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The immunomodulatory potential of *Commiphora gileadensis* and *Cynanchum radians* extracts in mice

[El potencial inmunomodulador de los extractos de *Commiphora gileadensis* y *Cynanchum radians* en ratones]

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Abstract: We aimed to investigate the effect of *Commiphora gileadensis* and *Cynanchum radians* extracts on T lymphocyte subsets. Fifty mice were divided into five groups: control group, *C. gileadensis* acetone-treated group, *C. gileadensis* sap-treated group, *C. radians* acetone-treated group, and *C. radians* methanolic-treated group. Blood was collected for flow cytometric analysis to measure various lymphocyte populations. A one-way ANOVA test was chosen to compare between-group differences. Data shows that there was a significant decrease in the number of total lymphocytes, $CD3^+$, $CD4^+$, and $CD8^+$ cells in all mice treated with all extracts compared with controls. In addition, the CD4/CD8 ratio was significantly higher in mice treated with *C. gileadensis* acetone extract. Furthermore, there was an elevation of $CD4^+/CD25^+$ and $CD8^+/CD25^+$ cells in all groups treated with all extracts as compared with the control group. This study concluded that *C. gileadensis* and *C. radians* have immunomodulatory properties.

Keywords: *Commiphora gileadensis*; *Cynanchum radians*; T lymphocyte; $CD4^+$; $CD8^+$

Resumen: Nuestro objetivo fue investigar el efecto de los extractos de *Commiphora gileadensis* y *Cynanchum radians* sobre los subconjuntos de linfocitos T. Cincuenta ratones se dividieron en cinco grupos: grupo control, grupo tratado con acetona de *C. gileadensis*, grupo tratado con savia de *C. gileadensis*, grupo tratado con acetona de *C. radians* y grupo tratado con metanol de *C. radians*. Se recolectó sangre para el análisis por citometría de flujo para medir diversas poblaciones de linfocitos. Se eligió una prueba ANOVA de un solo factor para comparar las diferencias entre grupos. Los datos muestran que hubo una disminución significativa en el número total de linfocitos, células $CD3^+$, $CD4^+$ y $CD8^+$ en todos los ratones tratados con todos los extractos en comparación con los controles. Además, el ratio CD4/CD8 fue significativamente mayor en los ratones tratados con el extracto acetónico de *C. gileadensis*. Asimismo, hubo un aumento de las células $CD4^+/CD25^+$ y $CD8^+/CD25^+$ en todos los grupos tratados con todos los extractos en comparación con el grupo control. Este estudio concluyó que *C. gileadensis* y *C. radians* tienen propiedades inmunomoduladoras.

Palabras clave: *Commiphora gileadensis*; *Cynanchum radians*; Linfocitos T; $CD4^+$; $CD8^+$

INTRODUCTION

The immune system plays an essential role in maintaining bodily homeostasis. Both the innate and adaptive immune systems are the two main components of the immune system (Wu *et al*., 2022). The vital function of the immune system in host protection is most evident when mishaps result in serious infections and malignancies (Huo *et al*., 2022). The immune system can be described as a complex interconnected network that relies on several immune cells, such as lymphocytes, dendritic cells, monocytes, natural killer (NK) cells, and others (Huo *et al*., 2022). T lymphocytes (T cells) play a major role in the adaptive immune process by facilitating cell-based immune responses that promote host health and prevent the onset of various diseases (Sun *et al*., 2023). T lymphocytes can be classified into two main categories: $CD4^+$ T helper cells as well as $CD8^+$ T cytotoxic cells (Sun *et al*., 2023). During activation, cytotoxic T lymphocytes (CTLs) can initiate the destruction of cancer cells via granule cytokinesis and apoptosis mediated by the Fas ligand (FasL). $CD4^+$ T cells are a type of antigen receptor T cell that plays an essential role in the activating and controlling of immune cells (Huo *et al*., 2022). It is currently believed that regulatory T cells (Tregs) provide an additional means of maintaining peripheral self-tolerance (Bienvenu *et al*., 2005). In addition to $CD4^+$ Tregs, $CD8^+$ cytotoxic T cells are becoming a significant subpopulation of Treg cells. Naturally occurring $CD4^+$/$CD25^+$ are the most well-characterized of these regulatory cell types, while $CD8^+$/$CD25^+$ cells have drawn less research attention (Churlaud *et al*., 2015). The precise regulatory role of these cells is unknown, but they impede a broad spectrum of inflammatory and autoimmune illnesses.

The primary source of contemporary pharmacological discoveries is medicinal plants. Both emerging and developed nations have come to recognize the medical and economic benefits of medicinal plants, leading to an upsurge in the cultivation of such plants (Qari *et al*., 2021). Saudi Arabia boasts many diverse floras on the Arabian Peninsula, with a wealth of agricultural and therapeutic plants (Elaidarous *et al*., 2022). *Commiphora gileadensis* (*C. gileadensis*), a member of the Burseraceae family, grows naturally in Saudi Arabia. Traditionally, *C. gileadensis* has been used for treating a variety of ailments and has been prescribed as an anti-inflammatory and antihypertensive agent, as well as an analgesic for fever and pain symptoms (Alhazmi *et al*., 2022; Bin Mokaizh *et al*., 2023; Mokaizh *et al*., 2024a). *Cynanchum radians* (*C. radians*) is one of several plants that also grow in various parts of Saudi Arabia (Qari *et al*., 2021; Elaidarous *et al*., 2022), but its potential biological effects have received only limited attention.

