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# *Zataria multiflora* hydroalcoholic extract: A triple-blind randomized controlled trial on immune genes, inflammation, and ulcerative colitis symptoms

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ABSTRACT

*Ethnopharmacological relevance: Zataria multiflora* Boiss. (Shirazi thyme) is traditionally used for digestive disorders and inflammatory conditions. Despite its known anti-inflammatory, immunomodulatory, and antioxidant properties, there is limited clinical evidence on its efficacy for ulcerative colitis (UC).

*Aim of the study:* To evaluate the effectiveness of *Zataria multiflora* Boiss. *(Z. multiflora)* extract in alleviating UC symptoms, reducing inflammatory markers, and modulating immune-related gene expression.

*Materials and methods:* In a multicenter, randomized, placebo-controlled, triple-blind trial in Iran, 92 participants received *Z. multiflora* extract (6 mg/kg/day) or a placebo for two months. Inflammatory markers and gene expression were analyzed from blood samples. Disease activity was assessed using the Partial Mayo Score (p-Mayo) and the Gastrointestinal Symptom Rating Scale (GSRS). Data were analyzed with SPSS software.

*Results:* The *Z. multiflora* group showed significant reductions in C-reactive protein (CRP) ($p < 0.001$), Interleukin-17 (IL-17) ($p = 0.001$), Interferon-gamma (IFN-γ) ($p = 0.002$), Nuclear Factor kappa B (NF-κB) ($p = 0.002$), T-box Transcription Factor T-bet (T-bet) ($p = 0.006$), and Retinoic Acid-Related Orphan Receptor gamma t (ROR-γt) ($p < 0.001$). No significant changes were observed in Erythrocyte Sedimentation Rate (ESR) ($p = 0.25$), GATA Binding Protein 3 (GATA3) ($p = 0.09$), and Forkhead Box P3 (FOXP3) ($p = 0.17$). Symptoms such as heartburn, acid reflux, bloating, diarrhea, and fecal urgency improved ($p < 0.05$). The GSRS score improved ($p < 0.001$), while the p-Mayo score did not show a significant change ($p = 0.24$).

*Conclusion: Z. multiflora* extract significantly alleviated UC symptoms and reduced inflammatory markers, indicating its potential as a complementary treatment for UC. However, the study was limited by its short intervention period and the absence of biopsy analysis to assess local tissue effects. Further longitudinal studies are required to validate these findings and determine long-term efficacy.

## 1. Introduction

Ulcerative colitis (UC) and Crohn's disease (CD) are the primary types of inflammatory bowel disease (IBD). Although they exhibit similarities, they differ in terms of genetic predisposition, risk factors, clinical presentation, endoscopic findings, and histological features (Ellinghaus et al., 2015). UC is more prevalent than Crohn's disease. Its global incidence is on the rise, with varying rates reported across different regions of North America and Europe (Du and Ha, 2020). The rising prevalence of IBD is also evident in Asian nations and Iran. This upward trajectory has persisted since 2017 and is anticipated to continue in Iran from 2020 to 2035. By 2035, compared to 2020, a 2.5-fold increase in prevalence is expected in Iran, reaching 69,000 cases (Olfatifar et al., 2021).

Symptoms of UC can include persistent diarrhea mixed with blood,

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**List of abbreviations**

| | |
|---|---|
| CRP | C-reactive protein |
| ESR | Erythrocyte Sedimentation Rate |
| FOXP3 | Forkhead Box P3 |
| GATA3 | GATA Binding Protein 3 |
| GI | Gastrointestinal |
| GSRS | Gastrointestinal Symptom Rating Scale |
| hs-CRP | high-sensitivity C-reactive protein |
| IBD | Inflammatory Bowel Disease |
| IFN-γ: | Interferon-gamma |
| IKK | IκB kinase |
| IL-17 | Interleukin-17 |
| IL-6 | Interleukin 6 |
| IκB | Inhibitor of kappa B |
| MAPK | Mitogen-Activated Protein Kinase |
| NF-κB | Nuclear Factor kappa B |
| ROR-γt | Retinoic Acid Receptor-Related Orphan Receptor Gamma t |
| T&CM | Traditional and complementary medicine |
| T-bet: | T-box Transcription Factor T-bet |
| Th cells | T helper cells |
| Tregs | Regulatory T cells |
| UC | Ulcerative Colitis |
| *Z. multiflora* | *Zataria multiflora* |

abdominal pain, cramping, rectal bleeding, urgency to have bowel movements, and, in some cases, constipation (Cardozo and Sobrado, 2022). Additionally, patients may experience fatigue, weight loss, and decreased appetite (Rozich et al., 2020). The exact cause of IBD remains unknown. However, disruptions in the mucosal immune response to the normal flora of the gastrointestinal tract can trigger bowel inflammation, particularly in individuals with genetic susceptibility (Ahluwalia et al., 2018; Lu et al., 2022). M cells in Peyer's patches activate immune responses by producing immunoglobulin A and T helper cells (Th cells), which can either protect the gut barrier or induce inflammation. This interaction is crucial in UC pathology (Iyer et al., 2023; Jiao et al., 2020). UC is an autoimmune disease. UC patients typically exhibit Th1 cells, driven by T-bet, producing IFN-γ, and Th17 cells, regulated by ROR-γt, secreting IL-17 and IL-6, in the lamina propria mucose, exacerbating inflammation. Conversely, regulatory T cells (Tregs) and Th2 cells normally help maintain immune tolerance and reduce inflammation, but their function is impaired in UC (Fu et al., 2020; Nakase et al., 2022; Zimmermann et al., 2016).

Treatment for UC combines conventional medications with complementary therapies, including dietary supplements and nutraceuticals (Iyengar et al., 2024; Spinelli et al., 2022). Conventional drugs such as amino salicylates and biologic agents target inflammation, while complementary approaches like probiotics, omega-3 fatty acids, herbal remedies, and nutraceuticals provide additional support (Maio et al., 2022; Park and Cheon, 2022; Sasson et al., 2021). *Zataria multiflora* Boiss L. (*Z. multiflora*), also known as " Shirazi thyme" or "Wild Thyme," is considered a nutraceutical due to its medicinal properties and potential health benefits. It belongs to the Lamiaceae family and is characterized by small, narrow, and ovate leaves. This plant grows exclusively in central and southern regions of Iran, Pakistan, and Afghanistan (Basti et al., 2016; Sajed et al., 2013). *Z. multiflora* is particularly noted for its use in gastrointestinal disorders, including IBD. In a cross-sectional study involving IBD patients, *Z. multiflora* was one of the most frequently used herbs to alleviate symptoms. The findings indicated a significant association between the use of herbal medicine including *Z. multiflora* and both improved quality of life and reduced disease severity scores (Shamsaddini et al., 2024). Also, another cross-sectional study conducted in Iran among adult patients with gastrointestinal (GI) disorders, *Z. multiflora* was identified as the most commonly used herb, with 60.7% of traditional and complementary medicine (T&CM) users reporting its use. Additionally, most of those who used T&CM, including *Z. multiflora*, reported its effectiveness in managing their gastrointestinal conditions (Ruyvaran et al., 2021).

Several compounds have been identified in the essential oil of *Z. multiflora*, with thymol, carvacrol, linalool, and p-cymene being its main components. Thymol and carvacrol have been studied more extensively than other components because of their anti-inflammatory, immunomodulatory, and antioxidant properties (Saedi Dezaki et al., 2016; Vassiliou et al., 2023; Zomorodian et al., 2011).

Most studies on *Z. multiflora*'s immunomodulatory effects focus on asthma models, showing its significant impact on the Th1/Th2 balance. It boosts IFN-γ while inhibiting IL-4 expression, shifting towards Th1 dominance via NF-κB pathways and transcription factors like T-bet and GATA3 (Boskabady et al., 2013). The extract also reduces IL-17 expression, dampening Th17-mediated inflammation, and increases FOXP3 expression, enhancing Treg function. This multifaceted approach holds promise for managing inflammatory and autoimmune conditions (Kianmehr et al., 2017c). *Z. multiflora* extract rebalances immune responses in asthma by reducing Th2 and Th17 cells while boosting Th1 cells, suppressing inflammation, and enhancing anti-inflammatory responses. It likely inhibits NF-κB, reduces Th17-related ROR-γt activity, and increases Treg-related FOXP3 expression (Kianmehr et al., 2017a). Additionally, it downregulates GATA3, upregulates T-bet, and inhibits pro-inflammatory pathways through thymol and carvacrol. Thymol and carvacrol inhibit the degradation of IκB and the activity of the IKK complex, preventing the release and nuclear translocation of NF-κB. This results in decreased transcription of NF-κB target genes, including those encoding pro-inflammatory cytokines (Gholijani et al., 2016).

