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Anti-Inflammatory Activity of *S. Marianum* and *N. Sativa* Extracts on Macrophages

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Abstract

Background: *Nigella sativa* (*N. sativa*) and *Silybum marianum* (*S. marianum*) are used to regulate macrophage polarization in lipopolysaccharide-induced RAW 264.7 cells and thioglycollate-elicited peritoneal inflammation.

Methods: Cytotoxicity assays and acute toxicity tests were performed to investigate the safe dose and toxicity of the prepared extracts. Also, nitric oxide production was determined by Griess assay on RAW264.7 and peritoneal macrophage supernatants. After RNA extraction from macrophages, real-time PCR was performed to measure the relative gene expression of tumor necrosis factor (TNF)-α, interleukin (IL)-6, transforming growth factor (TGF)-β, and IL-10. Finally, regulatory T cells (Treg cells) were counted by flow cytometry.

Results: *S. marianum* methanolic extract (SME), *N. sativa* ethanolic extract (NEE), and their mixture (SME+NEE) decreased NO levels significantly in RAW264.7 and peritoneal murine macrophages. *N. sativa* ethanolic extract significantly increased IL-10 gene expression and significantly decreased IL-6 and TNF-α expression in RAW264.7 cells. In mixture-treated peritoneal macrophages, IL-10 and TGF-β expression were significantly increased, while IL-6 and TNF-α were significantly decreased. Also, the percentage of Treg cells was significantly greater in the mixture-treated cells than in controls.

Conclusions: These results suggest that an SME and NEE mixture has anti-inflammatory and immunomodulatory activities and may be useful in the treatment of diseases of immunopathologic origin characterized by macrophage hyperactivation.

Keywords: Cytokine, Inflammation, *Nigella sativa*, Nitric oxide, *Silybum marianum*.

Introduction

Chronic inflammation has an important role in the creation, control, and exacerbation of a wide range of diseases including cancers and inflammatory and autoimmune diseases (1, 2). The main cells involved in inflammatory disease are macrophages, which participate in the inflammation process (2). Macrophages can be divided into two main groups referred to as classical (M1 or inflammatory) or alternative (M2 or anti-inflammatory) macrophages. Classical macrophages play important roles in many inflammatory diseases through the production of interferon-gamma (IFN-γ), nitric oxide (NO), interleukin- (IL) 1, IL-12, and tumor necrosis factor-α (TNF-α), while alternative macrophages act as anti-inflammatory cells by producing growth factor-beta (TGF-β) and IL-10 (2).

Although commercially available anti-inflammatory drugs, such as non-steroidal anti-inflammatory drugs (NSAIDs), are effective in reducing inflammation, they have a wide range of side effects without definite impacts on chronic inflammation (3). Therefore, safer, and more efficient alternative complementary therapies are needed. Researchers found that plant extract effects

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were due to more than one component or fraction because the plant constituents had synergistic and interactive effects with each other. Thus, whole plant extracts, in addition to suppressing the undesirable effects of the major toxic constituents, increased efficiency and performance (1). In addition, because of the synergistic effect between plants, researchers have highlighted the concept of polyherbalism (4).

According to studies, both *Silybum marianum* (*S. marianum*), or milk thistle, and *Nigella sativa* (*N. sativa*) have potent antioxidant and anti-inflammatory properties and have been used for years to treat of inflammatory disorders (5-7). Hence, this study aimed to investigate the attenuating effects of *S. marianum* methanolic extract (SME) and *N. sativa* ethanolic extract (NEE) on inflammation through macrophage polarization.

Materials and Methods

Chemicals and reagents

RPMI-1640 medium, fetal bovine serum (FBS), penicillin, streptomycin, dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS), MTT (3-(4, 5-dimethyl-2-yl)-2, 5 diphenyl tetrazolium bromide), brewer-modified thioglycollate, TPTZ (2, 4, 6-tripyridyl-s-triazine), and Griess reagent were provided by Sigma-Aldrich (Sigma, USA). Anti- CD4, CD25, and Foxp3 antibodies were supplied by eBioscience (eBioscience, USA). The total RNA Extraction Kit and the cDNA Synthesis Kit were purchased from Yekta Tajhiz (Yekta Tajhiz Azma, Iran). RAW 264.7 mouse macrophage cells were obtained from the Pasteur Institute (Tehran, Iran). N-hexane, methanol, and ethanol were purchased from Merck Company (Merck, Germany).

Preparation of the herbal extracts

S. marianum and *N. sativa* seeds were purchased from Tehran University of Medical Sciences. Dried *S. marianum* (30 g) was extracted in a Soxhlet system using n-hexane (75 mL; 6 h) and methanol (75 mL; 5 h) as the solvents. Powdered *N. sativa* seeds (60 g) were extracted with 95% ethanol (350 mL; 78 °C; 8 h). The extracts were filtered, the solvent was removed under vacuum in a rotary evaporator, and the residue was evaporated at 37 °C in an incubator (8, 9).

In vitro study on RAW 264.7 cells

Cell viability assay

RAW 264.7 cells were cultured at a density of 2×10^4 cells/well in a 96-well culture plate in complete RPMI-1640 supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin in 5% CO2 at 37 °C. Then, the cultured cells were treated with 50 - 800 µg/mL of SME, NEE, or the SME+NEE mixture, all in triplicate. After 4 hr of incubation, the cells were stimulated with LPS (1 µg/mL) for 24 hr. Finally, 10 µL of 5 mg/mL MTT was added to each well and the cells were incubated for an additional 4 hr. Subsequently, 100 µL/well of DMSO was added to each well and mixed for 30 min in the dark. Then, the optical density (OD) was measured at 570 nm on a microplate reader (Stat Fax 2100, USA) (10).

