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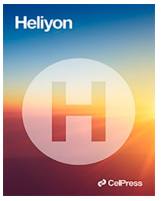

Research article

Anti-inflammatory effects of *Mentha pulegium* L. extract on human peripheral blood mononuclear cells are mediated by TLR-4 and NF-κB suppression

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ABSTRACT

There is great interest in evaluating the anti-inflammatory properties of new herbal products. Thus, the effects of *Mentha pulegium* L. extract on gene and protein expressions of pro-inflammatory mediators and transcription factors were determined.

The hydro-ethanolic extract of *Mentha pulegium* L. was obtained and optimal non-cytotoxic concentrations of the extract were determined by MTT assay. Then, three different concentrations of *Mentha pulegium* L. (10, 30, and 90 μg/mL) were used to pre-treat the lipopolysaccharide (LPS)-stimulated and non-stimulated peripheral blood mononuclear cells (PBMCs) of 10 healthy individuals. Finally, the tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, Toll-like receptor-4 (TLR-4), nuclear factor-kappa B (NF-κB) p65, activator protein-1 (AP-1), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) gene expressions and TNF-α, IL-1β, IL-6, TLR-4, prostaglandin E2 (PGE2), and COX-2 protein levels were measured.

MTT results showed that there is no significant difference in cell viability among 10, 20, 40, and 80 μg/mL concentrations of *Mentha pulegium* L. extract at 24, 48, and 72 h ($P > 0.05$). The IC50 values were 236.1, 147.0, and 118.0 μg/mL after 24, 48, and 72 h respectively. TNF-α, IL-1β, IL-6, TLR-4, iNOS, and NF-κB p65 mRNA levels in the pre-treated LPS-stimulated PBMCs were concentration-dependently reduced ($P < 0.01$ for TNF-α, TLR-4, and NF-κB p65; $P < 0.05$ for IL-1β, IL-6, and iNOS). Also, the protein levels of pro-inflammatory mediators decreased and these differences were significant for TNF-α, IL-1β, and TLR-4 ($P < 0.001$, $P < 0.01$, and $P < 0.001$, respectively).

Mentha pulegium L. extract decreased the expression and biosynthesis of pro-inflammatory mediators. These effects are mainly mediated by TLR-4 and NF-κB suppression. Thus, *Mentha pulegium* L. could be useful in treating or ameliorating chronic inflammatory diseases.

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1. Introduction

Inflammation is a protective response of the immune system against endogenous or environmental stimuli such as damaged or dead cells, pathogens, and toxic compounds [1–3]. Acute inflammation, which is short-term and under the control of the immune system, is considered beneficial. In contrast, chronic inflammation, which persists longer and is characterized by progressive tissue destruction and fibrosis, is considered detrimental [1–3]. Chronic inflammation underlies several diseases, including asthma, allergy, atopic dermatitis, Type I diabetes, cardiovascular diseases, neurodegenerative disorders, many forms of autoimmune diseases and disorders such as multiple sclerosis (MS), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Inflammatory bowel disease (IBD) (Crohn disease and ulcerative colitis), and even cancers [4]. Antigen-presenting cells, especially monocytes and macrophages, are important players in initiating inflammatory responses. They secrete several types of inflammatory mediators. These mediators include cytokines, chemokines, vasoactive amines, vasoactive peptides, fragments of the complement system, proteolytic enzymes, and lipid mediators. Among pro-inflammatory mediators, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, IL-8, and IL-11 are the main pro-inflammatory cytokines. Also, glutathione peroxidase (GPx), cyclooxygenase-2 (COX-2), superoxide dismutase (SOD), prostaglandin E2 (PGE2), NADPH oxidase (NOX), and inducible nitric oxide synthase (iNOS) are other important pro-inflammatory mediators [1–5].

Nowadays, the need for a reliable, least invasive, and inexpensive biomarker to investigate the inflammatory process remains, which has led researchers to investigate peripheral blood. Peripheral blood collection is simple, inexpensive, and less invasive than other samplings. In addition, a more suitable amount of sample can be obtained when using peripheral blood mononuclear cells (PBMCs) [6]. PBMCs mainly consist of lymphocytes (T cells, B cells, and natural killer cells) and monocytes which are all essential components of the immune system [7]. Analysis of PBMCs has defined gene expression changes associated with several chronic inflammatory and autoimmune diseases [8]. They are generally useful in identifying changes in the expression and activity of cytokines and inflammatory mediators. PBMCs interestingly express most of the human genome (over 80 %) and represent gene expression profiles of internal tissues [9,10]. Furthermore, PBMCs participate in physiological and pathological processes associated with immune responses. They also contribute to the knowledge of inflammatory gene expression patterns associated with mitogen-activated protein kinase (MAPK) (ERK, JNK, p38) and nuclear factor-kappa B (NF-κB) pathways [9,10]. Moreover, PBMCs are a more accessible source of cells that provide two further advantages: they can be kept in culture and they also allow us to use them in stimulation experiments [11]. Thus, the evaluation of gene expression in PBMCs is an interesting area for investigating responses to different therapies.

Lipopolysaccharide (LPS) is a potent stimulator of the innate immune system [12,13]. LPS interacts with Toll-like receptors (TLRs) on immune cells such as monocytes and macrophages and induces a pro-inflammatory response in them [12,13]. Among TLRs, TLR-4 is a major LPS receptor that leads to the activation of key transcription factors such as NF-κB and activator protein-1 (AP-1). These transcription factors participate in regulating the gene expression of pro-inflammatory mediators [12,13]. The NF-κB in mammals consists of five members including RelA (p65), RelB, c-rel, p105/p50 (NFκB1), and p100/p52 (NFκB2). A binding site for NF-κB is present in the promoter regions of the most pro-inflammatory mediators [12,13]. Managing and modulating NF-κB activity and production of pro-inflammatory mediators in activated monocytes and macrophages are necessary to control inflammation and improve chronic inflammatory diseases. Therefore, the inflammatory event is an essential target for the development of new approaches to pharmacological interventions.

The Labiatae family (Lamiaceae), which is widely used for various purposes, worldwide includes 220 genera and more than 3000 species [14,15]. Plants of this family have a wide range of compounds such as terpenoids, flavonoids, and polyphenolic compounds, and most of them are famous for their antioxidant properties [15–17]. *Mentha*, the genus of this family, includes 20 species that worldwide spread and represent six species in the flora of Iran. *Mentha pulegium* L. which is one of the *Mentha* species is native to Europe, North Africa, Asia Minor, and the Middle East [15–17]. *Mentha* varieties are commonly used as insect repellent, herbal tea, flavoring plant, and medicinal agent. The flowering aerial parts of the *Mentha pulegium* L. extract are traditionally used to treat colds, sinusitis, cholera, food poisoning, and bronchitis, as well as an antipyretic, anti-flatulent, anti-septic, anti-cough, expectorant, diuretic, menstrual medicine, and medicinal agent for treating inflammatory and autoimmune diseases [15–17]. Several pharmacological properties of *Mentha pulegium* L. such as its abortionist effect in rats, anti-microbial and anti-fungal properties, anti-oxidant, anti--hepatic, and cytotoxichttps://ascidatabase.com/result.php?searchin=Keywords&cat=&ascicat=ALL&Submit=Search&keyword=cytotoxic+activityactivity against different mice and human cell lines were previously reported [15–18]. Although the flowering aerial part of this plant is commonly used because of its several properties, no report comprehensively evaluated its anti-inflammatory properties so far. Thus, in the present study, we aimed to investigate the anti-inflammatory properties of the hydro-ethanolic extract of *Mentha pulegium* L. in human PBMCs to validate its use.

2. Materials and methods

2.1. Plant collection and extract preparation

The fresh flowering aerial parts of the *Mentha pulegium* L. plant were collected from the Sanandaj mountain (Kurdistan Province, Sanandaj-Iran). For maximum yields of high-quality extract, the leaves were harvested at the onset of the flowering period in spring 2020. The harvested plant was identified and dried in a dark place at room temperature and then powdered using an electric device. The dried powdered leaves were kept in a refrigerator at 4 °C until extraction. Hydro-ethanolic extract of *Mentha pulegium* L. was prepared using the macerated method [19,20]. Briefly, 100 g of dried powdered plant were suspended and macerated in 800 mL of 70 % ethanol (HPLC grade, Sigma-Aldrich Chemie GmbH, Germany) for 72 h with occasional shaking at room temperature. Then, the

obtained solution was passed through the filter paper and evaporated to dryness. The solution was concentrated using a rotary evaporator vacuum system (Rotavapor® R-100, BÜCHI) at 45 °C until the solvent evaporated and the extract was dried [20]. The dried extract was then kept in a closed, dark, airtight glass bottle, and stored at −20 °C until use. The extract was then directly dissolved in distilled water. By adding the distilled water to the dried extract, the final concentration was adjusted to 10 mg/mL. Finally, 10, 20, 30, 40, 80, 90, 160, and 320 μg/mL concentrations of the extract were prepared and used for each experiment. All the materials used for immunopharmacological studies including natural products, biologics, and synthetics were endotoxin-free.

2.2. Healthy controls selection

The study population comprised 10 healthy male volunteers with a mean age of 34.2 years. Subjects with no current status of inflammation, autoimmune diseases, viral and bacterial infections, and also using immunosuppressant and herbal medications were included in the study. Exclusion criteria were: metabolic diseases, smoking, and high blood pressure, as well as people under treatment with corticosteroids, immunosuppressants, and herbal medicines. The protocol of this study was approved by the ethics committee of the Kurdistan University of Medical Sciences, Sanandaj, Iran (IR.MUK.REC.1398.143). The study protocol was explained to each participant and informed consent from each of them was obtained for participating in this study and sample collection.

2.3. Peripheral blood sample collection and PBMCs separation

For each participant, 10 mL of whole blood samples were collected in EDTA-containing venepuncture vials. Immediately after sample collection, PBMCs were harvested and obtained by separating them through density gradient centrifugation. Briefly, the blood samples were mixed and diluted with cold phosphate buffer saline (PBS) at a 1:1 ratio in a sterile container. Diluted blood was placed on an equal volume of cell separation density gradient centrifugation media (Lympholyte®-H, Cedarlane, Ontario, Canada) and centrifuged at 2000 RPM (900×*g*) for 30 min at room temperature (18 °C–20 °C) with no brake. Then the white layer containing PBMCs was harvested and rinsed twice in cold PBS and centrifuged at 1200 RPM for 10 min at room temperature. The isolated cell pellets were resuspended in 1 mL of cold PBS [21]. PBMCs were then counted with a hemocytometer and their viability was measured by the trypan blue dye exclusion test [22–25]. Samples with viability greater than 95 % were selected for further analysis and experimentation.