In experimentally induced UC models, pretreatment with *Z. multiflora* and its components demonstrated a decrease in abdominal hyperalgesia, mucosal and histological damage, and clinical scores of UC. Additionally, inflammatory markers such as TNF-α, IL-1β, IL-6, and IL-1 were reduced (Arigesavan and Sudhandiran, 2015; Chamanara et al., 2019; de Santana Souza et al., 2017; Nakhai et al., 2007; Tahmasebi et al., 2019). Clinical trial studies on the effects of *Z. multiflora* and its components on inflammatory and immune markers have mostly been conducted on respiratory patients, including those with asthma, chronic obstructive pulmonary disease, and sulfur mustard-induced lung disorders. In these studies, intervention with *Z. multiflora* and its components led to a reduction in inflammatory markers such as high-sensitivity C-reactive protein (hs-CRP), TNF- α, IL-6, IL-2, and IL-8, and an increase in IFN- γ and IL-10 (Alavinezhad et al., 2018, 2020, 2022; Ghorani et al., 2021a; Khazdair et al., 2020b).

Despite its traditional use in treating digestive disorders and supporting evidence from laboratory and animal models on its beneficial effects for gastrointestinal issues, there is a limited number of clinical trials investigating *Z. multiflora*'s direct impact on ulcerative colitis, highlighting a significant gap in the literature (Jamalizadeh et al., 2022; Sajed et al., 2013; Shomali, 2019; Zamani et al., 2018). Specifically, there have been no studies examining its impact on UC, which limits its potential application in clinical settings. However, considering its potential advantages, particularly in managing chronic gastrointestinal conditions, further exploration of *Z. multiflora* as a complementary treatment is warranted. Moreover, its immunomodulatory and antioxidant properties may indirectly contribute to its effectiveness in improving gastrointestinal health (Khazdair et al., 2018, 2021). Overall, *Z. multiflora* exhibits qualities that suggest it could serve as a useful complementary treatment for gastrointestinal disorders (Mahboubi, 2019; Nosratabadi et al., 2023). This study aims to bridge this knowledge gap by investigating the effects of *Z. multiflora* extract on clinical

symptoms, inflammatory markers, and immune-related gene expression in UC patients. By elucidating its molecular mechanisms, this research seeks to assess *Z. multiflora*'s potential as a nutraceutical therapy for ulcerative colitis.

## 2. Materials and methods

### 2.1. Study design and patients

This multicenter, randomized, placebo-controlled, triple-blind clinical trial was conducted on patients with UC at the Alimentary Tract Research Center and two gastroenterology clinics affiliated with Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran, from October 2023 to May 2024. The trial utilized a parallel-group design with a 1:1 allocation ratio. The diagnosis of UC by a gastroenterologist relies on the patient's medical history, physical examination, and laboratory data (including stool and blood tests). Additionally, diagnostic criteria such as the Rome Diagnostic Criteria III for functional gastrointestinal disorders (bloating, constipation, and diarrhea), the Mayo grading scale, and findings from endoscopy and colonoscopy are considered. Inclusion criteria for the study encompass individuals aged between 18 and 65 years, with a body mass index (BMI) ranging from 18.5 to 30 kg/m2, and a confirmed diagnosis of UC by a gastroenterologist. This BMI range was selected to avoid participants who are underweight, as they may have acute inflammatory responses associated with malnutrition and severe disease activity. Additionally, individuals with obesity were excluded as excessive adipose tissue, particularly visceral fat, can disrupt normal immune regulation and contribute to chronic low-grade inflammation, potentially complicating the interpretation of the treatment's effects. By selecting this BMI range, the study aimed to assess the effects of *Z. multiflora* in individuals with a more stable inflammatory status, thereby improving the validity of the findings. The duration of the disease should be between 6 months and 5 years. Exclusion criteria comprise patients in the acute phase of the disease, those with autoimmune diseases and other inflammatory conditions (such as renal, cardiovascular, or hepatic diseases, various cancers, and HIV), thyroid disorders, a history of gastric surgeries, diabetes mellitus, pregnancy and lactation, and sensitivity to *Z. multiflora*. Patients who experienced the onset of the acute phase of the disease, underwent changes in medication, initiated anti-inflammatory and antioxidant supplements, or demonstrated non-compliance with the study (defined as acceptance and adherence to less than 80% of the intervention process) were excluded from the trial.

### 2.2. Preparation of Z. multiflora elixirs

The hydroalcoholic extract of *Z. multiflora* was obtained from Giah Essence Phytopharmaceutical Company, located in Gorgan, Golestan, Iran, and confirmed by the Food and Drug Organization of Iran (FDO). The extract contained 20% alcohol. The composition of the extract was analyzed in the central laboratory of Shahid Chamran University of Ahvaz. The carvacrol content in the elixir was determined using the gas chromatography (GC) method. For analysis, the gas chromatography-mass spectrometry (GC-MS) system (Agilent 7890B-597A) with Mass Hunter software was utilized. An HP-5MS fused silica capillary column facilitated the separation, following a temperature program starting at 67 °C and incrementally rising to 160 °C, 190 °C, 205 °C, 220 °C, and 270 °C. Varian workstation software (MSD Chemstation E. 02. 02. 1431) managed instrument control, data acquisition, and processing. The chromatographic profile, provided in Supplementary Material 1, displays a distinct carvacrol peak with a retention time of approximately 21.93 min, confirming its presence in the *Z. multiflora* extract. The preparation of *Z. multiflora* elixir and placebo were based on a previous study (Ghorani et al., 2020). The elixirs were by dissolving the extract in simple syrup containing only sucrose and purified water (28 mg/mL) at The Center for Pharmaceutical Technology Innovation at Ahvaz Jundishapur University of Medical Sciences. Additionally, the alcohol concentration in the final syrup was reduced to less than 5%. The placebo for the elixir was prepared by dissolving 5% alcohol in simple syrup (80% w/v sucrose). Its color was adjusted to mimic that of the *Z. multiflora* elixir using sunset color (FD&C Yellow #6). The composition of the placebo, including the alcohol, sugar, and other components, closely resembled that of the *Z. multiflora* elixirs. Due to the scent of the medicinal elixir, associated with thymol and carvacrol, a minimal amount of thymol was included in the placebo solution.

### 2.3. Randomization and blinding

Eligible individuals, based on the inclusion criteria, were randomly assigned to either the *Z. multiflora* supplement group or the placebo group. Randomization was performed using a balanced block randomization method with the help of specialized software (www.sealedenvelope.com), employing blocks of 4 with random sequences of group assignments (e.g., BABA, BBAA, ABAB). Allocation concealment was ensured by using sealed, opaque, and sequentially numbered envelopes prepared by an independent statistician. Participants were sequentially placed into these blocks according to their enrollment order and were randomly allocated to either the intervention or control group. Randomization was stratified based on disease severity, as assessed by the Partial Mayo Score (pMayo Score), to ensure balanced distribution of participants across groups. This trial was triple-blind, ensuring that patients, the investigator (who enrolled participants), and the data analyst were unaware of the group assignments. To achieve blinding, a pharmacology consultant coded the supplements and placebos, and the researcher administered the intervention in the order of patient enrollment without knowledge of the content (supplement or placebo).

### 2.4. Intervention

The prescribed daily dose for each patient was 6 mg/kg/day, calculated according to the elixir concentration (28 mg/mL) and administered based on the patient's weight three times a day over two months. To ensure accurate dosage, each patient was given a dropper. This dosage was selected based on previous clinical trials investigating the effects of *Z. multiflora* in respiratory diseases, which demonstrated significant reductions in inflammatory markers at doses ranging from 3 to 10 mg/kg/day (Alavinezhad et al., 2022; Ghorani et al., 2020). Specifically, the 6 mg/kg/day dosage provided a balance between efficacy and safety, as reported in prior studies, while minimizing the risk of adverse effects. The intervention duration of two months was chosen to allow sufficient time for the extract's anti-inflammatory and immunomodulatory effects to manifest, as observed in similar studies (Alavinezhad et al., 2022; Ghorani et al., 2020). This period aligns with recommendations for evaluating chronic conditions such as UC, where longer durations may offer a more reliable assessment of treatment efficacy. The *Z. multiflora* extract and placebo were packaged in dark-colored bottles and coded by a collaborating pharmacologist. Patient follow-up was conducted every ten days through phone calls and interviews. Patients with compliance below 80% were excluded from the study.

