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Anti-inflammatory Effects of *Matricaria chamomilla* Extracts on BALB/c Mice Macrophages and Lymphocytes

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ABSTRACT

Matricaria chamomilla (MC) was shown to have anti-inflammatory effects. Flavonoids are major groups of MC immunomodulators. The anti-inflammatory effects of apigenin as an MC flavonoid has already been demonstrated.

In this study, we aimed to report the amount of this compound by liquid chromatography-mass spectrometry (LC-MS) and measuring the total phenol content (TPC) in both the MC aqueous and alcoholic extracts. We also investigated the MC aqueous and ethanolic extracts effect on BALB/c separated macrophages and lymphocytes cell viability and macrophage nitric oxide production. Interferon-γ and interleukin-10 secretion were also measured in lymphocytes.

We found that the amount of apigenin was 0.078 and 0.25 mg/g per each of dry aqueous and alcoholic extracts, respectively. Also, the total phenol content was 2.99% in aqueous and 3.95% in alcoholic extracts. BALB/c separated macrophages cell viability significantly increased when treated with the MC aqueous extract but decreased when treated by the MC alcoholic extract in the presence of lipopolysaccharide. Also, the amount of nitric oxide production by macrophages and BALB/c separated lymphocytes cell viability in treatment with aqueous and alcoholic extracts significantly decreased. Interferon-γ increased, and interleukin-10 decreased in lymphocytes treated with the MC aqueous extract, which may suggest Th1 polarization. There was no significant change in the interferon-γ level in lymphocytes when treated with the MC alcoholic extract, but the level of IL-10 increased in these cells.

Altogether, besides the anti-inflammatory effect of MC extracts, we found MC aqueous extract effects as disrupting Th1/Th2 balance to Th1 upregulation. Overall, the anti-inflammatory effect of the MC alcoholic extract was higher than the MC aqueous extract.

Keywords: Apigenin; Lymphocytes; *Matricaria chamomilla*; Macrophages, Th1-Th2 balance

INTRODUCTION

Immunomodulators are the natural or synthetic substances that regulate the type, duration, and intensity of innate or adaptive immune responses.[1] Because of the side effects, high cost, and drug resistance of chemical immunomodulators, researchers have now focused on plants as a specific, safe, and inexpensive treatment.[2]

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Matricaria chamomilla (MC) is one of the most useful immunomodulatory plants with anti-inflammatory effects. MC also has antioxidant, antibacterial, antispasmodic,[3] wound healing, and anti-allergy effects.[4,5] It has long been used to treat bacterial infections, digestive, and respiratory diseases.[6]

There are two main categories of MC immunomodulators: essential oils and polyphenolic compounds. Sesquiterpenes, terpenoids, and azulenes are the main essential oils of MC. There are five main groups of phenolic compounds in chamomile extract: coumarins, phenylpropanoids, flavones, flavonols, and flavanone.[7,8]

Apigenin and apigenin 7-O-glucoside are the flavone compounds in MC. Its amount varies in different parts of MC but apigenin is the main compound in chamomile flowers.[3] Apigenin has many anti-inflammatory effects including inhibition of inducible nitric oxide synthase (iNOS),[9] cyclooxygenase-2 (COX-2) expression,[10] histamine-releasing in mast cells,[11] activator protein 1 (AP-1) and nuclear factor of activated T cells (NFAT) activation, suppression of T-helper-17s (Th-17), Interleukin-6 (IL-6), IL-8, Tumor necrosis factor-α (TNF- α) production, CD40 ligand expression and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) activation,[9,12,13] and decline in IL-4, IL-13, IgE, and IgG1 production.[14]

In this study, we aimed to isolate this agent with liquid chromatography-mass spectrometry (LC-MS) technique and measure the total phenol content (TPC) as the main MC anti-inflammatory agents in both MC aqueous and alcoholic extracts. Since there is not enough in vitro studies showing MC extracts effects on cytokines and viability of BALB/c immune cells, we investigated the immunomodulatory activity of MC aqueous and ethanolic extracts on mouse macrophages and lymphocytes cell viability and macrophages nitric oxide (NO) production. Then, the amount of IL-10 as an anti-inflammatory cytokine and interferon-gamma (IFN-γ) as the connection between innate and adaptive immunity production by lymphocytes was measured by enzyme-linked immunosorbent assay (ELISA). The comparison of MC aqueous and ethanolic extracts was novel in this study. All experiments were repeated three times under the same conditions to test the reproducibility of the results.

