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1,2,4-Triazine derivatives as agents for the prevention of AGE-RAGE-mediated inflammatory cascade in THP-1 monocytes: An approach to prevent inflammation-induced late diabetic complications

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A R T I C L E I N F O

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A B S T R A C T

Introduction: Monocytes mainly contribute to the development and progression of vascular inflammatory conditions *via* the M1 polarization. The elevated levels of advanced glycation end products (AGEs) in diabetic environment lead to severe inflammation, and the release of pro-inflammatory mediators. This shifts the balance towards the pro-inflammatory state of monocytes.
Objective: The current study was aimed to determine the antiglycation activity of 1,2,4-triazine derivatives, and study of their molecular basis in regulating the AGEs-mediated inflammatory responses in THP-1 monocytes.
Methods: Primarily, the antiglycation activity of a series of 1,2,4-triazine derivatives was evaluated against MGO-AGEs *in vitro*. The toxicity of antiglycation compounds was determined by a metabolic assay, using human hepatocyte (HepG2) and monocyte (THP-1) cell lines. DCFH-DA probe was used to evaluate the antioxidant potential of the compounds. Immunocytochemistry, Western blotting, and ELISA techniques were employed to determine the levels of pro-inflammatory markers (NF-κB, RAGE, COX-1, COX-2, and PGE_2) in THP-1 monocytes under *in-vitro* hyperglycemic conditions.
Results: Results indicate that the triazine derivatives **22**, and **23** were the most potent antiglycation agents among the entire series, while non-toxic to HepG2, and THP-1 cells. Both compounds inhibited the AGEs-induced upstream and downstream signaling of NADPH oxidase and inflammatory mediators p38 and NF-κβ, respectively, in THP-1 monocytes. They also inhibited the induction of COX-2 and its product PGE_2 by suppressing AGE-RAGE interactions. Moreover, compounds **22**, and **23** reversed the AGEs-mediated suppression of COX-1 in THP-1 monocytes.
Conclusion: In conclusion, 1,2,4-triazine derivatives **22**, and **23** have the potential to suppress inflammatory responses under the diabetic environment through AGE-RAGE-NF-κβ/p38 nexus in THP-1 monocytes. These findings identify triazines **22**, and **23** as compelling candidates for drug development, potentially beneficial for the diabetic patients with an elevated risk of vascular complications, such as atherosclerosis.

1. Introduction

Diabetic vasculopathy is one of the major complications associated with hyperglycemia, which causes retinopathy, nephropathy, neuropathy, and atherosclerosis. Several studies explain the mechanisms of hyperglycemic condition on neural, renal, and vascular tissues [1]. Intracellular hyperglycemia implicates oxidative stress through the polyol pathway, increased the expression of signaling molecules *via* PKC activation, and promote non-enzymatic glycation. This results in increased production of advanced glycation end products (AGEs) [2]. Non-enzymatic glycation involves the linkage between reducing sugars and the amino group of proteins, forming a Schiff base that undergoes several rearrangements to form a stable Amadori product [3–5]. The oxidation of Amadori products gives rise to the formation of irreversible

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AGEs. The synthesis of AGEs is catalyzed by the reactive oxygen species (ROS), where pentosidine is the major glycooxidative product, widely used as a marker for measuring AGEs in plasma and tissues [6–8]. Glyoxal and methylglyoxal are highly reactive carbonyl species that rapidly react with lysine side chains of proteins, causing the formation of cross-links, such as glyoxal-lysine dimer (GOLD) or methylglyoxal-lysine dimers (MOLD) [9]. Several studies indicate the implication of these molecules in diabetes, and inflammation, endothelial dysfunction, vascular complications, decreased vascular elasticity, atherosclerosis, etc. Therefore, various AGEs, including pentosidine, MOLD, GOLD, and carboxymethyllysine (CML), are used as biomarkers to detect the level of AGEs in diabetic patients [10].

The AGEs exert cellular effects by interacting with AGEs-specific receptors (RAGE), which are extensively expressed in different cell types, including monocytes, macrophages, endothelial, and smooth muscle cells under pathological conditions [11]. Though monocytes are the defensive immune cells, they also actively participate in developing vascular complications under the influence of AGEs. Depositions of AGEs in interstitial spaces lead to endothelial dysfunction, consequently increasing the vascular permeability and recruitment of monocytes at the site of inflammation [12]. AGEs-RAGE interaction at the cell surface generates oxidative stress through the NADPH oxidase enzymatic system, activating Ras- and redox-sensitive pathways. It also transduces multiple signaling mechanisms, including extracellular signal-regulated kinase (ERK ½), p38, mitogen-activated protein kinase (MAPK), and c-Jun kinase (JNK) which promote the transcription of NF-κB [13–16]. Activation of NF-κB modulates the expression of certain genes, causing the release of pro-inflammatory molecules and cytokines, such as IL-1, TNF-α, and COX-2, etc., consequently leading to inflammatory, thrombogenic, angiogenic, and apoptotic responses (Fig. 1) [12,17].

Therefore, inhibition of glycation at various stages is crucial to prevent the deleterious effects of AGEs. Literature surveys indicate that aminoguanidine, a prototype AGEs inhibitor, reduce atherosclerotic plaque, prevent angiogenesis, and improve sensory and motor nerve conductions in diabetic animal models [18–20]. Despite these promising results, the currently available drug candidates were found implausible to be used for therapeutic purposes due to their safety concerns [20]. Indeed, AGEs cross-link breaker, such as alagebrium, showed reversal of arterial and ventricular stiffness in randomized controlled clinical trials. However, it has insignificant effect on overall cardiovascular health. Similarly, azeliragon, a RAGE antagonist, reported to reduce the cognitive decline in Alzheimer's patients, has been tested against diabetic neuropathy. Unfortunately the development work on these drugs was discontinued due to unexplained reasons [21].

To this content, there is a need to investigate potential inhibitors of AGEs that avoid adverse effects. 1,2,4-triazine derivatives are heterocyclic compounds containing three nitrogen groups in their basic skeleton. 1, 2, 4-Triazine derivatives exhibit various biological activities, including anti-inflammatory, anticancer, antihypertensive, antifungal, antiviral, antimicrobial, and antimalarial properties [22–24]. Shahbaz *et al.* reported that 1,2,4-triazine derivatives are the potential inhibitors of α-amylase and α-glucosidase enzymes, and can act as a therapeutic agents for managing diabetes [25]. Considering the importance of the 1,2,4-triazine derivatives [25], the current study investigates their antiglycation effects against methylglyoxal-derived AGEs (MGO-AGEs). Studies highlight that AGE-RAGE ligation in high-glucose and S100b stimulated monocytes induce the expression of inflammatory cytokines, growth factors, receptors, and enzymes [26,27]. Monocytes instigate inflammatory responses and recruit a variety of cells in atherosclerotic plaques; thus their presence, along with lymphocytes, is the hallmark of atherosclerotic disease [27–29]. Therefore, an *in vitro* model of monocytes (THP-1) was used to study the impact of AGE inhibitors on the modulation of cell signaling molecules, and pro-inflammatory mediators. Metabolism-associated toxicity was determined by using human hepatocytes (HepG2) containing the intact drug metabolizing enzymatic system, cytochrome P450, to investigate the safety and effectiveness of lead inhibitors.

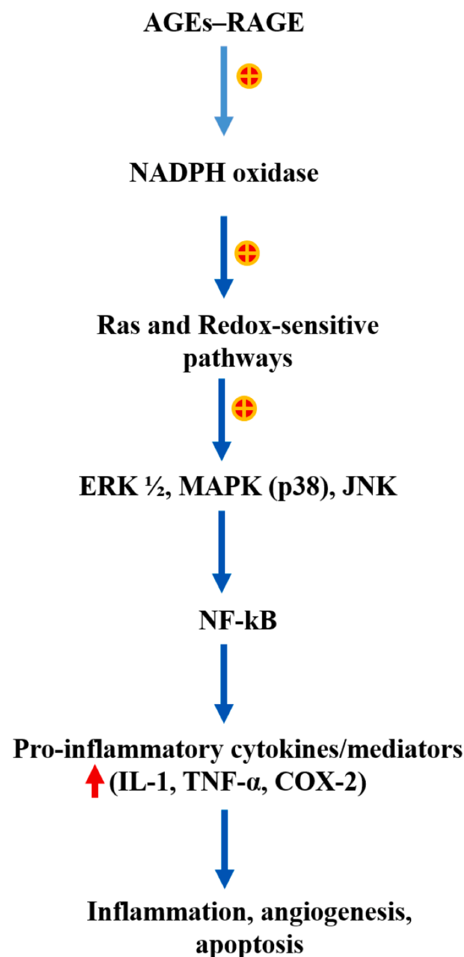

Fig. 1. Illustration of the signal transduction pathway, triggered by the interaction between AGEs and RAGE.