Nitric oxide determination

Nitric oxide was measured by the Griess reaction (11, 12). Briefly, RAW 246.7 cells were seeded in a 96-well culture plate at 2×10^4 cells/well, incubated overnight, and then treated with 50 - 800 µg/mL of the SME, NEE and SME+NEE extracts. Dexamethasone was used at a concentration of 1 µM/mL as a positive control (13). After 4 hr of incubation, the treated cells were stimulated with LPS (1 µg/mL) and again incubated for 24 hours. Finally, the cell supernatants were collected, and their NO levels were measured by the Griess method.

Cytokine gene expression assay

Briefly, RAW 264.7 cells were seeded in a 24-well culture plate at 1×10^5 cells/well. After overnight incubation, the macrophages were treated with 200 µg/mL of SME, 800 µg/mL of

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NEE and 400 µg/mL of SME+NEE extracts. After 4 hr, the treated cells were stimulated with LPS (1µg/mL) and incubated for 24 hr. The cells were harvested, total RNA was extracted, and cDNA was synthesized according to the manufacturer's instructions. Forward and reverse primers of the target genes (IL-10, IL-6, TGF-β, and TNF-α) and a housekeeping gene (GAPDH) were designed using Allele ID 6 software (Premier Bio soft, USA) and aligned in BLAST web sites (Table 1). Real-time PCRs were run in duplicate using SYBR Green I 2x Master Mix (Yekta Tajhiz, Iran) on a Roche light cycler® 96 (Roche, Switzerland). PCR products for each mRNA were verified by the melting curve assay. Also, 2% agarose gel electrophoresis was used to assess potential primer-dimer formation and non-specific product amplification for the data analysis. The Pfaffle method was used to calculate the relative gene expression. The ratios were considered as the final results for statistical analysis.

Table 1. PCR Primer Sequences.

Target	Amplicon (bp)	Primers	Sequences, 5'→ 3'
GAPDH	224	F	CGGTGTGAACGGATTTGG
		R	CTCGCTCCTGGAAGATGG
IL-6	157	F	GAAATGATGGATGCTACCAAACTG
		R	TCTGTATCTCTCTGAAGGACTCTG
TGF-β	193	F	AATTCCTGGCGTTACCTTGG
		R	GGCTGATCCCGTTGATTTCC
TNF-α	201	F	CCTCTTCTCATTCCTGCTTGTG
		R	ACTTGGTGGTTTGCTACGAC
IL-10	224	F	CACTGCTATGCTGCCTGCTC
		R	ACCCAAGTAACCCTTAAAGTCCTG

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; TGF-β: transforming growth factor-β; TNF-α: Tumor necrosis factor-alpha; IL: interleukin; F: Forward; R: Reverse.

In vivo study on peritoneal macrophages

Mice treatment

Experiments were performed using male BALB/c mice (18–22 g) obtained from the Pasteur Institute of Iran. The SME and NEE extracts were gavaged at 2000 mg/kg daily for 14 days according to the previous studies (14, 15). Another group was gavaged with the SME+NEE combination at a 50/50 ratio. The control group received 30% DMSO as a vehicle (16). The positive control group received dexamethasone intraperitoneally at 0.15 mg/kg (17).

Acute toxicity of the extracts

The acute toxicity test was performed based on the Organization for Economic Co-operation and Development guidelines. According to these guidelines the mice weights were measured every three days for 14 days (18). Moreover, the extracts' toxicities on splenocytes were evaluated by MTT assay.

Thioglycollate-induced peritonitis model

On the 10^{th} day of treatment, four days before the last gavage, peritoneal inflammation was induced by intraperitoneal injection of 1 mL of

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a sterile solution of Brewer-modified thioglycollate medium (3% w/v in PBS) (19). Peritoneal macrophages were collected by lavaging the peritoneal cavity with 10 mL of harvest medium (EDTA 5mM + PBS) (20). Isolated peritoneal exudate cells were washed twice with RPMI and centrifuged at 400 x G for 10 min at 4 °C. Finally, the cell suspension was dispensed in complete RPMI-1640 and allowed to adhere to the bottom of the 6 cm culture plate at 37 °C for 4 hr in 5% CO_2. The plates were then washed with warm PBS to remove non-adherent cells. The attached cells were considered as macrophages with 90% purity (20).

NO determination and cytokine gene expression

The scraped peritoneal macrophages at 2×10^4 cells/well were incubated overnight in a 24-well culture plate with 1µg/mL of LPS for 24 h for the NO assay and cytokine gene expression. The NO assay, total RNA extraction, cDNA synthesis, and real-time PCR were performed as described in sections 2.3.2 and 2.3.3.

Flow cytometry analysis of splenic Treg cells

The euthanized mice spleens were excised under sterile conditions, and single-cell suspensions isolated by perfusion with FACS buffer. After RBC removal with lysis solution (0.5 M NH_4Cl, 10mM $KHCO_3$, and 0.1mM disodium EDTA, pH 7.2), flow cytometry analysis was performed using a BD FACSCalibur (BD Biosciences, CA, USA). For this purpose, peridinin chlorophyll protein complex (PerCP)-labeled anti-mouse CD25 and PE-labeled anti-mouse CD4, and FITC-labeled anti-mouse forkhead box P3 (FoxP3) were used. All the experiments were performed according to the manufacturer's instructions. Acquisition and analysis were erformed with Flowjo software (Tree Star, Inc., OR, USA) by counting 10000 cells.