### 2.5. Side effects

In the *Z. multiflora* group, one patient with a history of constipation withdrew from the study due to exacerbation of constipation after two weeks of medication use. In the placebo group, no adverse effects were observed.

### 2.6. Demographic information, dietary intakes, and physical activity

Initially, a demographic information and medical history questionnaire was completed for the patients. Demographic characteristics were

obtained through the demographic information questionnaire. The demographic information included age, gender, marital status, family history of disease, medication use, education level, employment, smoking habits, and ethnicity. Dietary intake was assessed using a 24-h recall (one weekday and one weekend day). The dietary data were evaluated using the Nutritionist IV software. Physical activity was assessed using the International Physical Activity Questionnaire (IPAQ) short form to evaluate and control for differences between the intervention and control groups. All patients were advised not to alter their diet, physical activity, or other lifestyle-related factors throughout the study to maintain consistency and reduce potential confounding effects.

### 2.7. Anthropometric measurements

We utilized a standalone height-measuring device (Seca, Hamburg, Germany) to determine height. The individual stood upright, barefoot, with heels together, maintaining a straight back, and aligning the head with the Frankfort plane. Height was recorded to the nearest millimeter. Weight assessments were conducted using a calibrated scale (Seca, Hamburg, Germany) positioned on a flat surface, while the individual wore minimal clothing and remained shoeless. Before each measurement, we ensured the scale was properly zeroed, and weight was recorded to the nearest 0.1 kg. BMI was calculated using the equation: weight in kilograms divided by height in meters squared.

### 2.8. Inflammatory markers evaluation

Patients were instructed to fast overnight prior to blood collection as part of the patient preparation protocol. Fifteen milliliters of blood were drawn from each patient before and after intervention. The ESR was measured using the Westergren method. Blood samples collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes for measuring ESR were allowed to settle vertically for 1 h, after which the distance the red blood cells descended in millimeters was recorded as the ESR value. Serum separation and preparation involved centrifuging blood samples at a speed of 2000 g for 15 min to separate serum from cellular components. After centrifugation, the supernatant containing the serum was carefully collected and transferred to clean tubes. The serum samples were then aliquoted and stored at −80 °C until further analysis. The Enzyme-Linked Immunosorbent Assay (ELISA) kits were utilized to measure the levels of hs-CRP (Monobind Inc., United States), IL-17 (ZellBio GmbH, Germany), and IFN-γ (ZellBio GmbH, Germany) in patient serum samples.

### 2.9. Transcription factors expression analysis

Blood samples were collected into tubes containing EDTA as an anticoagulant and utilized for the isolation of Peripheral Blood Mononuclear Cells (PBMCs). Blood samples were prepared for density gradient centrifugation by allowing them to equilibrate to room temperature and diluting them 1:1 with sterile phosphate buffered saline (PBS). The diluted samples were layered onto Ficoll (BAHAR AFSHAN CO., Tehran, Iran) in 50 mL Falcon tubes and centrifuged at 400-500×*g* for 30–40 min at room temperature. Following centrifugation, PBMCs were isolated from the buffy coat layer located between the plasma and Ficoll. The buffy coat containing PBMCs was carefully aspirated and transferred to new Falcon tubes. For washing, the isolated cells were resuspended in 50 mL sterile PBS, centrifuged at 150-200×*g* for 10 min, and the supernatant was discarded. This washing step was repeated, and the cells were finally prepared for further experiments. RNA extraction was performed using the Total RNA Extraction kit according to the manufacturer's instructions (Parstous, Iran). For cDNA synthesis from RNA, the EasyTM cDNA Synthesis kit (Parstous, Iran) was used. Subsequently, the samples were stored at −20 °C until the polymerase chain reaction (PCR) reaction was conducted.

Real-time PCR was employed to evaluate the expression of the target genes. The list of primers used in this study, including their sequences, amplified fragment lengths, and GenBank accession numbers, is provided in Supplementary Material 2. Primer design was carried out using the Primer3 software. The 2X SYBR Green Real Time PCR kit (Parstous, Iran) and the Lava96 Real-time PCR Detection System (DaAn Gene Co. Ltd) were used to perform the real-time PCR reactions. The results were analyzed using the comparative Delta Delta Ct (ΔΔCt) method with the Lava96 3.2 software. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the calibrator gene. The results were reported based on the general formula $2^{-\Delta\Delta Ct}$.

### 2.10. Clinical assessments

In our study, we utilized the Partial Mayo Score (pMayo Score) to assess disease activity among patients diagnosed with UC. This scoring system focuses on three key parameters: stool frequency, rectal bleeding, and the physician's global assessment over the preceding three days. Each parameter is evaluated on a scale ranging from 0 to 3, and the total score offers an indication of the overall disease activity. Higher scores indicate more severe disease activity (Naegeli et al., 2021). Furthermore, the GSRS (Gastrointestinal Symptom Rating Scale) was utilized to assess clinical symptoms, covering a variety of gastrointestinal issues including abdominal pain, heartburn, acid reflux, hunger pains, nausea, rumbling, bloating, burping, flatulence, constipation, diarrhea, loose stools, hard stools, fecal urgency, and incomplete defecation. Each symptom was assigned a score according to the following scale: 1 = No discomfort at all, 2 = Minor discomfort, 3 = Mild discomfort, 4 = Moderate discomfort, 5 = Moderately severe discomfort, 6 = Severe discomfort, 7 = Very severe discomfort. Subsequently, the total score was calculated and reported as the final GSRS score (Lee et al., 2017).

### 2.11. Sample size estimation and statistical analysis

Based on the data obtained from the study by Ghorani et al., in 2020 (Ghorani et al., 2020), the sample size calculation was conducted using the CRP marker as the primary outcome measure. Considering the CRP marker values in the group receiving a dose of 6 mg per kg of body weight *Z. multiflora* extract before and after the intervention as two independent groups, the sample size for each group was calculated. The expected effect size (Cohen's d) was estimated at 0.75, derived from the mean difference and standard deviation of CRP values reported in the reference study. To achieve a statistical power of 95% and a type I error probability (α) of 0.05, the minimum required sample size was determined to be 46 participants per group. This calculation assumes a two-tailed hypothesis test and independent groups. G*Power software (version 3.1) was used for the sample size estimation.

Descriptive statistics and frequency distribution tables were used to present the data. The data obtained from the study were analyzed using SPSS software version 25.0 (SPSS Inc., Chicago, Illinois, USA). The normal distribution of quantitative data was assessed using the Kolmogorov-Smirnov test. Quantitative data were displayed as mean ± standard deviation. Changes in quantitative data before and after the intervention were compared using the paired *t*-test. If the assumptions for parametric tests were not met, non-parametric equivalents (the Mann-Whitney *U* test instead of the independent *t*-test and the Wilcoxon signed-rank test instead of the paired *t*-test) were used. To ensure robustness, non-parametric tests were selected for variables with skewed distributions or small sample sizes. The decision to use non-parametric methods was justified based on their ability to provide reliable results without requiring assumptions about data distribution. This approach ensures that the statistical analysis accurately reflects the data characteristics while maintaining the integrity of the results. Qualitative data were examined using the Chi-square test. To evaluate the effect of the intervention within each group, the paired *t*-test or Wilcoxon signed-rank test was employed. To compare the treatment effects between the two groups, the independent sample *t*-test or Mann-Whitney *U* test was

used. In this study, a change analysis was conducted to assess the significant effect of the intervention on the measured variable. It compared the changes between two points in time—before (pre-test) and after (post-test) the intervention—between two independent groups using a *t*-test. An intention-to-treat (ITT) analysis was performed, including all participants based on their initial group assignment, regardless of whether they completed the treatment or dropped out. To handle missing data, last observation carried forward (LOCF) was used to impute missing post-treatment values based on the last available measurement for each participant. Additionally, a sensitivity analysis was conducted by comparing the results of the ITT analysis (using LOCF) with those obtained from a complete-case analysis (excluding participants with missing data). No significant differences were observed between the two approaches, confirming the robustness of the findings. The level of significance for statistical tests was set at $p < 0.05$.