2.4. Experimental and studied groups

All experiments were examined and studied in the 8 different groups as follows.

1) Non-stimulated PBMCs (The PBMCs in complete RPMI-1640 were considered as a control for each group).
2) LPS-stimulated PBMCs. (In this group, PBMCs were treated with LPS at 1.0 μg/mL concentration).
3) Non-stimulated PBMCs treated with *Mentha pulegium* L. extracts at 10 μg/mL concentrations.
4) Non-stimulated PBMCs treated with *Mentha pulegium* L. extracts at 30 μg/mL concentrations.
5) Non-stimulated PBMCs treated with *Mentha pulegium* L. extracts at 90 μg/mL concentrations.
6) LPS-stimulated PBMCs pre-treated with *Mentha pulegium* L. extracts at 10 μg/mL concentrations.
7) LPS-stimulated PBMCs pre-treated with *Mentha pulegium* L. extracts at 30 μg/mL concentrations.
8) LPS-stimulated PBMCs pre-treated with *Mentha pulegium* L. extracts at 90 μg/mL concentrations.
(In groups 6, 7, and 8 PBMCs were pre-treated with 10, 30, and 90 μg/mL concentrations of the *Mentha pulegium* L. extract and then 2 h later incubated with LPS at 1.0 μg/mL concentration).

2.5. Cell culture and PBMCs stimulation

The isolated PBMCs were cultured in a complete culture medium RPMI-1640 comprising 10 % fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/mL penicillin, and 100 mg/mL streptomycin (Gibco BRL Co. Ltd., NY, USA) at a density of 1×10^6 cells/mL in a 24-well plate. PBMCs were pre-treated and incubated with three different concentrations of *Mentha pulegium* L. (10, 30, and 90 μg/mL) for 2 h before stimulation with 1.0 μg/mL of LPS (Escherichia coli O55: B5; Sigma-Aldrich, St. Louis, MO, USA). The contents of the well plate were mixed once with sampling after the ingredients were combined, followed by incubation at 37 °C in a 95 % humidified atmosphere incubator with 5 % CO_2 [24,25]. For gene expression analysis, 6 h later, cultured cells were harvested and centrifuged at 400×*g* for 10 min and transferred into the TRIzol™ reagent (Thermo Fisher Scientific Inc. Waltham, MA USA) for total RNA isolation. After 24 h, the supernatants of LPS-stimulated and pre-treated PBMCs were removed and stored at −70 °C until measuring the level of pro-inflammatory mediators.

2.6. Cell viability assay

The effects of *Mentha pulegium* L. on the viability of PBMCs were investigated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. This method is based on blue formazan formation from colorless MTT by mitochondrial dehydrogenases in live cells. The MTT assay was performed as previously described by Mosmann [26]. In brief, PBMCs were seeded into the 96-well flat-bottom culture plates (JET BIOFIL®, Guangzhou, China) at a density of 1×10^4 PBMCs/well at 37 °C in a 95 %

Table 1
Primer sequences used in quantitative RT-PCR for gene expression analysis.

Name	Primer Sequence	Tm (°C)	Accession number	Amplicon size
TNF-α	Forward primer: 5′-AGCCTCTTCTCCTTCCTGATC-3′	58.60	NM_000594.4	121 bp
	Reverse primer: 5′-CCAGAGGGCTGATTAGAGAGA-3′	58.04		
IL-1β	Forward primer: 5′-ACAGGATATGGAGCAACAAGTG-3′	58.39	NM_000576.3	146 bp
	Reverse primer: 5′-GCTGTAGAGTGGGCTTATCATC-3′	58.34		
IL-6	Forward primer: 5′-CAGACAGCCACTCACCTCTTCA-3′	61.92	NM_000600.5	134 bp
	Reverse primer: 5′- TTTCTGCCAGTGCCTCTTTGC-3′	61.69		
NF-κB	Forward primer: 5′-GGAGCACAGATACCACCAAGAC-3′	60.68	NM_021975.4	161 bp
	Reverse primer: 5′-CTCAGCCTCATAGAAGCCATCC-3′	60.29		
AP-1	Forward primer: 5′-CGGACCTTATGGCTACAGTAACC-3′	60.49	NM_002228.4	189 bp
	Reverse primer: 5′-CCGTTGCTGGACTGGATTATCA-3′	60.42		
TLR-4	Forward primer: 5′-CAACCAAGAACCTGGACCTGAG-3′	60.81	NM_138554.5	147 bp
	Reverse primer: 5′-GGTGGCTTAGGCTCTGATATGC-3′	60.87		
iNOS	Forward primer: 5′-CCGAGTCAGAGTCACCATCCT-3′	60.96	NM_000625.4	158 bp
	Reverse primer: 5′-CCAACAGCAGCCGTTCCT-3′	60.28		
COX-2	Forward primer: 5′-GCCCAGCACTTCACGCATCAG-3′	64.44	NM_000963.4	327 bp
	Reverse primer: 5′-GTTGTGTTCCCGCAGCCAGATTG-3′	64.82		
GAPDH	Forward primer: 5′-GGAGTCCACTGGCGTCTTC-3′	60.08	NM_001357943.2	159 bp
	Reverse primer: 5′-TTGCTGATGATCTTGAGGCTGTT-3′	60.56		

humidified atmosphere incubator with 5 % CO_2. After 24 h incubation and stabilization, the PBMCs were divided into seven different groups: a control group incubated with complete RPMI-1640 alone and six treatment groups incubated with six different concentrations of *Mentha pulegium* L. (10, 20, 40, 80, 160, and 320 μg/mL) for 24, 48, and 72 h. The treated supernatant at the end of the treatment times was removed and PBMCs were then incubated at 37 °C with a 5 mg/mL MTT solution (Sigma-Aldrich, St. Louis, MO, USA). After four additional hours of incubation at 37 °C with MTT, the supernatant from each well was discarded and replaced with 200 mL of dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) to dissolve the newly formed formazan crystals. All experiments were done in triplicate and the viability of non-treated PBMCs was considered as a control. The absorbance of the obtained color was measured at 570 nm wavelength using a PerkinElmer Multimode Microplate Reader; the viability of PBMCs was then calculated using the following formula and expressed as a percentage.

Cell Viability (%) = (Mean absorbance of the treatment group/Mean absorbance of the group without treatment) × 100.

2.7. Total RNA isolation, cDNA synthesis, and quantitative real-time PCR assay

After treatment and stimulation, total RNA from the PBMCs was isolated using the TRIzol™ reagent following the manufacturer's instructions. Then, the quality, purity, and concentration of extracted RNA samples were examined by agarose gel electrophoresis and UV absorbance. The purified total RNA was quantified at 260 nm using a nanodrop (Synergy™ HTX Multi-Mode Microplate Reader, BioTek Instruments, Inc.), and samples with an A260/A280 ratio of more than 1.8 were selected and used for cDNA synthesis. Then, approximately 2 μg of total RNA was converted to the cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc. Waltham, MA USA) according to the instructions described by the manufacturer.

Gene-specific primers were designed using Beacon Designer software version 7.9 (PREMIER Biosoft; Palo Alto, CA, USA) based on the standard criteria as described previously [27,28]. For these purposes, gene-specific sequences were obtained from the National Center for Biological Information website (http://www.ncbi.nlm.nih.gov). To avoid unwanted genomic DNA amplification, real-time PCR primers were designed on different exon or exon-exon boundaries [27,28]. The nucleotide sequence of designed specific primers is shown in Table 1.

The size of real-time PCR amplification products was verified by 2 % agarose gel electrophoresis alongside with 50 bp DNA size marker (Cinnaclon, Inc., Tehran, Iran). The real-time PCR reactions were performed in 10 μL, using real-time PCR SYBR Green Master Mix (Cinnaclon, Inc., Tehran, Iran) and Rotor gene 6000 instruments (QIAGEN, Hilden, Germany). The cycle parameters were as follows: 95 °C for 4 min, 45 × (95 °C for 15 s, 60 °C for 1 min). For all assays, PCR efficiency was tested to ensure an equivalent amplification rate. The relative quantification of mRNA was performed using the relative quantification method and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a standard internal control. Then, to evaluate the quality of the products and the rate of non-specific amplification, the melting curve analysis was performed. Finally, the expression levels of TNF-α, IL-1β, IL-6, NF-κB p65, AP-1, TLR-4, iNOS, and COX-2 were normalized to the GAPDH level for each sample (relative expression). After normalization, the results are expressed as differences in each group.

2.8. Enzyme-linked immunosorbent assay (ELISA)

The levels of TNF-α, IL-1β, IL-6, TLR-4, COX-2, and PGE-2 in cell culture supernatant were assayed by a commercially available ELISA kit (Karmania Pars Gene, Iran, and ZellBio GmbH, Germany); according to the instructions described by the manufacturers. All the controls and standards were done in triplicate and optical densities were measured using an ELISA reader (Synergy™ HTX Multi-Mode Microplate Reader, BioTek Instruments, Inc) under 450 nm wavelength. The results were calculated using standard curves generated by the standards provided by the manufacturer.

