001$) suppressed by compound **2** (4.07-fold) (Fig. 5E, and F) (SFig. 4). Similarly, PDTC, and rutin ($p < 0.05$) inhibited AGE-induced COX-2 protein levels by 1.00-fold. However, the effect of compound **2** was considerably better than both the reference compounds PDTC, and rutin.

We recently reported that the MGO-AGEs suppresses the COX-1 protein expression in THP-1 monocytes, which can be linked to gastrointestinal side effects, and other adverse conditions [15]. To address these concerns, the effect of compound **2** was examined on COX-1 protein in THP-1 cells, co-incubated with MGO-AGEs (200 μg/mL). Here, we observed a significant ($p < 0.001$) decrease in COX-1 levels (0.37-fold) in AGEs-treated THP-1 cells, as compared to the BSA treated, and untreated cells (Fig. 5D, and F). Whereas, pre-treatment of cells with compound **2** reversed ($p < 0.001$) the AGEs-mediated suppression by 4.26-fold. Similarly, treatment with PDTC, and rutin significantly regulate the AGEs-mediated decline of COX-1 protein levels (3.62 and 3.87-fold, respectively) in THP-1 monocytes (Fig. 5D, and F). However, the effect of compound **2** was significantly superior to the reference compounds.

3.5. Compound **2** inhibits AGEs-induced expressions of PGE_2

PGE_2 is an inflammatory agent, and a major product of COX-2 enzyme. Considering the effect of compound **2** in suppressing COX-2

Table 1
Cytotoxic of nitrovanillin derivatives against HepG2, and THP-1 cell lines.

Nitrovanillin Derivatives	Concentration (μM)	HepG2 cell % Inhibition ± SEM	THP-1 cell % Inhibition ± SEM
	100	46.67 ± 2.21	
	50	16.49 ± 2.31	
	30	1.00 ± 0.54	NA
	10	1.00 ± 0.01	
(*E*)-2-(4-Hydroxy-3-methoxy-5-nitrobenzylidene)hydrazine-1-carbothioamide (1)			
	100	18.60 ± 2.23	1.11 ± 0.01
	50	1.00 ± 0.87	1.12 ± 0.11
	30	0.10 ± 0.03	0.80 ± 0.02
	10	0.11 ± 0.003	0.90 ± 0.04
(*E*)-2-Methoxy-6-nitro-4-(((2-(trifluoromethyl)phenyl)imino)methyl)phenol (2)			
	100	75.54 ± 4.32	
	50	57.3 ± 4.12	
	30	40.00 ± 3.15	
	10	18.00 ± 1.67	NA
4,4'-((1*E*,1'*E*)-Hydrazine-1,2- diylidenebis(methaneylylidene))bis (2- methoxy-6-nitrophenol) (3)			
	100	35.20 ± 1.25	
	50	21.13 ± 2.22	
	30	20.00 ± 2.54	
	10	10.00 ± 1.15	NA
(*E*)-2-Methoxy-6-nitro-4-(((4-(trifluoromethyl)phenyl)imino)methyl)phenol (4)			
	100	55.34 ± 1.48	
	50	47.88 ± 2.21	
	30	30.00 ± 3.43	NA
	10	20.00 ± 1.23	
(E)-2-Methoxy-6-nitro-4-((2-phenylhydrazineylidene)methyl)phenol (5)			
	100	41.16 ± 3.43	
	50	2.39 ± 2.12	
	30	1.00 ± 2.98	NA
	10	0.50 ± 1.23	
(*E*)-2-Methoxy-6-nitro-4- ((phenylimino)methyl)phenol (6)			
	100	91.09 ± 2.57	
	50	46.67 ± 1.16	
	30	30.00 ± 2.54	NA
	10	20.00 ± 1.87	
(*E*)-4-(((2 Hydroxyphenyl)imino)methyl)-2-methoxy-6-nitrophenol (7)			
	100	45.70 ± 3.32	
	50	16.80 ± 2.45	
	30	8.00 ± 1.43	NA
	10	1.00 ± 0.009	
(*E*)-2-Methoxy-4-(((5-(methylthio)-2(trifluoromethyl)phenyl)imino)methyl)-6-nitrophenol (8)			
	100	46.81 ± 2.43	
	50	7.34 ± 3.21	
	30	5.00 ± 2.24	NA
	10	1.00 ± 2.10	
(*E*)-4-Hydroxy-3-methoxy-5-nitrobenzaldehydeoxime (9)			
	100	54.61 ± 1.43	
	50	32.32 ± 2.43	
	30	22.00 ± 2.22	NA
	10	1.00 ± 0.45	
(*E*)-4-(((3,4-Dichlorophenyl)imino)methyl)-2-methoxy-6-nitrophenol (10)			
	100	45.6 ± 2.24	
	50	3.0 ± 0.04	
	30	1.0 ± 0.001	NA
	10	1.0 ± 0.002	
(*E*)-2-Hydroxy-4-((4-hydroxy-3-methoxy-5-nitrobenzylidene)amino)benzoic acid (11)			
	100	72.47 ± 1.2	70.3 ± 1.2
	50	45.18 ± 1.7	58.5 ± 2.4
	30	45.21 ± 2.5	52.2 ± 1.4
Doxorubicin (Std)	10	40.21 ± 2.14	50.2 ± 1.16
	100	24.22 ± 2.2	22.22 ± 3.34
Rutin (Std)	50	14.62 ± 2.21	16.19 ± 1.10