Statistical Analysis

Statistical tests were performed using the SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA). The ANOVA method was used to evaluate differences between variables in each of the studied groups. The means between groups were compared by the post hoc Tukey test. P values less than 0.05 were considered significant. Data were expressed as means±standard errors of the means. All graphs were made using GraphPad Prism software version 6.07.

Results

Cytotoxicity of the extracts on RAW264.7 macrophages

Viabilities of cells treated with the NEE, SME, or SME+NEE extracts at all concentrations, either without or with LPS at 1 µg/mL were not significantly different from the controls (Figs. 1A and 1B, respectively).

Nitric oxide production in RAW264.7 macrophages

Nitric oxide concentrations were significantly greater in the LPS- (1µg/mL) stimulated than in the non-LPS-stimulated cells (p= 0.001) (Figs. 2A-C). Treatment of LPS-stimulated macrophages with 200-800 μg/mL SME resulted in a significantly less NO than in the control (p= 0.01) (Fig. 2A). Also, LPS-induced NO production was significantly less than control in NEE group at concentrations of 400, 600, and 800 μg/mL (p= 0.038, 0.040, and 0.004, respectively) (Fig. 2B). Nitric oxide production was also significantly less than control following incubation with the SME+NEE extract at 400, 600, and 800 μg/mL (p= 0.038, 0.018, and 0.015, respectively) (Fig. 2C).

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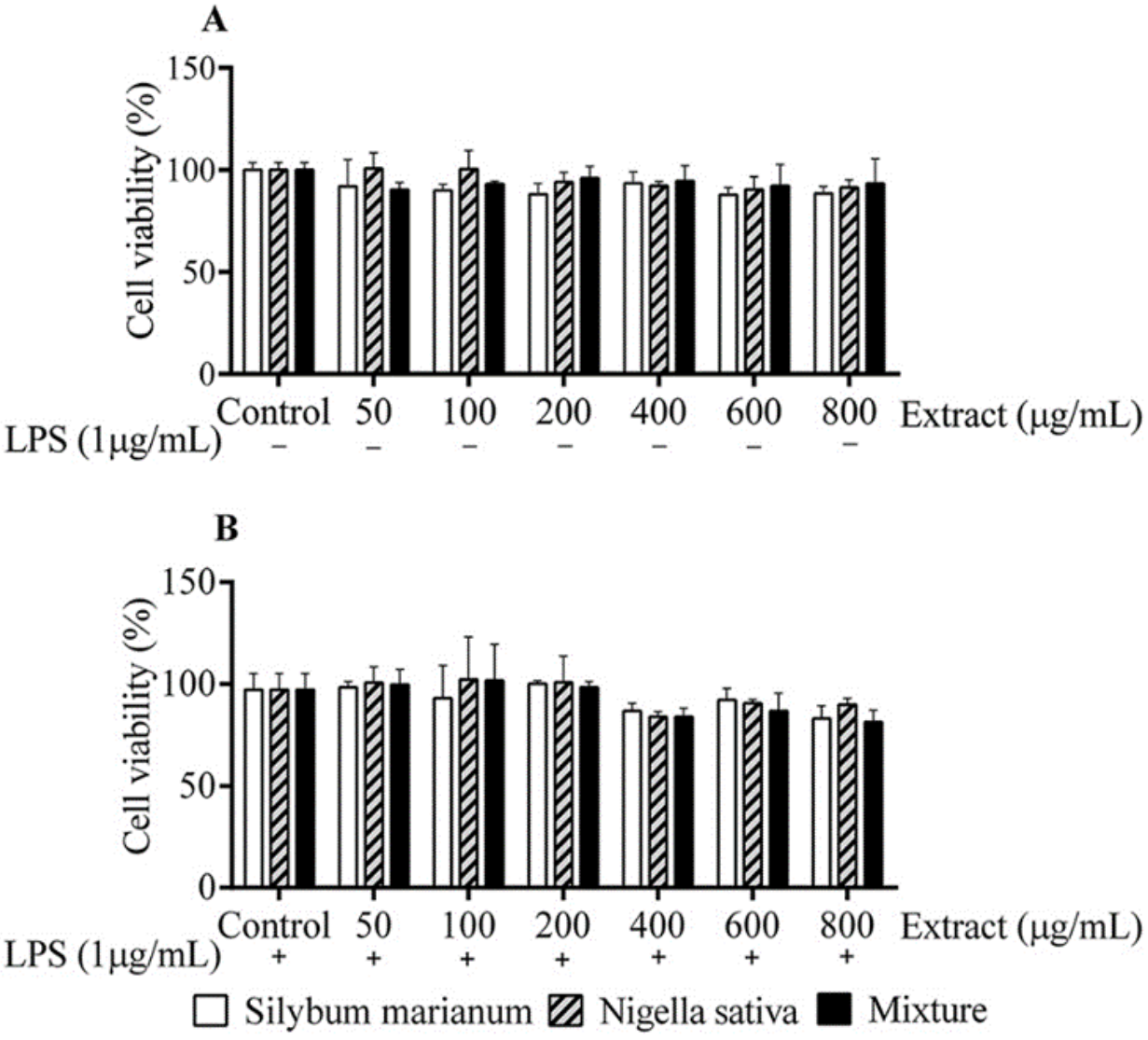

Fig. 1. Effects of NEE (*N. sativa*), SME (*S. marianum),* and SME+NEE (Mixture) on cell viability. Cell viability was evaluated by MTT assay 24 hours after treatment (A) without LPS, (B) with LPS (1 µg/mL) in RAW264.7 macrophage cells. Values are the means ± SEMs of three independent experiments. LPS: lipopolysaccharide.