2. Material and Methods

2.1. Antiglycation activity

The antiglycation activity of 1,2,4-triazine derivatives was determined by using an *in vitro* antiglycation assay [30]. The test compounds (1 mM) were dissolved in 10 % DMSO (Amresco LLC, USA). The mixture of 500 mM methylglyoxal (Thermo Fisher, USA) and 10 mg/mL BSA (Sigma-Aldrich, USA) was prepared in 0.1 mM sodium phosphate buffer containing 0.1 mM sodium azide (Merck, Germany). Rutin hydrate (1 mM) was used as a standard antiglycation agent. The test compounds were incubated with MGO and BSA for 24 h in a dark, and sterile conditions at 37 °C. Each test was performed in triplicate, and fluorescence was measured at 355 nm excitation /460 nm emission using a Varioskan Lux Microplate reader (Thermo Fischer, USA). Fluorescence emitted by MGO-AGEs was determined by using the following formula:

$$\%Inhibition\ of\ fluorescence = (1 - Fluorescence\ of\ test\ compounds/Fluorescence\ of\ glycated\ BSA) \times 100$$

Compounds that exhibited antiglycation potential were serially diluted at various concentrations, and IC_{50} (μM) was evaluated using the EZ-FIT Enzyme Kinetics Program (Perrella scientific Inc., USA).

2.2. Preparation of methylglyoxal (MGO)-derived AGEs

MGO-AGEs were prepared, as described earlier [30]. Sodium phosphate buffer (0.1 mM) was prepared by dissolving sodium hydrogen phosphate and sodium dihydrogen phosphate in distilled water, and the pH was adjusted to 7.2. MGO (500 mM) was incubated with BSA (10 mg/mL), the reagents were passed through a 0.22 μm filter, and incubated under sterile conditions at 37 °C for 24 h. Fatty acid-free-bovine serum albumin (BSA, Sigma-Aldrich, USA) (10 mg/mL) was dissolved in a sodium phosphate buffer, which was used as a control. The unbound MGO was removed by dialysing the mixture in sterile PBS for 2 h at 4 °C. AGEs-specific fluorescence was measured, as described above, and the levels of endotoxins were measured by LAL assay kit (Thermo Fisher Scientific, USA). The samples were stored in aliquots at −40 °C for further use.

2.3. Culturing THP-1, and HepG2 cells

Human monocytes (THP-1) were purchased from ATTC, and maintained in a sterile environment till 20 passages. The cells were cultured in a 75 cm^2 flask using RPMI-1640 medium (Gibco, Thermo Fisher Scientific, USA) containing 10% FBS, 1 mM Sodium pyruvate, and L-glutamate (Gibco, Thermo Fisher Scientific, USA), and 1 g/100 mL glucose anhydrous. Human hepatocytes (HepG2) (ATTC) were cultured in a minimal essential medium (MEM), containing 10 % FBS (Thermo Fischer Scientific, USA). Trypsin-EDTA (Gibco, Thermo Fisher Scientific, USA) was used for cell detachment. The cells were centrifuged at 1000 rpm for 10 min, the supernatant was discarded, and the cell pellet was dissolved in a freshly prepared medium. The cells were counted using trypan blue assay, and seeded in a tissue culture flask. The cells were incubated at 37 °C containing 5% CO_2 till they became 80–90% confluent.

2.4. Cytotoxic evaluation by metabolic assay

The cytotoxicity evaluation was carried out using HepG2, and THP-1 cell lines [30]. The HepG2 (8 x 10^4/mL) and THP-1 (20 x 10^4/mL) cells were seeded in a sterile 96-well tissue culture plate (Nest Biotech Co. Ltd., China) in a low-serum medium (2% FBS), and incubated at 37 °C and 5% CO_2. After overnight incubation, the cells were treated with various concentrations of test compounds (100, 70, 50, 30, and 10 μM), and incubated for 24 h under same experimental conditions. Later, the test medium was discarded, and HepG2 cells were incubated with 100 μL of MTT (0.5 mg/mL) for 3 h. After that the medium was removed, and the resulting formazan crystals in live cells were dissolved in DMSO (100 μL). Whereas, 20 μL WST-1 dye was added in THP-1 cells for 3 h. The plates were thoroughly shaken for 5 min on a 96-well plate shaker, and the optical density was recorded at 540 nm (HepG2), and 450 nm (THP-1) *via* Spectra max spectrophotometer (Thermo Fischer Scientific, USA).

The toxic effect of compounds was analyzed by calculating cell inhibition percentage by using the following formula:

$$\textit{Cell inhibition percentage} = 100 - [(\textit{Absorbance of test compound} - \textit{Absorbance of blank})/(\textit{Absorbance of control} - \textit{Absorbance of blank}) \times 100]$$

2.5. Assessment of ROS by DCFH-DA assay

The THP-1 cells (1 x 10^6/mL) were treated with 2,7-dichlorodihydrofluorescein diacetate (10 μM) (DCFH-DA; Sigma-Aldrich, USA) probe, and incubated for 45 min in a dark sterile environment at 37° C. Cells were washed twice with PBS, and treated with various concentrations (100, 50, 30, and 10 μM) of compounds for 1 h. The apocynin, NADPH oxidase inhibitor (100 μM), and rutin (antiglycation agent) (100, 50, 30, and 10 μM), were used as standard reference. Later, cells were exposed to MGO-AGEs (200 μg/mL), and kept in an incubator at 37 °C for 24 h. The ROS inducer H_2O_2 (100 μM) was taken as positive control, whereas untreated and BSA-treated cells were used as negative controls. The fluorescence intensity was measured at 490 nm excitation /520 nm emission using the Variosakn Lux microplate reader (Thermo Fischer Scientific, USA). The inhibition of MGO-AGEs-induced ROS was determined by using the formula:

% Inhibition = 100 – (Fluorescence of test compound − Fluorescence of blank/Fluorescence of control- Fluorescence of blank x 100).

2.6. Analysis of NF-κB by immunocytochemistry

The activation of NF-κB in THP-1 cells was detected by employing immunocytochemistry technique. The THP-1 cells (1 x 10^6 cells/mL) were pre-treated with test compounds, PDTC; NF-κB inhibitor (100 μM), and rutin (100 μM) at 37° C for 1 h, followed by the treatment with AGEs (200 μg/mL) for another hour. Later, the cells were fixed with 4% formaldehyde, and permeabilized using 0.2% triton X-100 for 10 min. The non-specific protein binding was prevented by incubating the cells in blocking solution (1-% BSA in PBS and 0.1% Tween) for 1 h at room temperature. Each step was followed by washing with chilled PBS thrice for 5 min. At last, the cells were kept at 4 °C overnight with primary antibody NF-κB p^{65} (Thermo Fischer Scientific, USA), followed by incubation with FITC conjugated anti-rabbit IgG polyclonal secondary antibody (Abcam, UK). The nuclei were stained with DAPI (Thermo Fischer Scientific, USA) for 2 min, and the images were obtained using a TE2000 fluorescence microscope (Nikon, USA) through NIE software. The fluorescence intensity of DAPI-positive cells (for each treatment) was measured from 10 different fields using Image J software (NIH, USA), and the mean fluorescence intensity was presented in the graph.

2.7. Protein expression through Western blotting

Expressions of COX-1, COX-2, p38, and RAGE, along with β-actin protein in THP-1 cells, were determined through the Western blotting technique. THP-1 cells (1 x 10^7 cells/mL) were pre-treated with PDTC and rutin (100 μM), and test compounds for 1 h, followed by incubation with MGO-AGEs for 6 h. The cells were lysed using RIPA lysis buffer for 30 min on ice, and were sonicated by giving 3 impulses (10 sec each) with the interval of 30 sec on ice. The lysate was centrifuged at 12,000 rpm for 30 min at 4° C, and the samples were stored at −20° C till further experimentation.

The proteins were separated by running the samples (50 μg/mL) on sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to the nitrocellulose membrane. The blot was treated with blocking (3% BSA) for 1 h at room temperature, followed by overnight incubation with COX-2, COX-1, p38, RAGE (ThermoFisher Scientific, USA), and β-actin (Cloud Clone Corp., China) antibodies at 4° C. Later, the membrane was washed with tris buffer saline tween (TBST) thrice with continuous shaking, and incubated with HRP mouse monoclonal anti-rabbit IgG secondary antibody (Abcam, UK) for 1 h. The bands were detected using a chemiluminescence (ECL) detection kit (Sangon Biotech, China), and visualized through a gel doc system. The densitometeric analysis was done using Image J software (NIH, USA).

2.8. Analysis of PGE_2 through ELISA

The THP-1 cells (5 x 10^6 cells/mL) were pre-exposed to test compounds, rutin, and PDTC (100 μM) at 37 °C for 1 h, and then stimulated with AGEs (200 μg/mL). After 6 h, the cells were centrifuged at 1000 rpm for 10 min. The supernatant was collected, and the concentration of PGE_2 was determined by using an enzyme-linked immunosorbent assay kit (Cloud-Clone Corp., China).

