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Original Article

Clove (*Syzygium aromaticum*) ingredients affect lymphocyte subtypes expansion and cytokine profile responses: An *in vitro* evaluation

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ABSTRACT

Clove (*Syzygium aromaticum*) has been used in folk medicine in many disorders. The present work aimed to investigate effects of clove essential oil as eugenol and water soluble ingredients on mouse splenocytes. Clove extracts were harvested and in different concentrations (0.001–1000 μg/mL) were affected to splenocytes and also phytohemagglutinin (PHA = 5 μg/mL) and lipopolysaccharide (LPS = 10 μg/mL) activated splenocytes; then splenocytes proliferation assayed using the MTT ([3-(4, 5-dimethylthiazole-2-yl) -2, 5-diphenyl tetrazolium bromide]) method were done. On the culture supernatant interferon (IFN)-γ, interleukin (IL)-4, IL-10, and transforming growth factor (TGF)-β cytokines were measured. Clove ingredients (100 μg/mL and 1000 μg/mL) reduced PHA stimulated splenocytes proliferation and enhanced LPS stimulated cells expansion. Treated splenocytes showed suppression of IFN-γ release and induction of IL-4, IL-10, and TGF-β secretion (in the range of 0.1–1000 μg/mL). The results of this study suggest clove extracts could suppress the T cell cellular immunity and enhance humoral immune responses. In clove affection cytokine pattern shifted toward modulatory and Th2 responses and accelerator of humoral immunity cytokines.

1. Introduction

Herbal medicine is used to treat different diseases in most of the world's cultures. Identification of the active component and mechanisms of action of traditional medicines on the immune system is highly desirable [1]. The immune system is divided into adaptive and innate systems and the adaptive immune system is divided into cellular and humoral responses. T and B lymphocytes are the main cells that are involved in adaptive immune phenomena. Many of the drugs and mediators in the treatment of the disease perform their functions by the influence of immune cells, especially lymphocytes. These agents could be body mediators, synthetic factors, or natural elements [2,3]. Cloves are dried flower buds derived from an evergreen tree *Syzygium aromaticum* (L.) Merr. & Perry (i.e., *Eugenia aromaticum* or *Eugenia caryophyllata*), a tree 10–20 m high that is indigenous to India, Indonesia, Zanzibar, Mauritius, and Iran [4]. It is commonly used in Africa, Asia, and other parts of the world in the preparation of various spicy rich dishes. It has a deep brown color, intense fragrance, and a burning taste [5]. Clove has several therapeutic properties; it is

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a well-known food flavor and a popular remedy for dental and respiratory disorders, headache, and sore throat in traditional medicines of Australia, and in Asian countries [6]. Clove possesses antidiabetic, anti-inflammatory, antithrombotic, anesthetic, pain-relieving, and insect-repellent properties [7,8]. The main ingredients of clove are eugenol (50–87%), eugenyl acetate, tanene, thymol, and β-cariophyllene [9]. These components are responsible for clove extract's effect when used under different conditions. In addition, these components have been shown to modulate some immune responses, including anti-inflammatory effects, although the mechanisms through which these effects are mediated remain unclear [10]. In addition to eugenol, which is extracted using alcoholic method [11], water-soluble ingredients also have an important function. Biological and therapeutic properties of clove have been reported, but its effect on the immune system is poorly investigated. Those who have studied the benefits of clove in traditional medicine propose its effects on the immune cells and immune system. The goal of this study was to investigate *in vitro* effects of the essential oil in eugenol (prepared by alcoholic extraction) and water-soluble elements of clove on splenocytes and mitogen-stimulated splenocytes for analysis of T and B lymphocyte subtypes [phytohemagluttinin (PHA (Sigma, St. Louis, MO)) mitogen for T cells and lipopolysaccharide (LPS (Sigma, St. Louis, MO)) mitogen for B cells] as the main effector cells in cellular and humoral immune responses. Moreover, the effect on cytokines secretion (IFN-γ, IL-4, IL-10, and TGF-β) by BALB/c splenic lymphocytes was assessed.