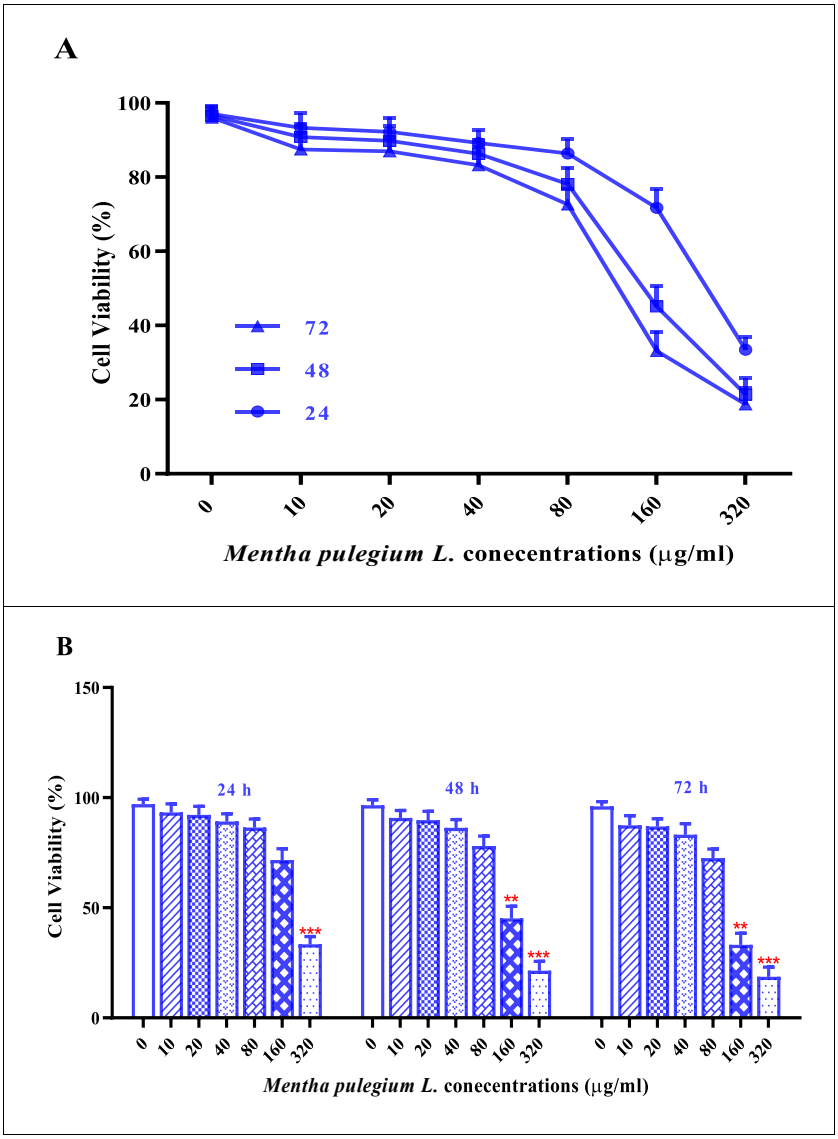

Fig. 1. Effect of Mentha pulegium L. on cell viability.
Graphs show the results of the MTT reduction assay. **a)** Percentage ± SEM of the cell viability of PBMCs treated with six different concentrations of Mentha pulegium L. during different times (24, 48, and 72 h). The following results were obtained for non-treated PBMCs as control and six different concentrations of Mentha pulegium L. (10, 20, 40, 80, 160, and 320 µg/ml): 97.0 ± 2.3, 93.3 ± 3.9, 92.2 ± 3.8, 89.2 ± 3.5, 86.40 ± 3.90, 71.70 ± 5.12, and 33.46 ± 3.50 in the 24 h; 96.5 ± 2.5, 90.80 ± 3.42, 89.80 ± 3.98, 86.29 ± 3.77, 78.10 ± 4.48, 45.16 ± 5.50, and 21.38 ± 4.32 in the 48 h, and 96.0 ± 2.15, 87.42 ± 4.40, 86.99 ± 3.44, 83.22 ± 4.98, 72.62 ± 4.11, 33.16 ± 5.22 and 18.72 ± 4.31 in the 72 h, respectively. **b)** Comparison of the percentage of cell viability among the different times with Mentha pulegium L. treatment (ANOVA with a post hoc Bonferroni's correction; $P < 0.05$). No significant difference was observed in cell viability among treatments with the 10, 20, 40, and 80 µg/ml concentrations relative to the non-treated cells as control at any time ($P > 0.05$). The 160 µg/ml concentration significantly decreased the cell viability at 48 and 72 h ($P < 0.01$), and the 320 µg/ml concentrations had significant effects on cell viability at all times ($P < 0.001$). Each bar graph shows the percentage ± SEM of the experiments. Significance was determined using ANOVA with a post hoc Bonferroni's correction; vs. the control group. *P < 0.05-0.01, **P < 0.01-0.001, ***P < 0.001-0.0001.

3. Statistical analysis

Statistical analysis was performed using version 20 software of SPSS (SPSS Inc., Chicago, IL, USA), and the graphs of this article were drawn using GraphPad Prism Software version 6.0 (GraphPad Software, San Diego, CA, USA). The one-sample Kolmogorov–Smirnov test for normality was used to determine the use of parametric versus non-parametric tests. The differences between multiple interest groups were analyzed using the Kruskal–Wallis non-parametric test. The analysis of variance (ANOVA) with a post hoc Scheffe test was used to compare variables between independent groups. Finally, data were expressed as the mean ± standard

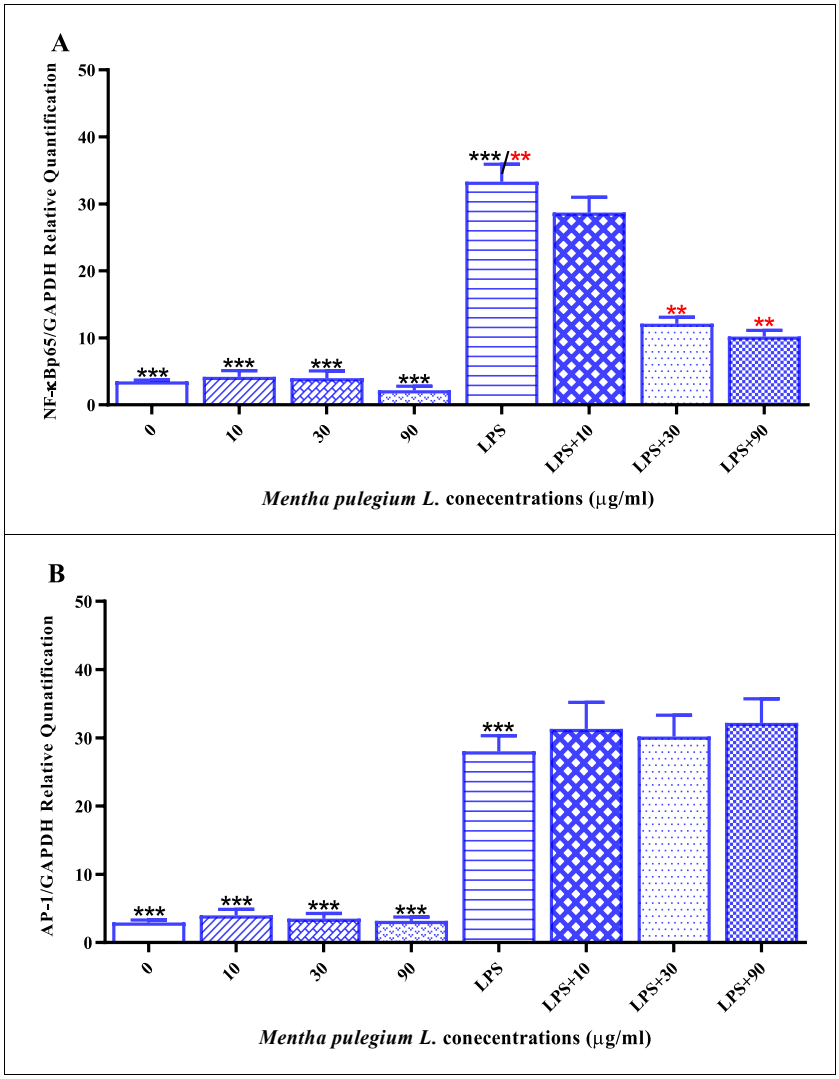

Fig. 2. Effects of Mentha pulegium L. on transcription factors gene expression.
Using the cyber green real-time PCR method, we examined the effect of Mentha pulegium L. on LPS-induced up-regulation of AP-1 and NF-κB p65 gene expression in PBMCs. AP-1 and NF-κB p65 subunit gene expression levels in the PBMCs after exposure to 10, 30, and 90 μg/mL concentrations of Mentha pulegium L. alone compared to the control group showed no significant differences ($P > 0.05$). The expression of AP-1 and NF-κB p65 in the PBMCs after exposure to LPS alone significantly increased ($P < 0.001$) (Fig. 2A and B). LPS-induced NF-κB p65 level in the PBMCs was reduced in a concentration-dependent manner by Mentha pulegium L. pre-treatment (Fig. 2B). NF-kB gene expression level was lower in the groups treated with 10, 30, and 90 μg/ml concentrations of Mentha pulegium L. compared to the LPS-treated group. This difference was statistically significant in the concentration of 30 and 90 μg/ml Mentha pulegium L ($P < 0.01$). However, the expression of the AP-1 transcription factor in PBMCs was not significantly affected upon treatment with different concentrations of Mentha pulegium L. ($P > 0.05$). Each bar graph represents the mean ± SEM of the experiments. Significance was determined using ANOVA with a post hoc Scheffe test vs. the LPS group in the absence of Mentha pulegium L. extract. *$P < 0.05$-0.01, **$P < 0.01$-0.001, ***$P < 0.001$-0.0001. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

error of the mean (SEM), and P-values less than 0.05 ($P < 0.05$) indicated statistically significant differences.

The results obtained in the MTT assay were expressed as the percentage ± SEM. The statistical analysis was performed using ANOVA with a post hoc Bonferroni's correction. A non-linear regression curve analysis was performed on the obtaining results of the MTT assay with the six different concentrations of *Mentha pulegium* L. (10, 20, 40, 80, 160, and 320 μg/mL) for 24, 48, and 72 h to determine the half-maximal inhibitory concentration (IC50).