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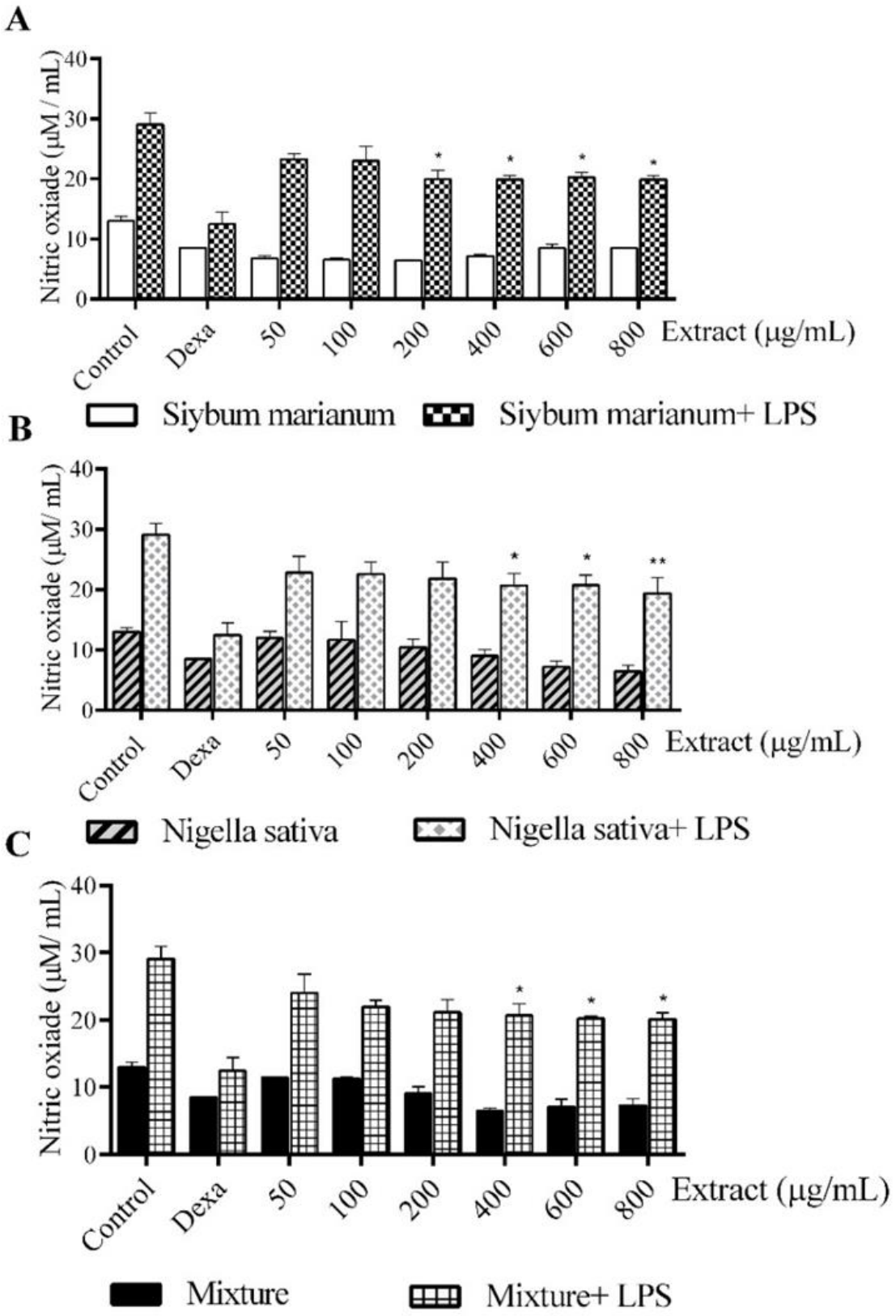

Fig. 2. Effects of **(A)** SME (*S. marianum*), **(B)** NEE (*N. sativa*), and **(C)** SME+NEE (Mixture) at various concentrations on NO production in RAW 264.7 macrophages with and without LPS. The data are representative of three experiments and expressed as means±SEMs. *p< 0.05 **p< 0.01 versus control.

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Cytokine gene expression in RAW264.7 macrophages

IL-10 gene expression was significantly greater in RAW 264.7 cells treated with 800 µg/mL of NEE than in controls (p= 0.0001, Fig. 3A). Also, NEE-treated cells expressed significantly less TGF-β, TNF-α, and IL-6 than control cells (p= 0.012, 0.001, and 0.0015, Figs. 3B, 3C, and 3D, respectively). IL-10, TGF-β, TNF-α, and IL-6 gene expression in RAW 264.7 cells was not significantly different between those treated with 200 µg/mL of SME and 400 µg/mL of SME+NEE extracts and controls (Figs 3A-3D).