## 3. Results

Fig. 1 illustrates the participant flow according to the CONSORT guidelines. Initially, 547 participants were assessed for eligibility, of whom 455 were excluded. This resulted in 92 participants randomized into either the *Z. multiflora* intervention group (n = 46) or the control intervention group (n = 46). In the *Z. multiflora* intervention group, 2 participants were lost to follow-up due to contact failure, while 3 participants discontinued the intervention due to constipation, change in medication, and noncompliance. Similarly, in the control intervention group, 2 participants were lost to follow-up due to contact failure, and 4 participants discontinued the intervention due to change in medication and noncompliance. Finally, an intention-to-treat analysis was performed on all 92 participants.

### 3.1. Baseline characteristics

The baseline characteristics of the participants in the *Z. multiflora* and control groups were generally comparable. The mean age and BMI of participants showed no significant differences between groups ($p = 0.52$ and $p = 0.07$, respectively). Disease duration, physical activity, sex distribution, smoking status, marital status, medical center, ethnicity, education level, job status, type of colitis, and medication use (aminosalicylates, corticosteroids, immunomodulators) were also similar between the groups, with all p-values greater than 0.05. These results indicate that the two groups were well-matched at baseline, as detailed in Table 1. Additionally, there were no significant differences between the groups regarding dietary intakes, including energy, carbohydrates, protein, fat, saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, fiber, and micronutrients (Table 2).

### 3.2. Inflammatory markers

As shown in Table 3, the results indicate changes in various inflammatory markers between the *Z. multiflora* and control groups over two months. While the ESR decreased slightly in the *Z. multiflora* group, the changes in both the *Z. multiflora* ($p = 0.24$) and control ($p = 0.67$) groups were not significant, nor was the difference in ESR between groups ($p = 0.25$). However, CRP values in the *Z. multiflora* group significantly decreased ($p < 0.001$), while the control group showed no significant change ($p = 0.93$), and the difference between groups was significant ($p < 0.001$). Similarly, IL-17 values significantly decreased in the *Z. multiflora* group ($p = 0.001$), but not in the control group ($p = 0.17$), with a significant difference between groups ($p = 0.007$). IFN-γ values also significantly decreased in the *Z. multiflora* group ($p = 0.002$), but the control group showed no significant change ($p = 0.29$), and the between-group difference was significant ($p = 0.004$).

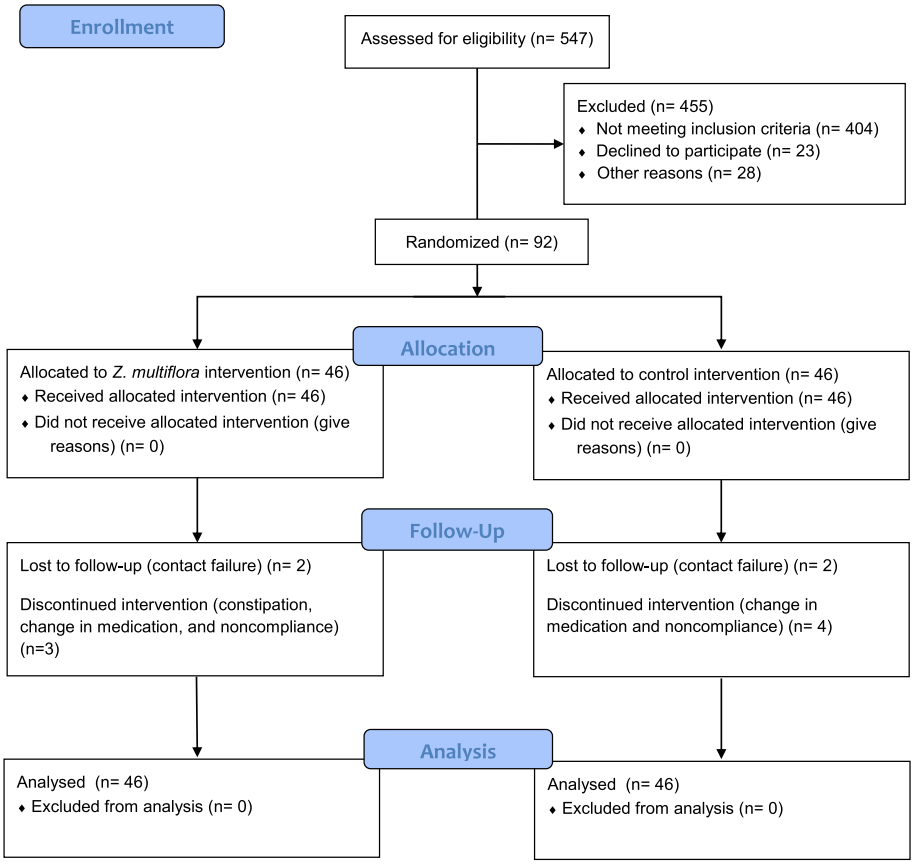


**Fig. 1.** CONSORT flow diagram.

**Table 1**
Baseline characteristics of participants.

| Variable | | | ZT (n = 46) | Control (n = 46) | p-value |
|---|---|---|---|---|---|
| **Age, years** | | | 38.0 ± 8.6 | 36.6 ± 11.6 | 0.52[a] |
| **BMI, kg/m$^2$** | | | 27.3 ± 4.1 | 25.9 ± 3.3 | 0.07[a] |
| **Disease duration, months** | | | 32.7 ± 16.8 | 33.9 ± 17.0 | 0.89[a] |
| **Physical activity, (MET-minute/week)** | | | 1126 ± 217 | 1317 ± 405 | 0.45[a] |
| **Sex** | **Male, n (%)** | | 18 (39.1) | 24 (52.2) | 0.20[b] |
| | **Female, n (%)** | | 28 (60.9) | 22 (47.8) | |
| **Smoking status** | **Yes, n (%)** | | 7 (15.2) | 11 (23.9) | 0.29[b] |
| | **No, n (%)** | | 39 (84.8) | 35 (76.1) | |
| **Marital status** | **Married, n (%)** | | 38 (82.6) | 31 (67.4) | 0.09[b] |
| | **Single, n (%)** | | 8 (17.4) | 15 (32.6) | |
| **Medical center** | **Alimentary tract research center, n (%)** | | 11 (23.9) | 9 (19.6) | 0.49[b] |
| | **Gastroenterology clinic 1, n (%)** | | 10 (21.7) | 15 (32.6) | |
| | **Gastroenterology clinic 2, n (%)** | | 25 (54.3) | 22 (47.8) | |
| **Ethnicity** | **Persian, n (%)** | | 8 (17.4) | 10 (21.7) | 0.68[b] |
| | **Arab, n (%)** | | 18 (39.1) | 20 (43.5) | |
| | **Lur, n (%)** | | 20 (43.5) | 16 (34.8) | |
| **Education** | **None, n (%)** | | 1 (2.2) | 1 (2.2) | 0.76[b] |
| | **Primary, n (%)** | | 8 (17.4) | 7 (15.2) | |
| | **Middle, n (%)** | | 7 (15.2) | 12 (26.1) | |
| | **Diploma, n (%)** | | 18 (39.1) | 17 (37.0) | |
| | **Higher education, n (%)** | | 12 (26.1) | 9 (19.6) | |
| **Job** | **None, n (%)** | | 23 (50.0) | 20 (43.5) | 0.45[b] |
| | **Self-employed, n (%)** | | 6 (13.0) | 7 (15.2) | |
| | **Employee, n (%)** | | 12 (26.1) | 8 (17.4) | |
| | **Worker, n (%)** | | 4 (8.7) | 10 (21.7) | |
| | **Retired, n (%)** | | 1 (2.2) | 1 (2.2) | |
| **Type of colitis** | **Ulcerative proctitis, n (%)** | | 14 (30.4) | 18 (39.1) | 0.68[b] |
| | **Left sided ulcerative colitis, n (%)** | | 15 (32.6) | 13 (28.3) | |
| | **Extensive ulcerative colitis, n (%)** | | 17 (37.0) | 15 (32.6) | |
| **Medications** | **Aminosalicylates, n (%)** | **Yes, n (%)** | 41 (89.1) | 40 (87.0) | 0.74[b] |
| | | **No, n (%)** | 5 (10.9) | 6 (13.0) | |
| | **Corticosteroids, n (%)** | **Yes, n (%)** | 13 (28.3) | 8 (17.4) | 0.21[b] |
| | | **No, n (%)** | 33 (71.7) | 38 (82.6) | |
| | **Immunomodulators, n (%)** | **Yes, n (%)** | 9 (19.6) | 11 (23.9) | 0.61[b] |
| | | **No, n (%)** | 37 (80.4) | 35 (76.1) | |

Data are presented as mean and standard deviation (SD) or median for quantitative variables, and as absolute (N) and relative frequencies (%) for qualitative variables.
Abbreviations: *Zataria multiflora* (ZT), Body mass index (BMI).

