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Modulatory effects of Micromeria Barbata and 3-acetyl-11-keto-boswellic acid on advanced glycation end products-induced inflammatory cytokine response in THP-1 cells

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Abstract: Advanced glycation end products (AGEs) contribute to diabetes complications. They activate inflammation by binding to Receptors for Advanced Glycation End Products (RAGE) on immune cells, triggering cytokine release. *Micromeria barbata* (MB), a medicinal herb with various biological actions, has not been examined for its anti-inflammatory effects on AGE. *Boswellia serrata* (BS) research on diabetes and AGE-related conditions is limited. This study examines the anti-inflammatory effects of 3-acetyl-11-keto-boswellic acid (AKBA), BS's main active component, and MB plant extract on AGE-stimulated THP-1 human monocytic cells. We investigated the impact of AGEs on pro-inflammatory cytokines and the effects of MB plant extract and AKBA on the gene expression of Interleukin-1β (IL-1β), Interleukin-6 (IL-6), Tumor Necrosis Factor-α (TNF-α), Interleukin-10 (IL-10), and Interleukin-4 (IL-4) in AGE-stimulated THP-1 cells. THP-1 cells were unaffected by MB, AKBA, and AGE-BSA at various doses. MB and AKBA may reduce AGE-stimulated THP-1 cell inflammation. Treatment with MB and AKBA significantly decreases IL-6 and TNF-α expression. AKBA (0.027 µg/mL) reduces IL-1β gene expression, while MB has no effect. Furthermore, at higher doses, both MB and AKBA significantly increase IL-10 and IL-4 gene expression. This is the first research to reveal MB and AKBA's anti-inflammatory effectiveness, shedding light on natural therapeutic agent development.

Keywords: *3-Acetyl-11-ketoβ boswellic acid (AKBA), Advanced glycation end products (AGE), Diabetes, Micromeria barbata (MB), Proinflammatory cytokines.*

1. Introduction

Advanced glycation end products (AGEs) are a diverse group of molecules resulting from the non-enzymatic glycation and oxidation of proteins, lipids, and nucleic acids. Their generation produces severe cell danger due to their ability to crosslink intracellular and extracellular matrix proteins, thus altering the functional and mechanical properties of tissues [1-3].

AGE is one of the key processes implicated in the onset and progression of diabetic complications, such as inflammation [4] nephropathy, retinopathy, and neuropathy [5]. Owed to its production from dicarbonyl precursors generated from glucose [6]. AGE accumulates intracellularly exerting direct

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activator effects on intracellular signaling pathways, and altering the intracellular function of proteins through different mechanisms including disordering molecular conformation, amending enzymatic actions, plummeting degradation capacity, and intervening with receptor recognition [7]. AGE molecules bind to their receptors (RAGE) found on the surfaces of immune cells such as macrophages and dendritic cells [8]. After binding, RAGE phosphorylates its downstream MAPK and stimulates NF-κB protein. Then, NF-κB enters the nucleus to stimulate the expression of proinflammatory cytokines such as TNF-α, IL-1β, IL-6 [9] and IL-8 [10]. When it comes to anti-inflammatory cytokine IL-4 and IL-10, there is neither apparent influence between AGE and IL-4 [11, 12] nor a substantial effect of AGE on the transcriptional level of IL-10[13].

Boswellia serrata (BS), a traditional medicinal plant [14] has been extensively known in pharmaceutical studies due to its potential therapeutic applications in various inflammatory conditions [15-19]. BS possesses anti-inflammatory, sedative, anti-hyperlipidemic, and antibacterial properties, making it a promising agent for treating conditions such as rheumatoid arthritis [20] osteoarthritis [21, 22] and Crohn's disease [23]. Additionally, BS exhibits analgesic [24] anti-proliferative [25] and anti-arthritic properties [26]. More recently, BS has been investigated for its potential to address chronic inflammatory conditions such as insulin resistance (IR) and type 2 diabetes (T2D) [27]. Studies have shown that BS can effectively inhibit the production of proinflammatory cytokines, including TNF-α and IL-1β [28]. Furthermore, BS displays preventive effects against diabetic complications [16]. An in-depth chemical examination of BS extracts uncovers their composition, covering terpenes, polysaccharides, and essential oils [29] where boswellic acid is the active functional group [30]. According to Cuaz-Pérolin, et al. [31] 3-acetyl-11-keto-β-boswellic acid (AKBA) can reduce oxidative stress and suppress the activity of NF-κB, a transcription factor activating pro-inflammatory cytokines [19] besides reducing the proliferation and activation of T cells without any cytotoxicity [32]. Lately, AKBA has shown the ability to modulate macrophage polarization in injured spinal cord [33]. According to research, extracts of BS gum resin affect the inflammatory cascade of both humoral and adaptive immune responses [34]. However, to the best of our knowledge, no research has examined the effect of AKBA on AGEs in the THP1 cell line.

Micromeria barbata (MB) is another aromatic plant with significant medicinal value thanks to the considerable amounts of essential oils and polyphenols, flavonoids [35]. This plant is found in the Mediterranean region and Arab countries, including Lebanon [36, 37]. The chemical analysis of MB raised in Lebanon revealed the presence of the following 17 elements. Pulegone (20.19%), Limonene (16.59%), Neomenthol (12.37%), Menthol (6.19%), β-pinene (3.29%), and Piperitone (4.22%) were the main constituents. The low content of pulegonee [38, 39] contributes to the low toxicity level of the Lebanese MB. The latter has also been known for its antibacterial, antioxidant, and anti-fungal properties [40] yet, the anti-inflammatory action has not yet been reported in AGE context and diabetes.

The purpose of this study is to investigate the potential modulatory effects of MB and AKBA on AGE-treated THP1 cells, for a better understanding of their inflammatory role. This will unravel the mechanisms of AGE-related pathologies while offering insight into the discovery of novel natural therapeutic strategies.

2. Materials and Methods

2.1. Materials

3-Acetyl -11-ketoβ Boswellic acid (AKBA), Fetal bovine serum (FBS), MTT reagent, Dimethylsulfoxide (DMSO), Phosphate buffered saline (PBS), RPMI 1640 with L glutamine, penicillin-Streptomycin, Dexamethasone, TRIzol, lipopolysaccharide (LPS) and Phorbol myristate acetate (PMA #P1585) were purchased from Sigma-Aldrich (St Louis USA). AGE-BSA was obtained from Abcam Ltd (Cambridge, UK) (ab51995), IScript synthesis kit, and ITaq universal SYBR green super mix from Bio-Rad (Hercules, California). Cells were imaged using an inverted microscope (OPTIKA). Human

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monocytic cell line THP-1 and primers were kindly provided by the American University of Beirut from Dr Nadine Darwish Lab, Faculty of Medicine, Biochemistry Department. *Micromeria barbata* (MB) plant was kindly provided by the Lebanese University (Doctoral School of Sciences and Technologies, Azm Center-Tripoli).

2.2. Preparation of Plant Extract

Sections of the leaves, flowers, and stem of MB were collected and dried at room temperature and then finely ground. 40 g of plant powder was soaked in 70% ethanol for 48 hours at room temperature. The solid phase was then removed by decantation and filtered using Whatman N°1 filter paper. The extract was transferred to a rotary evaporator where the ethanol was evaporated under reduced pressure at 60°C. The eluents were lyophilized and the dry samples were stored in tightly closed containers away from light and moisture. A stock solution of 0.5 mg/ml of the extract was prepared in DMSO.