2. Materials and methods

2.1. Animals

Six-to 8-week-old female inbred Balb/c mice were purchased from the Pasteur Institute of Iran (Tehran, Iran). They were kept in the animal house of Tarbiat Modares University, Tehran, Iran and were given standard mouse chow and sterilized water throughout the study. The study design was approved by the Ethical Committee of the Department (Tarbiat Modares University, Tehran, Iran) for Animal Care and Use.

2.2. Plant material

The flower buds of clove (*Syzygium aromaticum*) were collected from plants cultivated in the Center of Medicinal Plants Research 25 km north of Tehran, Iran, and confirmed by the Center of Agricultural Research, Tehran, Iran.

2.3. Preparation of clove extracts

Extraction of eugenol (in the essential oil) was done by the alcoholic extraction method [11]; in brief, dried powdered flower buds of clove (25 g) were soaked in 100 mL of ethanol to prepare the essential oil and in 100 mL of distilled water to prepare the aqueous extract. They were allowed to mix on a rotary for 24 hours and then filtered using Whatman No. 1 filter paper. The filtrate extract was poured into a special lyophilizing flask. The flask was connected to a vacuum pump and evacuated until drying. The isolates were recovered, weighed, and representative stock of 100 mg/mL was prepared in 5 mL of, accordingly, water or 50% ethanol for use in the various assays herein. Yields for both materials were routinely 2–3 g (8–12% [w/w] starting material).

2.4. Analyses of extract contents

Previous reports indicated that clove oil contained eugenol (~75%), β-caryophyllene (~5%), eugenyl acetate (~16%), and other components <1% [12]; analyses of the aqueous extracts showed it contained 49% eugenol [13]. The extracts in the current study were assessed using the same gas chromatography-mass spectrometry methods as that of Lee et al [13]; the data showed eugenol made up 74% of material in the ethanolic extract and 43% in aqueous extract. Although we did not assess directly the levels of β-caryophyllene and eugenyl acetate, we assume these are in line with the values reported in both Chaieb et al [12] and Lee et al [13] and thus comprise the bulk of the remaining materials in each extract.

2.5. Preparation and treatment of splenocytes

The mice were decapitated under mild diethyl ether anesthesia and the spleens were rapidly excised under sterile conditions. This tissue was subsequently homogenized in 10 mL cold RPMI 1640 complete medium (Sigma Chemical Company, St. Louis, MO) in a glass homogenizer. Homogenized spleen tissues passed through a fine steel mesh to obtain a homogeneous cell suspension. The erythrocytes were osmotically lysed by 0.75% NH4Cl in Tris buffer (0.02%, pH = 7.2). After centrifugation (360*g* at 4°C for 10 minutes), the pelleted cells were washed three times with phosphate buffered saline (PBS) and resuspended in RPMI 1640 complete medium supplemented with 11 mM sodium bicarbonate, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (all reagents were purchased from Gibco, Grand Island, NY). The total viable splenocyte cell count was determined using the trypan blue exclusion assay. Cell viability was more than 95%. Splenocyte treatment was assayed as follow: 5×10^6 cell/mL of the cells was seeded into each well of a 96-well flat-bottom microtiter plate (Nunc, Kamstrup, Denmark) in complete medium and PHA (final concentration 5 μg/mL), LPS (final concentration 10 μg/mL), or PBS was added to the wells. Clove ingredients (final concentration 0.001–1000 μg/mL) were added, giving a final volume of 200 μL (tetraplicate wells) and incubated for 48 hours at 37°C and 5% CO_2.

2.6. Cell proliferation assay

After 48 hours of incubation with various concentrations of clove extracts, cell proliferation was measured based on the MTT [3-(4, 5-dimethylthiazole-2-yl) -2, 5-diphenyl tetrazolium bromide] reduction assay [14]. In brief, after incubation, 20 μL of MTT (5 mg/mL in PBS) were added to 200 μL wells (in one tenth of the total volume) and incubated for 4 hours at 37°C and 5% CO_2. Then medium was removed and the formazan blue crystals, which formed by reacting MTT with mitochondrial dehydrogenase in the living cells, were dissolved by 100 μL of acidic isopropanol (0.04 M HCl in isopropanol). The