(*continued on next page*)

Table 1 (*continued*)

Nitrovanillin Derivatives	Concentration (μM)	HepG2 cell % Inhibition ± SEM	THP-1 cell % Inhibition ± SEM
	30	11.62 ± 2.13	13.17 ± 1.14
	10	10.62 ± 1.23	8.16 ± 1.13

*NA = not applicable.
Std = Standards/ Reference compounds.

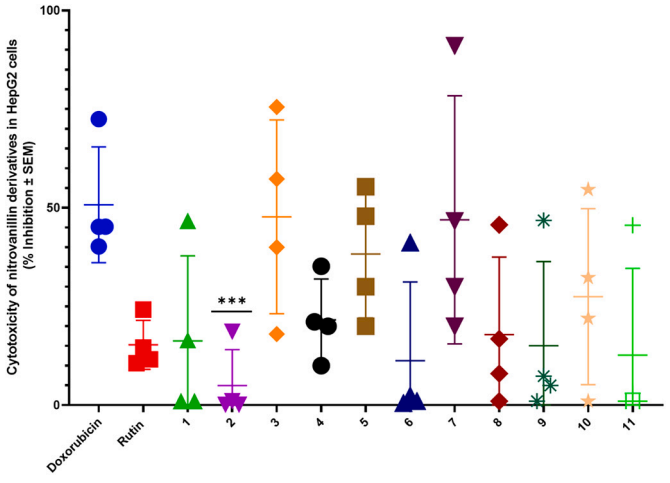


Fig. 1. The cytotoxic effect of nitrovanillin derivatives **1–11** on HepG2 cells. Compound **2** was found to be significantly ($p < 0.001$) non-toxic. It showed <30 % cell inhibition at all the tested concentrations (100, 50, 30, and 10 μM), as compared to standard, doxorubicin. Rutin was used as a reference compound. The data represent mean ± S.D. of three individual experiments.

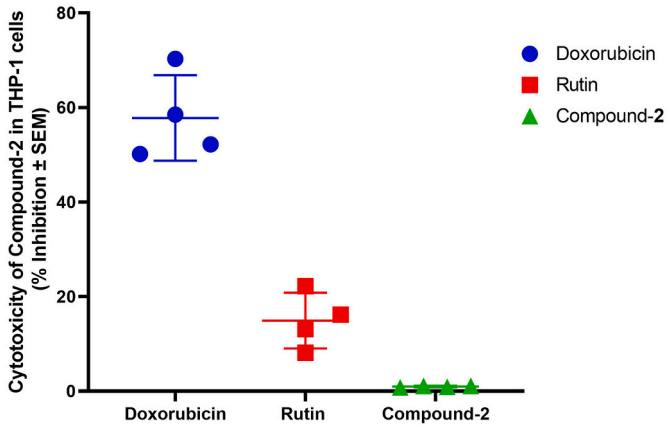


Fig. 2. The cytotoxic effect of nitrovanillin **2** on THP-1 monocytes. Compound **2**, and standard rutin exhibited a non-toxic behavior at all the concentrations (100, 50, 30, and 10 μM), as compared to doxorubicin ($p < 0.001$). The data represent mean ± S.D. of three individual experiments.

expression in AGEs-treated monocytes, its effect was further determined on the production of PGE_2 levels. Our results revealed that AGEs significantly increased the PGE_2 levels (268 pg/mL), as compared to BSA treated, and untreated cells. Whereas, pre-treatment of cells with compound **2**, PDTC, and rutin, substantially ($p < 0.001$) inhibited the PGE_2 production (78, 248, and 245 pg/mL, respectively), as compared to AGEs-treated cells. However, the effect of compound **2** was comparatively superior to PDTC, and rutin (Fig. 6).