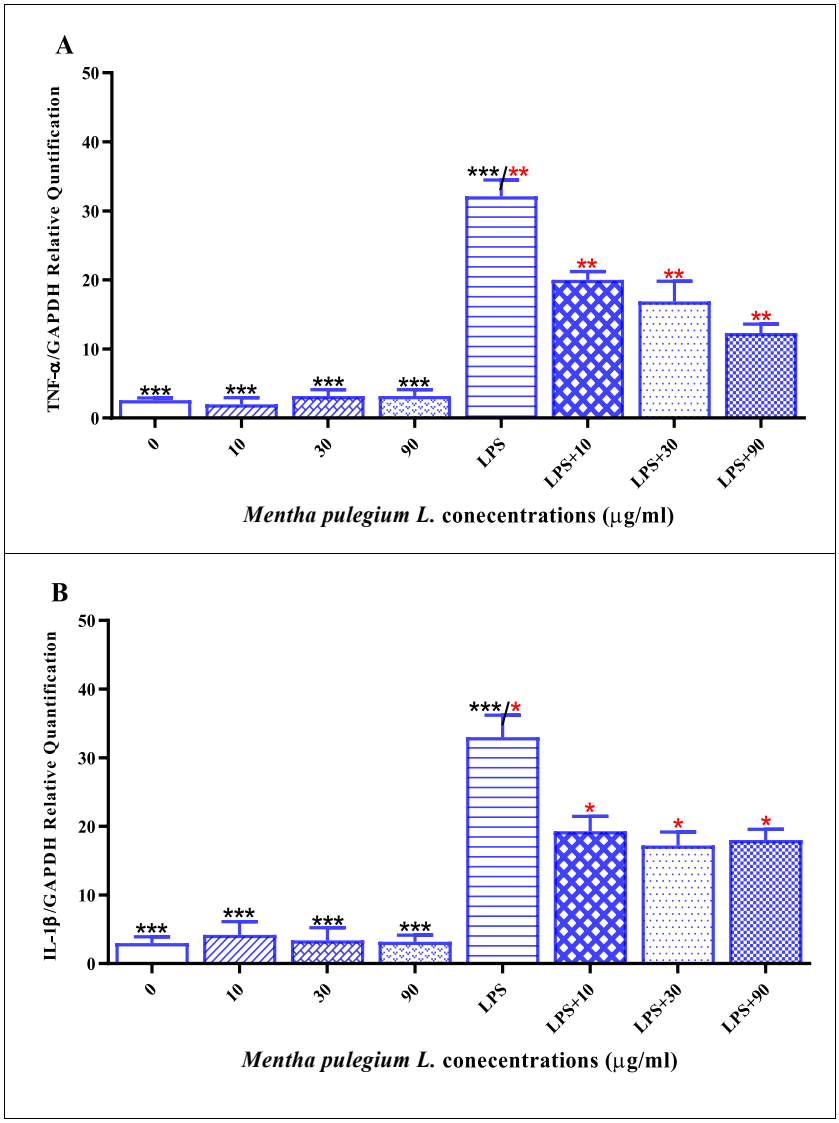

Fig. 3. Effects of Mentha pulegium L. on gene expression of pro-inflammatory mediators.
Intracellular activation of key transcription factors such as NF-κB and AP-1 by LPS leads to the expression of pro-inflammatory mediators. Thus, we examined the effect of different concentrations of Mentha pulegium L. extract on LPS-induced gene expression of pro-inflammatory mediators in human PBMCs. TNF-α, IL-1β, IL-6, TLR-4, iNOS, and COX-2 gene expression levels in the PBMCs after exposure to 10, 30, and 90 μg/mL concentrations of Mentha pulegium L. alone compared to the control group showed no significant differences ($P > 0.05$). TNF-α, IL-1β, IL-6, TLR-4, iNOS, and COX-2 expression in the PBMCs after exposure to LPS alone significantly increased ($P < 0.001$) (Fig. 3A–F). LPS-induced TNF-α, IL-1β, IL-6, TLR-4, iNOS, and COX-2 gene expression levels in the PBMCs were reduced by Mentha pulegium L. pre-treatment in a concentration-dependent manner. These differences were statistically significant for TNF-α and TLR-4 ($P < 0.01$) and also for IL-1β, IL-6, and iNOS ($P < 0.05$). But, the expression of the COX-2 in LPS-stimulated PBMCs was not significantly affected ($P > 0.05$). Each bar graph represents the mean ± SEM of the experiments. Significance was determined using ANOVA with a post hoc Scheffe test vs. the LPS group in the absence of Mentha pulegium L. extract. *P < 0.05-0.01, **P < 0.01-0.001, ***P < 0.001-0.0001.

4. Results

4.1. Cell viability assay and IC50 calculation

The MTT reduction assay was used for measuring the viability of PBMCs and the following results were obtained for the selected concentrations of *Mentha pulegium* L. (10, 20, 40, 80, 160, and 320 μg/mL) over different times (24, 48, and 72 h) relative to the non-treated PBMCs as control: 97.0 ± 2.3, 93.3 ± 3.9, 92.2 ± 3.8, 89.2 ± 3.5, 86.40 ± 3.90, 71.70 ± 5.12, and 33.46 ± 3.50 in the 24 h

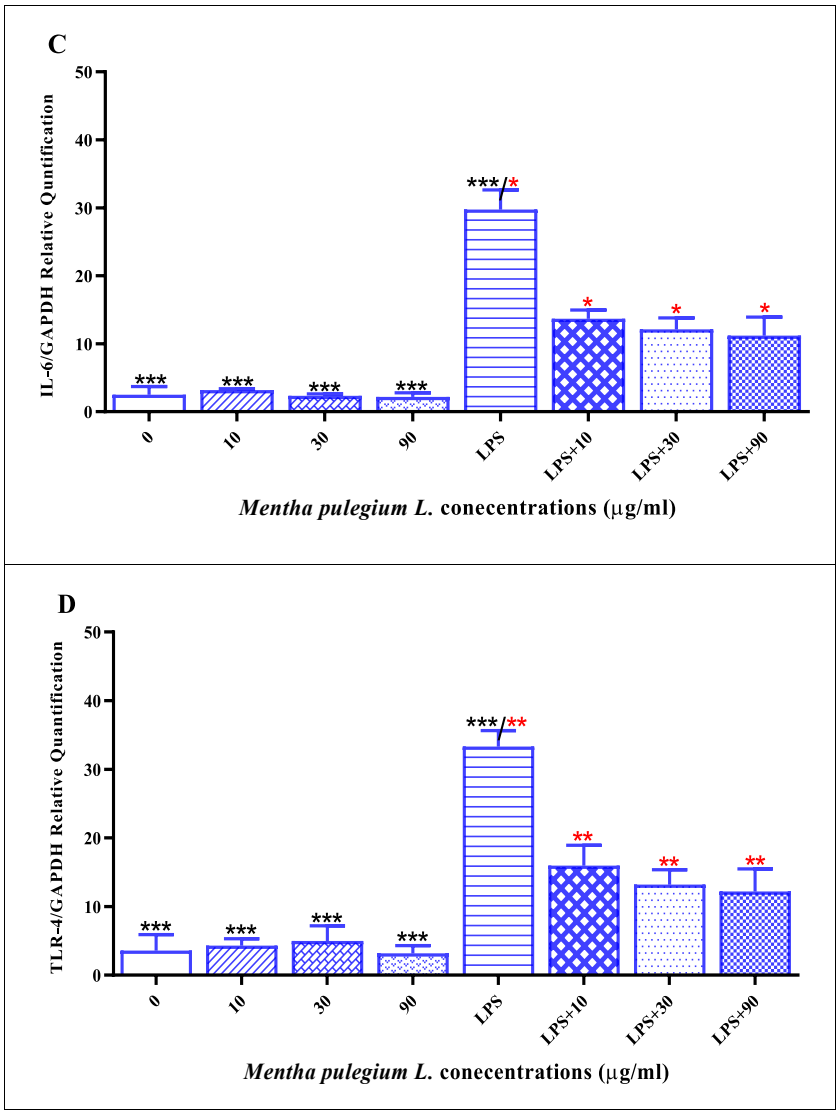

Fig. 3. (*continued*).

period; 96.5 ± 2.5, 90.80 ± 3.42, 89.80 ± 3.98, 86.29 ± 3.77, 78.10 ± 4.48, 45.16 ± 5.50, and 21.38 ± 4.32 in the 48 h period, and 96.0 ± 2.15, 87.42 ± 4.40, 86.99 ± 3.44, 83.22 ± 4.98, 72.62 ± 4.11, 33.16 ± 5.22 and 18.72 ± 4.31 in the 72 h, respectively. The ANOVA)/Bonferroni's correction test showed no significant differences in cell viability among treatment groups with the 10, 20, 40, and 80 μg/mL concentrations versus the control group at any time ($P > 0.05$). The cell viability significantly decreased at 160 μg/mL *Mentha pulegium* L. concentration after 48 and 72 h ($P < 0.01$). Moreover, the 320 μg/mL concentration of *Mentha pulegium* L. had significant cytotoxic effects on the viability of PBMCs at different times ($P < 0.001$) (Fig. 1A and B). These results showed that *Mentha pulegium* L. at 10, 20, 40, and 80 μg/mL concentrations did not affect cell viability at different periods. The IC50 was defined as the concentration of the *Mentha pulegium* L. extracts that produced a 50 % decrease in cell viability relative to the non-treated cells as control. The IC50 values of *Mentha pulegium* L. extract were 236.1, 147.0, and 118.0 μg/mL after 24, 48, and 72 h treatment, respectively.

4.2. Effects of Mentha pulegium L. On the expression of transcription factors

The inflammatory response to LPS depends on the presence of TLR-4, LPS-binding protein, and CD14 on various cells such as monocytes and macrophages. The binding of LPS to its receptor triggers the intracellular activation of NF-κB and AP-1 transcription factors [29]. Activation of these transcription factors finally leads to the expression of endogenous pro-inflammatory mediators. Thus, to determine the effect of *Mentha pulegium* L. on transcription factors, using real-time PCR, we examined its effect on LPS-induced up-regulation of AP-1 and NF-κB p65 subunit gene expression in human PBMCs.