2.9. Statistical analysis

The results are from three independent experiments. The data were presented as mean ± SD. One-way ANOVA, followed by Tukey's post hoc test was used to analyze the significant difference between activities of the compounds at various concentrations through SPSS 17. A statistically significant differences was considered for each analysis at $p \leq 0.05$.

3. Results

3.1. Antiglycation activity of 1,2,4-Triazine derivatives

The antiglycation activity of triazine derivatives (Fig. 2) was evaluated against MGO-AGEs *in vitro*. Several compounds of this series exhibited a good antiglycation activity, such as compounds **3** (IC_{50}= 183 ± 0.02 μM), **9** (IC_{50}= 103 ± 0.03 μM), **10** (IC_{50}= 135 ± 0.06 μM), **18**

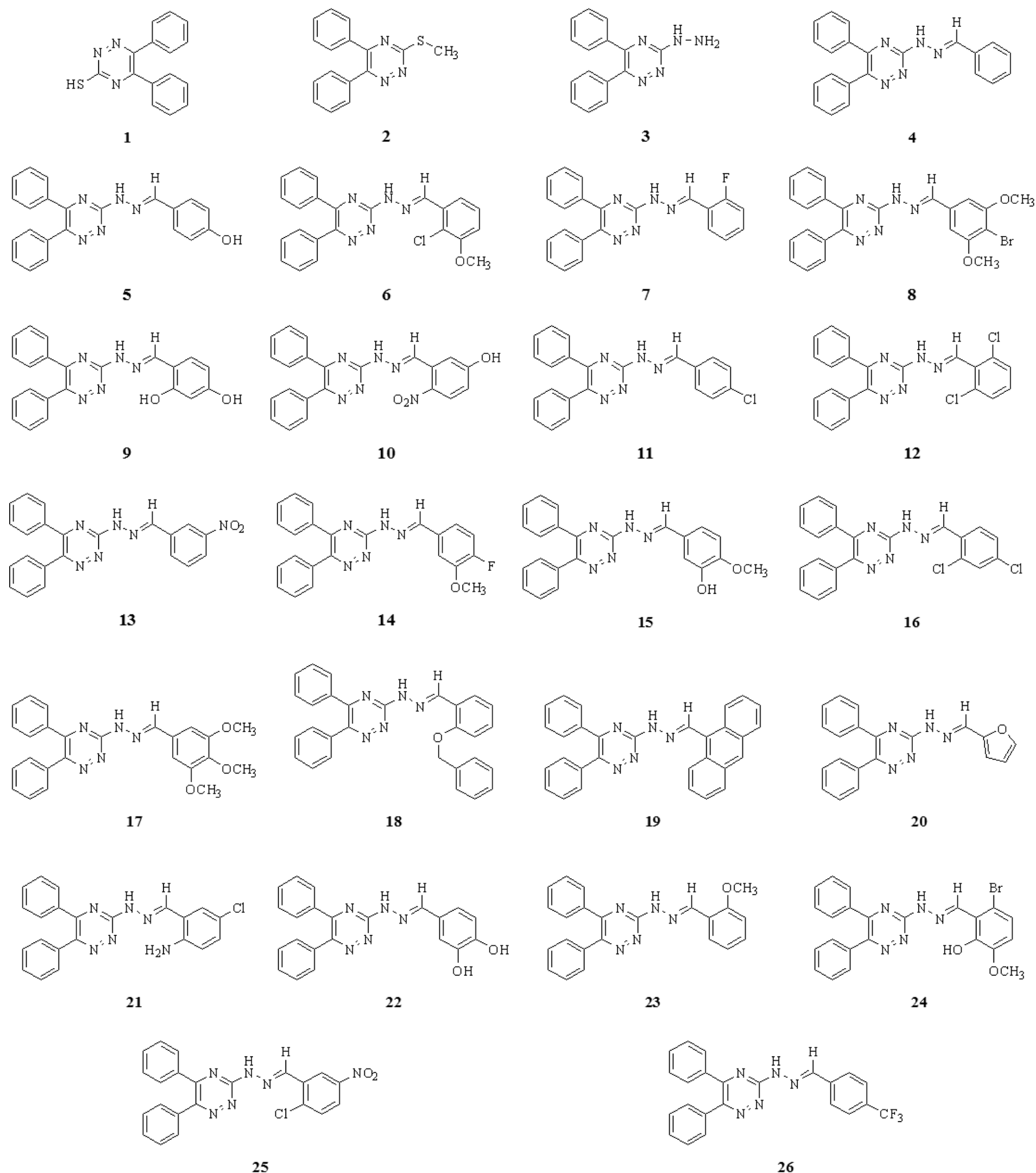

Fig. 2. Structures of 1, 2, 4-triaizine derivatives **1**–**26**.

(IC_{50}= 176 ± 0.02 μM), and **21** (IC_{50}= 194 ± 0.06 μM), **22** (IC_{50}= 93 ± 0.02 μM), **23** (IC_{50}= 91 ± 0.04 μM), and **24** (IC_{50}= 131 ± 0.04 μM), as compared to the reference compound rutin (IC_{50}= 180 ± 0.08 μM). Compound **20** exhibited a moderate antiglycation activity (IC_{50}= 259 ± 0.02 μM), whereas the rest of the compounds were found inactive, having less than 50% activity (Table 1).

3.2. Cytotoxicity of 1,2,4-Triazine derivatives

Compounds **3**, **9, 10**, **18**, and **20**—**24** showed a good antiglycation activity and, therefore, were evaluated for toxicity on HepG2, and THP-1 cell lines at various concentrations (10, 30, 50, 70, and 100 μM). Doxorubicin was used as a reference compound. Among them, compound **24** was found highly toxic (99% cell inhibition) to HepG2 cells at 100 μM, as compared to the standard doxorubicin, which showed 72% cell inhibition. Other compounds **3**, **9**, **10**, **18**, and **20** were moderately toxic, as they exhibited less inhibition of cells growth than standard compounds (Fig. 3). Compounds **21**–**23** showed less than 30% cell inhibition, and were considered least toxic among all the tested compounds. The reference antiglycation compound, rutin, showed 24% inhibition of HepG2 cells at the observed concentrations.

Considering the antiglycation activity, and least toxic behavior on HepG2 cells, compounds **22**, and **23** were further tested on THP-1 monocytes (Fig. 4). Both compounds were found to be non-toxic. Therefore, compounds have proceeded for further study.

3.3. Cell viability of THP-1 monocytes

Furthermore, the WST-1 assay was used to determine the safe concentration at which AGEs stimulate cell signaling without inducing cell death. To do so, THP-1 cells were grown in the presence of MGO-AGEs at various concentrations (10, 30, 50, 100, 200, and 500 μg/mL). The AGEs were shown to induce THP-1 cell growth in a concentration-dependent manner. A significant increase in cell viability was observed at higher concentrations. In contrast, 100% cell viability was observed at 200 μg/mL (Fig. 5). Therefore, AGEs (200 μg/mL) were used to stimulate the cell signaling in further experiments.

Table 1
Antiglycation activity of 1, 2, 4-triazine derivatives (**1**–**26**) against methylglyoxal-derived advanced glycation end products *in-vitro*.

Compounds	Glycation Inhibition %	IC_{50} (μM±SEM)
1	44.62 %	NA
2	21.86 %	NA
3	76.28 %	183 ± 0.02
4	32.19 %	NA
5	32.13 %	NA
6	32.21 %	NA
7	37.65 %	NA
8	21.98 %	NA
9	86.19 %	103 ± 0.03
10	89.56%	135 ± 0.03
11	18.56 %	NA
12	44.83 %	NA
13	22.95 %	NA
14	30.53 %	NA
15	32.23%	NA
16	48.02 %	NA
17	9.98 %	NA
18	68.72 %	176 ± 0.02
19	14.17 %	NA
20	59.97%	259 ± 0.02
21	69.86 %	194 ± 0.06
22	97.14 %	93 ± 0.02
23	70.29 %	91 ± 0.04
24	56.83 %	131 ± 0.04
25	44.17%	NA
26	36.97 %	NA
Rutin	62.88 %	180 ± 0.08

Note: NA=Not active.