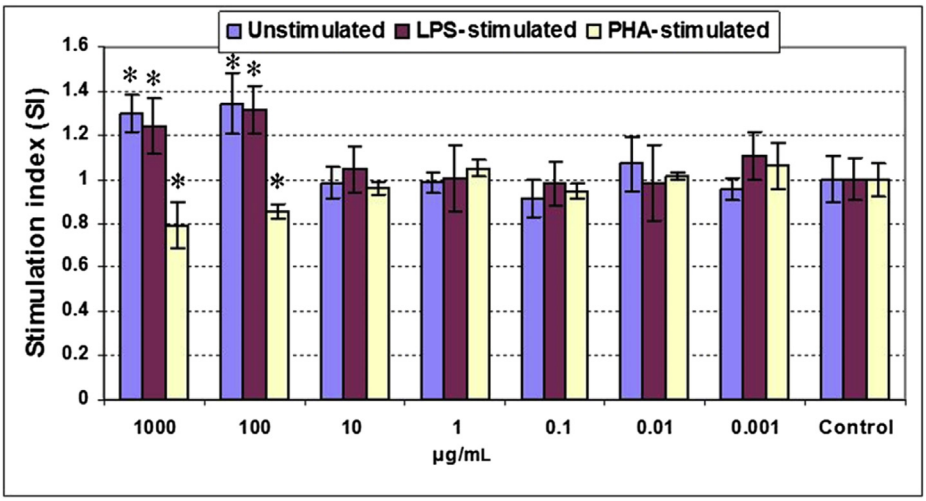

Fig. 1 – The values (mean ± standard deviation) of proliferation assay of LPS/PHA/unstimulated spelnocytes treated with various concentrations of clove alcoholic extract. One hundred µg/mL and 1000 µg/mL of extract-reduced PHA stimulated splenocytes (as T cells) proliferation and enhanced proliferation of LPS stimulated (as B cells) or unstimulated splenocytes. SI: OD540 of the group test samples/OD540 of each group negative control. *$p < 0.05$. LPS = lipopolysaccharide; PHA = phytohemagluttinin.

plates were read using a Multiskan MS microplate reader (Thermo Scientific Vantaa, Finland) at the wavelength of 540 nm. The result of the test was expressed as a Stimulation Index (SI), which is OD540 of the group test samples/OD540 of each group negative control.

2.7. *Cytokine enzyme-linked immunosorbent assay*

IFN-γ, IL-4, IL-10, and TGF-β cytokines in cell culture supernatants were determined using a commercially available enzyme-linked immunosorbent assay kit (eBiosciences, Frankfurt, Germany) according to the manufacturer's instructions. All samples were measured at least in duplicate.

2.8. *Data analysis*

Statistical analysis was performed using SPSS version 15 for Windows software (SPSS Inc., Chicago, IL, USA). For multiple comparisons, data were analyzed by one-way analysis of variance and followed by a least significant difference test. A p value <0.05 was considered to indicate a significant difference. Results are expressed as a mean ± standard deviation.

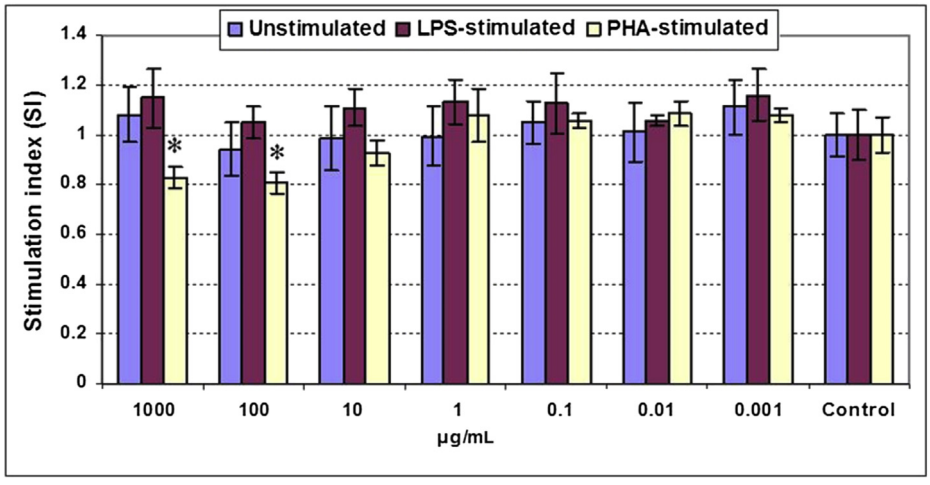

Fig. 2 – The values (mean ± standard deviation) of proliferation assay of LPS/PHA/un-stimulated splenocytes treated with various concentrations of clove aqueous extract. Only 100 µg/mL and 1000 µg/mL of extracts reduced PHA stimulated splenocytes (as T cells) proliferation. SI: OD540 of the group test samples/OD540 of each group negative control. *$p < 0.05$. LPS = lipopolysaccharide; PHA = phytohemagluttinin.