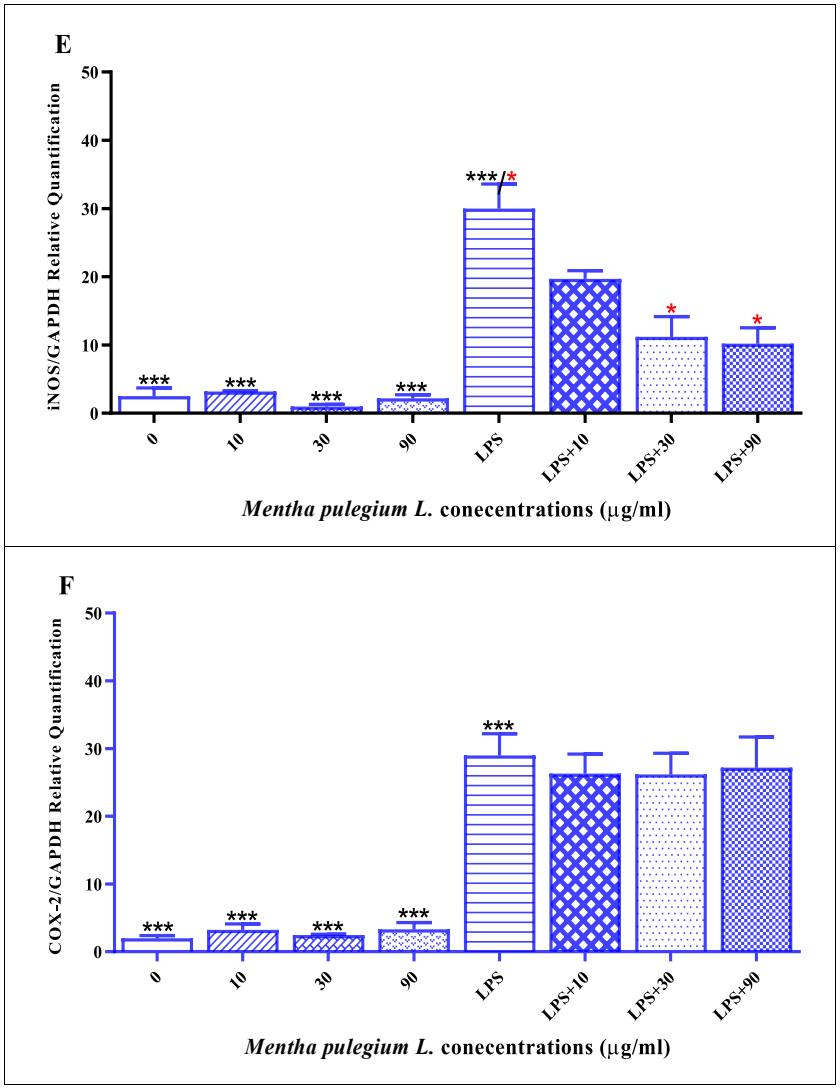

Fig. 3. (*continued*).

NF-κB p65 subunit gene expression levels in the PBMCs after exposure to 10, 30, and 90 μg/mL concentrations of *Mentha pulegium* L. alone compared to the control group showed no significant differences ($P > 0.05$). While results showed a significant increase in the amount of NF-κB p65 subunit expression in the PBMCs after LPS exposure alone ($P<0.001$); however, LPS-induced NF-κB p65 subunit expression level in the PBMCs was reduced in a concentration-dependent manner by *Mentha pulegium* L. pre-treatment. NF-kB p65 subunit gene expression level was lower in the groups treated with 10, 30, and 90 μg/mL concentrations of *Mentha pulegium* L. compared to the LPS-treated group. This difference was statistically significant in the concentration of 30 and 90 μg/mL *Mentha pulegium* L ($P<0.01$) (Fig. 2A).

The AP-1 gene expression levels in the PBMCs after exposure to 10, 30, and 90 μg/mL concentrations of *Mentha pulegium* L. alone compared to the control group showed no significant differences ($P > 0.05$). While the AP-1 gene expression was significantly increased in the LPS-treated group compared to the other treated and untreated groups ($P<0.001$). Expression of the AP-1 transcription factor in the LPS-stimulated PBMCs was not significantly affected upon pre-treatment with different concentrations of *Mentha pulegium* L. ($P > 0.05$) (Fig. 2B).

4.3. Effects of Mentha pulegium L. On the expression of pro-inflammatory mediators

As described above, intracellular activation of transcription factors is crucial for the induction of gene expression of the pro-inflammatory mediators such as TNF-α, IL-1β, IL-6, TLR-4, iNOS, and COX-2 in activated PBMCs; therefore, to clarify the effect of *Mentha pulegium* L. on LPS-induced gene expressions of pro-inflammatory mediators, PBMCs were treated with different concentrations of *Mentha pulegium* L. (10, 30, and 90 μg/mL).

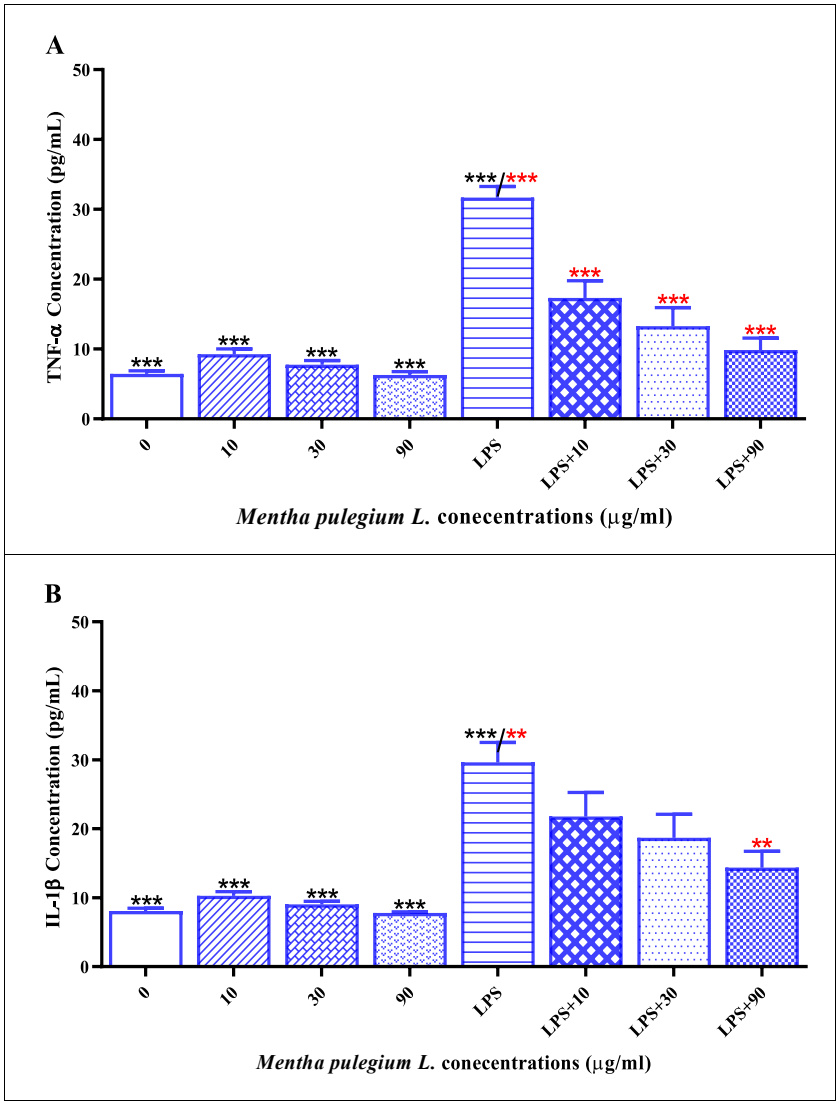

Fig. 4. Effects of Mentha pulegium L. on the production of pro-inflammatory mediators.
PBMCs were pre-treated with Mentha pulegium L. (10, 30, and 90 µg/mL) for 2 h before LPS treatment (1.0 µg/mL). After incubation for 24 h, levels of TNF-α (A), IL-1β (B), IL-6 (C), TLR-4 (D), COX-2 (E), and PGE2 (F) present in the cell culture supernatants were measured using the ELISA method. Results showed that TNF-α, IL-1β, IL-6, TLR-4, COX-2, and PGE2 levels in the PBMCs after exposure to 10, 30, and 90 µg/mL concentrations of Mentha pulegium L. alone compared to the control group showed no significant differences ($P > 0.05$). But, TNF-α, IL-1β, IL-6, TLR-4, COX-2, and PGE2 levels were significantly increased in the culture media of LPS-stimulated PBMCs alone ($P < 0.001$) (Fig. 4A–F). TNF-α, IL-1β, and TLR-4 levels were affected upon pre-treatment with selected concentrations of Mentha pulegium L. and these differences were statistically significant ($P < 0.001$, $P < 0.01$, and $P < 0.001$, respectively) (Fig. 4A, B, D). While, Mentha pulegium L. is not significantly effective in the suppression of IL-6, COX-2, and PGE2 production through alteration of their protein levels ($P > 0.05$). Each bar graph represents the mean ± SEM of the experiments. Significance was determined using ANOVA with a post hoc Scheffe test vs. the LPS group in the absence of Mentha pulegium L. extract. *$P < 0.05$-0.01, **$P < 0.01$-0.001, ***$P < 0.001$-0.0001.

As demonstrated in Fig. 3A–F, the expression of TNF-α, IL-1β, IL-6, TLR-4, iNOS, and COX-2 in the PBMCs after exposure to 10, 30, and 90 µg/mL concentrations of *Mentha pulegium* L. alone compared to the control group showed no significant differences ($P > 0.05$). The expression of TNF-α, IL-1β, IL-6, TLR-4, iNOS, and COX-2 in the PBMCs after exposure to LPS alone significantly increased ($P < 0.001$). LPS-induced TNF-α, IL-1β, IL-6, TLR-4, iNOS, and COX-2 gene expression levels in the PBMCs were reduced by *Mentha pulegium* L. pre-treatment in a concentration-dependent manner. These differences were statistically significant for TNF-α and TLR-4 ($P < 0.01$) and also for IL-1β, IL-6, and iNOS ($P < 0.05$), especially for the concentration of 30 and 90 µg/mL (Fig. 3A–F). However, the expression of COX-2 in the stimulated PBMCs was not significantly affected upon treatment with different concentrations of *Mentha pulegium* L. ($P > 0.05$).