[a] Independent sample *t*-test (parametric) or Mann-Whitney *U* test (nonparametric) was used for the comparison of quantitative variables between the two treatment groups.
[b] The chi-square test was used to compare qualitative variables between the two treatment groups.

**Table 2**
Dietary intakes of participants.

| Variable | ZT (n = 46) | Control (n = 46) | p-value |
|---|---|---|---|
| **Energy, kcal/day** | 2063.2 ± 409.0 | 1960.5 ± 304.5 | 0.17 |
| **Carbohydrate, g/d** | 261.1 ± 51.7 | 248.1 ± 38.5 | 0.51 |
| **Protein, g/d** | 71.4 ± 14.1 | 67.8 ± 10.5 | 0.48 |
| **Fat, g/d** | 57.8 ± 11.4 | 54.9 ± 8.5 | 0.25 |
| **SFA, g/d** | 17.1 ± 3.4 | 16.6 ± 3.6 | 0.47 |
| **MUFA, g/d** | 20.8 ± 4.1 | 19.8 ± 3.0 | 0.36 |
| **PUFA, g/d** | 19.2 ± 3.6 | 18.1 ± 3.2 | 0.12 |
| **Fiber, g/d** | 15.8 ± 3.1 | 15.0 ± 2.3 | 0.35 |
| **Vitamin A, μg/d** | 835.3 ± 165.5 | 793.7 ± 123.2 | 0.17 |
| **Vitamin E, mg/d** | 6.5 ± 1.3 | 6.1 ± 0.9 | 0.18 |
| **Vitamin D, μg/d** | 1.8 ± 0.4 | 1.7 ± 0.5 | 0.26 |
| **Vitamin C, mg/d** | 49.2 ± 9.7 | 46.8 ± 7.2 | 0.17 |
| **Zinc, mg/d** | 11.6 ± 3.5 | 11.2 ± 3.1 | 0.52 |
| **Magnesium, mg/d** | 135.2 ± 11.9 | 133.1 ± 9.6 | 0.64 |
| **Selenium, μg/d** | 18.9 ± 2.3 | 18.5 ± 2.1 | 0.46 |
| **Beta carotene, μg/d** | 294.2 ± 84.3 | 277.5 ± 66.9 | 0.29 |

Data are presented as mean and standard deviation (SD).
Abbreviations: *Zataria multiflora* (ZT), Saturated fatty acid (SFA), Monounsaturated fatty acid (MUFA), Polyunsaturated fatty acid (PUFA).
*Independent sample *t*-test was used for the comparison of quantitative variables between the two treatment groups.

### 3.3. Immune mediator genes

The findings of this study demonstrated that intervention with *Z. multiflora* resulted in a significant decrease in the expression levels of NF-κB ($p = 0.002$), T-bet ($p = 0.006$), and ROR-γt ($p < 0.001$) genes compared to the control group (Fig. 2). The mean difference in fold change for NF-κB expression in the *Z. multiflora* and control groups was $-0.14 \pm 0.15$ and $-0.03 \pm 0.16$, respectively. Similarly, the mean differences in fold change for T-bet and ROR-γt gene expression were $-0.12 \pm 0.20$ (*Z. multiflora* group) versus $-0.02 \pm 0.12$ (control group) for T-bet, and $-0.43 \pm 0.26$ (*Z. multiflora* group) versus $0.03 \pm 0.23$ (control group) for ROR-γt. In contrast, there were no significant changes in the mean differences of fold changes in the expression of GATA3 ($p = 0.09$) and FOXP3 ($p = 0.17$) genes. The mean difference in fold change for GATA3 expression was $-0.03 \pm 0.17$ in the *Z. multiflora* group compared to $0.01 \pm 0.16$ in the control group. Similarly, the mean difference in fold change for FOXP3 expression was $0.03 \pm 0.20$ in the *Z. multiflora* group compared to $0.02 \pm 0.11$ in the control group.

### 3.4. Clinical symptoms

Based on the GSRS rating scale, clinical symptoms including heartburn ($p < 0.001$), acid reflux ($p < 0.001$), bloating ($p = 0.01$), flatulence ($p = 0.02$), diarrhea ($p < 0.001$), loose stool ($p < 0.001$), and fecal urgency ($p = 0.03$) showed significant reduction following a two-month intervention with *Z. multiflora*. However, no significant changes were observed for abdominal pain ($p = 0.31$), hunger pain ($p = 0.46$), nausea ($p = 0.08$), rumbling ($p = 0.16$), burping ($p = 0.05$), constipation ($p = 0.31$), hard stools ($p = 0.66$), and incomplete defecation ($p = 0.57$). Furthermore, as shown in Fig. 3, a comparison of the mean differences between the *Z. multiflora* and placebo groups demonstrated significant reduction in the gastrointestinal symptoms of heartburn ($p = 0.002$), acid reflux ($p = 0.007$), bloating ($p < 0.001$), flatulence ($p = 0.003$), diarrhea ($p = 0.001$), loose stool ($p = 0.001$), and fecal urgency ($p < 0.001$). However, the mean differences in other symptoms between the *Z. multiflora* and placebo groups were not significant, including

**Table 3**
Inflammatory markers before and after the intervention.

| Variable | Group | Baseline | After two months | p-value | Difference | p-value[c] |
|---|---|---|---|---|---|---|
| **ESR, mm/hr** | **ZT** | 17.04 ± 5.12 | 16.52 ± 4.83 | 0.24[a] | −0.52 ± 3.39 | 0.25 |
| | **Control** | 17.19 ± 5.68 | 17.41 ± 5.76 | 0.67[a] | 0.22 ± 2.77 | |
| **CRP, mg/L** | **ZT** | 3.80 ± 1.17 | 3.06 ± 1.04 | **<0.001**[a] | −0.74 ± 0.62 | **<0.001** |
| | **Control** | 3.74 ± 1.36 | 3.75 ± 1.40 | 0.93[b] | 0.01 ± 0.55 | |
| **IL-17, pg/mL** | **ZT** | 45.74 ± 20.06 | 36.96 ± 17.80 | **0.001**[a] | −8.78 ± 11.89 | **0.007** |
| | **Control** | 46.32 ± 19.02 | 46.37 ± 17.33 | 0.17[b] | 0.05 ± 9.44 | |
| **IFN-γ, pg/mL** | **ZT** | 44.01 ± 19.56 | 34.04 ± 15.16 | **0.002**[a] | −9.97 ± 9.61 | **0.004** |
| | **Control** | 45.98 ± 22.76 | 47.73 ± 19.94 | 0.29[a] | 1.75 ± 9.29 | |

Data are presented as mean and standard deviation (SD).
Abbreviations: *Zataria multiflora* (ZT), Erythrocyte Sedimentation Rate (ESR), C-Reactive Protein (CRP), Interleukin-17 (IL-17), Interferon gamma (IFN-γ).
[a] The Wilcoxon signed-rank test was used to compare non-parametric variables before and after treatment.
[b] Paired sample *t*-test was used to compare parametric variables before and after treatment.
[c] Mann-Whitney *U* test was used to compare variables between two treatment groups.

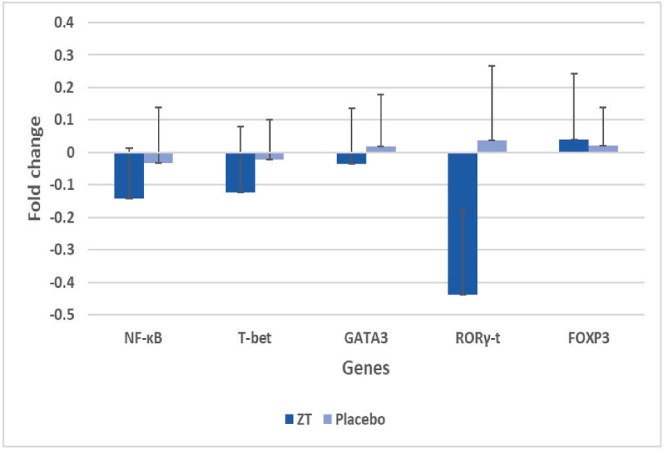


**Fig. 2.** The mean differences and standard deviations of immune mediator genes expression of *Z. multiflora* and placebo groups. The intervention involving *Z. multiflora* resulted in a significant decrease in the expression levels of NF-κB ($p = 0.002$), T-bet ($p = 0.006$), and RORγ-t ($p < 0.001$) genes compared to the placebo group. Statistical comparisons were conducted using the Independent sample *t*-test and Mann-Whitney *U* test to evaluate variables between the two treatment groups. Abbreviations: *Zataria multiflora* (*Z. multiflora*), Nuclear Factor-kappa B (NF-κB), T-box transcription factor (T-bet), GATA binding protein 3 (GATA3), Retinoic acid receptor-related Orphan Receptor gamma t (RORγ-t), Forkhead Box P3 (FOXP3).

abdominal pain ($p = 0.98$), nausea ($p = 0.32$), rumbling ($p = 0.141$), constipation ($p = 0.204$), burping ($p = 0.451$), hunger pain ($p = 0.44$), hard stools ($p = 0.981$), and incomplete defecation ($p = 0.85$).