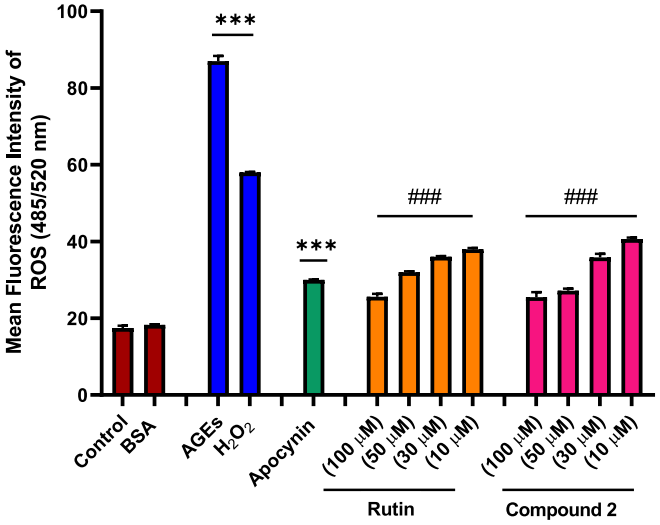


Fig. 3. Compound **2** inhibited the AGEs-induced ROS production in THP-1 monocytes. The cells were pre-treated with compound **2**, and reference compounds rutin (10, 30, 50, and 100 μM), and apocynin (100 μM) for 1 h, followed by the incubation with MGO-AGEs (200 μg/mL) for 24 h. The data represent mean ± S.D. of three individual experiments. *** Indicate significant increase ($p < 0.001$) in ROS induction by AGEs and H_2O_2 *vs* untreated cells and BSA control. ### Show significant decrease ($p < 0.001$) in ROS production with compound **2**, and reference compounds *vs* AGEs, and H_2O_2.

4. Discussion

Monocytes in their M1 state (pro-inflammatory) are associated with the development and progression of vascular complications *via* AGE-RAGE ligation. AGE-RAGE binding accelerates the formation of ROS and secretion of chemokines and cytokines by activating nuclear transcription factor, ultimately contributes to the progression of inflammation. Therefore, impeding the interaction of AGEs at the receptor level could be effective in blocking the proliferative and pro-inflammatory states of monocytes [11].

Recently, we have reported the promising antiglycation activity of novel derivatives of nitrovanillin using *in vitro* MGO-AGEs model [26]. Given their potency and efficacy (STable 1), the current study examines their role in alleviating the formation of ROS, and inhibition of inflammatory cascade in MGO-AGEs-treated THP-1 monocytes.

Several compounds, including aminoguanidine, azeliragon, alagebrium, and *N*-phenacylthiazolinium bromide (PTB), have been reported for their antiglycation potential, and their ability to mitigate complications associated with AGEs in clinical trials. However, some of them were excluded from drug development process due to their severe side effects [19]. In order to identify the safer drug leads, cytotoxic profile of nitrovanillin derivatives that showed good antiglycation activity (> 50 % glycation inhibition, STable 1), was examined on HepG2, and THP-1 cells lines (Table 1). HepG2 cells are human hepatocytes, containing a cytochrome P450 enzyme system, which is involved in drug metabolism. This makes them a suitable model for investigating the drug metabolism and toxicity [27]. Doxorubicin (anti-cancer drug), and rutin (antioxidant) were used as reference compounds.