3.4. 1,2,4-Triazine derivatives prevent ROS formation in THP-1 monocytes

AGEs produce cellular toxicity by inducing oxidative stress, following binding with RAGE receptors. It leads to the induction of NADPH oxidase and mitochondrial ROS formation. This study evaluated the role of triazine derivatives **22**, and **23** in inhibiting AGE-RAGE-mediated ROS production in THP-1 monocytes through the DCFH-DA technique. Hydrogen peroxide (H_2O_2) was used as a positive control, while BSA and untreated cells were used as negative controls. Our results showed that MGO-AGEs (200 μg/mL) significantly ($p < 0.001$) raised the intracellular ROS production, when compared to positive and negative controls (Fig. 6). apocynin, an NADPH oxidase inhibitor, was used as an internal control. The pre-treatment of cells with apocynin (100 μM) significantly ($p < 0.001$) reduced the ROS production by 65%. The antioxidant potential of rutin (reference compound), and compounds **22**, and **23** were investigated at different concentrations (10, 30, 50, and 100 μM). Compounds **22**, and **23** significantly ($p < 0.001$) reduced the ROS generation at all the tested concentrations (Fig. 6). Compound **22** exhibited antioxidant activity in a concentration-dependent manner. Whereas compound **23** showed stronger antioxidant activity at lower concentrations, slightly decreased activity was observed at higher concentrations. Comparatively, compound **22** was found to be better than compound **23**.

3.5. 1,2,4-Triazine derivatives inhibit activation of NF-κβ in THP-1 monocytes

AGEs-RAGE mediated ROS generation leads to the activation of nuclear factor kappa-B (NF-κβ) [16]. The translocation of p^{65} NF-κβ to the nucleus promotes the inflammation by triggering the release of several pro-inflammatory mediators, such as COX-2 [16]. As we observed, the ability of triazine derivatives **22**, and **23** to prevent the AGEs-induced ROS and their effect on inhibiting NF-κβ was investigated through immunocytochemistry. Our data revealed a significant increase in green fluorescence intensity in MGO-AGEs (200 μg/mL) treated THP-1 monocytes (Fig. 7A), as compared to the control BSA (See Figure S1). In contrast, PDTC (100 μM) and rutin (100 μM) suppressed the p^{65} NF-κβ ($p < 0.001$) activation. Pre-exposure of THP-1 monocytes to compounds **22**, and **23** at 100 and 10 μM concentrations, respectively, considerably halt the activation of p^{65} (RFU: 12, and 23, respectively), as compared to AGEs (RFU: 52), and reference compound rutin (RFU: 32) (Fig. 7B and Figure S2). However, the effect of rutin and both derivatives was comparatively lower than PDTC (RFU: 9, $p < 0.001$).

3.6. 1,2,4-Triaizne derivatives regulate the expressions of RAGE, p38, COX-1, and COX-2

To study the molecular mechanisms involved in inhibiting glycation-associated inflammatory reactions, the effect of triazines **22**, and **23** on the level of RAGE, p38, COX-2, and COX-1 proteins were investigated through western blotting. The quantification of protein (increase or decrease) was done in terms of fold change, whereas β-actin (housekeeping protein) was used to normalize the obtained signals (Fig. 8A).

Our results indicate that AGEs increased the levels of RAGE by 8.39 folds ($p < 0.001$), as compared to the control BSA and untreated cells. However, PDTC and rutin significantly reduced the RAGE levels by 2.60 and 4.79 folds, respectively. Compounds **22**, and **23** suppressed the RAGE ($p < 0.001$) by 8.49 and 2.62-fold, respectively. The inhibitory effect of compound **22** was stronger than PDTC, rutin, as well as compound **23**.

The AGE-RAGE interaction activates various signaling mechanisms, including mitogen-activated protein kinase-p38 (MAPK-p38). The p38 signaling further activates NF-κB, consequently producing pro-

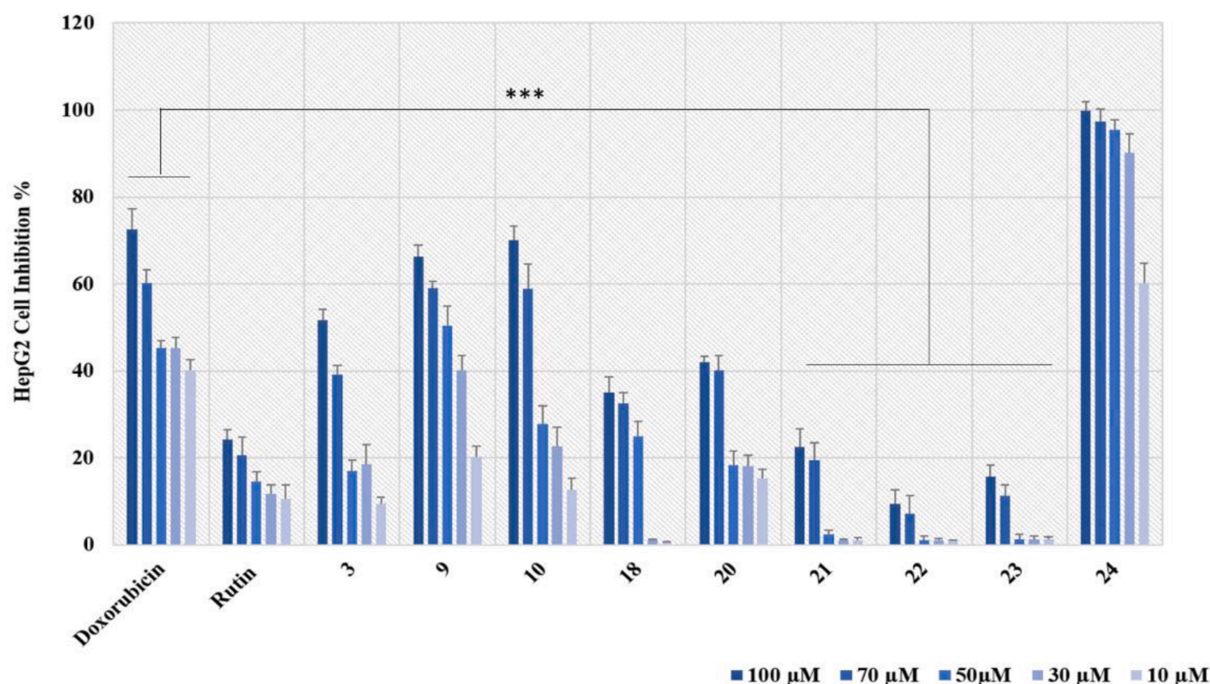

Fig. 3. The cytotoxic effect of selected 1, 2, 4-triazine derivatives on HepG2 cells. Compounds **21**–**23** were significantly ($p < 0.001$) non-toxic showing < 30% cell inhibition at all the tested concentrations (100, 70, 50, 30, and 10 μM), as compared to standard doxorubicin. The data represent mean ± S.D of three individual experiments.

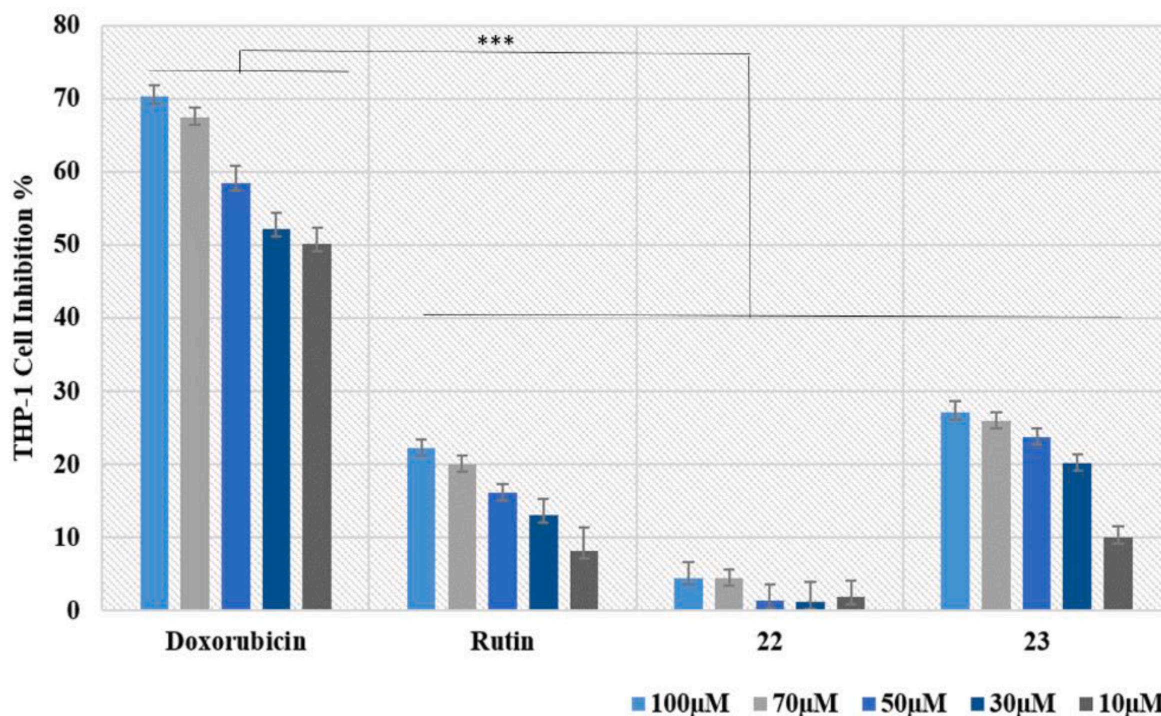

Fig. 4. The cytotoxic effect of 1, 2, 4-triazine derivatives (**22**, and **23**) on THP-1 cells. Compounds **22**,**23**, and standard rutin exhibited non-toxic behavior at all the concentrations (100, 70, 50, 30, and 10 μM), as compared to doxorubicin ($p < 0.001$). The data represent mean ± S.D. of three individual experiments.