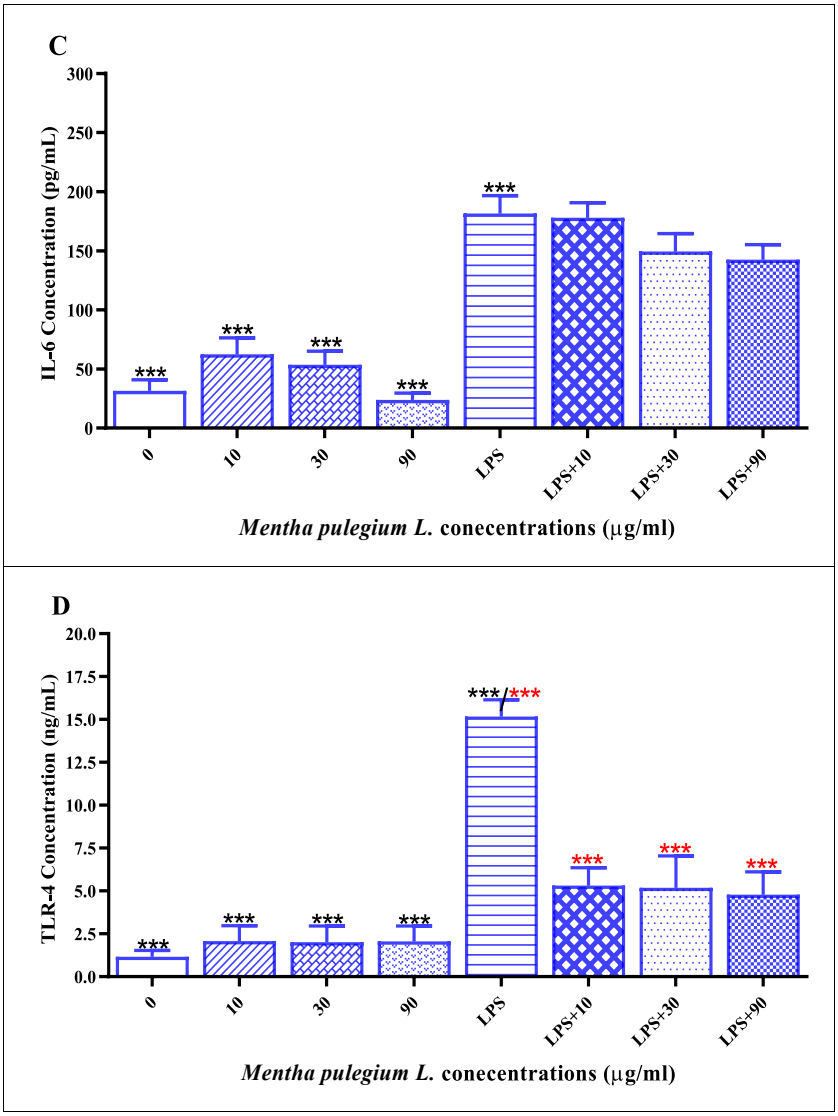

Fig. 4. (*continued*).

4.4. Effects of Mentha pulegium L. On the production of pro-inflammatory mediators

To further examine the question of whether the inhibition of transcription factors and pro-inflammatory mediator's gene expression is related to the reduction of protein levels, we performed the ELISA method. For this purpose, in a parallel experiment with the gene expression assay, PBMCs were treated with different concentrations of *Mentha pulegium* L. (10, 30, and 90 µg/mL) in the presence or absence of LPS (1.0 µg/mL) for 24 h. Then the levels of pro-inflammatory mediators' production in the cell culture supernatant were measured.

As demonstrated in Fig. 4A–F, the levels of TNF-α, IL-1β, IL-6, TLR-4, PGE2, and COX-2 in the PBMCs after exposure to 10, 30, and 90 µg/mL concentrations of *Mentha pulegium* L. alone compared to the control group showed no significant differences ($P > 0.05$). Results also showed that TNF-α, IL-1β, IL-6, TLR-4, PGE2, and COX-2 levels were significantly increased in the supernatant of LPS-stimulated PBMCs alone ($P < 0.001$). TNF-α, IL-1β, and TLR-4 were significantly affected upon treatment with selected concentrations of *Mentha pulegium* L. ($P < 0.001$, $P < 0.01$, and $P < 0.001$, respectively) (Fig. 4A, B, D). While, *Mentha pulegium* L. is not effective in the suppression of IL-6, COX-2, and PGE2 production through alteration of their protein levels. However, pre-treatment with *Mentha pulegium* L. decreased the release of pro-inflammatory mediators in the culture media of LPS-stimulated PBMCs.

5. Discussion

Based on the available evidence TNF-α, IL-1β, IL-6, TLR-4, NF-κB p65, AP-1, COX-2, PGE2, and iNOS play important roles in several

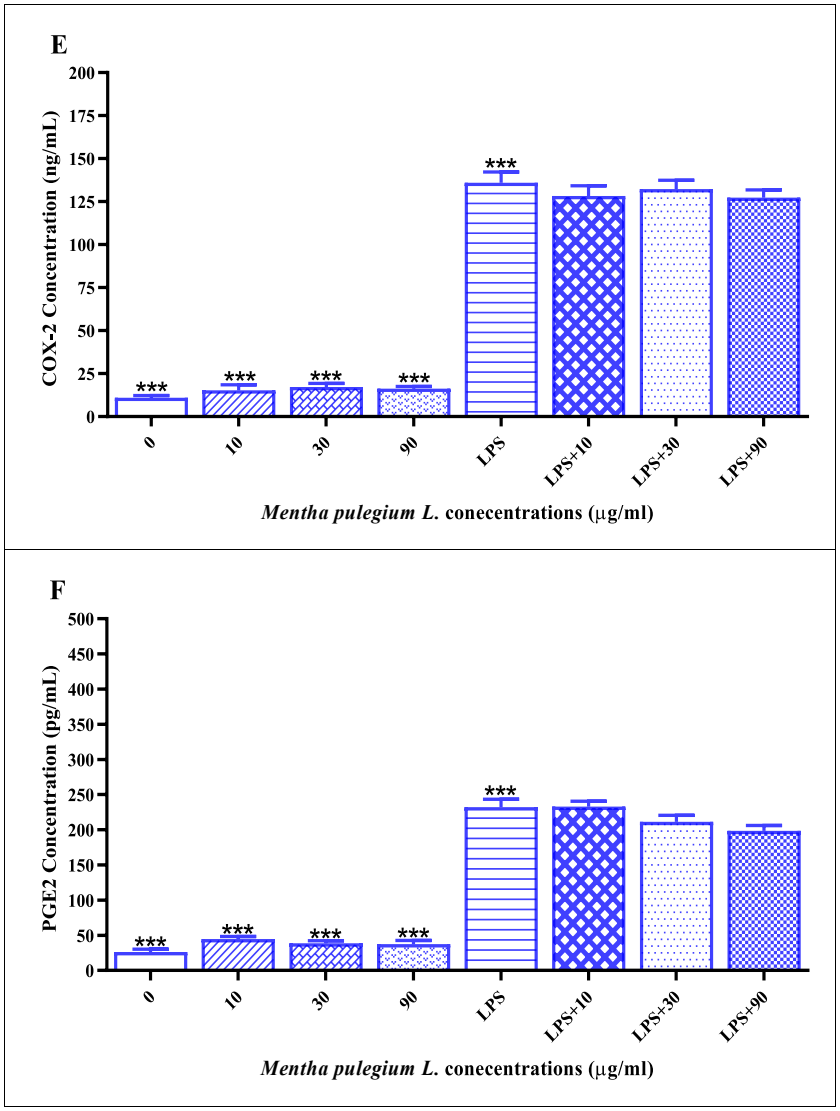

Fig. 4. (*continued*).

physiological and pathological conditions, thus they are major drug targets in treating autoimmune and inflammatory diseases [30,31]. Nowadays, increasing attention has been directed to the study of natural products used in traditional medicine that may counteract the harmful effects of inflammatory agents and stimuli. In this context, various medicinal plants such as curcumin, berberine, and other nutraceuticals have been evaluated, recognized, and used as valuable sources of anti-inflammatory agents [32–34]. Several studies have been conducted to identify the main ingredients of *Mentha pulegium* L. in different countries [35–37]. The results have shown that its constituents are similar, but their percentages are different depending on the geographical areas. Previous studies have shown that *Mentha pulegium* L. from Bulgaria origin contains pulegone (42.9–45.4 %); *Mentha pulegium* L. of Uruguay origin contains pulegone (73.4 %) and isomenthone (12.9 %); *Mentha pulegium* L. of Egypt origin contains pulegone (43.5 %) and piperitone (12.2 %); and *Mentha pulegium* L. from Tunisia, contains pulegone (41.8 %) and isomenthone (11.3 %). These studies have shown major constituents of *Mentha pulegium* L. with the following constituents: pulegone, piperitone/piperitenone, and isomenthone/neoisomenthol. The analysis of Iranian *Mentha pulegium* L. extract revealed that 12 compounds make up more than 92 % of *Mentha pulegium* L. extract. The other compounds found in *Mentha pulegium* L. extract respectively were γ-sitosterol, Benzene, 1-methoxy-4-(2-propenyl), 3-octanol, α-amyrin, α-pinene, α-terpineol, β–pinene, Germacerene-D, and Mint furanone. The phytochemical studies also showed that flavonoid, phenolic, and terpenoid compounds were seen in the water and methanolic extracts [35–37]. Thus, like in other countries, the major components of Iranian *Mentha pulegium* L. extract were pulegone (40.5 %), isomenthone/neoisomenthol (35.4 %), and piperitone/piperitenone (5.2 %). So far, the chemical compositions and biological activities of *Mentha pulegium* L. extracts have been reported, especially in a limited number of studies [38,39]. These biological activities are mostly related to cell growth, mitochondrial effects, and induction of apoptosis [38,39]. However, no comprehensive study has been conducted regarding the

anti-inflammatory effects of this substance on the immune system. Therefore, for the first time in this study, we examined the anti-inflammatory effects of this substance on human PBMCs. For this purpose, the hydro-ethanolic extract of *Mentha pulegium* L. was obtained, and optimal and non-cytotoxic concentrations of the extract were determined by MTT assay. Then, three concentrations (10, 30, and 90 μg/mL) of *Mentha pulegium* L. were selected based on the MTT results and used to treat the LPS-stimulated and non-stimulated PBMCs of 10 healthy individuals. Finally, the TNF-α, IL-1β, IL-6, TLR-4, NF-κB p65 subunit, AP-1, iNOS, and COX-2 mRNA expressions and TNF-α, IL-1β, IL-6, TLR-4, PGE2, and COX-2 protein levels were determined.