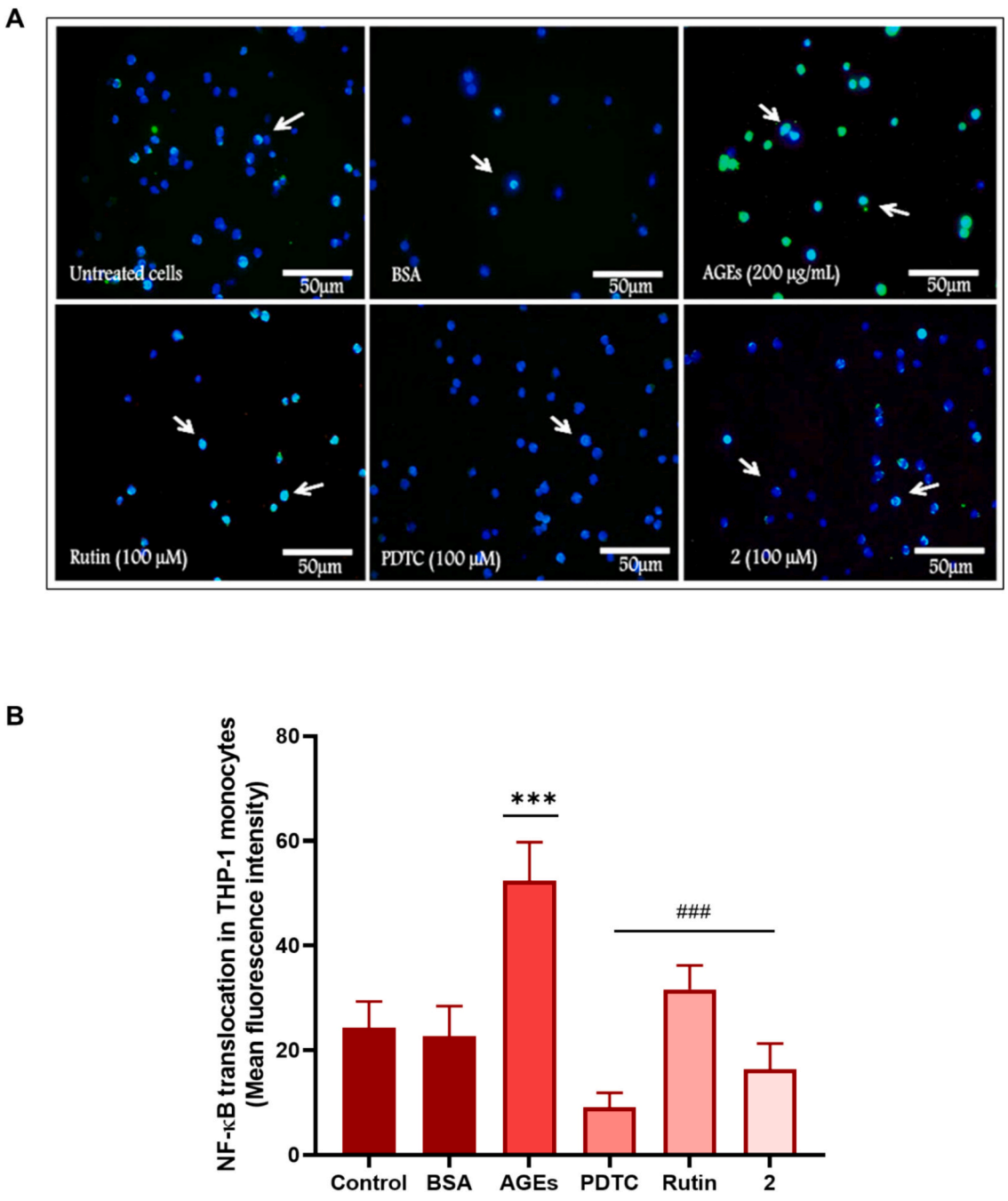


Fig. 4. Compound **2** blocked AGEs-induced translocation of NF-κB (p^{65}) in THP-1 monocytes. The cells were pre-treated with compound **2** and reference compounds (PDTC, and rutin) (100 µM) for 1 h, followed by the exposure with MGO-AGEs (200 µg/mL) for 1 h. (**A**) Immunocytochemical study showed significant increase in green fluorescence intensity of NF-κB in MGO-AGEs treated cells. While, compound **2** and reference compounds noticeably suppressed the AGEs-induced activation of κB (p^{65}) in THP-1 cells. (**B**) The graph illustrates significant increase ($p < 0.001$, ***) in mean fluorescence intensity of NF-κB in AGEs-treated cells, as compared to untreated cells and BSA control. ### Indicate significant decrease ($p < 0.001$) in κB intensity by rutin, PDTC, and compound **2**, as compared to AGEs-treated THP-1 cells. The data represent mean ± S.D. of three individual experiments.

Our results showed that doxorubicin (100 µM) inhibited >70 % growth of HepG2 cells, while rutin was found non-toxic across all the tested concentrations (10, 30, 50, and 100 µM). Whereas, nitrovanillin derivatives **1**, **4**, **5**, and **8**–**11** (Table 1) were found moderately toxic, exhibiting over 57 % inhibition of HepG2 cells at 100 µM concentration, as compared to the standard drug doxorubicin. The toxicity of these compounds might be due to their functional groups (hydrazine carbothioamide, phenylhydrazineylidene, methylthio-triflurometyl, nitrobenzaldehydeoxime, dichlorophenyl, and benzoic acid, respectively) attached to the parent group (nitrovanillin), rendering them potentially toxic even at a concentration as low as 100 µM. Compounds **3**, and **7** containing bismethoxy nitrophenol, and hydroxylphenyl imino groups, respectively, were also found highly toxic than the doxorubicin (Table 1). However, compound **2** with tri-fluoro carbon at *ortho* position (SFig. 1) exhibited a least toxic behavior in HepG2 cells. Trifluoromethyl phenyl appears to be associated with low cytotoxicity of compound **2**. A study demonstrated that adding a trifluoromethyl group to triphenylphosphonium-conjugated compound has reduced toxicity in mice, while preserving its antitumor efficacy [34]. Similarly, another study highlighted the protective effects of a curcumin analog, bearing a