2.3. Cell Culture

THP-1 cells were grown in RPMI 1640 culture medium with L-glutamine from Sigma-Aldrich (St Louis USA) supplemented with 10% fetal bovine serum (FBS), from Sigma-Aldrich (St Louis USA), 1% penicillin-streptomycin from Sigma-Aldrich (St Louis USA). Cells were seeded in 75 cm^2 tissue culture flasks (Sigma-Aldrich, St Louis, USA) until confluency. For MTT assay THP-1 cell number was counted and adjusted to 2×10^5 cells/ml, and 100 µl of cell suspension was inoculated in 96-well flat-bottom cell culture plates for 24 hours at 2×10^4 cells/well with 50 ng/ml Phorbol Myristate Acetate (PMA) from Sigma-Aldrich (St Louis USA) to differentiate cells into mature macrophage-like cells. For RT-PCR Cells were cultured at a density of 1×10^6 cells/ml in fresh culture medium in the presence of 25 nM of PMA, then 1 ml of cell suspension was inoculated in 12-well flat-bottom cell culture plates at 1×10^6 cells/well for 72 hours.

2.4. MTT Cell Viability Assay

Cell viability was evaluated by MTT assay as reported previously Morisi, et al. [41]. 24 hours after seeding, the cells were washed with fresh culture medium, then treated with increasing concentrations of AKBA (0.01, 0.03, 0.125, 0.5, 2, and 20 µg/mL) (Sigma-Aldrich, St Louis, USA), AGE-BSA (10, 25, 50, and 100 µg/mL) (Abcam ab51995), and MB-Ethanolic extract (5, 10, 20, and 40 µg/mL). Each setup was tested in triplicate. Moreover, cells were cultured in a mixture of AKBA and MB (AKBA-MB) using increasing concentrations of AKBA (0.01, 0.03, 0.125 and 0.5 µg/mL) and MB (5, 10, 20, and 40 µg/mL). Then, the cells were incubated for 24-48 hours at 37°C (5% CO2 and 95% humidity). Untreated cells in a complete RPMI-1640 medium were used as a control. After incubation with the different stimulants, cells were supplemented with 10 µl MTT reagent (Sigma-Aldrich, St Louis, USA) at 37°C for 4 hours. Next, 100 µl stop solution (DMSO 1%) was added to each well, to solubilize the formed formazan as an indicator of cell viability, and the cells were incubated overnight. The extent of formazan production was determined by an ELISA (enzyme-linked immunosorbent assay) reader at 570 nm. Cell viability percentage was calculated using the following formula:

$$\%\ viability\ = \frac{Absorbance_{\ treated\ cells}}{Absorbance_{\ Control}} \times 100$$

2.5. Measurement of Cytokine mRNA by RT-PCR

The adherent cells seeded after 72 hours were washed with a fresh culture medium and treated with different agents to study their effects on secreted cytokines levels. After the incubation time, the supernatant was kept at -80°C, and adherent cells were treated by TRIzol Reagent (Sigma-Aldrich, St Louis, USA). mRNA expression was assessed by real-time PCR using specific primers Table 1.

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Table 1.
Primer sequences used in real-time PCR.

Gene	Primers	Reference
IL-1β (NG_008851.1)	F: 5′-TGGCATTGATCTGGTTCATC-3′ R: 5′-GTTTAGGAATCTTCCCACTT-3′	Karakaxas, et al. [42]
IL-6 (NG_011640.1)	F: 5′-CACCGAGCTCACCCCACTACC-3′ R: 5′-CTACATTATCCGAACAG-3′	Figueiredo, et al. [43]
IL-8 (NG_029889.1)	F: 5′-GTGCAGTTTTGCCAAGGAGT-3′ R: 5′TTATGAATTCTCAGCCCTCTTCAAAAACTTCTC-3′	Xu, et al. [44]
TNF-α (NG_007462.1)	F: 5′-CTGGGGCCTACAGCTTTGAT-3′ R: 5′-GGCTCCGTGTCTCAAGGAAG-3′	Saxena, et al. [45]
IL-4 (NG_023252.1)	F: 5′-TGGCCCGAAGAACACAGATG-3′ R: 5′-C TTGAGGTTCCTGTCCAGTCC-3′	Saini, et al. [46]
IL-10 (NG_012088.1)	F: 5′-TCTGCCCTGTGAAAATAAGAGC-3′ R: 5′-GTCAAACTCACTCATGGCTTTG-3′	Saini, et al. [46]
CXCL-1 (NC_000004.12)	F: 5′-AGTGGCACTGCTGCTCCT-3′ R: 5′-TGGATGTTCTTGGGGTGAAT-3′	Kim, et al. [47]
CXCL-2 (NC_000004.1)	F: 5′-CTGCTCCTGCTCC TGGTG-3′ R: 5′-TCTGCAAGCACTGG-3′	Chui and Dorovini-Zis [48]
CXCL-3 (NG_029076.1)	F: 5′-ATGCAGGTCTCCACTGCTGCCCTT-3′ R: 5′-GCACTCAGCTCCAGGTCGCTGACAT-3′	Chui and Dorovini-Zis [48]
GAPDH (NG_007073.2)	F: 5′-CCATGTTCGTCATGGGTGTGAACCA-3′ R:5′-GCCAGTAGAGGCAGGGATGATGTTC-3′	Chui and Dorovini-Zis [48]

Total RNA was isolated using TRIzol (Sigma-Aldrich, T9424). The amount and quality of RNA were determined using a Spectrophotometer Nanodrop (Thermo Fisher Scientific) after resuspending in 20 µl of RNase and DNase-free water. Reverse transcription and PCR were designed in a two-step reaction. Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Bio-Rad, Cat) with 2 µg total RNA as template and the reaction was performed on the RT-PCR machine (Bio-Rad Laboratories, California, USA) as follows: 10 min at 25°C, 2 hours at 37°C followed by 5 min at 85°C and ends at 4°C. PCR was carried out using SYBR Green Supermix (Bio-Rad Laboratories, California, USA). Then, according to the following protocol: one cycle at 94°C for 15 minutes, 50 cycles at 94°C for 15 seconds, 56°C for 9 seconds each, ended by one cycle at 72°C for 30 minutes, the reaction was carried out using the CFX384 system (Bio-Rad Laboratories, California, USA). The results were quantified and analyzed using the Delta-Delta CT method [49] and normalized to GAPDH, the housekeeping gene.