Peripheral blood monocytes and tissue-resident macrophages initiate and regulate the immune responses. Exposure and stimulation of these immune cells through TLR-4, which binds to bacterial products such as LPS with the aid of accessory proteins LPS-binding protein and CD14, results in the activation of the NF-κB and AP-1 transcription factors [40,41]. These transcription factors are the primary regulators of genes that induce the production of pro-inflammatory mediators and are involved in several inflammatory processes. Activation of these transcription factors also induces or suppresses a wide range of genes that regulate inflammation, cell survival, proliferation, and migration. Blockades of NF-κB transcriptional activity suppress the expression of pro-inflammatory cytokines and mediators such as TNF-α, IL-1β, and IL-6. This process is highly regulated while loss of control can result in conditions such as acute or chronic inflammatory diseases, autoimmune disorders, multiple organ failure, stimulating tumor cell growth, and even cancers [40,41]. In the present study, mRNA expressions of NF-κB p65 subunit and AP-1 transcription factors were determined. This study showed that the expression of AP-1 and NF-κB p65 in the PBMCs after exposure to 10, 30, and 90 μg/mL concentrations of *Mentha pulegium L.* alone compared to the control group showed no significant differences while their expression in the PBMCs after exposure to LPS alone significantly increased (Fig. 2A and 2B). LPS-induced NF-κB p65 level in the PBMCs was reduced by *Mentha pulegium* L. pre-treatment in a concentration-dependent manner (Fig. 2B). NF-κB gene expression level was lower in the groups treated with 10, 30, and 90 μg/ml concentrations of *Mentha pulegium* L. compared to the LPS-treated group. This difference was statistically significant in the concentration of 30 and 90 μg/ml *Mentha pulegium* L. But, the expression of the AP-1 transcription factor in PBMCs upon treatment with different concentrations of *Mentha pulegium* L was not significantly affected. Also, gene expression levels of TNF-α, IL-1β, IL-6, and TLR-4 were determined. The expression of TNF-α, IL-1β, IL-6, and TLR-4 in the PBMCs after exposure to 10, 30, and 90 μg/mL concentrations of *Mentha pulegium* L. alone compared to the control group showed no significant differences but their expression in the PBMCs after exposure to LPS alone significantly increased (Fig. 3A–D). LPS-induced TNF-α, IL-1β, IL-6, and TLR-4 gene expression levels in the PBMCs were reduced concentration-dependently by *Mentha pulegium* L. pre-treatment. Also, results showed that TNF-α, IL-1β, IL-6, and TLR-4 protein levels after exposure to 10, 30, and 90 μg/mL concentrations of *Mentha pulegium* L. alone compared to the control group showed no significant differences. But, TNF-α, IL-1β, IL-6, and TLR-4 protein levels were significantly increased in the culture media of LPS-stimulated PBMCs alone (Fig. 4A–D). Upon pre-treatment with selected concentrations of *Mentha pulegium* L., TNF-α, IL-1β, and TLR-4 levels were significantly reduced (Fig. 4 4A, B, and D). While *Mentha pulegium* L. is not significantly effective in the suppression of IL-6 production through alteration of its protein level. Although the decrease in IL-6 level was not statistically significant, its amount dose-dependently decreased (Fig. 4C).

Signaling through TLR-4 also controls the activation of the COX-2-PGE2 pathway in macrophages and several cell types. COX-2 up-regulation is associated with increased cell adhesion, resistance to apoptosis, and tumor angiogenesis under pathological conditions [42–45]. PGE2 is a potent bioactive lipid mediator produced by the metabolism of arachidonic acid via the COX-1 and COX-2 pathways. PGE2 has several important biological and physiological functions including immune surveillance, immune regulation, both anti- and pro-inflammatory action, and fever generation [42–45]. Nitric oxide (NO) is implicated in several biological processes including platelet aggregation inhibition, neurotransmission regulation, vasorelaxation, inflammation, and immune responses. NO is synthesized by the three-enzyme family including inducible nitric oxide synthase (iNOS). iNOS is constitutively expressed in kidneys, while in other tissues is induced by bacterial LPS, growth factors, and cytokines such as TNF-α and IL-1β [46–49]. Therefore, based on their important role in inflammatory processes, iNOS and COX-2 mRNA expressions and PGE2 and COX-2 protein levels were determined in this study. iNOS and COX-2 gene expression levels in the PBMCs after exposure to 10, 30, and 90 μg/mL concentrations of *Mentha pulegium* L. alone compared to the control group showed no significant differences (Fig. 3E and F) while in the PBMCs after exposure to LPS alone significantly increased. *Mentha pulegium* L. pre-treatment attenuated iNOS and COX-2 expression in LPS-stimulated PBMCs and this difference was statistically significant for iNOS (Fig. 3E and F). Our data also showed that *Mentha pulegium* L. reduced LPS-induced COX-2 and PGE2 protein levels in human-stimulated PBMCs but these differences were not statistically significant (Fig. 4E and F). Although the differences are not statistically significant for some pro-inflammatory mediators, in general, the expression and protein levels of pro-inflammatory mediators decreased after *Mentha pulegium* L. treatment in a dose-dependent manner. This difference may be due to differences in the accuracy and sensitivity of the commercial kits used. Two other studies have similarly investigated the anti-inflammatory effects of *Mentha pulegium* L. and our results are similar to their results. In a study by Bahrami et al. [50], the anti-inflammatory properties of ethanolic extracts of three *Mentha* species including *Mentha spicata* L., *Mentha pulegium* L., and *Mentha rotundifolia* L. in a murine RAW264.7 macrophage cell line were investigated. The effects of ethanolic extracts on TNF-α, IL-6, IL-10, and IFN-γ cytokine secretion, and Monocyte Chemoattractant Protein (MCP)-1 were determined at 24 h in LPS-treated RAW264.7 cells. Comparatively, the synergic effects of the *Mentha* extracts were observed on cytokine secretion, and all the *Mentha* extracts strongly reduced IL-6 secretion. Besides, *Mentha pulegium* L. and *Mentha rotundifolia* L. also decreased MCP-1 and TNF-α secretion. Also, in another study by Rocha and colleagues [51], the effects of a *Mentha pulegium* L. phenolic extract in the impairment of inflammatory processes in IBD were evaluated. For this purpose, the effect of *Mentha pulegium* L. extract administration in a model of TNBS-induced colitis in mice was investigated. Administration of the *Mentha pulegium* L. extract in a rat model of carrageenan-induced paw edema exhibited significant anti-inflammatory effects. Further results also showed a beneficial effect of the *Mentha pulegium* L. extract in the attenuation of experimental colitis. A reduction in several markers of colon inflammation was observed following the extract administration to colitis-induced mice, including functional and histological indicators. Significant

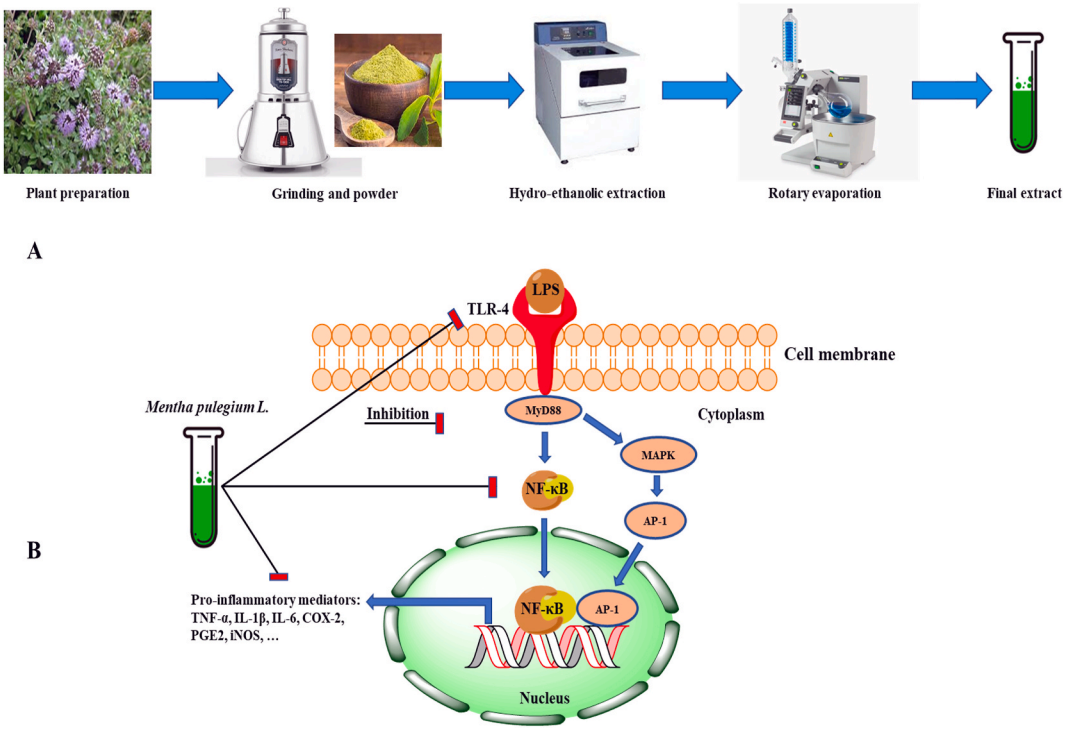

Fig. 5. Summary of the present study. Schematic representation of the Mentha pulegium L. collection, general extraction, isolation, and purification (Fig. 5A). Anti-inflammatory effects of Mentha pulegium L. extract on the LPS-induced inflammation in human PBMCs (Fig. 5B). Mentha pulegium L. attenuated LPS-induced inflammation by preventing TLR-4 and NF-κB expression.

inhibition of crucial mediators of intestinal inflammation, iNOS/COX-2 expression, in the colon of colitis-induced mice was observed.

Collectively our results showed that *Mentha pulegium* L. concentration-dependently attenuated TNF-α, IL-1β, IL-6, TLR-4, iNOS, and NF-κB p65 subunit gene expression in LPS-stimulated PBMCs. Also, *Mentha pulegium* L. attenuated the release of pro-inflammatory mediators, including TNF-α, IL-1β, IL-6, TLR-4, COX-2, and PGE2. These results suggest the involvement of the TLR-4 and NF-κB pathways in the anti-inflammatory effect of *Mentha pulegium* L. treatment. The summary of the present study is illustrated in Fig. 5A and B. Our study had some limitations: it would be better to check NF-κB protein through western blotting, NK-κB luciferase assay, etc. It is also better to identify the chemical composition of *Mentha pulegium* L. extract using high-performance liquid chromatography (HPLC) fingerprinting or ultra-performance liquid chromatography-mass spectrometry (UPLC-MS/MS) analysis. However, since this was a preliminary screening study, we used the total extract and we only examined gene expression of key transcription factors NF-κB and AP-1.

6. Conclusions and future perspectives

In conclusion, our data clearly showed the marked inhibition of pro-inflammatory mediators' expression and release. Furthermore, *Mentha pulegium* L. acted at the level of transcription mainly by the prevention of TLR-4 and NF-κB expression. Although several studies regarding the role of *Mentha pulegium* L. have been performed, we provide the first evidence that *Mentha pulegium* L. decreases the expression and biosynthesis of pro-inflammatory mediators in human LPS-stimulated PBMCs. These primary findings provide support for further studies on these compounds to develop anti-inflammatory agents. More confirmatory studies are recommended to verify our results.However, further investigations are also needed to clarify the precise mechanism by which *Mentha pulegium L.* decreases the TLR-4 expression. Furthermore, the pharmacology and mode of action of its active components must be further investigated. It is also better to investigate our results in animal models of inflammation and autoimmunity or human clinical trials.