inflammatory mediators [16,31]. Here, we investigated the potential of compounds **22**, and **23** in the suppression of p38 signaling. Our results indicate a significant increase of p38 protein (1.89-fold) in AGEs-treated THP-1 monocytes ($p < 0.001$), as compared to BSA and untreated controls (Fig. 8A). In contrast, compounds **22**, and **23** potentially halt the activation of p38. The expression levels were decreased by 1.51- and 1.27-fold, respectively (Fig. 8B). The reference compound PDTC also declined the p38 signals by 1.12 fold. However, insignificant reduction in p38 expressions was observed with rutin treatment.

COX-2 is an inducible gene commonly expressed as an inflammatory mediator and aggravates the condition by stimulating the expressions of other inflammatory molecules [16,32]. Considering the promising

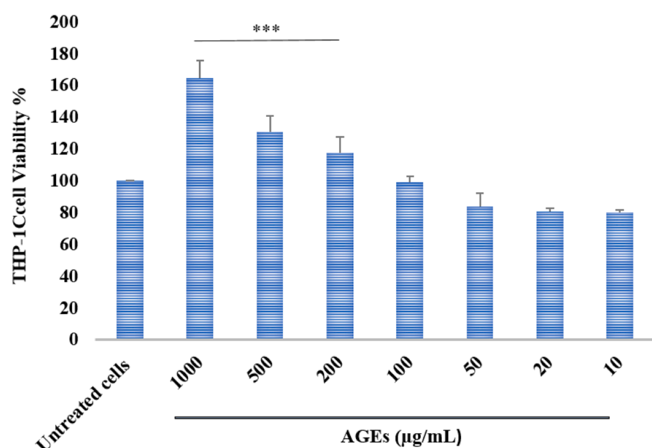

Fig. 5. The effect of MGO-AGEs on the viability of THP-1 cells. The cells were incubated with various concentrations of AGEs (1000, 500, 200, 100, 50, 30, and 10 µM) for 24 h. The cell viability was determined by using WST-1 assay. Concentration dependent increase in cell growth was observed with AGEs treatment. *** Represent significant increase ($p < 0.001$) in THP-1 cell growth in the presence of AGEs, as compared to untreated cells. The data represents mean ± S.D. of three different experiments.

effects of compounds **22**, and **23** on the inhibition of ROS, P^{65}, and p38 proteins, we further evaluated their role in alleviating the AGE-RAGE-NF-κB-induced COX-2 expression in THP-1 monocytes (Fig. 8A and B). Our results indicate that compounds **22**, and **23** significantly ($p < 0.001$) reduced the AGEs-mediated COX-2 induction by 3.86-and 2.34-fold, respectively. In contrast, reference compounds PDTC and rutin decreased the COX-2 ($p < 0.01$) levels by 1.00-fold, as compared to the AGEs-treated cells.

COX-1 is a housekeeping gene that promotes tissue homeostasis by stimulating the release of certain prostaglandins ($PGH_2/PG1_2$) in the GI tract [16]. The decrease in COX-1 levels may lead to life-threatening conditions, such as gastrointestinal bleeding, thrombosis, etc. [16,32]. Considering the initiative towards safe drug discovery, the effect of compounds on the modulation of COX-1 levels was investigated. Surprisingly, we found that AGEs significantly decreased the extent of COX-1 protein ($p < 0.001$) by 0.37-fold, which was significantly restored by the treatment with compounds **22**, **23**, and references, PDTC and rutin (Fig. 8). Compounds **22**, and **23** showed promising effects in blocking the AGEs-mediated suppression of COX-1 ($p < 0.001$) by increasing its levels to 4.95- and 4.84-fold, respectively. Similar results were found with PDTC, and rutin, with a fold change increase of 3.95, and 3.87, respectively.

3.7. 1,2,4-Triazine derivatives inhibited the AGEs-induced levels of PGE_2

COX-2 catalyzes the formation of prostaglandin E_2 from arachidonic acid, which contributes to inflammatory conditions, such as pain and edema [16,32]. Considering the involvement of monocytes in the progression of inflammatory responses to vascular dysfunction under hyperglycemic conditions, the effect of test compounds was analyzed in the suppression of AGEs-induced PGE_2 through competitive ELISA (Fig. 9). The obtained results indicated a significant ($p < 0.001$) increase in PGE_2 concentration (268 pg/mL) in AGEs-treated cells. Consistent with the COX-2 inhibition, compounds **22**, and **23** showed a relatively stronger inhibition ($p < 0.001$) of PGE_2 (82, and 144 pg/mL, respectively) than PDTC, and rutin ($p < 0.001$, 248, and 245 pg/mL, respectively).

4. Discussion

Monocytes are the pro-inflammatory cells that expedite the vascular complications in diabetic conditions or AGEs-rich environments. AGEs

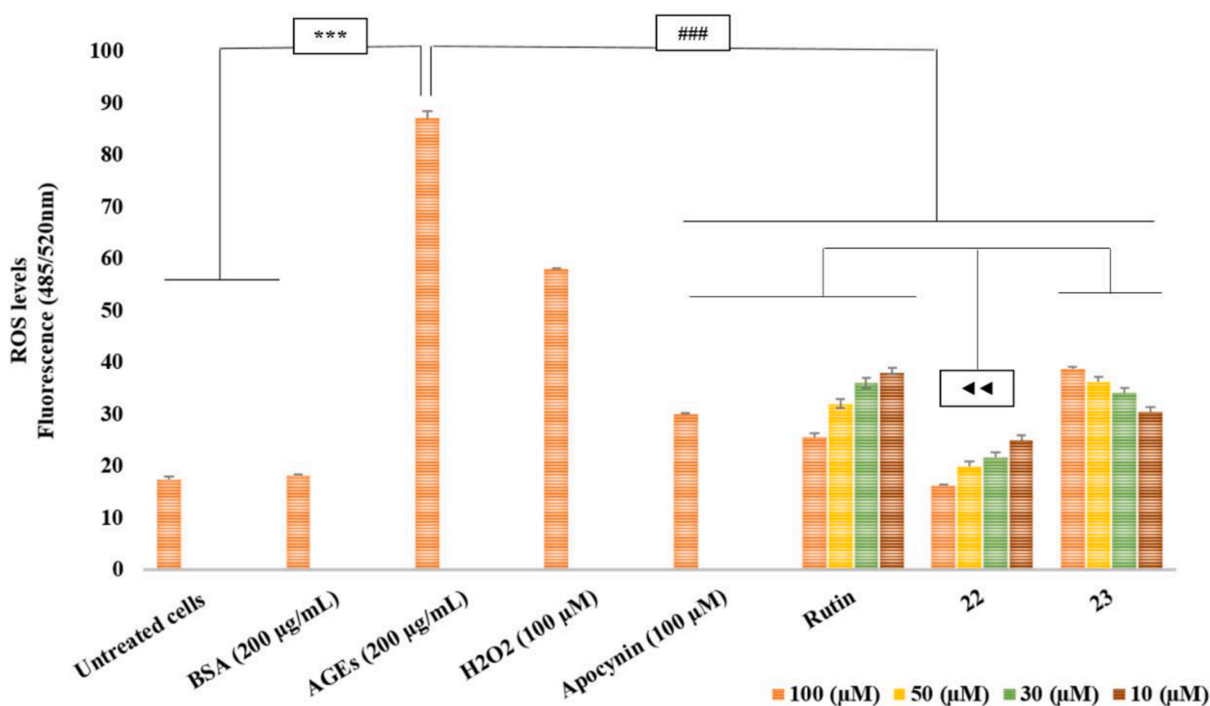

Fig. 6. 1, 2, 4-Triazine derivatives **22**, and **23** inhibited AGEs-induced ROS production in THP-1 cells. The cells were pre-treated with compounds **22**, and **23** (10, 30, 50, and 100 µM) and standards; apocynin, and rutin (100 µM), followed by the exposure to MGO-AGEs (200 µg/mL). Compounds **22**, **23**, and standards exhibited different extents of inhibitory potential against AGEs-induced ROS at indicated concentrations. The data represent mean ± S.D. of three individual experiments. *** Showed ROS induction by AGEs ($p < 0.001$) *vs.* controls (untreated and BSA treated cells). ### Indicate significant decline ($p < 0.001$) in ROS *via* compounds **22**, **23**, and standards (rutin and apocynin) *vs.* AGEs. ◄◄ Expressed significant ($p < 0.001$) activity of compounds **22** *vs.* **23**, rutin, and apocynin at all the tested concentrations.