2.6. Statistical Analysis

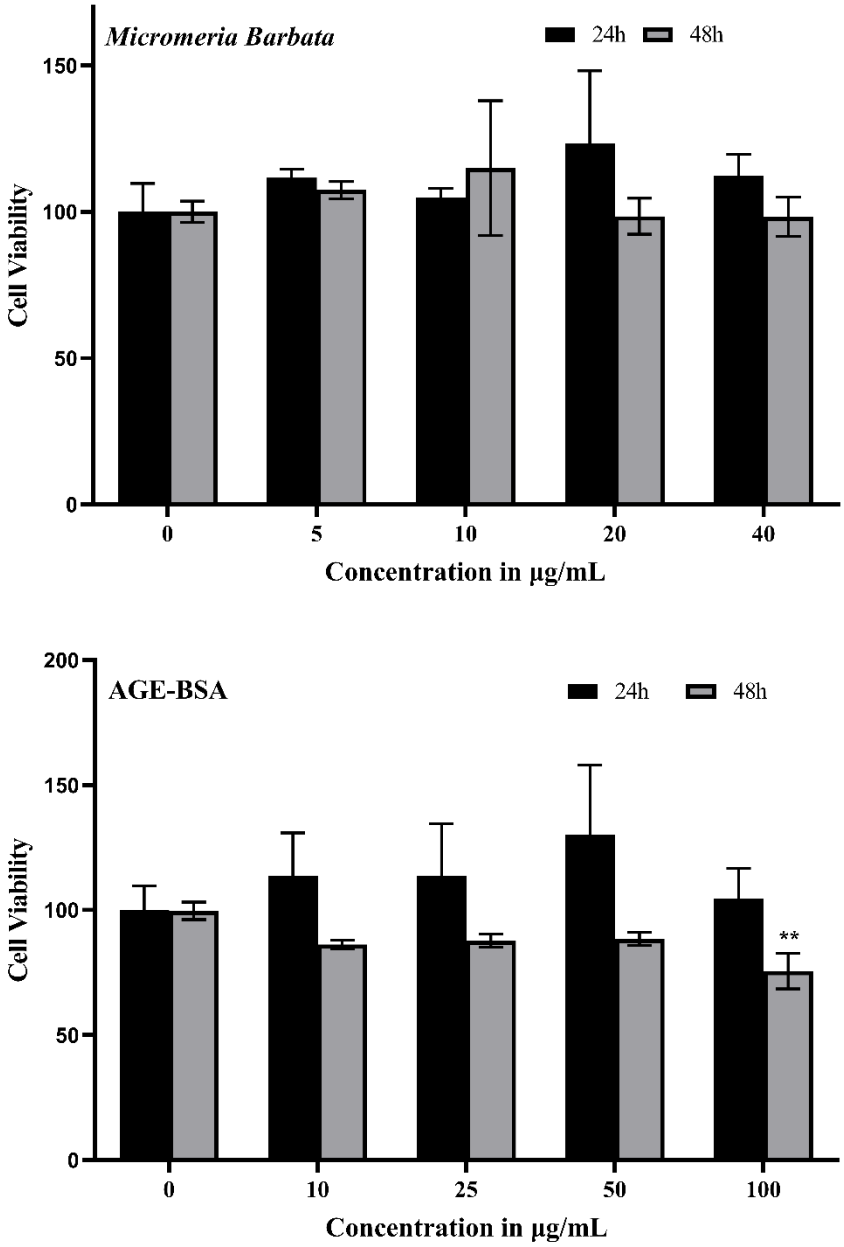
The analysis was performed using the GraphPad Prism 9 (GraphPad Software, San Diego, CA). All values are expressed as a mean of a triplicate experiment ± SEM. Student's unpaired t-test was performed for comparison of paired samples, and ONE-WAY ANOVA was used for multiple-variable comparisons. A probability value of $p < 0.05$ was considered significant.

3. Results

3.1. Cytotoxic Effect of AGE, MB, and AKBA on THP-1 Cell Line

The assessment of the cytotoxicity of AGE, MB, and AKBA on the THP-1 cell line revealed that MB-ethanolic extract exhibited no discernible cytotoxic effects regardless of both concentration and duration (Figure 1). AKBA within the concentration range of 0.01 to 2 µg/mL, demonstrated no significant cytotoxic effects after 24 hours of treatment, however, a significant cytotoxic effects was observed after 48 hours. AGE-BSA, within the concentration range of 10, 25, 50, and 100 µg/mL did not induce any detectable cytotoxic effects on THP-1 cell after 24 hours. However, at 48 hours, a

notable cytotoxic effects was observed with a dose of 100 µg/mL. The absence of cytotoxicity at 24 hours within the studied concentrations for MB-Ethanolic extract, AKBA, and AGE-BSA suggests their potential safety for further investigations.



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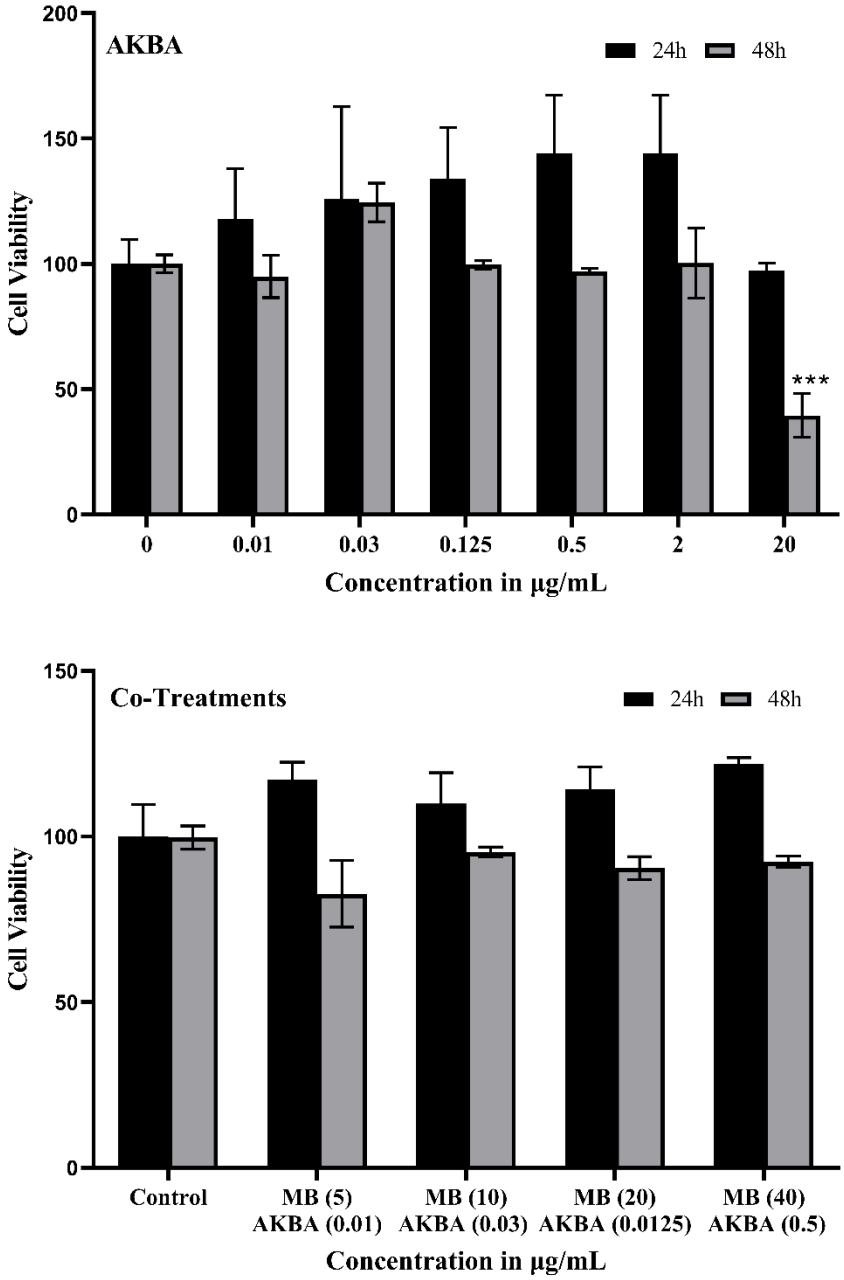

Figure 1.
Cytotoxicity and cell viability. Cytotoxic effects of *Micromeria barbata*-Ethanolic extract, AKBA, AGE-BSA, and combination (AKBA - *Micromeria barbata*) at different concentrations on THP-1 cells treated for 24 hours and 48 hours Cell viability was determined by MTT assay. Data are expressed as mean ± SEM of triplicate samples. $^{**}p<0.01$, $^{***}p<0.001$. and are expressed as absorbance units and normalized to the values in untreated cells (100%).