Data availability

Data will be available on request.

CRediT authorship contribution statement

Firouz Mohammadi: Methodology, Formal analysis, Data curation. **Kaveh Rahimi:** Methodology, Investigation, Formal analysis, Data curation. **Abbas Ahmadi:** Visualization, Supervision, Project administration, Methodology. **Zahra Hooshmandi:** Validation,

Investigation, Formal analysis. **Sabrieh Amini:** Writing - review & editing, Writing - original draft, Funding acquisition, Conceptualization. **Asadollah Mohammadi:** Writing - review & editing, Writing - original draft, 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.

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References

[1] R. Medzhitov, Origin and physiological roles of inflammation, Nature 454 (7203) (2008) 428–435.
[2] R. Medzhitov, Inflammation 2010: new adventures of an old flame, Cell 140 (6) (2010) 771–776.
[3] L. Chen, et al., Inflammatory responses and inflammation-associated diseases in organs, Oncotarget 9 (6) (2018) 7204–7218.
[4] A.H. Katsanos, et al., Biomarker Development in Chronic Inflammatory Diseases, Biomarkers for Endometriosis, 2017, pp. 41–75.
[5] A. Shapouri-Moghaddam, et al., Macrophage plasticity, polarization, and function in health and disease, J. Cell. Physiol. 233 (9) (2018) 6425–6440.
[6] B. Arosio, et al., Peripheral blood mononuclear cells as a laboratory to study dementia in the elderly, BioMed Res. Int. 2014 (2014) 169203.
[7] C.R. Kleiveland, Peripheral blood mononuclear cells, in: K. Verhoeckx, et al. (Eds.), The Impact of Food Bioactives on Health: in Vitro and Ex Vivo Models, Springer International Publishing, Cham, 2015, pp. 161–167.
[8] A.S. Santos, et al., Prevalence of inflammatory pathways over Immuno-Tolerance in peripheral blood mononuclear cells of Recent-onset type 1 diabetes, Front. Immunol. 12 (2021) 765264.
[9] C.-C. Liew, et al., The peripheral blood transcriptome dynamically reflects system wide biology: a potential diagnostic tool, J. Lab. Clin. Med. 147 (3) (2006) 126–132.
[10] B. Reynés, et al., Peripheral blood cells, a transcriptomic tool in nutrigenomic and obesity studies: current state of the art, Compr. Rev. Food Sci. Food Saf. 17 (4) (2018) 1006–1020.
[11] O. Pansarasa, et al., Biomarkers in human peripheral blood mononuclear cells: the state of the art in amyotrophic lateral sclerosis, Int. J. Mol. Sci. 23 (5) (2022).
[12] A. Ciesielska, M. Matyjek, K. Kwiatkowska, TLR4 and CD14 trafficking and its influence on LPS-induced pro-inflammatory signaling, Cell. Mol. Life Sci. 78 (4) (2021) 1233–1261.
[13] M.A. Dobrovolskaia, S.N. Vogel, Toll receptors, CD14, and macrophage activation and deactivation by LPS, Microb. Infect. 4 (9) (2002) 903–914.
[14] W. Evans, Trease and Evans' Pharmacognosy, sixteenth ed., Saunders Ltd., 2009.
[15] F. Naghibi, et al., Labiatae family in folk medicine in Iran: from ethnobotany to pharmacology, Iran. J. Pharm. Res. (IJPR) 4 (2) (2022) e128228.
[16] B. Nickavar, A. Alinaghi, M. Kamalinejad, Evaluation of the Antioxidant Properties of Five Mentha Species 7 (3) (2022) e128592.
[17] S. Miraj, S. Kiani, Study of pharmacological effect of Mentha pulegium: a review, Der Pharm. Lett. 8 (9) (2016) 242–245.
[18] Farshad H. Shirazi, Neda Ahmadi, Mohammad Kamalinejad, Evaluation of Northern Iran Mentha Pulegium L. Cytotoxicity, DARU Journal of Pharmaceutical Sciences, 2004, p. 5.
[19] A.R. Abubakar, M. Haque, Preparation of medicinal plants: basic extraction and fractionation procedures for experimental purposes, J. Pharm. BioAllied Sci. 12 (1) (2020) 1–10.
[20] V.R. Askari, et al., The influence of hydro-ethanolic extract of Portulaca oleracea L. on Th(1)/Th(2) balance in isolated human lymphocytes, J. Ethnopharmacol. 194 (2016) 1112–1121.
[21] M.E. Kanof, P.D. Smith, H. Zola, Isolation of whole mononuclear cells from peripheral blood and cord blood, Curr. Protoc. Im. (2001) **(Chapter 7)**: p. Unit 7.1.
[22] W. Strober, Trypan blue exclusion test of cell viability, Curr. Protoc. Im. (2001). **Appendix 3**: p. Appendix 3B.
[23] W. Strober, Trypan blue exclusion test of cell viability, Curr. Protoc. Im. 111 (2015) p. A3.b.1-a3.b.3.
[24] V.R. Askari, et al., Evaluation of the anti-oxidant and anti-inflammatory effects of the methanolic extract of Ferula szowitsiana root on PHA-induced inflammation in human lymphocytes, Drug Chem. Toxicol. 43 (4) (2020) 353–360.
[25] V. Baradaran Rahimi, et al., Crocetin regulates Th1/Th2 and Th17/Treg balances, nitric oxide production, and nuclear localization of NF-κB in Th2-provoked and normal situations in human-isolated lymphocytes, Biofactors 49 (3) (2023) 699–711.
[26] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1–2) (1983) 55–63.
[27] A. Mohammadi, et al., HTLV-1-infected asymptomatic carriers compared to HAM/TSP patients over-express the apoptosis- and cytotoxicity-related molecules, Med. Microbiol. Immunol. 208 (6) (2019) 835–844.
[28] A. Mohammadi, et al., Modulatory effects of curcumin on apoptosis and cytotoxicity-related molecules in HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients, Biomed. Pharmacother. 85 (2017) 457–462.
[29] T. Liu, et al., NF-κB signaling in inflammation, Signal Transduct. Targeted Ther. 2 (1) (2017) 17023.
[30] A. Mohammadi, et al., Macrophage plasticity, polarization and function in response to curcumin, a diet-derived polyphenol, as an immunomodulatory agent, J. Nutr. Biochem. 66 (2019) 1–16.
[31] A. Mohammadi, et al., Manipulating macrophage polarization and function using classical HDAC inhibitors: implications for autoimmunity and inflammation, Crit. Rev. Oncol. Hematol. 128 (2018) 1–18.
[32] K.R. Kahkhaie, et al., Curcumin: a modulator of inflammatory signaling pathways in the immune system, Inflammopharmacology 27 (5) (2019) 885–900.
[33] S.M. Haftcheshmeh, et al., Berberine as a natural modulator of inflammatory signaling pathways in the immune system: focus on NF-κB, JAK/STAT, and MAPK signaling pathways, Phytother Res. 36 (3) (2022) 1216–1230.

[34] S.M. Haftcheshmeh, et al., Therapeutic potency of curcumin for allergic diseases: a focus on immunomodulatory actions, Biomed. Pharmacother. 154 (2022) 113646.
[35] F.Z. El Hassani, Characterization, activities, and ethnobotanical uses of Mentha species in Morocco, Heliyon 6 (11) (2020) e05480.
[36] M. Mahboubi, G. Haghi, Antimicrobial activity and chemical composition of Mentha pulegium L. essential oil, J. Ethnopharmacol. 119 (2) (2008) 325–327.
[37] A. Kamkar, et al., The antioxidative effect of Iranian Mentha pulegium extracts and essential oil in sunflower oil, Food Chem. Toxicol. 48 (7) (2010) 1796–1800.
[38] S. Amalich, et al., Chemical Composition of the Essential Oil and Isolation of Two Main Constituents of Mentha Pulegium *L.* Vegetos, 2023.
[39] F. Brahmi, et al., Evidence of biological activity of Mentha species extracts on apoptotic and autophagic targets on murine RAW264.7 and human U937 monocytic cells, Pharm. Biol. 55 (1) (2017) 286–293.
[40] O. Sharif, et al., Transcriptional profiling of the LPS induced NF-kappaB response in macrophages, BMC Immunol. 8 (2007) 1.
[41] A.A. Steiner, et al., Bacterial lipopolysaccharide fever is initiated via Toll-like receptor 4 on hematopoietic cells, Blood 107 (10) (2006) 4000–4002.
[42] F.A. Fitzpatrick, Cyclooxygenase enzymes: regulation and function, Curr Pharm Des 10 (6) (2004) 577–588.
[43] R. Nørregaard, T.H. Kwon, J. Frøkiær, Physiology and pathophysiology of cyclooxygenase-2 and prostaglandin E2 in the kidney, Kidney Res Clin Pract 34 (4) (2015) 194–200.
[44] M. Nakanishi, D.W. Rosenberg, Multifaceted roles of PGE2 in inflammation and cancer, Semin. Immunopathol. 35 (2) (2013) 123–137.
[45] J.Y. Park, M.H. Pillinger, S.B. Abramson, Prostaglandin E2 synthesis and secretion: the role of PGE2 synthases, Clin Immunol 119 (3) (2006) 229–240.
[46] L. Rochette, et al., Nitric oxide synthase inhibition and oxidative stress in cardiovascular diseases: possible therapeutic targets? Pharmacol. Ther. 140 (3) (2013) 239–257.
[47] F. Aktan, iNOS-mediated nitric oxide production and its regulation, Life Sci. 75 (6) (2004) 639–653.
[48] M.A. Cinelli, et al., Inducible nitric oxide synthase: regulation, structure, and inhibition, Med. Res. Rev. 40 (1) (2020) 158–189.
[49] U. Förstermann, W.C. Sessa, Nitric oxide synthases: regulation and function, Eur. Heart J. 33 (7) (2012) 829–837, 837a-837.
[50] F. Brahmi, et al., Evaluation of antioxidant, anti-inflammatory and cytoprotective properties of ethanolic Mint extracts from Algeria on 7-ketocholesterol-treated murine RAW 264.7 macrophages, Antioxidants 7 (12) (2018).
[51] J. Rocha, et al., Reduction of inflammation and colon injury by a Pennyroyal phenolic extract in experimental inflammatory bowel disease in mice, Biomed. Pharmacother. 118 (2019) 109351.