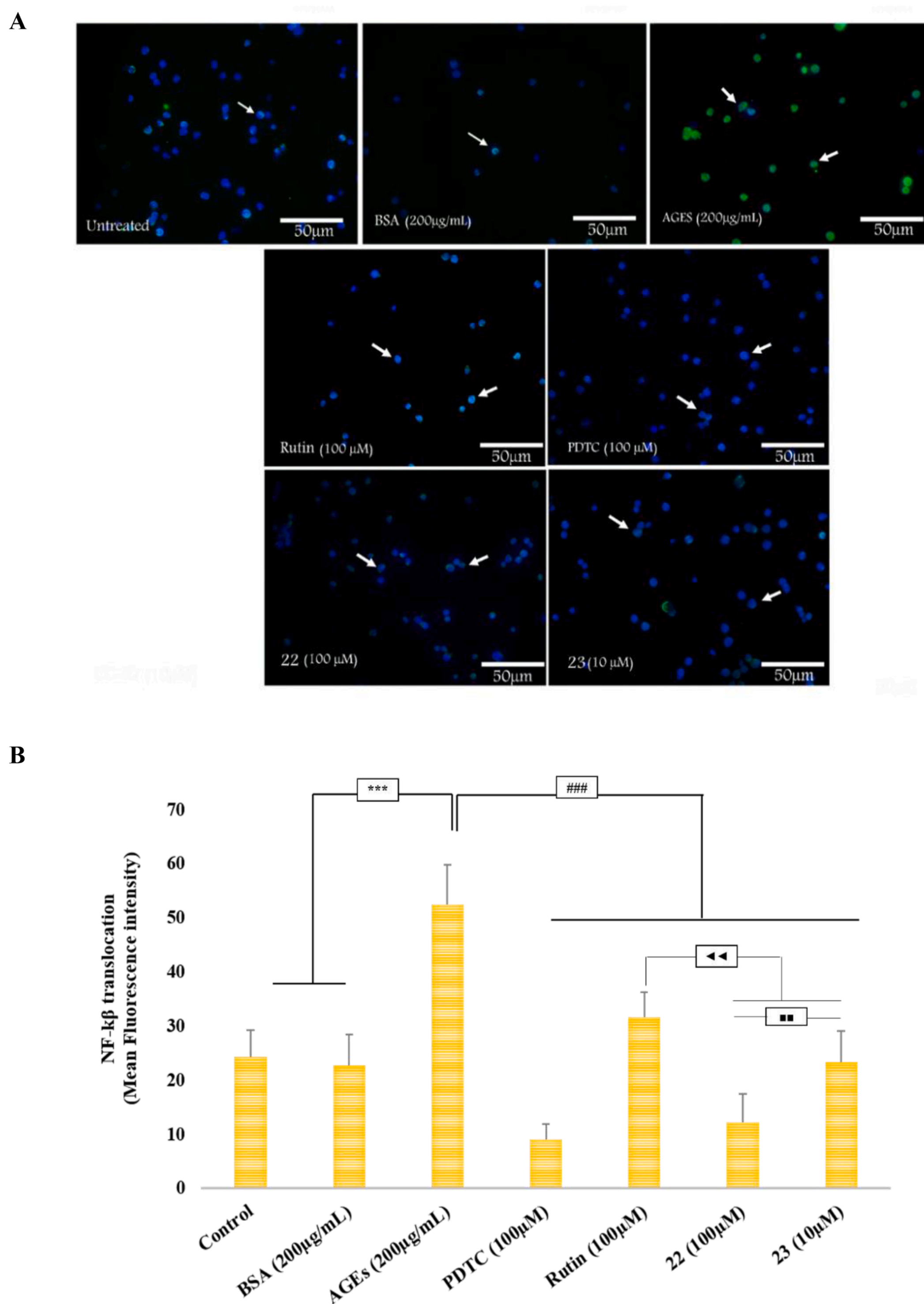

Fig. 7. 1, 2, 4-Triazine derivatives restricts AGEs- induced translocation of NF-κB in THP-1 cells (**A**). Immunocytochemical studies showed a significant increase in green fluorescence intensity of NF-κB in MGO-AGEs (200 μg/mL)-treated cells. While, pre-treatment of cells with compounds **22**, **23**, and reference (PDTC and rutin) noticeably suppressed the expression of NF-κB (**B**) The graph represents difference in mean fluorescence intensity of NF-κB by inhibitors. The data represents mean ± S.D. of three individual experiments. *** Exhibit significant increase ($p < 0.001$) in NF-κB expression by AGEs *vs.* BSA and untreated cells. ### Depict a significant reduction ($p < 0.001$) in NF-κB expression with the treatment of rutin, PDTC, and compounds **22**, and **23** *vs.* AGEs. ◀◀ Indicate significant ($p < 0.001$) decline in NF-κB levels by compounds **22**, and **23**, as compared to the reference rutin, while ■■ showed comparatively better inhibition by compound **22**. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

trigger the migration of monocytes at the site of inflammation, and accelerate the secretion of chemokines, cytokines, and reactive oxygen species *via* activation of specific signaling molecules and transcription factors [33]. In the current study, we investigated the antiglycation activity of 1,2,4-triazine derivatives against MGO-AGEs *in vitro*, and their role in suppressing the AGEs-mediated ROS production, and inflammatory reactions in THP-1 monocytes.