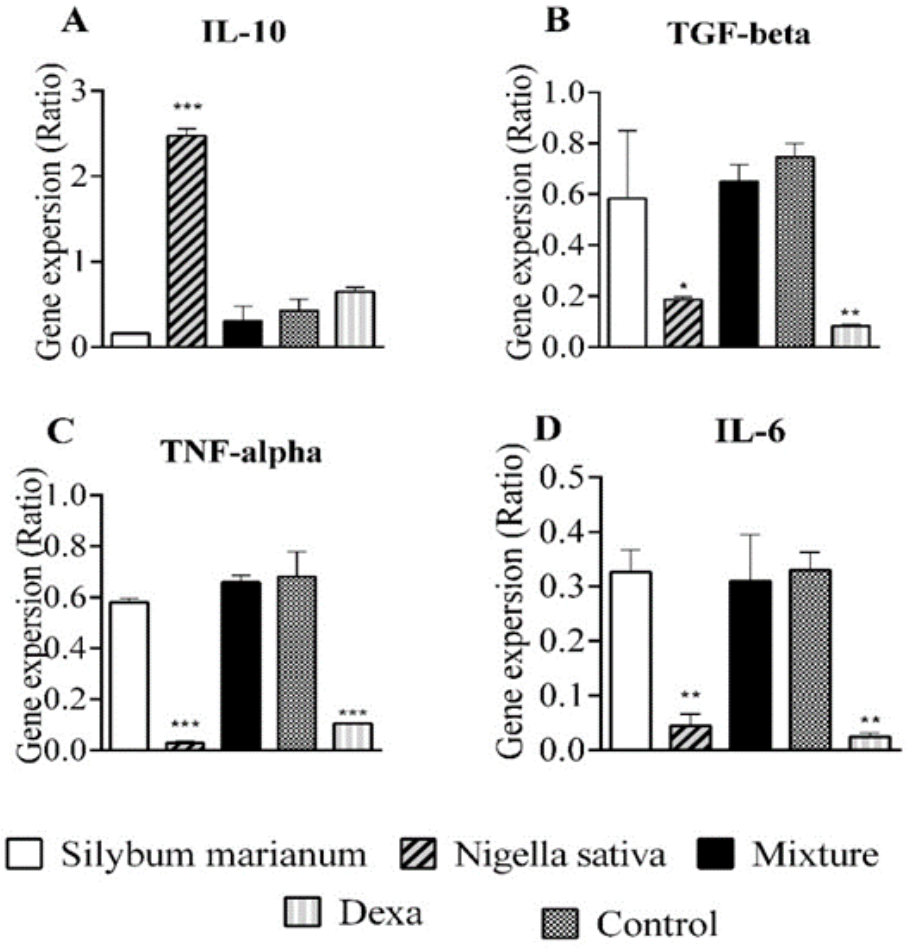

Fig. 3. The effects of SME (*S. marianum*) (200 µg/mL), NEE (*N. sativa*) (800 µg/mL), and SME+NEE (Mixture) (400 µg/mL), on **(A)** IL-10, **(B)** TGF-β, **(C)** TNF-α, and **(D)** IL-6 expression in RAW 264.7 cells. The controls cells were treated with DMSO+LPS. Data represent means±SEMs of duplicate independent experiments. *p< 0.05, **p< 0.01, ***p< 0.001 versus DMSO control.

Acute toxicity of the extracts on the animal model

Mice weights did not differ between those treated with the extracts and controls, indicating the non-toxic nature of extracts at the tested dosage (Fig. 4A). In confirmation of the non-toxicity of these extracts, spleen lymphocytes viability from treated mice was not significantly different from control (Fig. 4B).

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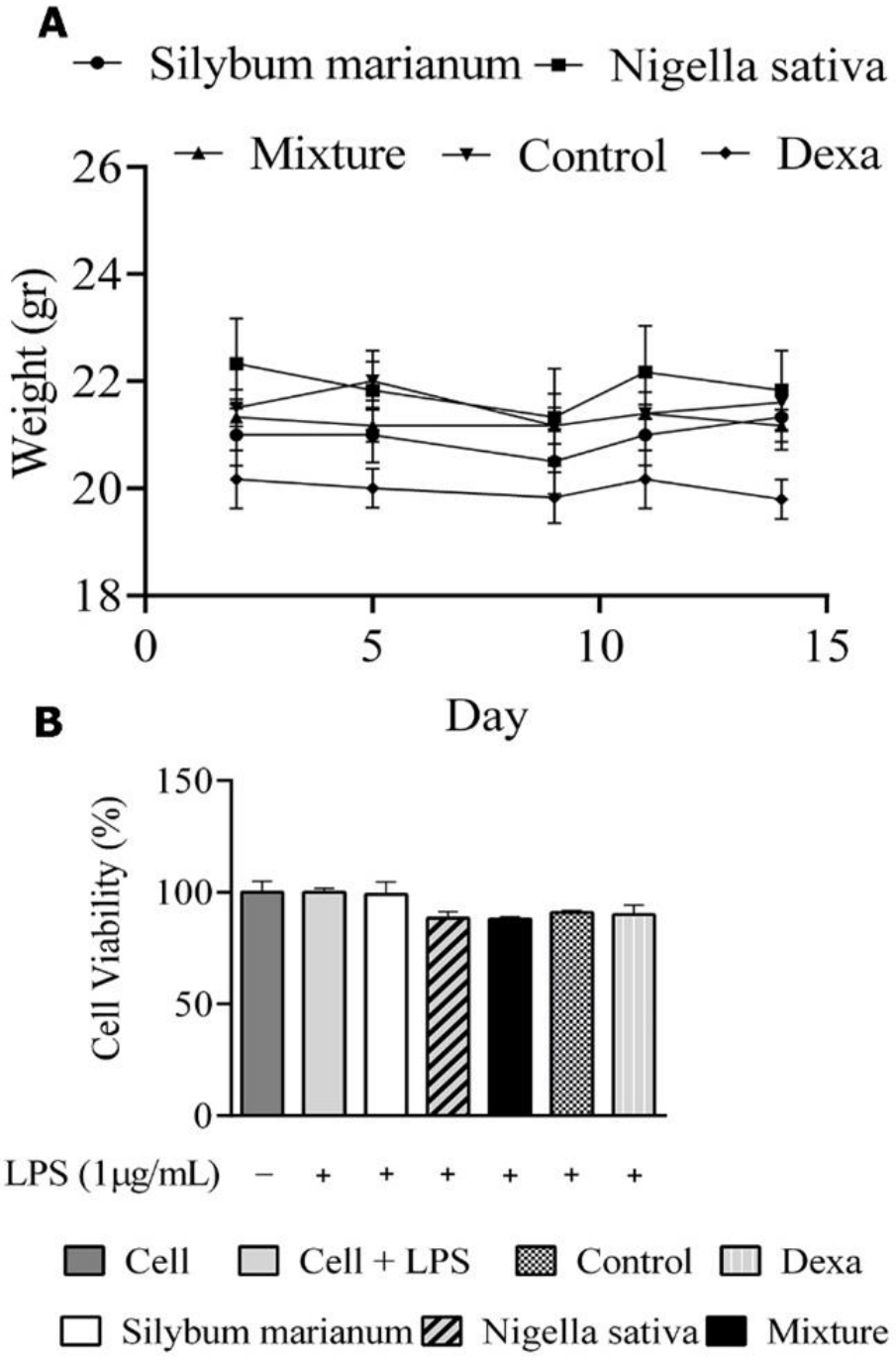