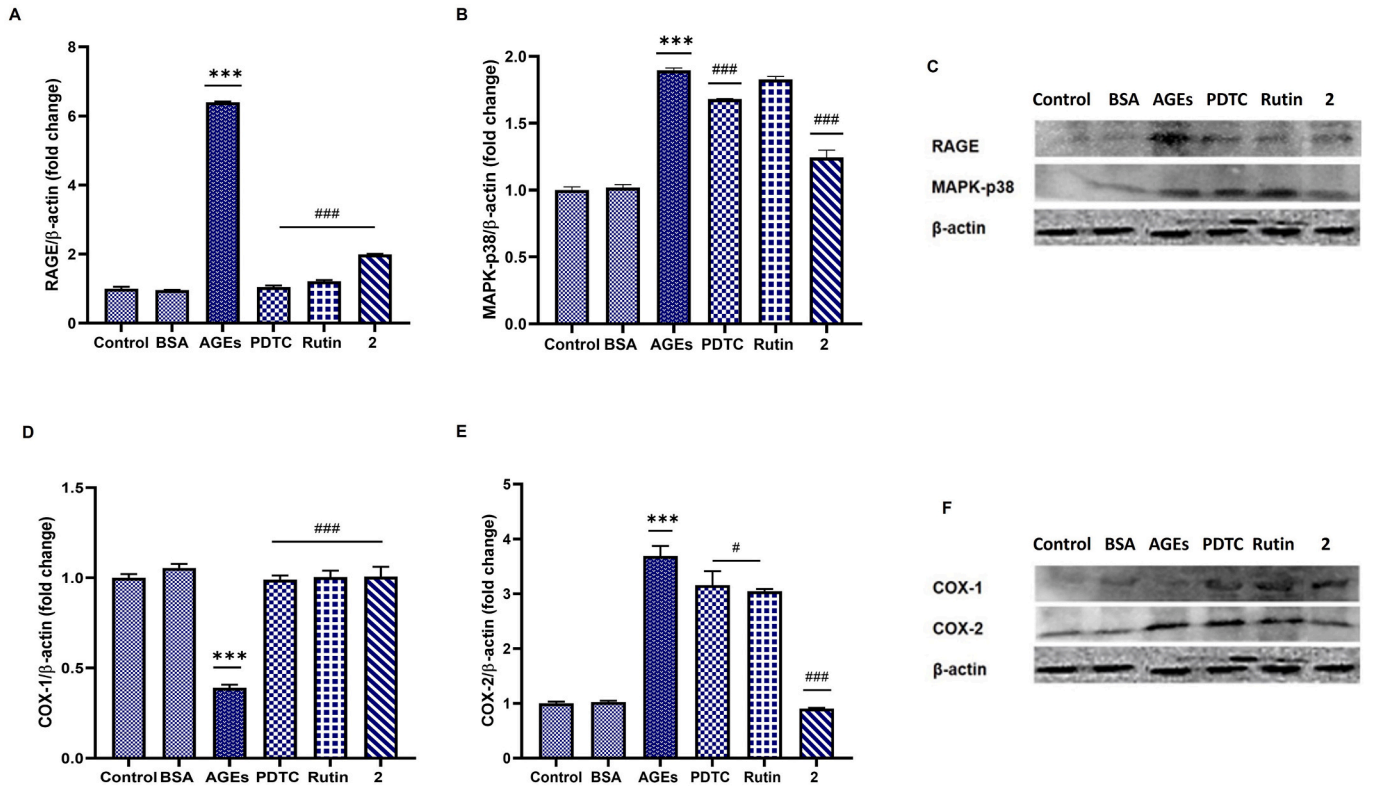


Fig. 5. Effect of compound **2** on the regulation of RAGE, p38, COX-1, and COX-2 protein levels in AGEs-treated THP-1 monocytes. The cells were pre-treated with compound **2**, and reference compounds (PDTC, and rutin) (100 μM) for 1 h, followed by incubation with MGO-AGEs (200 μg/mL) for 6 h. (**A**, and **B**) The graphs show the increase ($p < 0.001$, ***) in RAGE and p38 proteins levels in MGO-AGEs-treated THP-1 cells, as compared to untreated cells and BSA-treated control. While compound **2** and reference compounds significantly decreased ($p < 0.001$, ###) their levels, as compared to AGEs-treated cells. (**C**) Western blot showing a remarkable increase in RAGE and p38 signals in AGEs-treated cell, which were suppressed by the treatment of compound **2** and reference compounds. (**D**, and **E**) Exhibit significant decrease and increase ($p < 0.001$, ***) in COX-1, and COX-2 proteins levels, respectively, in AGEs treated cells *vs* untreated cells and BSA control. Whereas, compound **2** and reference compounds reversed the COX-1 suppression, and decreased ($p < 0.001$ and 0.05, ### and #, respectively) COX-2 protein levels in cells, co-incubated with MGO-AGEs. (**F**) The blot shows a noticeable decrease in COX-1, and increase in COX-2 protein expression in AGES-treated cell, which was substantially decreased with the treatment of compound **2**, and reference compounds. (**C**, and **F**) β-Actin was used as a loading control, and from the same protein lysates, and run altogether on the same gel under similar experimental conditions.

trifluoromethyl moiety, against acrolein-induced toxicity in retinal pigment epithelial cell line (ARPE-19) [35]. In agreement with these findings, the low or negligible toxicity of compound **2** may be due to the presence of trifluoromethyl phenyl moiety. Additionally, our findings further reveal the influence of trifluoromethyl group at the *meta* position, as seen in compound **4**, is associated with moderate toxicity, whereas its substitution at the *ortho* position, as in compound **2**, exhibited no toxicity to the cells. Compound **2** was further evaluated on THP-1 monocytes, and found non-cytotoxic at all the tested concentrations (Table 1), and hence further studied to assess the cell receptor response, and signaling pathways in AGEs-treated THP-1 monocytes.