MATERIALS AND METHODS

Preparation of Plant Extract

The dry chamomile flower of Iranian origin was purchased from Pakan Bazr Isfahan company (Iran). Then, the powder (10 g) was macerated in 100 mL of distilled water or 100 mL of 70% ethanol. Extraction was done using the maceration method.[15] The dried plant material was kept at a temperature of 4°C until use. The dried aqueous and ethanolic extracts were weighed and suspended in RPMI 1640 culture medium (Sigma-Aldrich, Germany), to obtain a stoke solution, sterilized by a 0.22 μm filter and then attenuated to reach desired concentration according to relevant literature.[16]

Total Phenolic Content Measurement

The total phenolic content (TPC) of MC aqueous and ethanolic extract was determined by the Folin-Ciocalteu method. The calibration curve was drawn by mixing 1.6 mL of 40, 80, 120, 200, 400, and 800 mg/L of gallic acid solutions with 100 μL Folin-Ciocalteu reagent. After 3 minutes, 300 μL of 7% (w/v) sodium carbonate solution was added. The mixture was kept for a further 2 h in the dark at 20°C, and the absorbance rate was measured at 765 nm.[17]

A 20 mg/ml solution of each MC aqueous and ethanolic extract was prepared. The Folin-Ciocalteu method was performed for each solution as mentioned above. The TPC as mg of gallic acid equivalent per g dry weight of extract was obtained using the gallic acid standard curve equation.

Measurement of the Apigenin Amount in Extracts

Quantification of apigenin was performed by using the LC-MS method on a Waters Alliance 2695 HPLC-Micromass Quattro micro API mass spectrometer. We used an eclipse XDB-C18 5 μ, 4.6×150 mm column whose temperature was 35°C throughout the run. The mobile phase was comprised of H_2O plus 0.1 formic acid and acetonitrile plus 0.1% formic acid with a flow rate of 0.5 mL/min. Acetonitrile plus 0.1% formic acid was held at 30% for 10 min when it increased to 90%. This gradient was maintained for more than 10 min. The injection volume was 25 μL.

Next, the selected ion recording (SIR) was performed. In this method, a specific mass to charge ratio range is detected by the instrument instead of a full sample scan. For optimizing the instrument

parameters at m/z 269 as apigenin negative mode, a solution contained the commercial standard of apigenin dissolved in DMSO: H_2O (1:10) was injected into the mass spectrometer. The scan was performed for m/z 100-400. The instrument was optimized at: cone voltage 35 V, capillary voltage 4 kV, flow gas 250 L/h, source temperature 120°C, and desolvation temperature 300°C. The amount of apigenin was obtained using single-point calibration. A serial dilution of the apigenin standard sample was provided, and the same amount of these solutions was injected. The MC aqueous and ethanolic extracts were used as a reference to compare with the LC-MS analysis of the apigenin standard sample. After measuring every peak area, the best peak of the standard was chosen in terms of area amount, then the exact amount of apigenin was calculated with a simple proportional formula.

$$\frac{Ru}{Cu} = \frac{Rs}{Cs}$$

Harvesting and Culture of Mouse Peritoneal Macrophages

BALB/c male mice (weight: 22-25 g) were purchased from Pasteur Institute (Karaj, Iran). This research was done with the Ethics Committee approval code: IR.SHAHED.REC.1397.024.

Mice were anesthetized, and their peritoneal macrophages were extracted.[18] The collected cells were seeded in 96-well plates at a density of 400000 cells per well. After incubating for 2 hours and washing nonadherent cells by phosphate-buffered saline, the macrophages were incubated with various concentrations (25, 50, 100, 200, 400, 800 and 1600 µg/mL) of MC aqueous and ethanolic extracts in the presence of Lipopolysaccharide (LPS) (10 µg/mL) for 16 hours according to literature.[16] Positive control wells only received LPS. To determine the toxicity of the extracts, macrophages were cultured with each concentration of two extracts without LPS.

Preparation of Murine Lymphocytes

Then, the mice were anesthetized, their spleens were removed, and their lymphocytes were isolated.[19] The cells were seeded in 96-well plates at a density of 200000 cells per well. Then, the lymphocytes were incubated with various concentrations (25, 50, 100, 200, 400, 800 and 1600 µg/mL) of MC aqueous and ethanolic extracts in the presence of concanavalin A (12.5 µg/mL) as mentioned in literature for 72 hours. The positive control wells only received concanavalin A (Con A). For determination of the toxicity of extracts, the lymphocytes were cultured with each concentration of two extracts without Con A.