3.2. Effect of MB, AKBA, and AGE-BSA on THP-1 Cells Morphology

Cells were divided into 4 groups. Group1 stimulated with AGE 25 µg/mL, Group 2 pre-treated with MB (5 µg/ml, 40 µg/ml) and then stimulated with AGE 25 µg/mL, Group 3 treated with AKBA

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(0.027 µg/ml, 0.3 µg/ml), and then stimulated with AGE 25 µg/mL. Group 4 was pre-treated with dexamethasone and then stimulated with AGE 25 µg/mL. The macrophages after being stimulated with AGE-BSA, polarize from M0 (round shape) to M1 macrophages adopting an elongated spindle-like shape with increased cytoplasmic volume. This morphological change contributes to cell motility. When treated with AKBA or MB, the predominant cell phenotype changed to a more rounded and spread morphology of M2, as an indication of cellular remodeling (Figure 2).

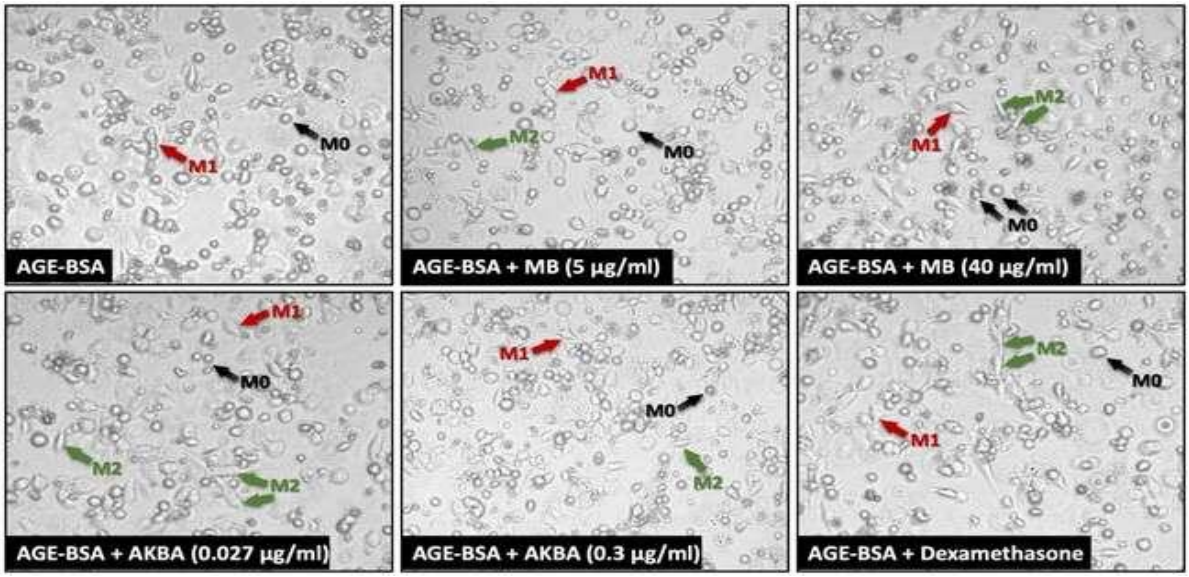
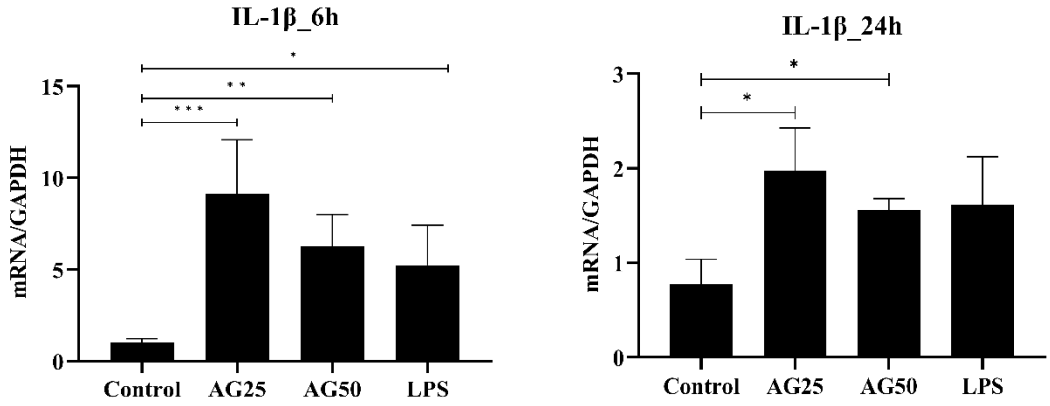


Figure 2.
Effect of anti-inflammatory compounds (MB -ethanolic extract or AKBA or AGE-BSA) on the phenotypic response of THP-1 cells.



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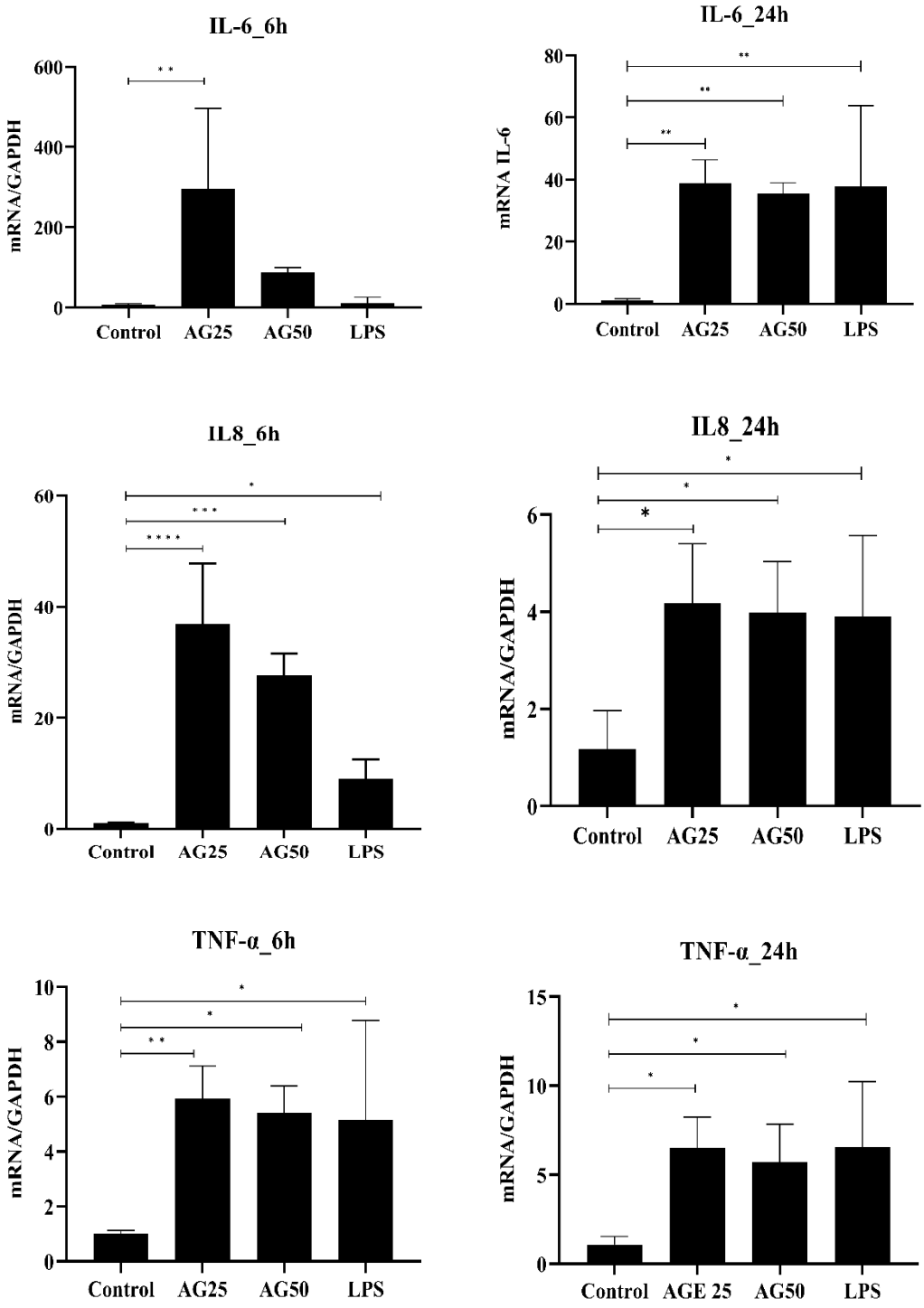