The mean GSRS final scores before (40.26 ± 11.51) and after (30.82 ± 8.55) the *Z. multiflora* intervention showed significant reduction ($p < 0.001$). In contrast, there were no significant changes before (37.54 ± 10.67) and after treatment (37.43 ± 10.18) with the placebo ($p = 0.80$). The comparison between the mean differences of GSRS final scores in the *Z. multiflora* (−9.44 ± 6.63) and placebo (−0.11 ± 2.10) groups revealed a significant difference ($p < 0.001$). The mean p-Mayo score before the intervention in the *Z. multiflora* group was 3.56 ± 2.25, and after the intervention, it decreased to 3.45 ± 2.02. However, this change was not statistically significant ($p = 0.48$). Similarly, in the placebo group, the mean p-Mayo score before the intervention was 3.17 ± 2.19, and it remained 3.17 ± 1.69 after the intervention ($p = 0.93$), indicating no significant change. The mean difference in p-Mayo score was −0.11 ± 0.48 in the *Z. multiflora* group and 0.00 ± 0.21 in the placebo group, with no significant differences observed between the groups ($p = 0.24$).

## 4. Discussion

The study investigated the impact of *Z. multiflora* hydroalcoholic extract on the clinical symptoms, inflammatory markers, and gene expression of immune mediators in individuals diagnosed with UC. The *Z. multiflora* group showed notable enhancements, including decreases in clinical symptoms such as heartburn, acid reflux, bloating, flatulence, diarrhea, loose stool, and fecal urgency. In addition, there were significant reductions in the levels of inflammatory markers CRP, IL-17, and IFN-γ, as well as a noticeable decrease in the expression of the NF-κB, T-bet, and ROR-γt genes. This study is the initial investigation into the possible therapeutic benefits of *Z. multiflora* as a nutraceutical for the complementary treatment of patients with UC.

### 4.1. The expression of immune mediator genes

Our investigation showed that the administration of *Z. multiflora* significantly reduced the expression of NF-κB, T-bet, and ROR-γt genes in PBMCs of patients with UC compared to the control group. Although the current literature lacks clinical trials specifically addressing this issue, other *in vivo* and *in vitro* research provides evidence that supports similar conclusions. For example, thymol and carvacrol, key components of *Z. multiflora*, have demonstrated anti-inflammatory properties by modulating the NF-κB pathway. Carvacrol enhances NF-κB p65 phosphorylation and reduces phosphorylated inhibitor of kappa B alpha (IκBα), crucial for NF-κB activation, while thymol blocks NF-κB nuclear translocation by stabilizing IκBα (Gholijani et al., 2016; Tahmasebi et al., 2019). These effects suppress the inflammatory signaling cascade, as observed in models of acetic acid-induced colitis (Chamanara et al., 2019). Additionally, *Zataria multiflora* essential oil (ZMEO), encapsulated in dendrosomes, has shown dual activity by inhibiting NF-κB activation and promoting the nuclear factor erythroid 2-related factor 2 (NRF2) pathway. This dual modulation suggests potential anti-inflammatory and antioxidant effects (Aminizadeh et al., 2020). Our findings are consistent with those of previous studies, which demonstrated that *Z. multiflora* administration and its constituents reduced NF-κB gene expression. However, since these studies were conducted on animal models or *in vitro* environments, there are limitations in comparing the results and their generalizability.

Thymol and carvacrol also modulate T-helper cell responses by downregulating transcription factors such as T-bet (TH1), GATA-3 (TH2), and ROR-γt (TH17), while not significantly altering FoxP3 (Treg) expression (Gholijani and Amirghofran, 2016). In contrast, studies have reported mixed results depending on the disease model. For instance, Ariaee et al. (2018) observed a decrease in FoxP3 expression in allergic rhinitis patients treated with *Z. multiflora* syrup, while Kianmehr et al. (2017a) found an increase in FoxP3 expression in asthmatic mice treated with *Z. multiflora* extract (Ariaee et al., 2018; Kianmehr et al., 2017a). Additionally, another experimental study demonstrated that

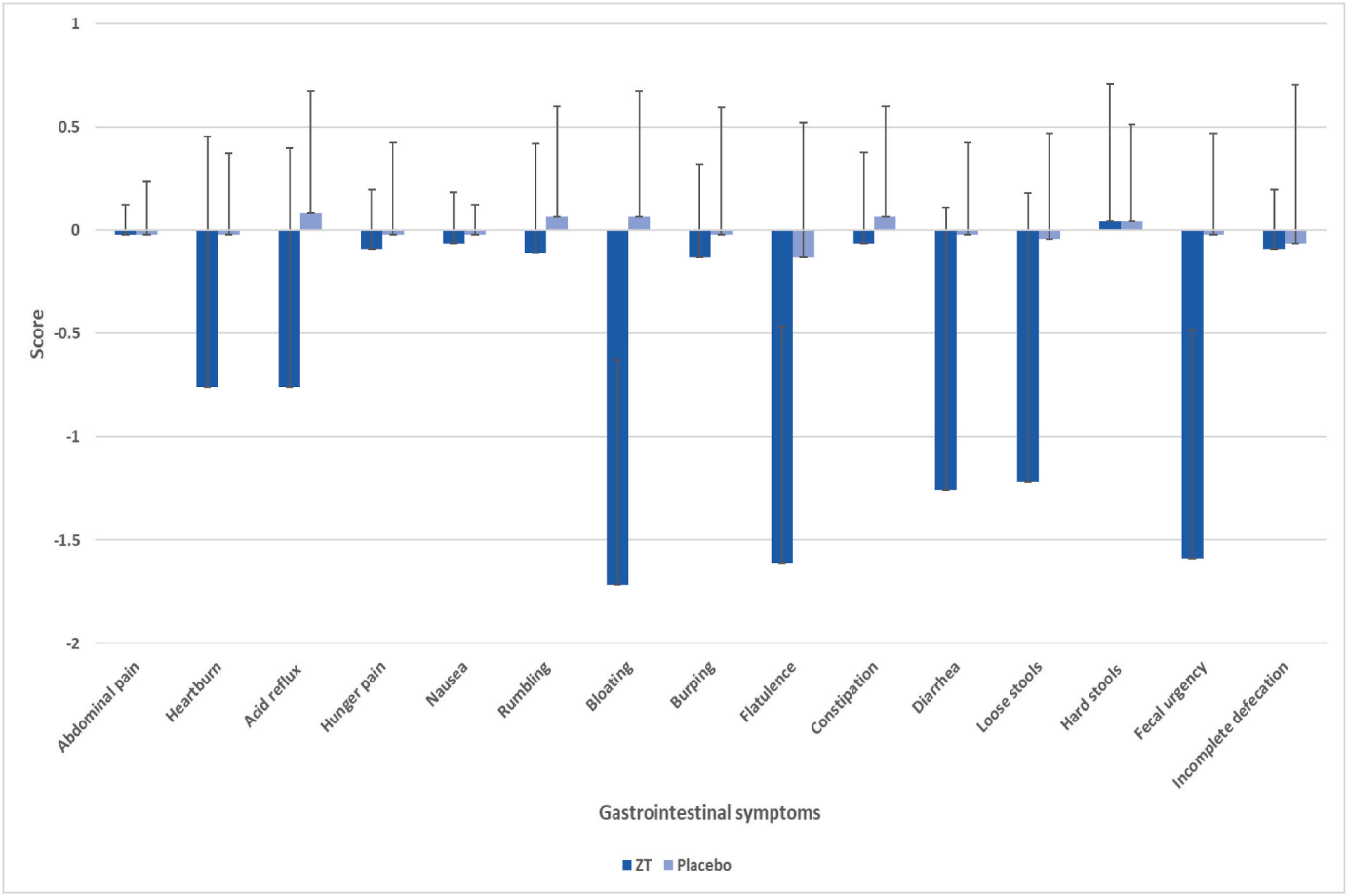


**Fig. 3.** Comparison of the mean differences and standard deviations of gastrointestinal symptoms between the *Z. multiflora* and placebo groups. The intervention with *Z. multiflora* resulted in significant reductions in gastrointestinal symptoms, including heartburn ($p = 0.002$), acid reflux ($p = 0.007$), bloating ($p < 0.001$), flatulence ($p = 0.003$), diarrhea ($p = 0.001$), loose stool ($p = 0.001$), and fecal urgency ($p < 0.001$). Statistical comparisons were conducted using the Independent sample *t*-test and Mann-Whitney *U* test to evaluate variables between the two treatment groups. Abbreviations: *Zataria multiflora (Z. multiflora)*.