Cell Viability Assay

Cell viability was measured as cell respiration. In this assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is reduced to formazan in a mitochondrial-dependent path. The supernatant of cell culture was collected after 16 hours for macrophages and after 72 hours for lymphocytes. Then cells viability was measured by MTT assay.[16]

Nitric Oxide Measurement

Macrophage culture supernatant was collected after 16 hours. The amount of NO (nitric oxide) production by macrophages was measured by the Griess test.[20] Sodium nitrite (0, ‹5, 10, 20, 30, 40, 50, 60 nmol/mL) was used to obtain standard curve.

Enzyme-Linked Immunosorbent Assay for Interleukin-12, Interleukin-10, and Interferon-γ

We measured IL-10, and IFN-γ secretion in the supernatant from the treated lymphocytes by ELISA according to the manufacturer's instructions.

Statistical Analysis

The obtained data were expressed as mean±SD and analyzed by 1-way analysis of variance (ANOVA) with Tukey's post hoc test in SPSS version 24. P values of less than 0.05 were set as significant.

RESULTS

Total Phenolic Content

The TPC of each extract was measured by the Folin-Ciocalteu method. The absorption of a 20 mg/mL solution was 0.405 for aqueous and 0.56 for ethanolic extract. This number is inserted in the equation derived from gallic acid standard curve (Figure 1). The exact amount of TPC was 29.9 in aqueous and 39.5 mg gallic acid per g dry weight of the extract.

Measurement of the Apigenin Amount in Extracts

To measure the apigenin amount in MC aqueous and ethanolic extract, we did LC-MS. Figure 2a

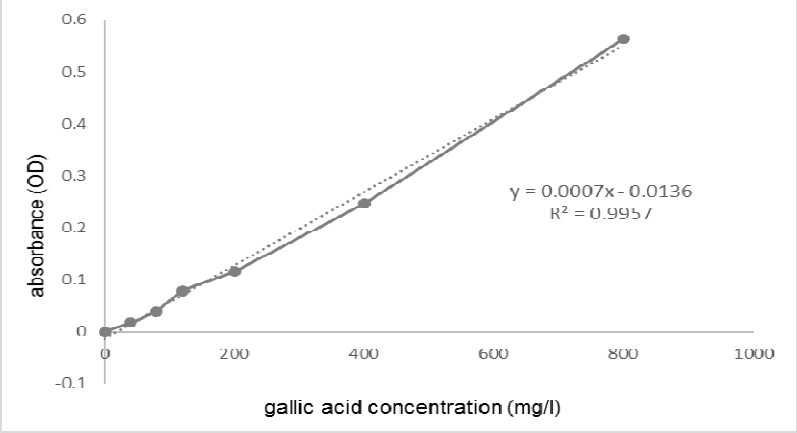

Figure 1. Gallic acid standard curve at 765 nm for the determination of total phenol content (TPC).