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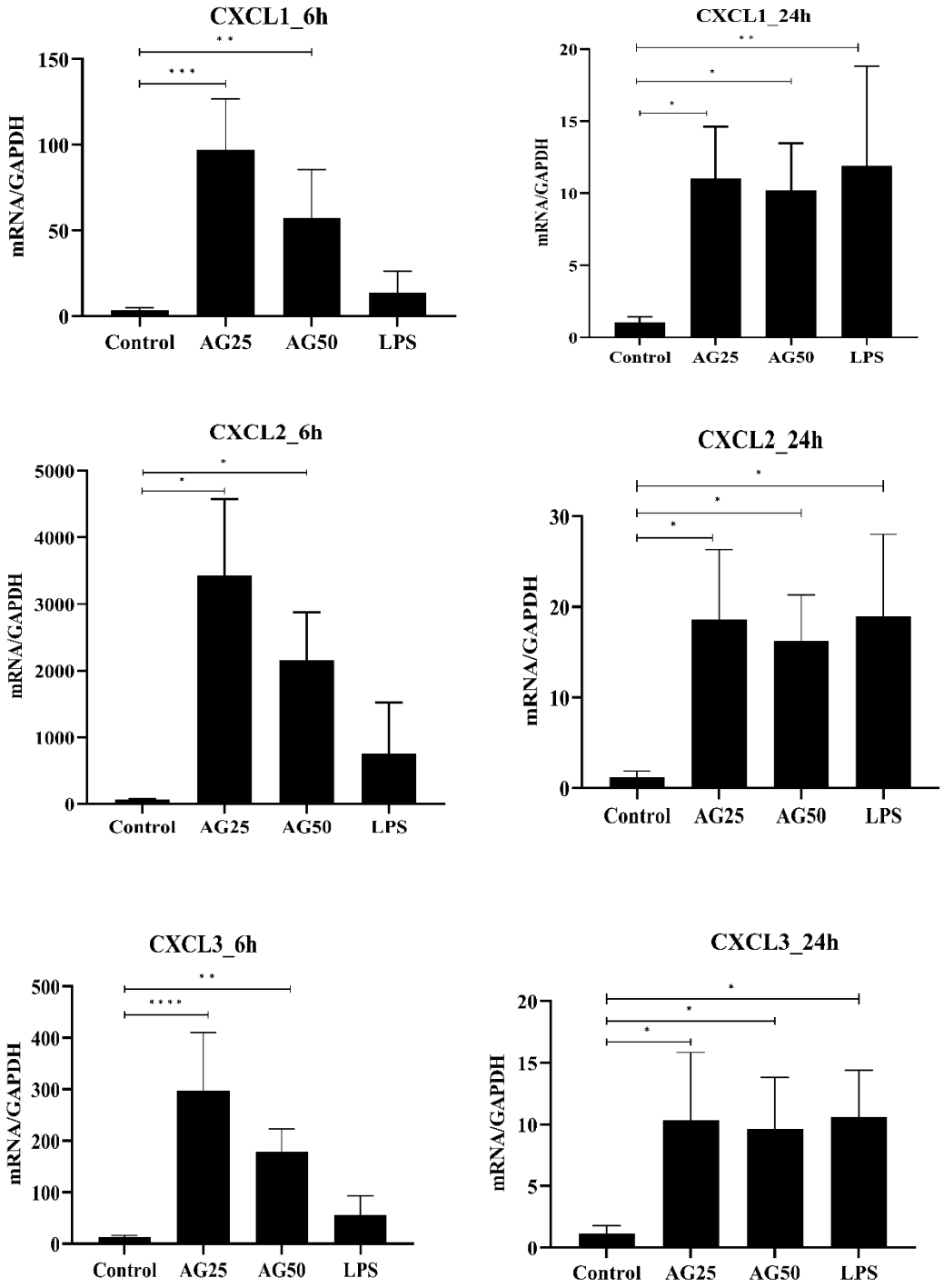

Figure 3.
Time course of pro-inflammatory cytokines levels in THP-1 cell line treated with 25 or 50 µg/mL of AGE. Representative RT-PCR analysis showing the effect of LPS (positive control, 6- and 24-hours incubation) and of 25 or 50 µg/mL of AGE on levels of pro-inflammatory cytokines or chemokines in THP-1 cell line after 6 hours and 24 hours of incubation as compared to untreated cells (control). Data are expressed as mean ± SEM of triplicate samples. *p<0.05, **p<0.01, ***p<0.001.

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3.3. Effects of AGE on Proinflammatory Cytokines and Chemokines mRNA Levels in THP-1 Cell Lines

Following incubation with AGE at a concentration of 25 µg/mL for 6 and 24 hours, the gene expression levels of pro-inflammatory mediators (IL-1β, IL-6, IL-8, TNF-α, CXCL-1, CXCL-2, CXCL-3) were analyzed. The expression of these pro-inflammatory mediators exhibited a significant increase at 6 and 24 hours compared to the levels expressed in untreated control cells. Six hours after incubation with AGE at 25 µg/mL, gene expression increased by 9-fold for IL-1β, 295-fold for IL-6, 36.9-fold for IL-8, 6-fold for TNF-α, 96.9-fold for CXCL-1, 3429-fold for CXCL-2, and 297-fold for CXCL-3. Similarly, when treated with AGE at 50 µg/mL, gene expression significantly increased at 6 hours, with fold changes of 6.28-fold for IL-1β, 87.1-fold for IL-6, 27.6-fold for IL-8, 5-fold for TNF-α, 57.2-fold for CXCL-1, 2153-fold for CXCL-2, and 179-fold for CXCL-3. After 24 hours of incubation with AGE 25 µg/ml, gene expression decreased to 2-fold for IL-1β, 40-fold for IL-6, 4-fold for IL-8, remained 6-fold for TNF-α, 11-fold for CXCL-1, 18-fold for CXCL-2, and 10-fold for CXCL-3. Similarly, when treated with AGE at 50 µg/mL, gene expression significantly decreased after 24 hours to 1.5-fold for IL-1β, 38-fold for IL-6, 4 for IL-8, remained 5-fold for TNF-α, 10-fold for CXCL-1, 16-fold for CXCL-2, and 9-fold for CXCL-3. Thus, the findings reveal that the expression of proinflammatory mediators exhibited a significant peak at 6 hours after treatment with 25 µg/ml of AGE, except TNF-α remaining the same.