AGE-RAGE ligation activates multiple signaling pathways, including JAK2-STAT1, MAPK, PI3K-AKT, and NADPH oxidase. These further lead to the transcription of pro-inflammatory cytokines, genes, and growth factors through NF-κB activation, and formation of AGEs through a positive feedback loop [36,37]. Inhibition of AGEs can effectively block these signaling cascades, and the transcription of inflammatory molecules. Rasheed *et al.* reported that blocking MAPK-p38 and ERK signaling ceased the AGE-induced production of IL-1β, and IL-6 in osteoarthritis chondrocytes [38]. Another study reported a significant reduction in TNF-α, IL-6, and IL-1β secretion, which persist even after 24 h removal of AGE-albumin in LPS-treated macrophages [31]. Furthermore, the traditional Chinese medicine (TCM) *Zhen-wu-tang* (ZWT) had shown to decrease serum levels of AGEs, and inhibited the release of inflammatory mediators, including TNF-α, IL-1β, and IL-6, in a rat model of membranous glomerulonephritis [39].

AGE-RAGE interaction triggers the activation of mitochondrial respiratory chain, NADPH oxidases, xanthine oxidase, and arachidonic acid signaling pathways [40]. Thallas *et al.* suggested that inhibition of NADPH oxidases (NOX 4) and downstream signaling could prevent diabetes-related micro-vascular complications [41]. In the current study, MGO-AGEs found to stimulate ROS in THP-1 monocytes, which was considerably higher than the controls (BSA, and untreated cells). However, we observed a significant decrease in AGEs-dependent ROS production by compound **2**. This may be due to the inhibition of NADPH oxidase activity, and by scavenging ROS production through mitochondrial electron transport chain reactions. The radical capturing ability of compound **2** can be attributed to the electron withdrawing effect of trifluoromethyl substituent [42]. This substituent may help in stabilizing the ROS, thus reducing the oxidative stress. This is in agreement with the previous studies which demonstrate that vanillin, and its derivatives, have radical-scavenging ability, and exhibited antioxidant effects by neutralizing free oxygen radicals within the mitochondrial membranes of rat liver cells, thereby preventing membrane damage [43], and elevating antioxidant levels in mice blood plasma [44]. Furthermore, apocynin (NADPH oxidase inhibitor) exhibited a 65 % (100 μM) decrease in ROS production. However, its effect was comparatively lower than that of compound **2**, and the reference compound rutin (71 % at 100 μM).

The transcription factor NF-κB plays a crucial role in regulating immune and inflammatory responses. NF-κB (p65:p50 heterodimer) is primarily activated through the canonical pathway by pathogens and

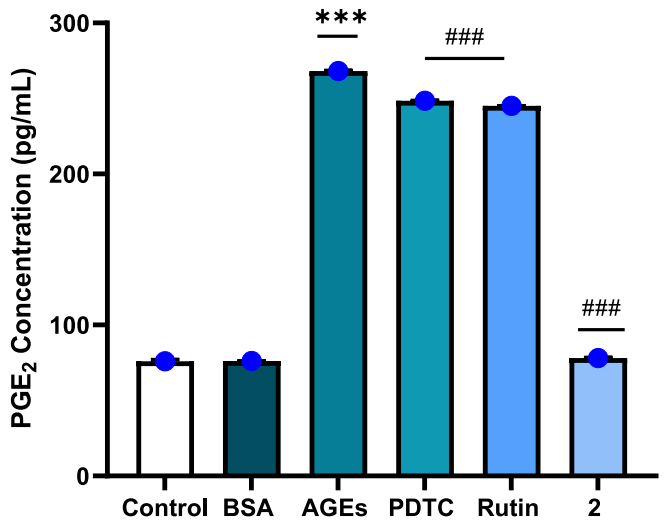


Fig. 6. Compound **2** inhibited the AGEs-induced PGE_2 levels in THP-1 monocytes. The cells were pre-treated with compound **2**, and reference compounds (PDTC, and rutin) (100 μM) for 1 h, followed by the treatment with MGO-AGEs (200 μg/mL) for 6 h. The graph (***) indicates a significant increase ($p < 0.001$) in PGE_2 concentration in AGEs-treated cells, as compared to untreated cells and BSA control. Whereas pre-exposure to compound **2**, and reference compounds considerably inhibited ($p < 0.001$, and $p < 0.05$, respectively, ###) the PGE_2 production, as compared to AGEs-treated cells. The data indicate mean ± S.D. of three individual experiments.

inflammatory mediators. Excessive increase in activated p65, and subsequent stimulation of effector molecules is central to the development of several chronic inflammatory conditions [45]. A study showed that AGEs activate p65, and inflammatory gene expression (TNF, IL-6, and IL-1 beta) in macrophages for up to 24 h [31]. Additionally, AGEs-induced ROS formation reported to activate NF-κB (p65) signaling in THP-1 monocytes [28]. Similarly, we observed a significant rise in p65 expression in AGEs-treated THP-1 monocytes (Fig. 4), which was markedly decreased by the treatment with PDTC (an NF-κB inhibitor), rutin (a known antioxidant), and compound **2**. Antioxidants are reported to scavenge ROS, and interfere with proteins and kinases, thereby inhibiting NF-κB signaling [46]. Zabad *et al.* showed that the oral administration of vanillin suppressed renal expression of NF-κB and cytokine secretion in diabetic model of nephropathy in rats [47]. Similarly, the ability of compound **2** to suppress p65 activation, and inhibit NF-κB activity can be attributed to its capacity to hinder ROS formation through its radical stabilizing properties in AGEs-treated THP-1 monocytes.