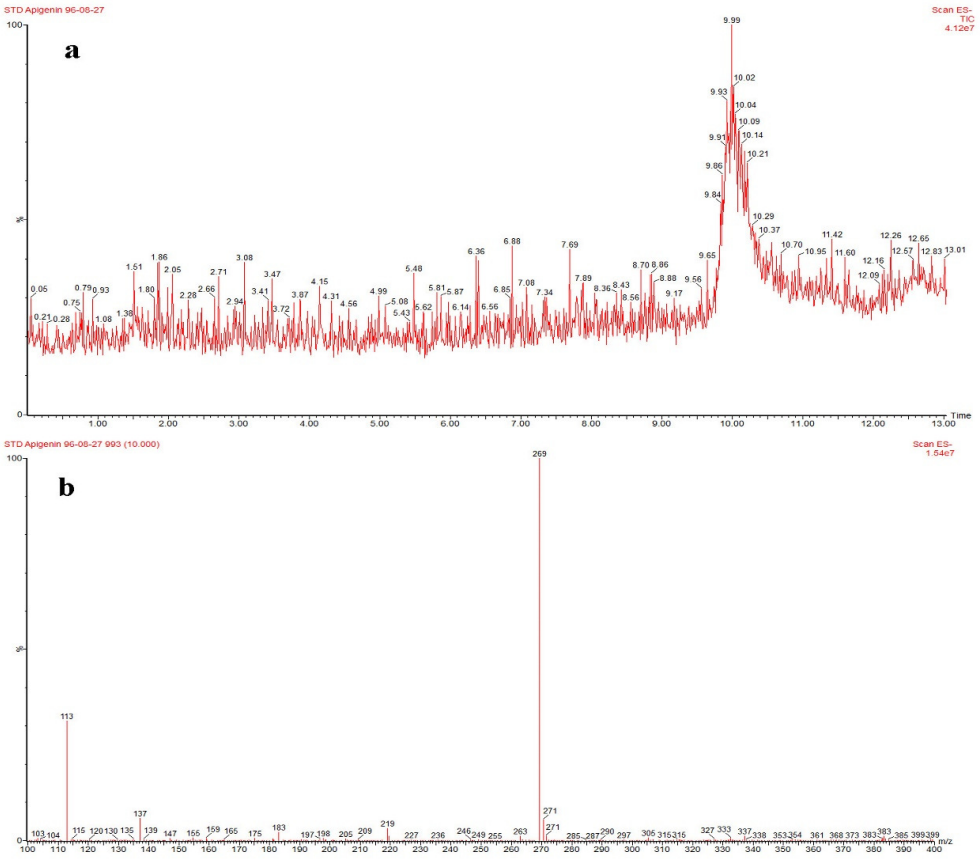

Figure 2. (a) Total ion chromatogram (TIC) of the standard sample of apigenin (ES, negative ion mode). The peak at 10.00 represents apigenin. (b) Mass spectrometry (MS) spectrum of the standard of apigenin and m/z 269.2 as apigenin negative mode.

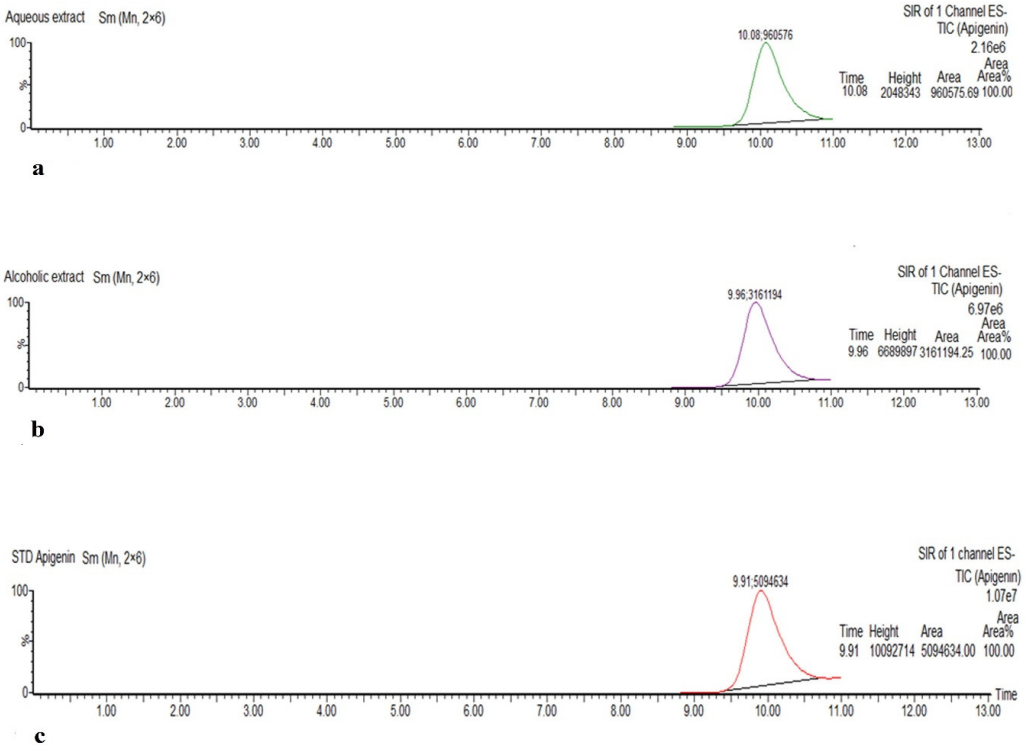

Figure 3. (a) Selected ion recording (SIR) model chromatogram of *Matricaria chamomilla* (MC) aqueous extract, (b) ethanolic extract and (c) standard sample of apigenin.

displays the total ion chromatogram (TIC) of the standard sample of apigenin. Figure 2b shows the mass spectrometry of the negative mode of apigenin with m/z of 269.2.

In the first step of SIR, the apigenin chromatogram in each sample of extracts was obtained (Figure 3). Considering the area of apigenin peak in each extract, we chose a 12.5 mg/L standard sample of apigenin to achieve a pick area that is close to the area of Apigenin apigenin peak in each extract. Figure 3shows the standard chromatogram. The apigenin amount was derived in a one-point manner using the equilibrium. There were 0.078 and 0.25 mg/g apigenin in aqueous and ethanolic extract.

Macrophages Nitric oxide Production

NO production of macrophages was measured using the Griess test after 16 h. Significant decline ($p\leq0.05$) in endogenous LPS-induced NO production was detected in the macrophage supernatant after treatment with 200, 400, 800 and 1600 µg/mL MC aqueous extract. The MC ethanolic extract decreased NO amount in the macrophages supernatant in all concentrations except for the 25 µg/mL ($p\leq0.05$). Results showed a concentration-dependent manner.