3. Results

3.1. Lymphocyte subtypes proliferation

Proliferation assay of LPS/PHA/unstimulated splenocytes treated with various concentrations of clove essential oil and aqueous extracts are shown in Figs. 1 and 2. One hundred μg/mL and 1000 μg/mL of clove essential oil reduced PHA stimulated splenocytes (as T cells) proliferation ($p < 0.05$) and enhanced proliferation of LPS stimulated (as B cells) or unstimulated splenocytes ($p > 0.05$). One hundred μg/mL and 1000 μg/mL of aqueous extract only reduced PHA stimulated splenocytes (as T cells) proliferation ($p < 0.05$) and had no effect on two other populations.

3.2. Cytokine production

Cytokine assay on treated splenocytes supernatant are shown in Figs. 3–6. Clove eugenol rich essential oil downregulated

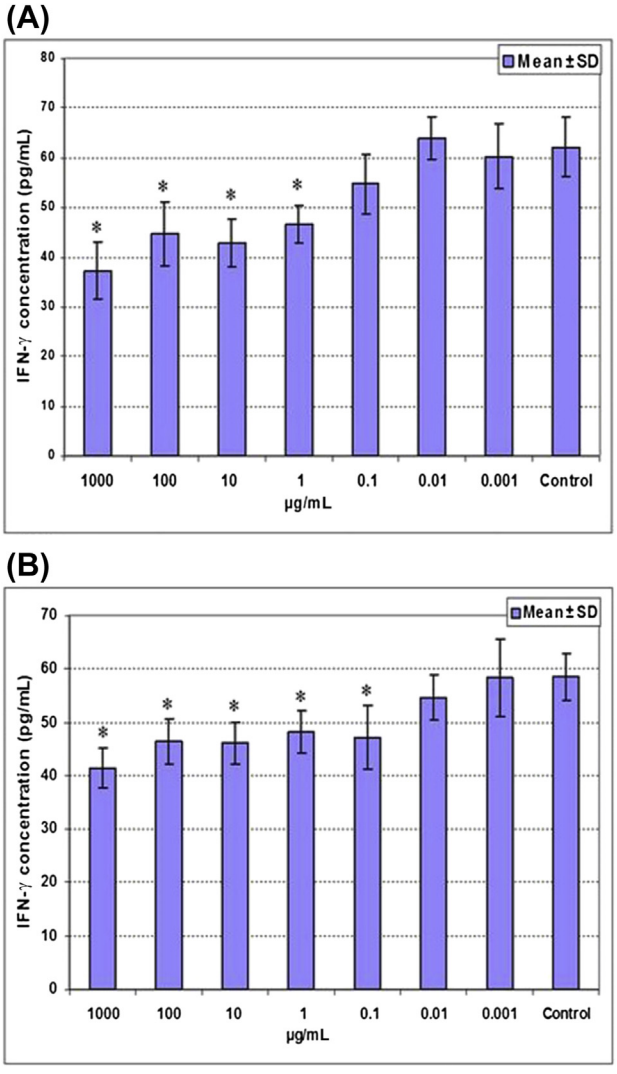

Fig. 3 – Interferon (IFN)-γ production splenocytes treated with various concentrations clove (A) alcoholic and (B) aqueous extracts. *$p < 0.05$. SD = standard deviation.