Fig. 4. Effects of SEM (*S. marianum*), NEE (*N. sativa*) and SME+NEE (Mixture) on mice body weights and splenocyte viability. **(A)** Weights of treated groups **(B)** Spleen lymphocyte viability. Viability was determined by MTT assay. Values are the means±SEMs of three independent experiments. Cell: group that received no treatment, control: group were treated with 30% DMSO for 14 days as solvent for extract.

NO production in LPS-stimulated peritoneal macrophages

Nitric oxide concentrations in the supernatants of peritoneal macrophages treated with SME, NEE, SME+NEE, and dexamethasone (0.15 mg/kg) were all significantly less than in the control (p= 0.001, Fig. 5).

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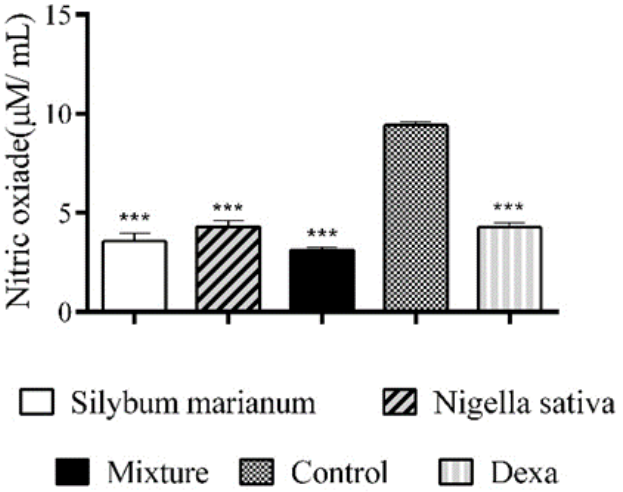

Fig. 5. Effect of SEM (*S. marianum*), NEE (*N. sativa*) and SME+NEE (Mixture) on NO production in the supernatants of cultured peritoneal macrophages from mice with thioglycollate-induced peritonitis. The data are expressed as means±SEMs. ***p< 0.001 versus DMSO control.

Gene expression in LPS-stimulated peritoneal macrophages

IL-10 and TGF-β expression were significantly greater in the SME+NEE-treated cells than in the control cells (p= 0.0045 and 0.037, Figs. 6A and 6B, respectively), while TNF-α and IL-6 expression were significantly less than control (p= 0.034 and 0.010, Figs. 6C and 6D, respectively). IL-6 expression in NEE extract and LPS-stimulated peritoneal macrophages was significantly less than control (p= 0.026, Fig. 6D).