The comparison of these two extracts' effects on macrophage NO production showed that ethanolic extract was significantly more effective than aqueous one in all concentrations except for the 25 µg/mL (Figure 4).

Macrophages Cell Viability

The macrophages cell viability was measured by MTT assay. After 16 h, the average of light absorption in co-incubated cells with MC aqueous extract and LPS increased significantly ($p\leq0.05$) in 200 and 800 µg/mL but decreased in 800 and 1600 µg/mL and increased in 400 µg/mL of MC ethanolic extract in comparison with

the control group.

The comparison between two groups of macrophages showed that in high concentrations, the ethanolic extract is an effective agent to decrease macrophage cell viability (Figure 5).

Lymphocytes Cell Viability

The lymphocyte viability was measured with MTT assay after 72 h of incubation with each extract. The cell viability decreased in all concentrations of aqueous extract except 25 µg/mL and all concentrations of ethanolic extract.

The comparison of MC aqueous and ethanolic treated lymphocytes showed no significant differences between these two groups except in the concentration of 1600 µg/mL (Figure 6).

ELISA Assay

We employed sandwich ELISA to measure the level of IFN-γ production in lymphocytes supernatant. The amount of IFN-γ increased in 100, 200, and 1600 µg/mL of aqueous extract in comparison with the control group. But no statistically significant differences were seen in the presence of ethanolic extract.

The comparison of the effect of MC of two extracts showed no significant differences between them. Although a general increase in IFN-γ could be seen in treatment with ethanolic extract, it was not significant (Figure 7).

The amount of IL-10 production by lymphocytes was also measured. The results showed that these cells produced less concentration of IL-10 in 100, 800, and 1600 µg/mL in comparison with the control group after treatment with aqueous extract. IL-10 production increased in 25 and 50 µg/mL in the presence of ethanolic extract.

Comparison of MC ethanolic and aqueous extracts treated cells in IL-10 production showed that ethanolic extract is a significantly effective stimulator in IL-10 production at the low concentration of 25 and 50 µg/mL. But aqueous extract reduced IL-10 production in 800 µg/mL of concentration in comparison with ethanolic extract (Figure 8).

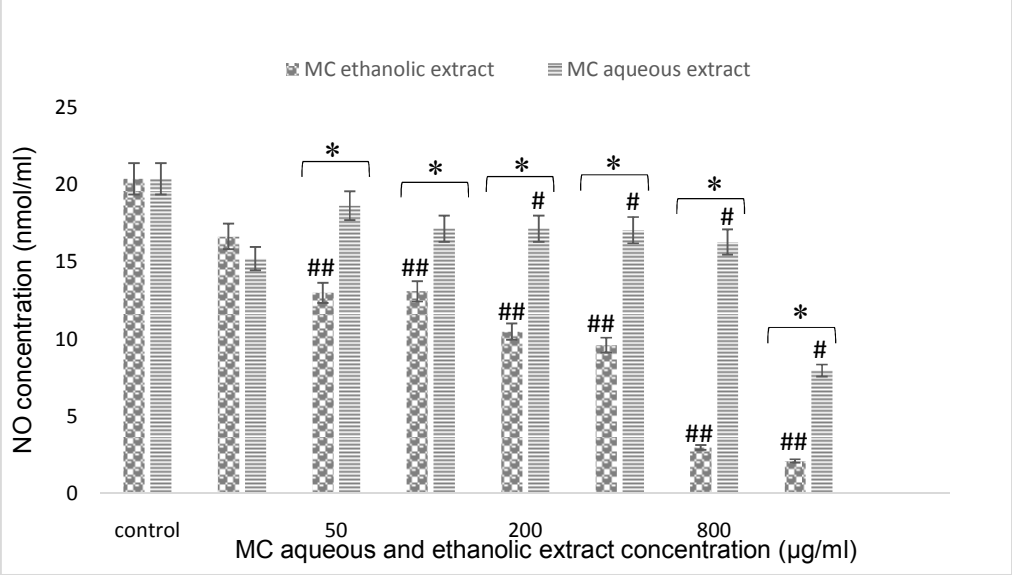

Figure 4. The effect of *Matricaria chamomilla* (MC) aqueous and ethanolic extracts on LPS-induced (10 µg/mL) NO production in BALB/c peritoneal macrophages. One-way ANOVA was performed to compare these two groups (* $p \leq 0.05$). #$p \leq 0.05$ aqueous extract and ##$p \leq 0.05$ ethanolic extract in comparison with the control group. The data is representative of at least 3 independent experiments