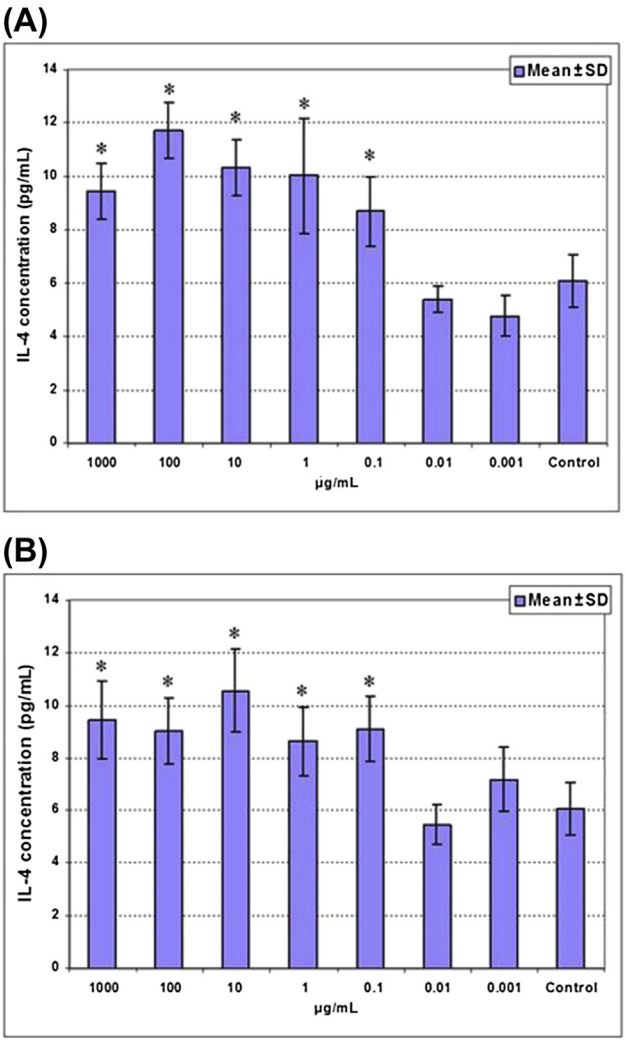

Fig. 4 – Interleukin (IL)-4 production splenocytes treated with various concentrations clove (A) alcoholic and (B) aqueous extracts. *$p < 0.05$. SD = standard deviation.

IFN-γ production in range of 1–1000 μg/mL (62.17 ± 5.93 ng/mL vs. <46.5 ng/mL of control group vs. 1–1000 μg/mL of clove oil). Water-soluble ingredients also suppressed IFN-γ production in the range of 0.1–1000 μg/mL (62.17 ± 5.93 ng/mL vs. <48.2 ng/mL of control group vs. 0.1–1000 μg/mL of aqueous extract). IL-4 release was enhanced by both alcoholic and aqueous extracts in the range of 0.1–1000 μg/mL (6.06 ± 0.98 ng/mL vs. >10.3 ng/mL of untreated splenocytes vs. 0.1–1000 μg/mL ingredients affected by splenocytes). Both extracts also had stimulatory effect on IL-10 production (an approximate twofold increase in the range of 1–1000 μg/mL for eugenol-rich essential oil and 0.1–1000 μg/mL for water-soluble ingredients). The strongest stimulatory effect of clove components on IL-10 production was seen in 10 μg/mL concentrations (2.34-fold for essential oil and 2.22-fold for aqueous extract in comparison with untreated lymphocytes). In addition, 0.01–1000 μg/mL of essential oil increased TGF-β release (32.11 ± 7.44 ng/mL vs. >80 ng/mL of untreated splenocytes vs. 0.1–1000 μg/mL ingredients affected splenocytes). Clove in higher examined concentrations as 10 ug/mL,