Initially, a series of 1,2,4-triazine derivatives were evaluated for their

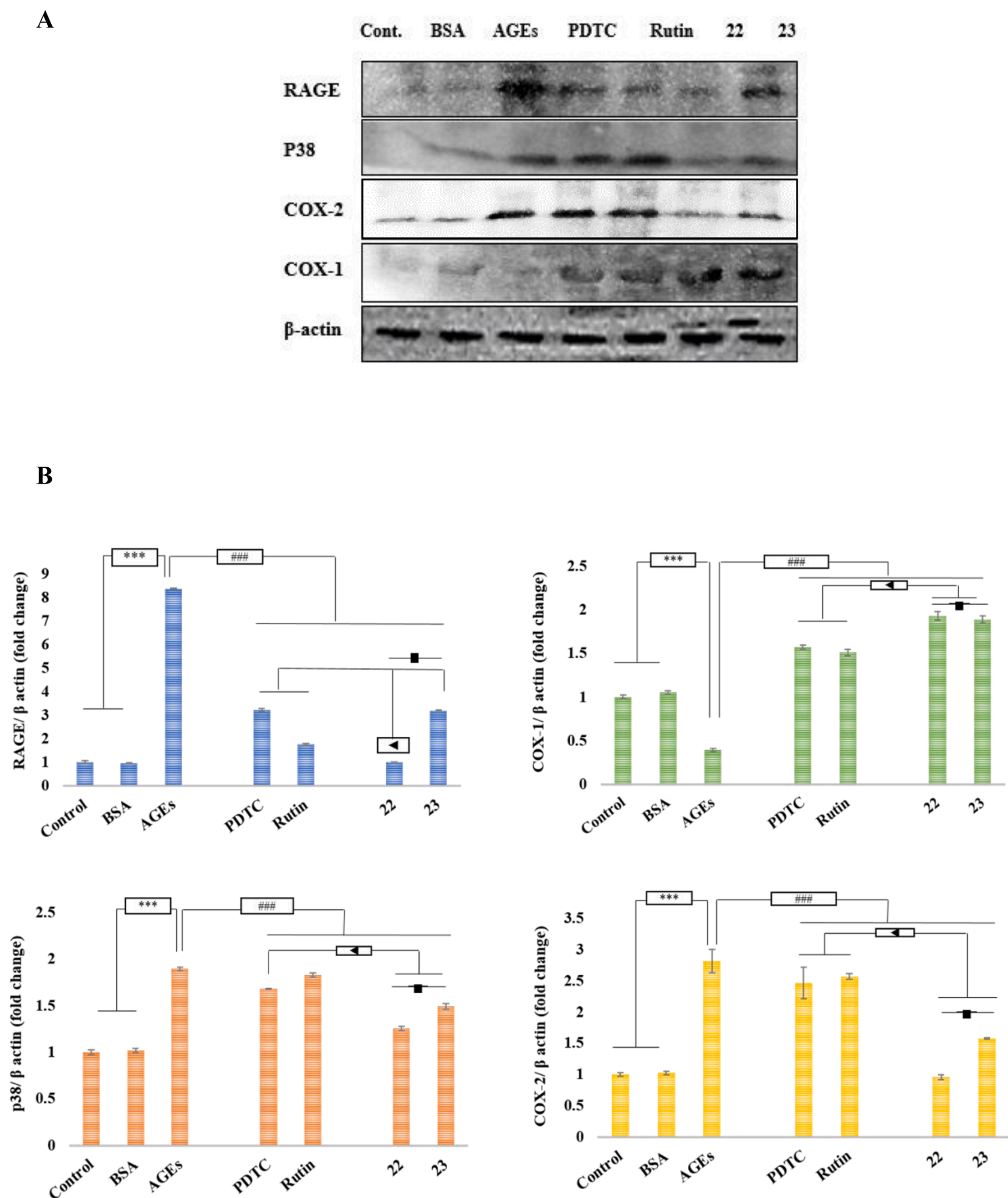

Fig. 8. Effect of 1, 2, 4-triazine derivatives **22**, and **23** on the regulation of AGEs-induced alteration in protein expression of RAGE, p38, COX-1, and COX-2 in THP-1 monocytes (**A**) Western blot showed increased concentrations of RAGE, p38, and COX-2 proteins in MGO-AGEs treated THP-1 monocytes. While compounds **22**, **23**, and standards (PDTC and rutin) decreased the AGEs-mediated signal of RAGE, p38, and COX-2. COX-1 levels were significantly reduced in AGEs-treated cells, while triazine derivatives, and standards (PDTC, and rutin) inhibited the AGEs-mediated suppression of COX-1 in THP-1 monocytes. (**B**) The graph represents fold change in protein expression of RAGE, p38, COX-1, and COX-2. The data represent mean ± S.D. of three individual experiments. *** Exhibit significant increase ($p < 0.001$) in RAGE, p38, and COX-2, and decrease in COX-1 expression in AGEs-treated cells *vs.* control and BSA. ### Represent decrease in RAGE, p38, and COX-2, while increase in COX-1 by standards (rutin and PDTC), and compounds **22**, and **23** *vs.* AGEs. ◄ Indicate significant change in the levels of proteins by compounds **22**, and **23**, as compared to references PDTC, and rutin, while ■ showed significance difference in the activity of compounds **22** *vs.* **23**.

antiglycation activity *in-vitro*, where several compounds exhibited an excellent inhibitory potential against MGO-AGEs. The antiglycation property of 1,2,4-triazine derivatives is attributed to the hydrazine ring that was further augumented by other substituents. It agrees with a study that co-relates the antiglycation activity of aminoguanidine with the hydrazine and guanidine groups involving the trapping of MGO, and thus inhibition of AGEs formation [34].

Among the tested derivatives, compound **3** showed a good antiglycation activity (IC_{50}= 183 ± 0.02 µM), possibly due to the presence of a hydrazine ring and amino group. A study by Lo, C. Y. *et al.* reported

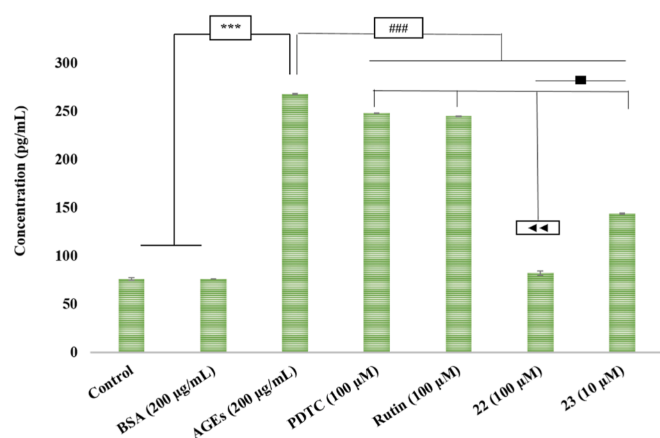

Fig. 9. 1, 2, 4-Triazine derivatives **22**, and **23** inhibited the AGEs-induced levels of PGE_2 in THP-1 monocytes. The graph represents significant increase in PGE_2 in AGEs-treated cells, whereas pre-exposure of cells to compounds **22**, and **23**, and standards (PDTC and rutin) significantly decreased PGE_2 production in THP-1 cells. The data indicates mean ± S.D. of three individual experiments. *** Depict significant increase ($p < 0.001$) in PGE_2 levels by AGEs *vs.* control and BSA treated cells. ### Showed PGE_2 reduction ($p < 0.001$) by compounds **22**, **23**, and standards ($p < 0.05$) *vs.* AGEs. ◄◄ Exhibit significant ($p < 0.001$) activity of compound **22**, as compared to reference PDTC, rutin, and compound **23**, whereas ■ indicates efficacy of compounds **22**, *vs.* **23**.

that the presence of numerous hydroxyl groups in the vicinity of the phenol ring enhances the antiglycation ability of the compound [35]. To this notion, the promising effects of compounds **9** (IC_{50}= 103 ± 0.03 µM), and **22** (IC_{50}= 93 ± 0.02 µM) can be due to the hydroxyl groups, attached to the phenyl ring in the basic triazine skeleton. Moreover, the inhibitory potential of compound **10** (IC_{50}= 135 ± 0.06 µM) can be attributed to the hydroxyl and nitro moieties. Compound **23** with an alkoxy group was the most potent antiglycation member of the series (IC_{50}= 91 ± 0.04). The presence of the methoxy group possibly promotes the hemiacetal formation, and facilitate the MGO derivatization [36]. Similarly, compound **18** (IC_{50}= 176 ± 0.02 µM) with a benzyloxy ring showed relatively better inhibition than reference, rutin (IC_{50}= 180 ± 0.08 µM). The benzyloxy ring apparently contributes to the MGO-capturing ability of the compound. Furthermore, we observed that the halogenation and the amido group in compound **21** increased the inhibitory potential (IC_{50}= 194 ± 0.06 µM). The antiglycation activity of compound **24** (IC_{50}= 131 ± 0.04 µM) can be attributed to the alkoxy, hydroxyl, and bromo substituents. The moderate activity of compound **20** (IC_{50}= 259 ± 0.02 µM) possibly was due to the electronegative nature of the oxygen group at the furan ring.

Other compounds of the series were found inactive against MGO-induced glycation. Compounds **1**, and **2**, containing thiol, and thiomethyl groups, respectively, showed no antiglycation activity (Table 1). Presence of a benzyl and phenolic rings, respectively, in compounds **4**, and **5** could not inhibit AGEs formation in the MGO-glycation model. It indicates that one hydroxyl group cannot trap the carbonyl of methylglyoxal [34,35]. Furthermore, with halogens (chloro, bromo, and fluoro) and alkoxy (methoxy) groups of compounds **6**, **8**, and **14** were inactive in BSA-MGO-glycation model. Interestingly, compound **15** with methoxy and hydroxyl substituents exhibited no antiglycation behavior, which indicates the importance of the position of substituents on the compounds phenyl ring for the antiglycation activity. Likewise, simple halogenation in compounds **7**, **11**, and **12** showed no potential against MGO-glycation. Additionally, compounds **16** (dichloro), **17** (trimethoxy), **19** (benzyl rings), **25** (chloro and nitro), and **26** (trifluoro carbon) were found inactive.

Considering the good antiglycation activity of some of the triazine derivatives, these derivatives were further evaluated for cytotoxic effects on the HepG2 cell line. Literature showed that an increased number of phenolic rings is responsible for the cytotoxic activity of the compounds [37]. The number and position of free hydroxyl groups and the unsaturated substituted chains are the basic structural motifs for the anticancer activity of the phenolic compounds. In this context, numerous compounds were found toxic to HepG2 cancer cells. Compound **24** substituted with methoxy hydroxyl, and bromo groups was the most toxic (99%) compound of the series. Compounds **9,** and **22**, with similar structural motifs, showed 60%, and 30% cell inhibition of HepG2 cells, respectively. The difference is most likely due to the different positions of hydroxyl groups, which indicates that the presence of hydroxyl group at the *ortho* and *para* positions (as in compound **9**) activates the apoptotic activity of the compound. Hydroxyl, nitro, and benzyloxy ring of the compounds **10**, **18**, and **20,** respectively, seems to be responsible for a moderate toxicity of these compounds (<70% inhibition). Whereas, compounds **21**, **22**, and **23** with chloro and amide, hydroxyl, and methoxy groups, respectively, exhibited the least cytotoxic activity (<30% cell inhibition) at all the tested concentrations (Fig. 2). Since compounds **22**, and **23**, with higher antiglycation activity, are associated with lower toxicity on HepG2 cells, they were further assessed on THP-1 monocytes (Fig. 3). Both the compounds were found to be non-toxic, and thus further studied.