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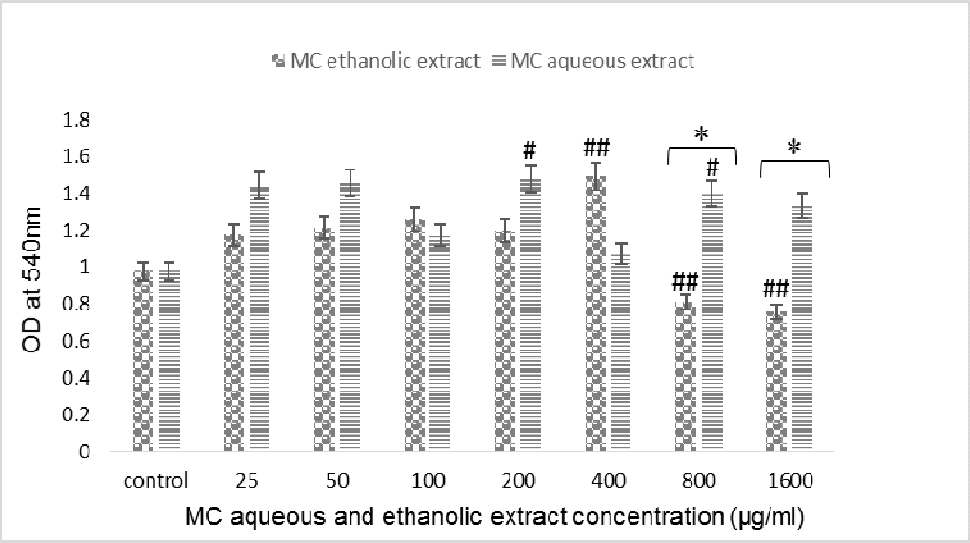

Figure 5. Macrophages viability treated with LPS (10 µg/mL) and different concentrations of *Matricaria chamomilla* (MC) aqueous and ethanolic extracts. One-way ANOVA results of comparisons of these two groups are also shown (*$p \leq 0.05$). #$p \leq 0.05$ and ##$p \leq 0.05$ significant difference between the aqueous and ethanolic treated group with the control group, respectively. The data is representative of at least 3 independent experiments.

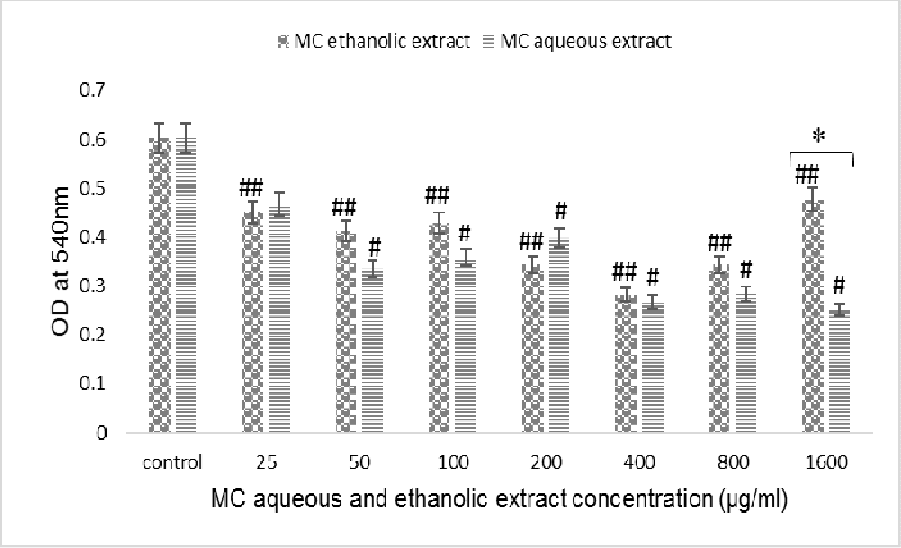

Figure 6. Murine lymphocytes viability in response to Con A (12.5 µg/mL) and different concentrations of *Matricaria chamomilla* (MC) aqueous, and ethanolic extract: The comparison of the cell viability by 1-way ANOVA is also shown (*$p \leq 0.05$). #$p \leq 0.05$ and ##$p \leq 0.05$ significant difference between the aqueous and ethanolic group with the control group. The data is representative of at least 3 independent experiments.