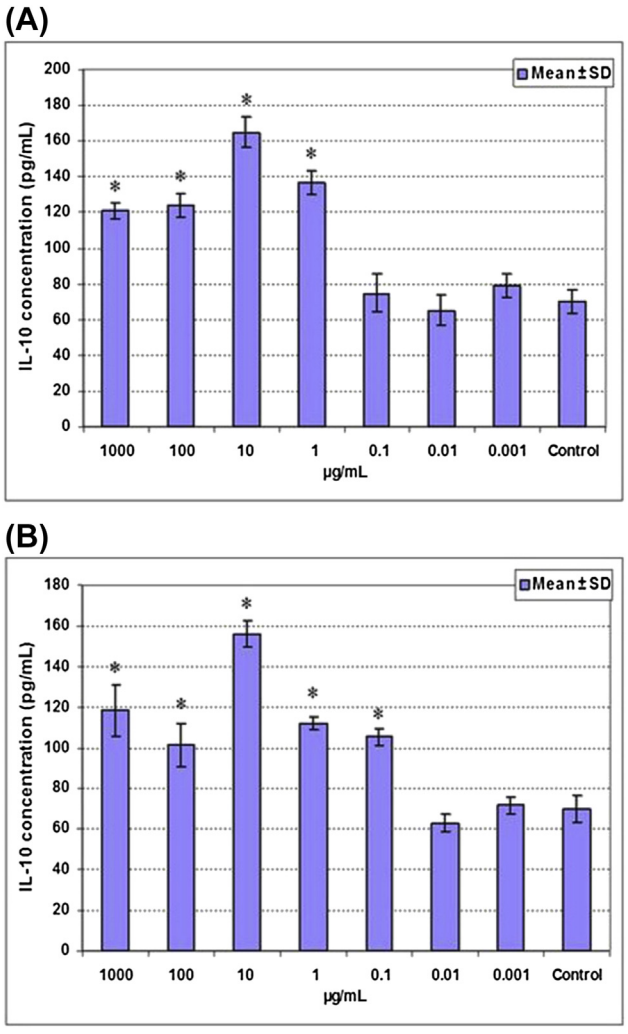

Fig. 5 – Interleukin (IL)-10 production splenocytes treated with various concentrations clove (A) alcoholic and (B) aqueous extracts. *$p < 0.05$. SD = standard deviation.

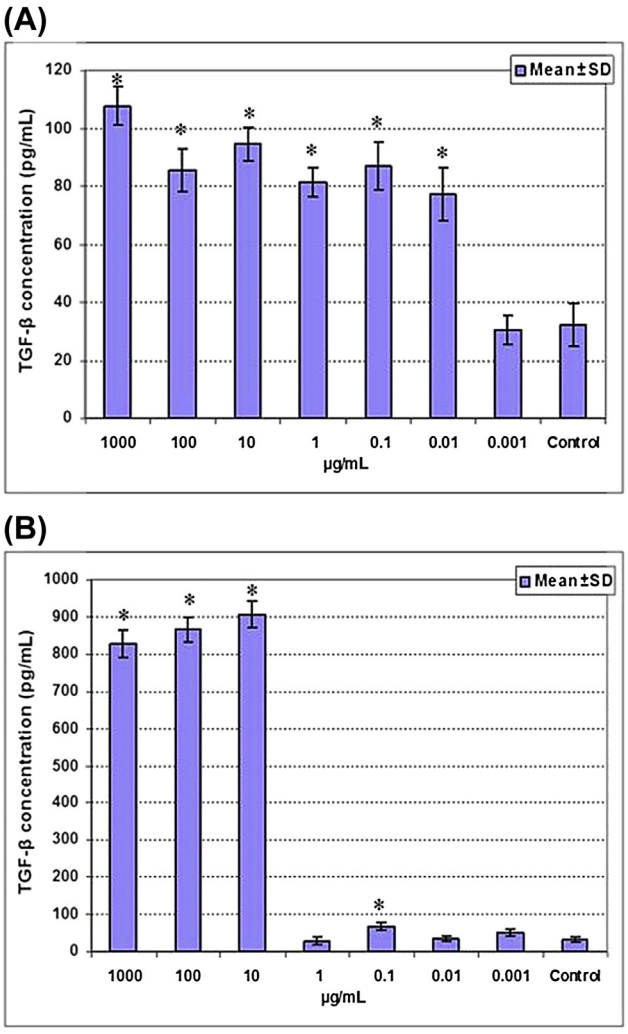

Fig. 6 – Transforming growth factor-beta (TGF-β) production splenocytes treated with various concentrations of clove (A) alcoholic and (B) aqueous extracts. *$p < 0.05$. SD = standard deviation.

100 ug/mL, and 1000 ug/mL showed stimulatory effects on TGF-b release (>800 ng/mL vs. 32.11 ± 7.44 ng/mL of control splenocytes). Cytokine significant differences were considered as $p < 0.05$.