Numerous receptors for AGEs have been recognized in vascular and other cell types, where the RAGE gained much attention. Under the influence of AGEs or in hyperglycemic conditions, NADPH oxidase and mitochondrial respiratory chain are the major sources of reactive oxygen species (ROS) *via* AGE-RAGE ligation [15,38,39]. In the current study, the treatment of monocytes with MGO-AGEs exhibited a significant ROS production, as compared to controls (BSA, and untreated cells). However, the apocynin treatment (NADPH oxidase inhibitor) resulted in a marked reduction in ROS levels, which confirmed that MGO-AGEs mainly trigger oxidative stress through the enzymatic reactions. The study by Quintela *et al.* showed that the triazine derivatives have the inhibitory activity against inducible nitric oxide synthase and, therefore, inhibited the production of oxidative stress in murine macrophages [40]. Similarly, compounds **22**, and **23** significantly suppressed the AGEs-mediated induction of ROS in THP-1 monocytes. The antioxidant activity of compounds can be attributed to their MGO scavenging property, and the ability to inhibit the mitochondrial- and NADPH oxidases- associated ROS production in AGEs-stimulated THP-1 monocytes.

Previous studies showed that treating monocytes with AGEs activates the inflammatory responses through oxidative stress, and activation of redox-sensitive transcriptional factor NF-κβ [36,41,42]. In this study, AGE-RAGE-mediated ROS production has activated the downstream signaling of NF-κB in THP-1 monocytes that was blocked by the NF-κB inhibitor, PDTC, which can prevent the NF-κB at protein [16] or mRNA levels [43]. The triazine derivatives **22**, and **23** inhibited the AGE-ROS-induced activation of NF-κB in THP-1 monocytes. It agrees with the earlier studies, which reported the inhibition of p^{65} in RAW 264.7 and PC12 cells by 1, 3, 5-triazine and 1,2,4-triazine derivatives, respectively [23,44,45]. Whereas the activity of compound **22** was better than compound **23**. Reference compound rutin also diminished the NF-κB expression, manifested by decreased green fluorescence intensity in THP-1 cells (Fig. 7).

Several factors, including oxidative stress, hypoxia, inflammation, and AGEs, contribute to the overproduction of RAGE in different cell types and start viscous cycle of AGEs. A study showed that the exogenous AGEs treatment elevates the RAGE expression in THP-1-derived macrophages *in vitro*. Another study demonstrates that the overexpression of RAGE is associated with vascular oxidative stress injury in diabetic mice. At the same time, the inhibition of RAGE led to the attenuation of cardiac oxidative stress, and hence atherosclerosis [46]. Protein expression of RAGE was determined to check whether the MGO-AGEs induce RAGE in THP-1 monocytes. The AGEs-treated cells exhibited a significant increase in RAGE expression, as compared to the controls (BSA, and untreated cells).

In contrast, rutin, and PDTC greatly suppressed the AGEs-mediated upregulation of RAGE. Similarly, compounds **22**, and **23** considerably blocked the RAGE in THP-1 monocytes. The effect of compound **22** was superior to both reference compounds (PDTC, and rutin), while compound **23** was comparatively less efficient than rutin, but equivalent to PDTC. The activity of triazine derivatives is possibly related to their radical scavenging and carbonyl trapping properties, enabling them to inhibit the formation of AGEs, and suppress the expression of RAGE in THP-1 monocytes. It is relevant to a study that suggests that the metal chelating ability of the AGE inhibitor prevented the glycoxidation, and blocked the expression of RAGE in S100b stimulated monocytes [47]. Studies highlighted that RAGE is an important pathogenic factor in the development and progression of diabetes-related complications [16]. Our findings substantiate that triazine derivatives **22**, and **23** are capable of inhibiting the AGE-mediated effects at the receptor level by attenuating the AGE-NF-κB nexus.

Guglielmo Sorci and co-workers explained that AGE-RAGE interaction stimulates multiple signaling cascades, including AKT, ERK1/2, and p38, through ROS in various cell types under pathological conditions, such as atherosclerosis, inflammation, and arthritis [5,48]. It was shown that ROS-mediated p38 activation in diabetic, and AGEs-treated non-diabetic monocytes leads to cellular dysfunctions [49]. Likewise, we found that AGE-RAGE interaction activated the p38 signaling in THP-1 monocytes. Moreover, the AGE-RAGE/ROS-mediated activation of p38 was significantly reduced by the compounds **22**, **23**, and PDTC. By inhibiting p38 signaling, compounds **22**, and **23** also limited the pro-inflammatory signaling in AGE-stimulated monocytes.

A previous study by our group has shown that up-regulation of COX-2 protein in THP-1 monocytes observed through AGE-RAGE-NF-κB nexus [30,50]. In this study, we confirmed that AGE-RAGE-mediated activation of NF-κB induces COX-2 expression in THP-1 monocytes. Blocking the NF-κB signaling through PDTC, rutin, or triazine derivatives suppressed the COX-2 induction. However, compounds **22,** and **23** elicited the major inhibitory activity on COX-2 expression, possibly due to their capability to inactivate upstream signaling pathways, involving AGE-RAGE-mediated ROS-dependent activation of MAPKs, leading to NF- κB activation [16].

COX-1 is constitutively expressed in various cells, and involved in tissue homeostasis. Several studies have reported that AGE-RAGE interaction induces COX-2 at a protein, and transcript levels [16,51], while there is no effect of AGEs (D-ribose and L-lysine) [52], S-100b [16], and high glucose [43] on COX-1 mRNA levels in THP-1 cells. In contrast, our data revealed that MGO-AGEs decreased the COX-1 levels in THP-1 monocytes which was first reported by our research group. Moreover, the modulatory effect of AGEs over COX-1 was significantly reversed by compounds **22**, and **23**, which can be co-related to their antiglycation activity, and NF-κB inhibition. These effects of compounds **22**, and **23** was equivalent to PDTC, and rutin.

Prostaglandin E_2 (PGE_2) is the enzymatic product of COX-2. It plays a key role in the progression of diabetes-related inflammatory conditions, such as atherosclerosis. Treatment of THP-1 monocytes with AGEs significantly elevates COX-2 mRNA, and protein levels [16,30,43,53]. In agreement with this, we found a significant increase in PGE_2 levels in MGO-AGEs treated THP-1 cells, comparable to the controls, as reported by our research group [19]. However, pre-treatment of cells with compounds **22**, and **23** significantly abrogated the COX-2 induced PGE_2 production. The inhibition of PGE_2 by PDTC, and rutin further indicated the stimulation of PGE_2 through NF-κB mediated COX-2 signaling. However, the effect of compound **22** was comparatively superior than compound **23**, and the reference compounds. Therefore, we identified that compounds **22**, and **23** possess antiglycation properties, and they are also capable of inhibiting inflammatory signaling at the receptor levels.

The identification of 1,2,4-triazine derivatives **22**, and **23** as potent antiglycation agents represents a novel therapeutic strategy for addressing diabetes-associated vascular complications. These compounds specifically inhibited the AGE-RAGE-NF-κB/p38 signaling pathway, thereby reducing the inflammation and oxidative stress. Their *in vitro* safety and efficacy indicate significant potential for further development of AGEs-targeted treatments of late diabetic complications. This will may thus offer improved therapeutic outcomes with minimized side effects.

This study lays the foundation for future research towards the treatment of chronic diabetic conditions.

5. Conclusion

Numerous metabolic abnormalities, intrinsic to diabetes, contribute to the development of vascular complications. One pivotal mechanism in this pathogenesis involves the induction of M1 (pro-inflammatory) state in monocytes by MGO-AGEs. This phenomenon significantly accelerates the progression of atherosclerosis in individuals with diabetes. Therefore, targeting the pro-inflammatory impact of AGEs holds promise for preventing diabetic vasculopathies. Our research reveals that triazine derivatives **22**, and **23** effectively suppress the M1 state by obstructing the AGE-RAGE-induced ROS production, and subsequent activation of MAPK-p38 and NF-κB pathways, ultimately diminishing COX-2 and PGE_2 production in THP-1 monocytes *in-vitro*.

Furthermore, compounds **22**, and **23** mitigate AGEs-mediated COX-1 levels, enhancing their safety as inhibitors of AGEs. These findings emphasize that triazines **22**, and **23** are leads for drug development, potentially benefiting the diabetic patients with an elevated risk of vascular complications. Nonetheless, further investigations are necessary to validate the inhibitory effects of triazines **22**, and **23** on the M1 phenotype of monocytes through an appropriate *in-vivo* diabetes model.

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CRediT authorship contribution statement

Humera Jahan: Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Priya Tufail:** Writing – original draft, Methodology, Investigation. **Shahbaz Shamim:** Methodology, Compounds synthesis. **Khalid Mohammad Khan:** Methodology, Organic Synthesis. **Michele Gennari:** Writing – review & editing. **Marina Pizzi:** Writing – review & editing. **M. Iqbal Choudhary:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2024.113145.

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