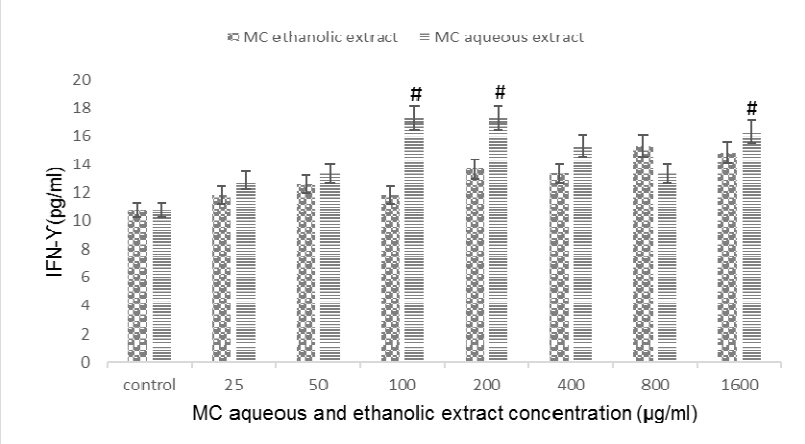

Figure 7. Comparison of IFN-γ production by murine lymphocytes incubated with con A (12.5 µg/mL) and *Matricaria chamomilla* (MC) aqueous and ethanolic extract (One-way ANOVA) for at least 3 independent experiments. No significant differences were seen between the two groups. #P≤0.05 significant difference in response to aqueous extract.

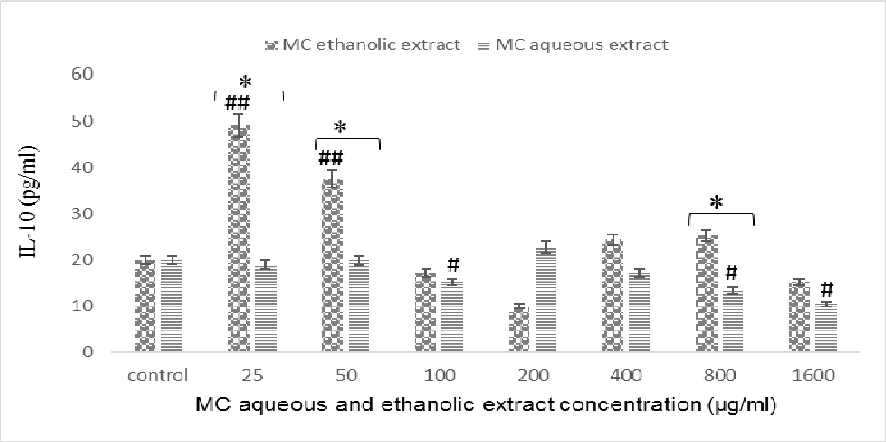

Figure 8. Comparing IL-10 production by murine lymphocytes incubated with con A (12.5 µg/mL) and *Matricaria chamomilla* (MC) aqueous and ethanolic extract by 1-way ANOVA (*$p \leq 0.05$) for at least 3 independent experiments. #$p \leq 0.05$ and ##$p \leq 0.05$ significant difference between aqueous and ethanolic extract with the control group.

DISCUSSION

The TPC values of MC were 2.99% and 3.95% in the aqueous and ethanolic extract, respectively. This finding is consistent with the previous studies that reported TPC of MC as 3.9% and 4.9% in aqueous and ethanolic extracts, respectively. The small differences are because of the extraction method and the region of the plant.[21] Apigenin amounts were 0.078 mg/g and 0.25 mg/g per g dry extract in the aqueous and ethanolic extract, respectively. According to the studies, the free apigenin concentration is very low in chamomile and the glycoside forms are almost seen in high concentrations. Apigenin 7-O-glycoside and other glycosides are the main MC polyphenols.[21,22]

Apigenin amount and TPC of the ethanolic extract

are more than those in the aqueous extract. Generally, the phenolic contents extraction yield depends on the kind of solvent being used for extraction. Ethanol is a high yield solvent for phenolic compounds extraction, although it is not specific for phenolic compounds. Mixing ethanol with water is more effective than one part solvent in phenolic compounds extraction. Ethanol 70% is the best solvent. Using water more than 30% causes other compound extraction so it can reduce the final concentration of phenolic compounds. Pure water has the lowest yield in phenolic compounds extraction.[21-23]

The immunomodulatory effects of MC aqueous and ethanolic extracts were investigated in BALB/c macrophages and lymphocytes in vitro. Cells treatment with these extracts without any mitogen had no effects on cell viability. In previous studies, it is shown that MC extracts had no toxicity on normal cells. Our finding confirms these studies.[7,22]