4. Discussion

Despite different studies on clove and its vast uses in traditional medicine, data are sparse on its effects on the immune system and especially lymphocytes. The most important constituent of clove is eugenol, which gives this spice its pungent, distinctive aroma. Eugenol makes up 70–90% of the essential oil and 15% of the dry weight of clove buds [15]. Cloves contain volatile oil (14–21%), tannin (10–13%), phenol, sesquiterpene ester, and alcohol [16]. These components are responsible for clove's consequent effects under various conditions. In this study, we evaluated essential oils rich in eugenol as alcoholic extracts and aqueous extract's effects on expansion of mitogen-stimulated lymphocytes (T and B cells); also, we have measured changes in cytokine production. Evaluation of the proliferation of lymphocyte subtypes showed that higher concentrations (100 µg/mL and 1000 µg/mL) of clove essential oil reduced PHA stimulated splenocytes (as T cells) proliferation and enhanced expansion of LPS stimulated (as B cells) or unstimulated splenocytes. The same concentrations of aqueous extract only reduced PHA stimulated splenocytes (as T cells) proliferation whereas there was no effect on the other two lymphocyte populations. In comparison with these results, another study has the same results in various methods. Halder et al [17] assessed humoral immunity by the measure of the hemagglutination titer to sheep red blood cells and delayed-type hypersensitivity (DTH) was examined by the measure of foot-pad thickness, and their data have shown that clove oil can modulate the immune response by augmenting humoral immunity and decrease cell mediated immunity. Another study has reported some enhancement of humoral and cellular responses as some contradictory results. Carrasco et al [18] showed that in cyclophosphamide-immunosuppressed mice, clove essential oil increased the total white blood cell count and enhanced

the DTH responses while restoring cellular and humoral immune responses in these mice. In this study, mice were immunocompromised and enhancement of T cell responses involves restoration of function. Therefore, these data indicate that clove modulates T cell expansion and function; moreover, clove can restore cellular immunity that would lead to beneficial applications. Similar to previous reports that have shown enhancement of antibody secretion [17] and restoration of B cells [18], our data revealed expansion of B cells and humoral immune responses with clove ingredients. After evaluation of lymphocyte expansion responses, we analyzed the cytokine profile in the treated cells. Our results showed that effective concentrations of both ingredients (0.1–1000 μg/mL) affected splenocytes cytokine production. Both clove essential oil and aqueous extracts reduced IFN-γ production and induced release of IL-4, IL-10, and TGF-β. Grespan et al [19] also showed a reduction of IFN-γ secretion by eugenol in mice and in accordance with decreased secretion of IFN-γ from T cells, Park et al [20] showed that eugenol and its structural analog, isoeugenol, inhibit IL-2 gene expression that is another typical T cell cytokine. IL-10 cytokine production by eugenol was enhanced in another study [19]. A different work investigated the administration effect of clove water extract over a short term to BALB/c mice on T helper 1 (Th1;IFN-γ and IL-2) and Th2 (IL-4 and IL-10) cytokine production. These results indicated that clove treatment for mice did not influence the Th1/Th2 cytokine balance [21]. Grespan et al [19] also mentioned that eugenol administration in mice induces significant reduction in TNF-α and TGF-β levels. In addition to these findings, other studies show that clove ingredients could affect immune responses; for instance, a study on isoeugenol and its analogues (eugenol and allylbenzene) clove ingredients showed inhibition of nitric oxide production and inducible nitric oxide synthetase expression in a dose-dependent manner in LPS-stimulated RAW 264.7 murine macrophages [8]. Another study showed that clove could inhibit IL-1β and IL-6 production after LPS challenge from macrophages [22]. Eugenol in essential oil also showed reduction of TNF-α production from Kupffer cells [23].

In conclusion, our study showed that clove ingredients could suppress T cell proliferation and enhance B cells expansion, findings compatible with those of previous studies for reduction of cellular T cell responses and increase in humoral functions. Cytokine release evaluation showed suppression of IFN-γ as Th1 and proinflammatory mediators and increase of IL-4, IL-10, and TGF-β as Th2 and anti-inflammatory cytokines. Studies on macrophages and other cells showed the same anti-inflammatory and modulatory functions of clove ingredients. Therefore, clove could suppress T cells and their functions and enhance B cell expansion, function, and humoral responses. Induction of cytokine pattern is geared toward Th2 responses, modulation of inflammation, and acceleration of humoral immunity.

Conflicts of interest

The authors report no conflicts of interest.

Acknowledgments

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