carvacrol has immunomodulatory effects, showing reduced expression of FOXP3 alongside decreased IFN-γ and increased IL-4 and IL-17 levels (Kianmehr et al., 2016). Although similarities have been observed in the results of studies, there are also many differences. The discrepancies between our study and others may be due to several reasons. Different diseases (UC, allergic rhinitis, asthma) involve distinct immune pathways, leading to variable effects of treatments. Studies on mice may only partially translate to human immune responses due to species-specific differences. Variations in the cell types studied (PBMCs vs. splenocytes) can lead to different gene expression outcomes. The specific components of *Z. multiflora* (e.g., thymol, carvacrol) may have distinct effects when used in isolation versus as part of a compound mixture. Finally, differences in dosage, treatment duration, and experimental settings can result in variable outcomes.

### 4.2. Inflammatory markers

The study's findings indicate that consuming *Z. multiflora* extract for two weeks significantly decreased hs-CRP, IL-17, and IFN-γ serum levels in UC patients. However, it did not significantly impact ESR. The effects of *Z. multiflora* on inflammatory markers have been thoroughly researched in respiratory conditions. However, so far, no clinical trials have investigated its impact on inflammatory bowel diseases such as UC.

C-reactive protein (CRP) is an acute-phase protein produced in response to inflammation and is a significant biomarker in UC (Malakar, 2020). In a study evaluating asthmatic patients, a two-month treatment with 5 and 10 mg/kg/day of *Z. multiflora* significantly decreased serum hs-CRP levels (Alavinezhad et al., 2022). Similarly, a placebo-controlled trial in COPD patients revealed reductions in hs-CRP following a two-month intervention with 3 and 6 mg/kg/day of *Z. multiflora* (Ghorani et al., 2020). Additionally, carvacrol, a major component of *Z. multiflora*, reduces hs-CRP levels by suppressing cyclooxygenase-2 (COX-2) expression, thereby inhibiting prostaglandin synthesis (Alavinezhad et al., 2018). Thymol, another active compound, further supports this by downregulating pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α, which stimulate CRP production (Yu et al., 2016). Furthermore, thymol diminishes inflammation caused by free radicals in the myocardium, resulting in reduced CRP levels in rats with isoproterenol-induced myocardial infarction (Meeran et al., 2015). In our research, administering 6 mg/kg/day of *Z. multiflora* to UC patients resulted in a notable decrease in hs-CRP levels, consistent with the described mechanisms. This outcome corresponds with earlier studies showing reduced hs-CRP levels following *Z. multiflora* treatment.

IL-17, a pro-inflammatory cytokine produced by Th17 cells, plays a central role in UC by promoting chronic inflammation in the colonic mucosa (Cătană et al., 2015; Jiang et al., 2014). Our study results showed that after a two-month intervention with *Z. multiflora*, serum IL-17 levels decreased in patients with UC. These results align with prior studies, including one in allergic rhinitis patients, where *Z. multiflora* reduced IL-17 expression (Ariaee et al., 2018). Similarly, in an asthma model, *Z. multiflora* extract suppressed Th17 activity, reducing IL-17 expression in splenocytes (Kianmehr et al., 2017a). Thymol and carvacrol have also demonstrated IL-17-lowering effects in experimental models, supporting their potential as modulators of inflammatory

pathways (Badr et al., 2022; Kianmehr et al., 2016; Mahmoodi et al., 2019). The results from these studies on various diseases concerning the effects of *Z. multiflora* and its components show a decrease in gene expression and serum levels of IL-17, along with modulation of TH17, which corroborates our study's findings. However, it is essential to note that most of these studies were experimental, and due to study constraints, comparing them with clinical trial studies related to ulcerative colitis was impossible.

IFN-γ contributes to UC pathogenesis by enhancing immune activation and compromising the epithelial barrier (Bevivino and Monteleone, 2018; Popov et al., 2021). Modulating IFN-γ and its pathways presents potential therapeutic advantages, making it a significant subject of current research in IBD (Wallace et al., 2021). Our study results demonstrated that a two-month intervention with *Z. multiflora* reduced serum IFN-γ levels in patients with UC. The primary focus of clinical trial studies in this area has been on respiratory diseases. In contrast, studies in conditions such as asthma and mustard gas-induced lung disorders found that *Z. multiflora* increased IFN-γ levels, reflecting its protective role in infections and Th1 immunity (Alavinezhad et al., 2020; Khazdair et al., 2020a). Additionally, carvacrol treatment in asthma patients was associated with elevated levels of IFN-γ, a cytokine produced by Th1 cells that combat infections (Ghorani et al., 2021a). The impact of *Z. multiflora* on IFN-γ levels varies between UC and respiratory diseases due to differences in immune mechanisms. In UC, IFN-γ promotes inflammation by stimulating immune activation and compromising the epithelial barrier, making its reduction beneficial for alleviating inflammation and improving outcomes (Naschberger et al., 2023; Saadh et al., 2023). Conversely, in respiratory diseases like asthma or mustard gas-induced lung disorders, IFN-γ supports Th1 immunity and combats infections, with increased IFN-γ levels following *Z. multiflora* treatment contributing to improved respiratory symptoms (Bergeron et al., 2023; Khazdair and Boskabady, 2022). Our findings suggest that *Z. multiflora* suppresses Th1 cell activation and proliferation, leading to decreased IFN-γ production. This effect may occur through the disruption of signaling pathways and inhibition of transcription factors like T-bet, which is critical for IFN-γ gene expression. Discrepancies across studies likely reflect disease-specific immune responses, influenced by factors such as local versus systemic effects, epithelial barrier integrity, and variations in disease stage and severity.

ESR is a widely used laboratory test to measure inflammation in the body. In ulcerative colitis, ESR is a valuable indicator for assessing disease activity and severity (Cioffi et al., 2015). Our study did not show significant effects of *Z. multiflora* consumption on ESR levels in UC patients. There are limited clinical trials in this area. A randomized trial evaluating the safety and tolerability of carvacrol in healthy subjects at two doses (1 and 2 mg/kg/day) found that treatment led to a significant decrease in ESR levels, which remained within the normal range (Ghorani et al., 2021b). Similarly, an experimental study on a rheumatoid arthritis model demonstrated that treatment with carvacrol-loaded nanoparticles significantly lowered ESR levels compared to untreated rats (Gholijani et al., 2020). ESR is a nonspecific indicator of inflammation and may not accurately reflect acute changes in UC due to its sensitivity to other factors such as age, anemia, and comorbid conditions. Unlike CRP, which promptly responds to inflammation, ESR is less reliable in assessing real-time inflammatory changes in UC (Assasi et al.; Turner et al., 2011).

### 4.3. Clinical symptoms

Gastrointestinal symptoms, including heartburn, acid reflux, bloating, flatulence, diarrhea, loose stools, and fecal urgency, improved significantly following *Z. multiflora* treatment. Additionally, the mean GSRS score showed a significant reduction. Jamalizadeh et al. demonstrated the efficacy of a *Zataria multiflora* and *Trachyspermum copticum* combination in alleviating IBS symptoms, such as pain, bloating, and constipation (Jamalizadeh et al., 2022). Similarly, a randomized trial involving ICU nurses reported a reduction in gastrointestinal symptom scores after one month of *Z. multiflora* treatment, though no significant differences were observed between the treatment and placebo groups at the end of the trial period (Vafaarani et al., 2015). Several experimental studies suggested mechanisms for *Z. multiflora* and its components, carvacrol and thymol, to alleviate clinical symptoms of UC. They reduce oxidative stress markers, inhibit inflammatory cytokine pathways, and exhibit antimicrobial properties against colitis-associated pathogens (Nakhai et al., 2007). Carvacrol and thymol specifically demonstrate gastroprotective effects in animal models of colitis, reducing mucosal damage, inflammatory markers like myeloperoxidase (MPO) activity and cytokines, and alleviating abdominal hyperalgesia. These findings suggest that *Z. multiflora* and its components could be valuable natural treatments for UC, potentially offering alternatives to conventional therapies (Arigesavan and Sudhandiran, 2015; de Santana Souza et al., 2017; Tahmasebi et al., 2019).