Macrophages LPS-induced NO production was measured in treatment with MC extracts. Macrophages produce reactive nitrogen species mainly NO with the iNOS enzyme. It is an inducible enzyme that has very low activity in resting macrophages, but it can be induced in response to a microbial product like LPS.[10] Aqueous extract in the concentrations of 200, 400, 800, and 1600 µg/mL and ethanolic extract in all concentrations except 25 µg/mL could decrease NO production in macrophages. MC aqueous extract could significantly reduce the NO production in RAW 264.7 macrophages in the presence of LPS by a reduction in iNOS gene expression.[16] However, the effect of the ethanolic extract is higher than the aqueous extract. There are some compounds in MC flowers like apigenin, luteolin, quercetin, naringenin, and flavones that inhibit the induction of iNOS in activated macrophages and diminish NO production.[3,9] NO production decreased in a concentration manner in treatment with MC ethanolic extract, and this is more effective than an aqueous extract because of its higher amount of phenolic compounds.

Macrophages cell viability significantly increased in treatment with MC aqueous extract in concentrations of 200 and 800 µg/mL. The cell viability of macrophages decreased in 800 and 1600 µg/mL in treatment with MC ethanolic extract. This finding was consistent with the previous findings by Hatami. In this study, mice were intraperitoneally and orally treated with MC aqueous and ethanolic extracts. MC ethanolic extract effectively reduced the in vivo viability of macrophages as compared with the aqueous extract (unpublished data).

Murine lymphocyte cell viability measurement revealed that ethanolic extract in all concentrations and aqueous extract in all concentrations except 25 µg/mL reduced lymphocyte cell viability. With regard to previous studies, the polyphenolic and other compounds of MC like α-bisabolol, apigenin, and luteolin can inhibit lymphocytes proliferation.[24,25] So it is expected that MC extracts inhibit proliferation in response to Con A.

In lymphocytes cytokine determination, we found that the amount of IFN-γ increased in concentrations of 100, 200, and 1600 µg/mL MC aqueous extract in comparison with those in the control group. On the other hand, IL-10 decreased in concentrations of 100, 800, and 1600 µg/mL in these cells. BALB/c lymphocytes treatment with ethanolic extract had no significant effect on IFN-γ production by these cells. However, IL-10 production increased in 25 and 50 µg/mL concentrations.

Among T lymphocytes, Th1 is a good producer of IFN-γ and a good stimulator for Th1 polarization.[12] There is a balance between Th1 and Th2. This balance disruption is the cause of some immune disorders like allergy.[26] IFN-γ released by Th1 can suppress Th2 and alters Th1/Th2 balance.[12] Th1 is also a producer of IL-10 in the late stage of the immune response. IL-10 can inhibit Th1 polarization as negative feedback. IL-10 is also produced by some other cells like Th2 and regulatory T cells.[27] It is found that chamomile is an anti-allergic plant, and its purified polyphenolic compounds like quercetin and luteolin are Th2 suppressor. Multiple sclerosis models of mouse lymphocytes treated with apigenin can inhibit autoimmune lymphocytes proliferation and IFN-γ production.[28] Apigenin could moderate Th1/Th2 balance to Th2 cells through decreasing of IFN-γ and TNF-α as Th1 cytokines and increase of IL-4 and IL-10 as Th2 cytokines in myocarditis BALB/c mice.[26] Dietary apigenin alleviates IgE and IFN-γ in NC/Nga mice serum in human atopic dermatitis model.[29] Apigenin and apigenin 7-O-glucoside are the most abundant compounds in MC aqueous extract.[10] In the mentioned investigations, apigenin suppresses IFN-γ production. The extract is a combination of various compounds. Apigenin and TPC in the aqueous extract are lower in ethanolic one.

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With regard to the increase of IFN-γ production and decrease in IL-10 production with aqueous extract treatment, it seems that this extract disrupts the Th1/Th2 balance by Th1 upregulating. It seems that the other aqueous extract compounds moderate apigenin effect in the matter of IFN-γ production. Although the in vitro investigation cannot be generalized to in vivo studies, the higher amount of apigenin in ethanolic extract could lead to IFN-γ production decrease and IL-10 increase.

In conclusion, these two extracts showed different effects. The results of this study indicate that the anti-inflammatory effects of MC ethanolic extract on macrophages were related to a decrease in NO production and cell viability. But the anti-inflammatory effect of this extract on lymphocytes is a result of the induction of IL-10 production as an anti-inflammatory cytokine and cell viability decrease. MC aqueous extract can be a good suppressor of Th2 by disrupting the Th1/Th2 balance. Its effects on macrophages are NO production decrease and cell viability increase. The differences between these two extracts can be a result of different compounds extracted.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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