3.4. Effect of MB and AKBA Treatment on Gene Expression of IL-6

The pre-incubation of THP-1 cells with MB (5 and 40 µg/mL) for 18 hours followed by the activation with AGE for 6 hours induces a significant decrease in gene expression by 0.23 and 0.923-fold (p<0.0001) respectively. However, AKBA causes a non-significant decrease in gene expression by 0.14-fold when treated with 0.027 µg/mL, and a significant 0.4-fold decrease (p<0.05) when treated with 0.3 µg/mL (Figure 4).

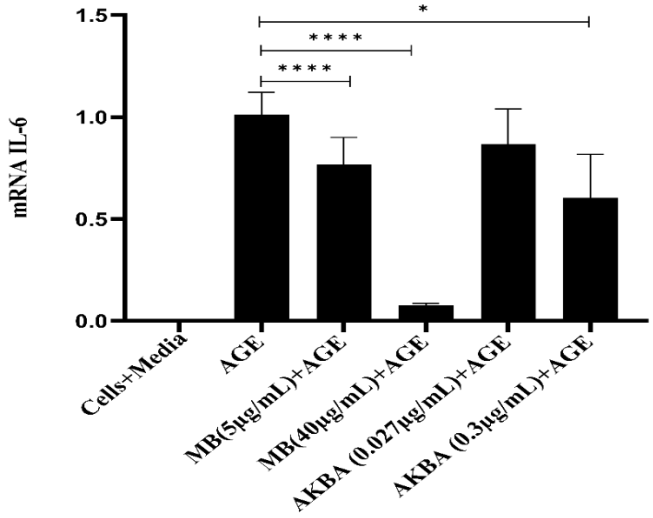

Figure 4.
Analysis of MB-ethanolic extract and AKBA on IL-6 gene expression in AGE-BSA-treated THP-1 cell line. THP-1 cells were preincubated with various concentrations of MB or AKBA for 18 hours, then stimulated with AGE-BSA (25 µg/ml) for 6 hours. The inhibitory effect of MB-ethanolic and AKBA on IL-6 expression was studied by RT-PCR analysis. Data are expressed as mean ± SEM of triplicate samples. *p<0.05, ****p<0.0001.

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3.5. Effect of MB and AKBA Treatment on Gene Expression of TNF-α

Pre-incubation of THP-1 cells with MB (5 and 40 µg/mL) for 18 hours, followed by activation with AGE for 6 hours caused a significant decrease in gene expression level by 0.3 and 0.64-fold (p<0.05) respectively, whereas AKBA causes significant decrease by 0.5-fold when treated with 0.027 µg/mL, and 0.62-fold when treated with 0.3 µg/mL(p<0.05) (Figure 5).

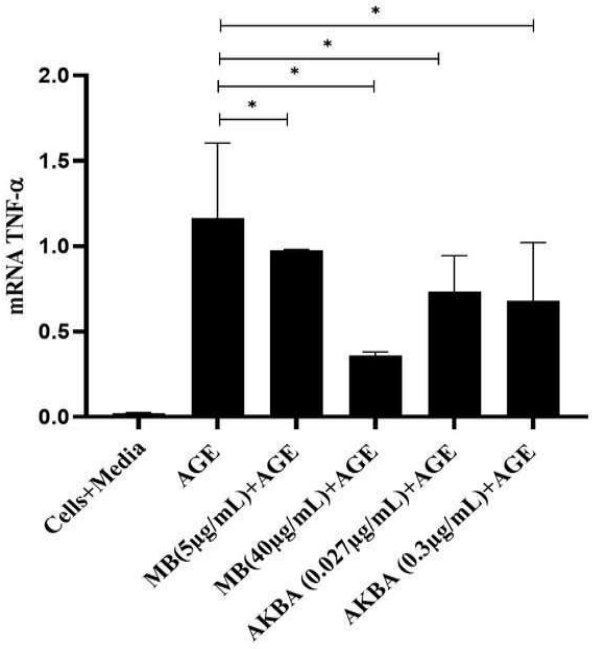

Figure 5.
Analysis of MB-ethanolic extract and AKBA on TNF-α gene expression in the AGE-BSA-treated THP-1 cell line. THP-1 cells were preincubated with either MB or AKBA for 18 hours and then stimulated with AGE-BSA (25 µg/ml) for 6 hours. The inhibitory effect of MB-ethanolic and AKBA on TNF-α expression was studied by RT-PCR analysis. Data are expressed as mean ± SEM of triplicate samples. *p<0.05.

3.6. Effect of MB and AKBA Treatment on Gene Expression of IL-1β

Pre-incubation of THP-1 cells with MB (5 and 40 µg/mL) for 18 hours, followed by activation with AGE for 6 hours induced a negligible decrease in gene expression. In contrast, the response was different with AKBA treatment. At AKBA doses of 0.027 µg/mL, but not with 0.30 µg/mL, a notable decrease in gene expression folds of approximately 0.4 (p<0.05) was noted (Figure 6).

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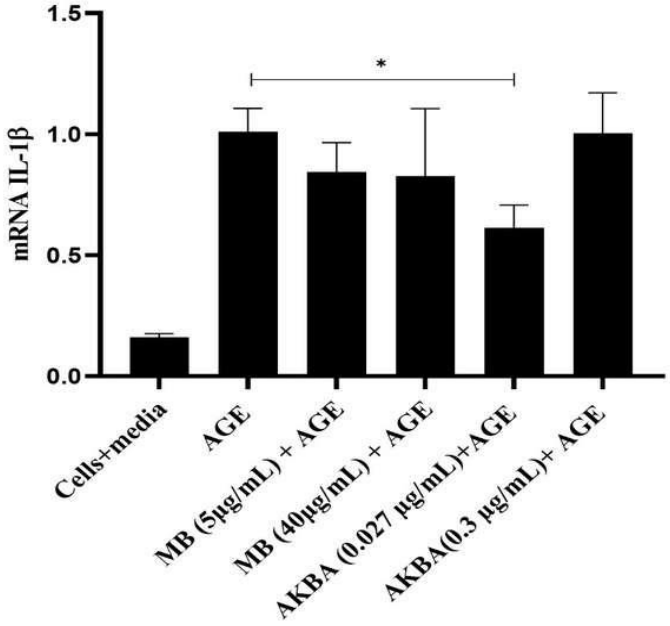

Figure 6.
Analysis of MB-ethanolic extract and AKBA on IL-1β gene expression in the THP-1 cell line treated with AGE-BSA. THP-1 cells were preincubated with various concentrations of MB or AKBA for 18 hours, then stimulated with AGE-BSA (25 µg/ml) for 6 hours. The inhibitory effect of MB-ethanolic and AKBA on IL-1β expression was studied by RT-PCR analysis. Data are expressed as mean ± SEM of triplicate samples. *p<0.05.

3.7. Effect of MB and AKBA Treatment on Gene Expression of IL-4

A Pre-incubation of THP-1 cells with MB (5 and 40 µg/mL) for 18 hours, followed by activation with AGE for 6 hours resulted in a negligible increase in IL-4 gene expression. In contrast, AKBA led to a significant increase of approximately 2-fold, when treated with both concentrations of 0.027 µg/mL and 0.3 µg/mL (Figure 7).