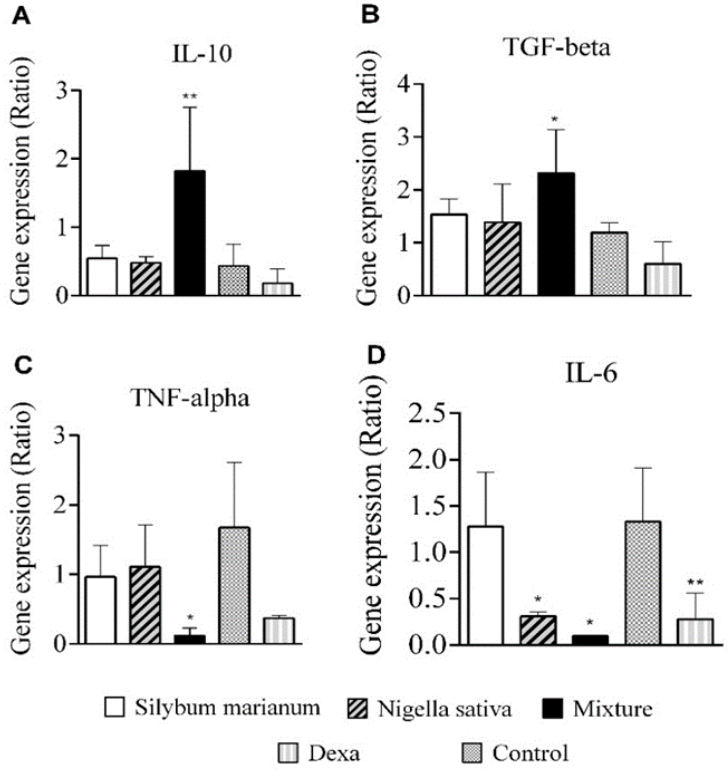

Fig. 6. The effects of SEM (*S. marianum*), NEE (*N. sativa*) and SME+NEE (Mixture) on (**A)** IL-10, **(B)** TGF-β, **(C)** TNF-α, and **(D)** IL-6 expression in LPS-stimulated macrophages. Data represent means±SEMs of duplicate independent experiments. *p< 0.05, **p< 0.01 versus DMSO control.

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Effect of herbal extracts on splenic Treg cells

Regulatory T cells were analyzed by flow cytometry, in which firstly lymphocytes were isolated through forward and side scatter characteristics (Fig. 7A). Then separated CD4+ T lymphocytes were separated based on CD4 marker (Fig. 7B), and finally, Treg cells were reported based on the proportion of FoxP3+, CD25+ markers in the population of CD4+ T cells (Fig. 7C). The percentage of Treg cells was more significant in the SME+NEE treatment group than in those treated with the other extracts or controls (p= 0.015, Fig. 7D).

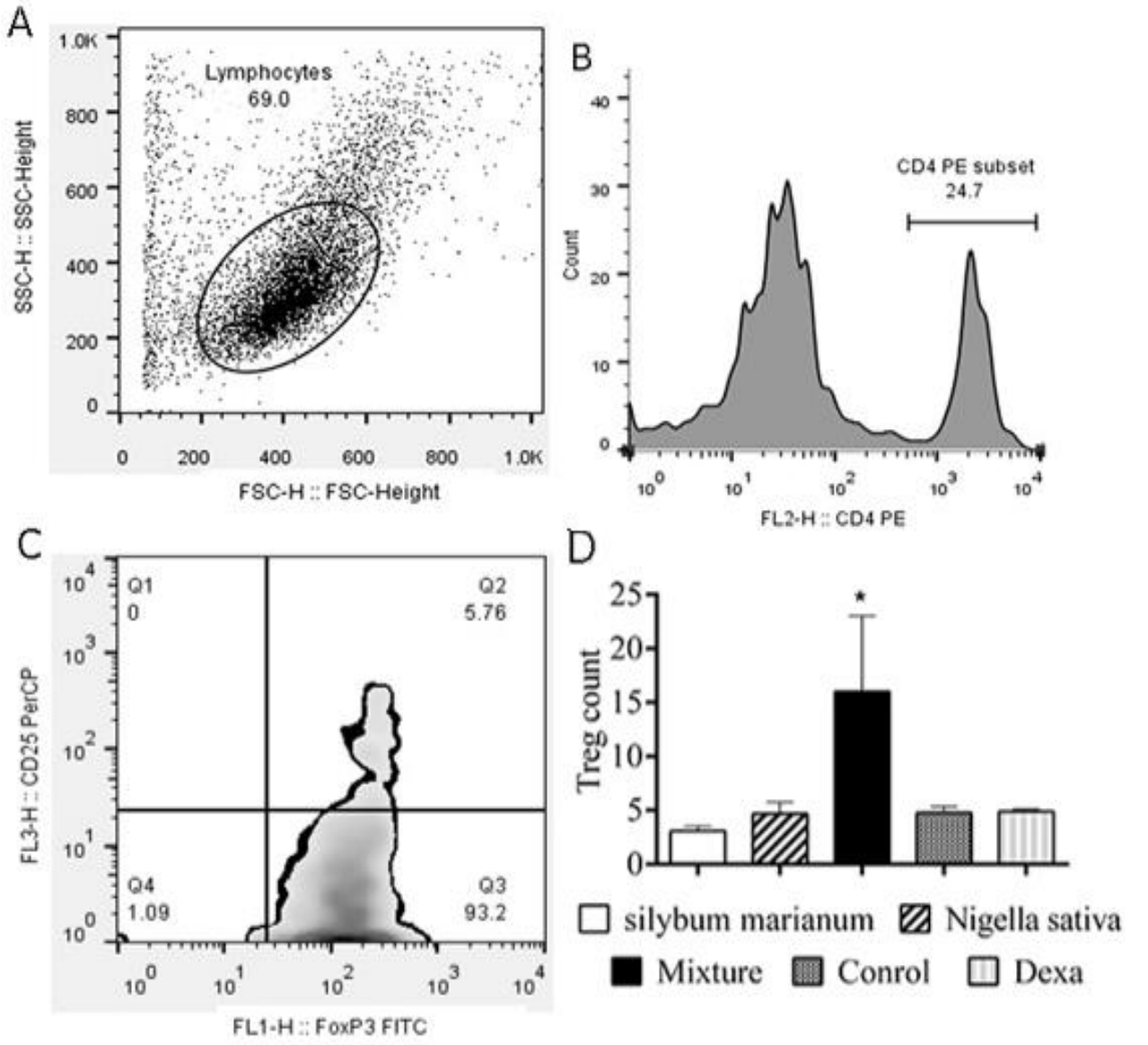

Fig. 7. Results of flow cytometry measurement and gating strategy. **(A)** Identification of cellular subsets based on forward and side scatter characteristics. **(B)** The histogram shows the count of separated lymphocytes based on the CD4 marker. **(C)** The percentage of FoxP3+, CD25+ T cells in CD4+ T cells population which was stained with PerCP and FITC fluorochromes **(D)** Effect of SEM (*S. marianum*), NEE (*N. sativa*), and SME+NEE (Mixture) on splenic T-reg cell number (*p< 0.05).