RAGE plays an important role in activating AGEs-associated ROS signaling, and causes the release of pro-inflammatory molecules in THP-1 cells. While excessive generation of ROS triggers the RAGE expression through NF-κB activation through a positive feedback loop [48]. Thus, suppression of RAGE can limit the AGEs-mediated inflammation, and cell apoptosis. In current study, we observed a significant increase in RAGE protein levels in AGEs-treated monocytes, which was markedly decreased by compound **2** treatment. Earlier studies demonstrate that vanillic acid possesses anti-inflammatory and antioxidant properties, therefore, inhibited LPS-induced RAGE/NF-κB signaling in glial cells [49]. Likewise, this study indicated that compound **2** inhibited RAGE expression by blocking the AGEs binding site on the RAGE [50], and redox signaling *via* NF-κB inhibition in THP-1 monocytes. Similarly, pre-treatment of cells with PDTC, and rutin, significantly decreased the RAGE expression. This agreed with the prior studies, supporting the anti-inflammatory effects of PDTC in macrophages *via* RAGE/NF-κB nexus [51], and suppression of RAGE (mRNA) levels in high glucose-exposed THP-1 monocytes by rutin [52].

AGE-RAGE ligation also initiates the MAPK-p38 signaling through ROS generation, which further leads to the activation of NF-κB [36]. The p38 downstream signaling also regulates the release of cytokines (IL-1β, and TNFα), growth factors, and cell adhesion molecules (ICAM, and VCAM) in monocytes [53]. These events eventually contribute to inflammation, and cellular dysfunction. In agreement with the existing literature, we observed a significant increase in phospho-p38 levels in AGEs-treated THP-1 monocytes. Whereas, p38 signaling was considerably suppressed by the pre-treatment with PDTC, which can be co-related with its ability to intercept AGE-RAGE ligation *via* NF-κB inhibition. However, standard (rutin) did not alter p38 expression, which indicates that p38 modulation *via* rutin is independent of ROS signaling [52], and might be regulated through other upstream signaling pathways. On the other hand, compound **2** inhibited p38 activation in THP-1 monocytes, apparently *via* AGE-RAGE/ROS/NF-κB nexus, indicating its efficacy against AGE-induced inflammation.

Furthermore, the current study showed that inhibition of AGE-RAGE binding can significantly alter the temporal expression patterns of COX proteins. COX-1 is a housekeeping protein, which is normally expressed in various cells and tissues. COX-1 inhibition reduces the synthesis of thromboxane A2 (a vasoconstrictor and platelet aggregator), thus inhibiting the formation of a platelet plug [54,55]. COX-1 inhibitors are widely used for the prevention of cardiovascular complications in high-risk population. However, long-term use of COX-1 inhibitors is not recommended [54] due to its adverse effects, including stomach distress, GI bleeding, and ulcers.

Recent studies have exhibited that MGO-AGEs markedly suppressed the COX-1 expression in THP-1 monocytes [15], indicating that AGEs exposure can lead to COX-1 related side effects. We again observed a significant decrease in COX-1 levels in MGO-AGEs treated THP-1 cells. Whereas, the pre-treatment of cells with PDTC, rutin, and compound **2** significantly reversed the AGEs-mediated abrogation of COX-1, and raised their levels equivalent to the untreated, and BSA-treated controls (Fig. 5).

The expression of COX-2 is induced in response to inflammatory stimuli, and is associated with the production of pro-inflammatory prostaglandins. AGEs-induced activation of COX-2 (an inducible gene) is linked with inflammation through various signaling pathways (p38, ERK1/2, PKC, JNK, and NF- κB) [56,57]. In this study, we observed that MGO-AGEs significantly raised the COX-2 levels in THP-1 monocytes, most likely through the activation of AGE-RAGE/NF-κB/p38 pathway. However, PDTC, and compound **2** reduced the COX-2 protein expression by inhibiting the AGE-RAGE binding, and by suppressing p38, and NF-κB signaling. On the other hand, rutin seems to implicate the AGE-RAGE/ROS/NF-κB nexus [52]. However, the effect of compound **2** was found relatively superior than the rutin, and PDTC. Thus, blocking the AGE-RAGE interaction not only affects the inducible expression of COX-2, but also reversed COX-1 expression, thereby reducing the inflammatory response, and the COX-1 associated adverse effects.