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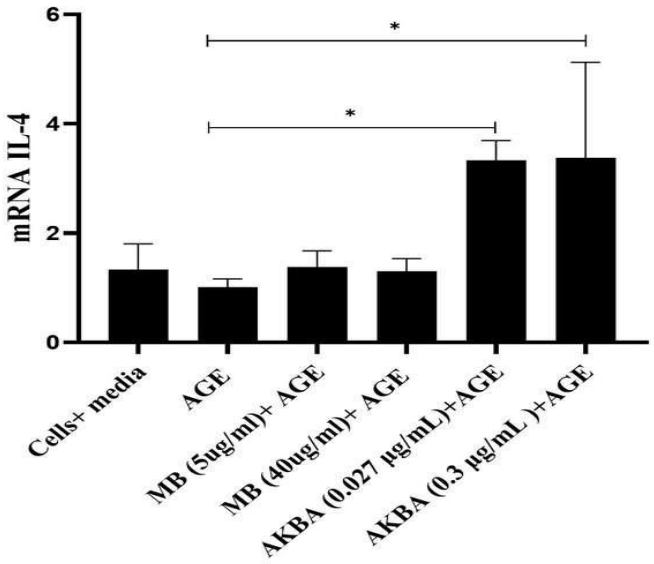

Figure 7.
Analysis of MB-ethanolic extract and AKBA on IL-4 gene expression in the AGE-BSA treated THP-1 cell line. THP-1 cells were preincubated with various concentrations of MB or AKBA for 18 hours, then stimulated with AGE-BSA (25 µg/ml) for 6 hours. The effect of MB-ethanolic and AKBA on IL-4 was studied by RT-PCR analysis. Data are expressed as mean ± SEM of triplicate samples. *p<0.05.

3.8. Effect of MB and AKBA Treatment on Gene Expression of IL-10

After being stimulated with AGE-BSA (25 µg/mL), then pre-treated with MB only at doses of 40 µg/mL, a notable increase in IL-10 gene expression was observed by approximately 3.5-fold. Likewise, with AKBA, a significant increase was evidenced only at the highest concentration of 0.3 µg/mL by around 3.9-fold (Figure 8).

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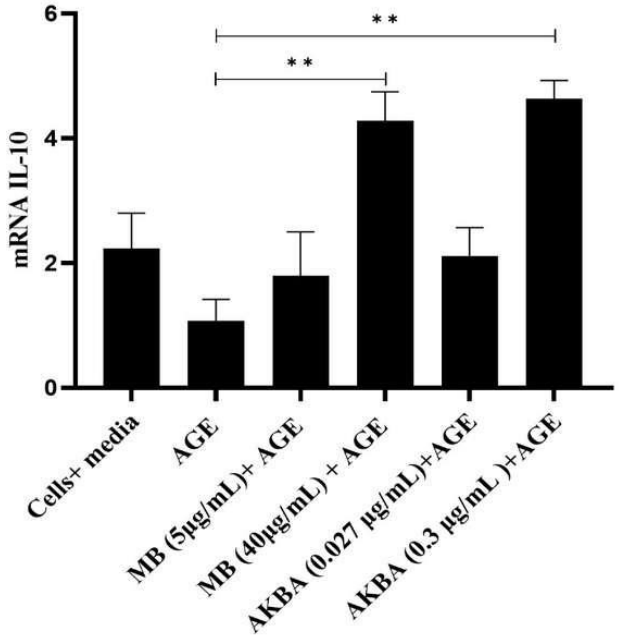

Figure 8.
Analysis of MB-ethanolic extract and AKBA on IL-10 gene expression in the AGE-BSA treated THP-1 cell line. THP-1 cells were preincubated with various concentrations of MB or AKBA for 18 hours, then stimulated with AGE-BSA (25 µg/ml) for 6 hours. The effects of MB-ethanolic and AKBA on IL-10 were studied by RT-PCR analysis. Data are expressed as mean ± SEM of triplicate samples. **p<0.01.

4. Discussion

AGEs constitute a diverse group of compounds formed through non-enzymatic reactions between reducing sugars and proteins, lipids, or nucleic acids [1]. AGEs have been shown to suppress macrophage phagocytosis activity [50]. Macrophages are key players in regulating the innate immune system and serving as antigen-presenting cells in adaptive immunity [51]. In response to invasive antigens, they play a crucial role in both initiating and ending inflammation. In contrast to toll-like receptors (TLRs) such as TLR2 and TLR4, which identify pathogen-associated molecular patterns (PAMPs) or host-derived damage-associated molecular patterns (DAMPs), the innate immune receptor protein. The receptor for Advanced Glycation End Products (RAGE) exhibits a distinct ability to recognize specifically a diverse range of DAMPs [52]. Ligands such as HMGB1, AGEs (van Beijnum et al., 2008), and S100 proteins exemplify molecules that engage in crosstalk between TLR4 and RAGE [52]. The expression of TLR4 has been established in endothelial cells, monocytes, and macrophages [53-55].

Elevated levels of AGEs are particularly implicated in chronic diseases such as diabetes and its associated complications, substantially contributing to inflammation and oxidative stress [56-61]. The interaction between AGEs and RAGE initiates downstream signaling pathways, leading to inflammation, the release of pro-inflammatory cytokines, and the activation of the transcription factor nuclear factor-κB (NF-κB). This activation is consistently observed in various cell types exposed to AGEs, including RAW 264.7 cells [62-64] peritoneal macrophages [65] bone marrow-derived macrophages (BMDM) [9] THP-1 cells [44, 62] and human peripheral blood mononuclear cells PBMCs [66, 67]. Understanding these complicated

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molecular interactions provides useful insights into potential treatment targets for treating chronic inflammatory illnesses related to elevated AGE levels. The interaction of RAGE and TLR4, as well as its effect on immune responses, highlights the complexities of molecular pathways involved in diabetes-related problems. This realization creates opportunities to develop tailored treatments to reduce the effects of AGEs on inflammation and immunological response in the context of diabetes. Natural compounds obtained from medicinal plants have recently attracted more attention because of their potential anti-inflammatory properties [30, 68-73].

Among compounds displaying promise in this regard, MB and AKBA have shown encouraging results. However, its specific efficacy in alleviating AGEs-induced inflammation remains insufficiently explored. Here we used the human monocytic cell line, THP-1, as an *in vitro* approach. Initially, we examined the possible inflammatory effects of AGE-BSA. Subsequently, we investigated the effect of MB-ethanolic extract and AKBA on the gene expression of proinflammatory mediators (IL-6, TNF-α, IL-1β) and anti-inflammatory mediators (IL-4, and IL-10) in THP-1 cells stimulated with AGE-BSA. MB-ethanolic extract showed safety profiles and did not have any cytotoxic effects on THP-1 cells. For AKBA, at 24 hours it demonstrated remarkable safety at different concentrations of 0.01 to 20 µg/mL, however after 48 hours, a notable cytotoxic impact was observed at the higher dose (20 µg/mL). Regarding AGE-BSA, the doses range (10 to 100 µg/mL) for 24 hours, showed no cytotoxic effects, and only over 48 hours, the highest dose (100 µg/mL) showed cytotoxic effects.