Discussion

According to our result a mixture of SME+NEE has anti-inflammatory and immunomodulatory activities without cytotoxic effects on inflammatory macrophages. Our findings agree with a study that showed classical macrophages play key roles in inflammation by producing

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inflammatory mediators such as nitrite oxide and inflammatory cytokines (21). Previous evidence showed the anti-inflammatory and immunomodulatory properties of NEE and SME in rheumatoid arthritis, preeclampsia, asthma, and respiratory diseases (5, 22). In this study, we investigated the synergistic effects of NEE and SME extracts and their mixture on immunomodulation of inflammatory macrophages *in vitro* and *in vivo*.

The extracts had no toxic effects on RAW264.7 cells or splenic lymphocytes either in the presence or absence of LPS. Acute toxicity results also demonstrated their safety at the doses administered to mice. Therefore, the inhibitory effects of these extracts on inflammatory macrophages were not due to toxic or cell-killing effects (23, 24). LPS is an important stimulus that leads to classical macrophage activation. The appropriate dose of LPS without apoptosis induction was chosen based on previous studies (1 μg/mL) (25, 26). Cho et al. showed no cytotoxic effect of deoxysilybin from SME even at the relatively high concentration of 750 μg/mL on RAW264.7 cells (27). In parallel studies the hydroalcoholic extract of NEE and thymoquinone had no cytotoxic effects at concentrations greater than that used in our study (28, 29). Extensive in vivo studies have shown a lack of acute and chronic toxicity of SME and NEE extracts on laboratory animals even at high doses (30, 31).

Our results also indicated that the extracts significantly inhibited LPS-induced NO production in a concentration-dependent manner on RAW264.7 cells and peritoneal macrophages. Moreover, the inhibitory function of the mixture was stronger than the dexamethasone, which confirmed their polyherbalism and synergistic effects. In line with our study, thymoquinone showed an anti-inflammatory effect by inhibiting NF-κB signaling and iNOS mRNA expression in RAW264.7 macrophages (32). In contrast, thymoquinone increased NO levels and induced inflammatory macrophages (33). This discrepancy might be due to the responsiveness of the different cell lines.

According to studies, silibinin from SME has been reported to inhibit NO production by blocking iNOS expression and blocking the P38, MAPK, and NF-κB signaling pathways, thus inhibiting the activity of stimulated mouse peritoneal macrophages and RAW 264.7 cells (34-36). *In vivo* studies confirmed the results of this study; aqueous NEE extract on peritoneal macrophages stimulated with LPS and IFN-γ suppressed NO production (24). In parallel, carvacrol, from NEE extract, significantly decreased serum NO in patients with respiratory inflammation (37). Therefore, the anti-inflammatory effect of NEE in the present study was likely due to the combined activity of multiple components in the extract mixture.

In this study, SME extract had no significant effect on inflammatory gene expression in RAW264.7 and peritoneal macrophages; however, in agreement with previous studies, NEE extract inhibited proinflammatory cytokine production. In this regard, the aqueous NEE extract and thymoquinone suppressed TNF-α and IL-6 in peritoneal macrophages stimulated by LPS and IFN-γ (24) and TNF-α in RAW264.7 mouse macrophages stimulated by LPS (24, 32). Others have observed an ethanolic extract of NEE decreased IL-6 expression in macrophages derived from human monocytes (9). El-Dakhakhny et al. reported that NEE oil has a greater inhibitory effect than thymoquinone on the inflammatory cascade. Hence, it is likely that oil components may also be involved in the extracts in our study (38, 39).

In the present study, SME had no significant effect on IL-10 and TGF-β expression in RAW264.7 and peritoneal macrophages, whereas a significant increase in IL-10 was observed in NEE-treated RAW264.7 macrophages. In this regard, evidence has shown that thymoquinone in NEE increases IL-10 production in RAW264.7 and peritoneal macrophages through attenuation of the NF-κB signaling pathway (40, 41). The current study demonstrated that the combination of the two extracts significantly increased IL-10 and TGF-β, and decreased TNF-α and IL-6 expression. This finding indicates the combined synergistic effect of these two plants *in vivo*.

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One valuable property of both NEE and SME is their regulatory effect on the immune system. We observed a significant increase in the number of splenic regulatory lymphocytes in the combination group. In contrast, silymarin and NEE extract increased Treg cell numbers in rats exposed to gamma-rays and ovalbumin, respectively. This discrepancy might be due to the lower concentration of the extracts that we used (42).

In conclusion, the LPS-activated mouse macrophage cell model is an economic approach for basic screening of the extracts' anti-inflammatory activity. Our results demonstrated a combination of SME+NEE may prevent inflammation. Future studies will be directed towards clarifying the signaling pathways inhibited by the combined extracts.

These results suggest that the SME+NEE mixture has anti-inflammatory and immunomodulatory activity, and thus, may be of value in the treatment of diseases of immunopathological origin characterized by macrophage hyperactivation.

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The authors hereby state that there are no conflicts of interest in the present research.

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