In addition, the impact of AGEs on PGE2 levels, a major product of COX-2 that is involved in inflammation, was also evaluated. Our results indicate a significant increase in PGE_2 levels in AGEs-stimulated THP-1 monocytes [15]. However, the effect of AGEs on PGE_2 production was significantly reduced with the pre-treatment of cells with PDTC, rutin, and compound **2**. Whereas, the effect of compound **2** was comparatively superior than the reference compounds. This suppression is attributed to the efficacy of compound **2** in mitigating the AGEs response at the cell receptor level, and cell signaling at various steps, particularly COX-2 production. Specifically, our results suggest that compound **2** has the potential to inhibit AGEs-mediated inflammatory responses in THP-1 monocytes, by capturing reactive oxygen and carbonyl compounds, interfering the binding between AGE and RAGE, and inhibiting the activation of NF-κB /p38/COX-2 signaling.

The AGE-RAGE-NF-κB axis serves as a central mechanism underlying chronic inflammation in various diseases, including diabetes, arthritis, neuroinflammation, and cancer [10]. Our findings indicated that compound **2** possesses antiglycation, antioxidant, and anti-inflammatory

properties, and suppresses AGE-RAGE-ROS-NF-κB nexus in THP-1 monocytes, demonstrating a potential protective mechanism against diabetes-related vasculopathies. Given its potential, and the implication of this pathway in chronic inflammatory conditions, the therapeutic effects of compound **2** may extend for the management of aforementioned conditions. For instance, AGE-RAGE-mediated PI3K/AKT signaling is involved in the transcription of inflammatory molecules and genes (such as, IL-1β, Il-6, TNF-α) *via* NF-κB activation in neuroinflammation, and neurodegenerative diseases [58]. Additionally, AGE-RAGE-mediated AMPK signaling is implicated in reduced insulin sensitivity in various tissues, impairing glucose metabolism [59]. Therefore, by inhibiting the AGE-RAGE interaction, and subsequent inhibition of PI3K/AKT or AMPK, compound **2** may also prevent the neuroinflammation, and improve glucose metabolism in diabetes.

Previous studies have examined the effects of several antiglycation agents for their potential to inhibit AGEs formation at various levels. However, their clinical trials were primarily failed due to adverse effects, lack of efficacy, and stability issues. Aminoguanidine, for instance, was withdrawn from clinical trials due to severe adverse effects, including flu-like symptoms, anemia, glomerulonephritis, and autoimmune responses. Similarly, azeliragon, a RAGE inhibitor, caused confusion and cognitive decline in Alzheimer's disease patients [60]. Alagebrium, an AGE inhibitor and AGE cross-link breaker has demonstrated efficacy in reducing arterial and left ventricular stiffness, but failed to exhibit significant improvements in overall cardiac function [61]. Whereas, compound **2** was studied in THP-1 monocytes to determine its inhibitory effects in AGEs-mediated vascular inflammation *in vitro*. Compound **2** exhibited a safe cytotoxicity profile most likely due to the presence of a trifluoromethyl phenyl moiety among all the tested compounds **1**–**11**. It has shown promising attributes, such as inhibits the AGEs formation, blocks the AGE-RAGE interaction, decreases intracellular ROS production, and downregulates pro-inflammatory molecules, and reversed suppressive effect of AGEs on COX-1. These attributes make compound **2** as a promising antiglycation and anti-inflammatory agent, however, it is difficult to predict it's *in vivo* effects in preclinical models and in clinical trials. The identification of compound **2** as anti-inflammatory and cardiovascular protective agent is the first stage of drug discovery process, which needs to be validated in *in vivo* models of cardiovascular and inflammatory diseases.

5. Conclusion

MGO-AGEs stimulate the pro-inflammatory M1 state of monocytes, exacerbating the progression of micro- and macro-vascular complications, associated with diabetes, including atherosclerosis. Therefore, preventing the pro-inflammatory effects of AGEs could also slow down the progression of diabetic vasculopathies. The current study reveals that compound **2** (*E*)-2-methoxy-6-nitro-4-(((2-(trifluoromethyl)phenyl) imino)methyl)phenol effectively suppress the pro-inflammatory effects in monocytes by blocking the AGE-RAGE-mediated redox signaling. Compound **2** also ameliorates pro-inflammatory state, and restored the COX-1 levels in THP-1 monocytes. Given the potential of compound **2** as a glycation inhibitor and anti-inflammatory agent, further investigations are warranted to validate its efficacy in appropriate *in-vivo* models. These may include the carrageenan-induced paw edema model for acute inflammation, the collagen-induced arthritis model for chronic inflammation, and the AGE-induced vascular inflammation model. Such studies are essential towards its validation as a drug lead.

CRediT authorship contribution statement

Priya Tufail: Writing – original draft, Methodology, Investigation, Formal analysis. **Sajjad Anjum:** Methodology. **Bina Shaheen Siddiqui:** Methodology. **Marina Pizzi:** Writing – review & editing. **Humera Jahan:** Writing – original draft, Validation, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization. **M. Iqbal Choudhary:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2025.167784.

Data availability

The data supporting this study's findings are available from the corresponding authors upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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