### 4.4. Potential mechanisms of Action and clinical outcomes

The therapeutic effects of *Z. multiflora* in UC appear to result from its ability to modulate interconnected molecular pathways and improve clinical outcomes. A significant finding of this study was the down-regulation of NF-κB, T-bet, and ROR-γt gene expression, along with reductions in serum levels of IL-17, IFN-γ, and CRP. These molecular changes directly target central inflammatory mechanisms involved in UC pathogenesis and are reflected in clinical improvements.

NF-κB plays a pivotal role in activating inflammatory cascades by driving the transcription of pro-inflammatory cytokines such as IL-17 and IFN-γ, which exacerbate intestinal inflammation. The observed inhibition of NF-κB likely disrupted this cycle, reducing cytokine production and immune cell infiltration in the colonic mucosa (Barnabei et al., 2021). Additionally, the suppression of T-bet and ROR-γt indicates modulation of Th1 and Th17 immune responses. T-bet promotes Th1 differentiation and IFN-γ secretion, contributing to epithelial barrier disruption and chronic inflammation. ROR-γt drives Th17 cell activity, producing IL-17, which recruits neutrophils and worsens inflammation (Ohara et al., 2024; Stadhouders et al., 2018).

The bioactive components of *Z. multiflora*, such as thymol and carvacrol, further enhance its therapeutic potential through their antioxidant and anti-inflammatory properties. These compounds are known to inhibit the degradation of IκB, thereby preventing NF-κB activation. Experimental studies have also demonstrated their ability to reduce oxidative stress and suppress cyclooxygenase-2 (COX-2) expression, reinforcing their role in inflammation regulation (Imran et al., 2022; Khazdair et al., 2018; Nagoor Meeran et al., 2017).

Clinically, these molecular changes translated into significant reductions in GSRS scores and improvements in symptoms such as diarrhea, loose stool, and fecal urgency. The alignment of molecular modulation with clinical benefits underscores *Z. multiflora*'s potential as a complementary treatment for UC. However, future studies are needed to confirm these effects over the long term and explore additional mechanisms underlying its efficacy.

### 4.5. Strengths and limitations

This study is notable for its novelty, as it is the first to investigate the potential effects of *Zataria multiflora* hydroalcoholic extract as a complementary treatment for patients with UC. The innovative nature of this research provides a valuable foundation for future studies in this area. The triple-blind, randomized, placebo-controlled design enhances the validity and reliability of the findings. Additionally, the comprehensive analysis conducted in this study, which examines the effects of *Z. multiflora* on clinical symptoms, inflammatory markers, and the expression of immune mediator genes, offers a holistic understanding of the impact of *Z. multiflora* on UC.

However, the study also has some limitations. The two-month

intervention period and limited sample size present important limitations that may impact the generalizability and long-term applicability of the findings. While the sample size of 92 participants was sufficient to detect statistically significant changes in the primary and secondary outcomes, it may not fully capture the variability in treatment response across diverse populations. Larger-scale studies involving participants from different demographic and clinical backgrounds are needed to confirm the findings and ensure their broad applicability.

Additionally, the two-month duration of the intervention, though adequate for observing short-term effects, may not provide sufficient insight into the sustainability of symptom relief or the potential for long-term immune modulation. Ulcerative colitis is a chronic condition characterized by periods of relapse and remission; therefore, extended follow-up studies are necessary to determine whether the observed benefits of *Z. multiflora* persist over time and to evaluate its potential in reducing relapse rates. Future research should also explore the cumulative effects of prolonged *Z. multiflora* use and assess its safety profile over longer durations. These efforts would provide a more comprehensive understanding of its role as a complementary treatment in managing UC and inform its integration into clinical practice.

Another limitation is the absence of biopsies from the lesion site, which restricts the ability to directly assess the local inflammatory and immunological response in the colonic tissue. While serum markers and PBMC analysis provide valuable systemic insights, they may not fully reflect the localized effects of *Z. multiflora* at the site of inflammation. Future studies should incorporate endoscopic evaluations and biopsies to measure the impact on mucosal healing and tissue-specific immune responses.

Furthermore, due to financial constraints, we were unable to conduct follow-up tests at multiple time points during the two-month period. As a result, trends in symptom progression and molecular changes over time could not be explored. Longitudinal analyzes would provide a more detailed understanding of how *Z. multiflora* influences disease activity over time and help identify early versus late responses to treatment.

### *4.6. Research implications and recommendations*

Future studies should prioritize conducting larger-scale clinical trials with extended intervention periods to validate further and substantiate the therapeutic potential of *Z. multiflora* in managing UC. Longitudinal studies are essential to assess the sustainability of symptom relief and explore the potential long-term effects of *Z. multiflora* treatment. Additionally, there is a need for deeper investigation into the underlying mechanisms by which *Z. multiflora* affects inflammatory markers and immune mediator gene expression in UC patients. Exploring various formulations or dosages of *Z. multiflora* could provide valuable insights into optimizing its efficacy and ensuring its safety for clinical use. Ultimately, confirming these initial findings could facilitate the integration of *Z. multiflora* into mainstream treatment approaches for UC, offering patients a broader range of potentially effective therapeutic options.

## 5. Conclusion

Our study revealed that the hydroalcoholic extract of *Zataria multiflora* demonstrated significant improvements in clinical symptoms and reduced inflammatory markers and immune mediator gene expression in patients with UC. The treatment effectively alleviated symptoms such as heartburn, bloating, diarrhea, and fecal urgency, while also reducing serum levels of CRP, IL-17, and IFN-γ and downregulating the expression of NF-κB, T-bet, and ROR-γt genes. While these findings suggest that *Z. multiflora* holds promise as a complementary treatment for UC, it is important to acknowledge the limitations of this study, including the relatively small sample size and the short duration of the intervention. The study's results may not be broadly applicable to all UC populations, especially those with different disease severities, comorbidities, or demographic characteristics. Therefore, further research is needed in diverse populations, including larger, multicenter trials with longer follow-up periods, to confirm these findings and assess the long-term effects of *Z. multiflora* in UC management.

## CRediT authorship contribution statement

**Mehrnaz Morvaridi:** Writing – review & editing, Writing – original draft, Visualization, Resources, Project administration, Methodology, Investigation, Data curation. **Naheed Aryaeian:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **Pezhman Alavinejad:** Validation, Resources, Methodology, Conceptualization. **Seyed Saeed Seyedian:** Validation, Resources, Methodology, Conceptualization. **Mehri Ghafourian:** Validation, Resources, Methodology, Conceptualization. **Nima Bakhtiari:** Validation, Resources, Methodology, Investigation, Data curation, Conceptualization. **Maryam Seyedtabib:** Validation, Software, Formal analysis.

## Ethics approval and consent to participate

The present study was approved by the Medical Ethics Committee of Iran University of Medical Sciences *(IR.IUMS.REC.1402.207)*, ensuring compliance with ethical standards set forth in the Declaration of Helsinki. Furthermore, the study was registered in the Iranian Registry of Clinical Trials *(IRCT20120415009472N27)*, a registry that promotes transparency and accountability in clinical research. This process involved explaining the purpose, procedures, potential risks, and benefits of the study to the participants, ensuring that they understood the information and voluntarily agreed to participate. All patients involved in this study signed and filled informed consent forms, ensuring their voluntary participation and understanding of the research process.

While placebo-controlled trials are the gold standard for assessing the efficacy of interventions, we acknowledge that the use of a placebo in conditions like ulcerative colitis can raise ethical concerns, particularly in terms of withholding active treatment from patients. To mitigate these concerns, we ensured that all participants were fully informed about the study design during the informed consent process, including the possibility of being randomized to the placebo group. Additionally, participants in the placebo group were provided with standard care for ulcerative colitis, in line with current clinical guidelines, to ensure that they were not deprived of necessary medical treatment. We also monitored all participants closely throughout the trial, ensuring that any adverse events were promptly addressed.

## Consent for publication

Not applicable in the declarations section.

## Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

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## Declaration of Competing interest

I have nothing to declare.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2025.119527.

## Data availability

Data will be made available on request.

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