The results obtained from the MTT assay after 24 hours for the MB-ethanolic extract, AKBA, and AGE-BSA indicate the absence of cytotoxicity with the tested concentrations. These findings establish a promising safety profile, laying the groundwork for our research to proceed with the doses tested. It is noteworthy to mention that our study is not the first to assess the toxicity levels of AKBA and AGE. A study by Takahashi, et al. [67] supports our findings on AKBA [74] and the study by Li et al. strengthens our safety assessments on AGE-BSA [75]. Their work indicates that exposure of human umbilical vein endothelial cells (HUVECs) to AGEs at a concentration of 100 µg/ml for 24 hours resulted in a decrease in proliferation over time and the appearance of cytotoxic effects. At 100 µg/ml, our results are consistent with others [75]. Regarding the ethanolic extract of MB, our investigation represents the first exploration of its safety profile in the range of tested concentrations. THP-1 cells were activated with different doses of AGE-BSA (25-50 µg/mL) for 6 and 24 hours. After 6h at AGE at 25 µg/mL, the gene expression levels exhibited a significant increase in the proinflammatory cytokine (IL-1β, IL-6, IL-8, TNF-α, CXCL-1, CXCL-2, CXCL-3) reaching peak levels for all mediators. Nevertheless, during a 24-hour incubation period at 25 µg/mL of AGE, there was a reduction in gene expression levels of all proinflammatory mediators (IL-1β, IL-6, IL-8, CXCL-1, CXCL-2, CXCL-3) in contrast to the 6-hour peak suggesting a time-dependent impact, except for TNFα which exhibited constant expression level at the two-time points. Similar patterns were observed when 50 µg/mL of AGE was added to cells. The observed prolonged expression of TNFα may suggest the presence of a regulatory mechanism specifically related to TNFα expression. The observed changes in mRNA levels correlate with the peak production of mRNA during the 4 to 8-hour time frame, which was then followed by protein production peaking at 24 to 48 hours [76].

According to conventional views, macrophages are divided into three subsets: pro-inflammatory (M1), non-activated (M0), and anti-inflammatory (M2), with each having a distinctive function in inflammation initiation and resolution [77]. Polarisation and phenotypic transitions in macrophages require extensive alterations in the cell transcriptome and proteome, which are closely controlled by both internal and external cues. M1 activity inhibits cell proliferation and increases tissue damage, whereas M2 activity promotes tissue regeneration and cell proliferation. M0 macrophages initially exhibit a round shape. After activation and treatment with AGE-BSA, it polarizes into M1. M1 macrophages with a spindle-shaped elongated form and increased cytoplasmic volume. This physical

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alteration, followed by increased motility, indicates accelerated macrophage differentiation into the proinflammatory M1 phenotype, possibly mediated by the activation of the RAGE/NF-κB pathway [9] leading to cytokines and chemokines production that are essential for activating and attracting lymphocytes to inflamed areas, as well as performing antigen-presenting tasks that initiate the humoral immune response [78]. Previous research has shown that AGEs cause macrophages to release proinflammatory cytokines [79, 80]. Our findings support this, indicating that AGEs may prime macrophages into the proinflammatory M1 phenotype by increasing the production of proinflammatory molecules [9].

Micromeria species are known for their antibacterial and antioxidant properties [81, 82]. A study that evaluated the antibacterial efficiency of the essential oil of MB against both gram-negative and gram-positive bacterial strains found significant activity against both wild strains and those with various resistance mechanisms. This essential oil exhibited significant activity in all tested microorganisms [35]. In another investigation, MB oil completely prevented the development of all mycobacterial strains tested at low concentrations [83]. The considerable antibacterial and antioxidant properties of the oil revealed the potential therapeutic effects of MB. In this regard, we evaluated the effects of MB-ethanolic extract after AGE-BSA stimulation on IL-6, TNF-α, IL-1β, IL-4, and IL-10 mRNA levels in THP-1 cells. The results showed considerable inhibition of TNF-α, and IL-6 when compared to control cells. Our findings revealed a dose-dependent reduction in IL-1β, although not being significant. Furthermore, IL-4 exhibited an increase in expression levels, yet also in a non-significant manner. Notably, a significant increase in the expression level of IL-10 was observed at the 40 µg/mL dose. This investigation represents the first exploration of the potential anti-inflammatory properties of MB. The results obtained indicate promising therapeutic benefits associated with MB, thus underscoring the need for additional research to fully elucidate its anti-inflammatory attributes.

AKBA treatment did not result in a downregulation in IL-6 at 0.027 µg/mL. However, at 0.3 µg/mL downregulation was detected. Although it has not yet been investigated with AGE pathology, AKBA, the major component of BS, exhibits inflammatory activities, as shown by pre-treating H9C2 cardiomyocytes with AKBA (2.5, 5, and 10 µM for 24 hours) in an in vitro study that investigated the protective effects of AKBA against LPS-induced cardiac dysfunction [74]. AKBA treatment caused a downregulation in the TNF- α gene expression level. Our results are consistent with Taherzadeh, et al. [74] which showed that the levels of proinflammatory cytokines (IL-β, IL-6, and TNF- α), significantly decreased after AKBA treatment. The anti-inflammatory activity of AKBA can be attributed to the inhibition of lipoxygenase (LOX) enzymes [68] and the inhibition NF-κB signal pathway [68]. In addition, the biological activity of AKBA has been studied in different body systems, such as nervous system diseases [84] and the gastrointestinal part [17]. In the aforementioned study, the exploration of AKBA's impact on AGE pathology marks the first investigation of its kind. Notably, a recent publication has reported that AKBA mitigates experimental pancreatitis by inhibiting oxidative stress in macrophages through the Nrf2/HO-1 pathway [85]. This may support our findings and open the research to study the potential effect of AKBA in the treatment of diabetes by targeting the Nrf2/HO-1 and NF-κB pathways. Here, upon treatment with either AKBA or MB, a notable transformation in the cellular phenotype was observed, with cells adopting a predominantly M2 phenotype characterized by a more rounded and spread morphology, allowing resolution of inflammation and cellular remodeling (Fig. 2).

Moreover, the effects of AKBA on IL-1β production have been studied before and the findings are conforming with our findings, as AKBA reduces the level of IL-1β [86]. Interestingly our results reveal that at the low dose, AKBA exhibits a greater impact on IL-1β, which requires further investigation to understand the cause. As for the anti-inflammatory cytokines, IL-4 and IL-10, Boswellia acids have

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demonstrated the ability to enhance their production in murine splenic T cells [87]. Notably, in rats subjected to lipopolysaccharide treatment, pre-treatment with AKBA resulted in an improvement in IL-10 levels [88].

5. Conclusion

Our findings reveal that both MB and AKBA can modify pro-inflammatory mediator gene expression, with MB having a stronger dose-dependent impact. Although different agents have been implemented to prevent AGE formation and activity, most of them are still in the early phase of clinical studies [89]. Therefore, understanding the specific mechanisms of action and the general influence of MB-ethanolic extract and AKBA on AGE-induced inflammation will help to further understand their potential therapeutic uses in inflammatory and autoimmune diseases such as diabetes. There are several perspectives included in the current study that need to be considered. The lack of information about the phytochemical composition of the MB extract requires a future analysis to establish a connection between the effects and the available metabolites in the extract. In addition, the critical use of *in vivo* experimental animal models is required for gaining valuable insights into the efficiency and safety of the plant. Furthermore, understanding the lipid mediators of inflammation on the protein level and dissecting different signaling pathways that may be involved, all of which may give further insight into the mechanism of action of the natural compounds investigated in the current study.

Transparency:

The authors confirm that the manuscript is an honest, accurate, and transparent account of the study; that no vital features of the study have been omitted; and that any discrepancies from the study as planned have been explained. This study followed all ethical practices during writing.

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