Natural compounds originating from plants possess diverse biological characteristics that have the potential to elicit or maintain immune responses (Gomez-Cadena *et al*., 2020). However, the effects of these two plants (*C. gileadensis* and *C. radians*) on T lymphocyte subsets have not been investigated. Thus, the aim of this research is to elucidate the effect of *C. gileadensis* and *C. radians* extracts on T lymphocyte subsets such as $CD4^+$, $CD8^+$, $CD4^+$/$CD25^+$, and $CD8^+$/$CD25^+$ cells using flow cytometric analysis.

MATERIALS AND METHODS

Plant material and preparation of extracts

Sampling site for Commiphora gileadensis

C. gileadensis was obtained in December 2023 from the Alaab Valley, a high mountain region close to the Al-Madinah region in the western region of Saudi Arabia.

Preparation of Commiphora gileadensis sap extract

The *C. gileadensis* branches were wounded, the growing tips were clipped off at approximately 5 mm from the ends, and the gushing sap was collected. The sap was agitated for 15 min at room temperature before being diluted in an equivalent volume of ethanol and centrifuged for 10 min at 10,000 rpm. The supernatant was then kept at -20℃ until it was analyzed (Wineman *et al*., 2015).

Preparation of *Commiphora gileadensis* acetone extract

The *C. gileadensis* leaves and branches were dried at 60℃ for six hours in a vacuum oven before being broken into small bits with a razor blade to create a powder. Then, 10 g of the raw *C. gileadensis* powder was immersed in 200 mL of acetone for three days at room temperature, with the acetone being replaced every day and stirred using a magnetic stirrer. The extract was dried using a rotary evaporator to remove acetone residues. The sample was then stored at -20℃ for subsequent analysis (Suleiman, 2015).

Sampling site for Cynanchum radians

C. radians samples were collected from the northern region of Saudi Arabia in November 2023. The roots and branches of the plants were cleaned with water, dried, and ground into fine powder.

Preparation of Cynanchum radians methanolic extract

The finely ground pieces of the plant were mixed with 450 ml of methanol using sterile filter papers for 3 days with continuous agitation at room temperature. A rotary evaporator was used to dry the sample to remove any leftovers. The sample was then kept for additional testing at -20℃.

Preparation of Cynanchum radians acetone extract

Fine ground plant material was combined with acetone, and these compositions were allowed to stand at room temperature. The supernatant (i.e., the clear liquid on top) was then poured into glass containers. This procedure was carried out three times. The resulting extract was spread out on aluminum foil and allowed to dry at ambient temperature to eliminate acetone.

Experimental design

Fifty male BALB/c mice weighing between 20 g and 25 g at birth were acquired from the Umm Al-Qura University animal house at two months of age. The mice were kept in a large, well-ventilated room with a 12-hour light-dark cycle, a standard rodent cage, and woodchip bedding at a temperature of 25℃. For the entire duration of the study, all the mice were given tap water and a conventional rodent meal. After acclimating for two weeks, the 50 mice were randomly divided into five groups. The first group (control group) was not treated. The second group was the *C. gileadensis* acetone-treated group. These mice were fed and hydrated in the same manner as the group under negative control. The third group was the *C. gileadensis* sap-treated group. The fourth group was the *C. radians* acetone-treated group. The *C. radians* methanolic treatment group was the fifth group. All treated mice received 200 mg/kg body weight of *C. radians* acetone daily via intragastric gavage. From all groups, blood samples were withdrawn in ethylenediamine tetraacetic acid (EDTA) tubes for flow cytometry analysis at the conclusion of the experiment. These experimental procedures were conducted from February to March 2024 at Taif University.

Flow cytometry staining and analysis

The EDTA blood samples were stacked over a Ficoll-Paque density gradient medium in a conical tube. The mixture was centrifuged at 400 × g for 30 min at 4°. After cautious aspiration, the mononuclear cell interface layer was moved to a fresh tube. Phosphate-buffered saline (PBS) was used twice to wash the cells. Fluorochrome-conjugated monoclonal antibodies of CD3 (PE), CD4 (BV510), CD8 (PerCP), and CD25 (APC) were incubated with cells for 30 min at 4℃, with great care taken to limit light exposure. The samples were then reconstituted in a fluorescence-activated cell sorting (FACS) buffer after being washed with PBS and were evaluated using a flow cytometer (a BD FACSCanto II system). The data was analyzed via FACS Diva software. The lymphocytes were identified and gated in accordance with their side scatter (SSC) and forward scatter (FSC). The CD4/CD8 ratio was calculated by dividing the CD4 count by the CD8 count.

Statistical analysis

GraphPad Prism software (version 9) was used for the statistical testing. We used the one-way ANOVA to compare the levels of various lymphocyte subsets among the groups, with the data expressed as the mean ± standard deviation (SD). The data was set at $p<0.05$ for significant results.

RESULTS

To investigate the immunomodulatory effects of *C. gileadensis* and *C. radians*, different T lymphocyte populations were assessed using flow cytometry. The results show that the total number of lymphocytes decreased in all mice treated with *C. radians* acetone ($p<0.01$), methanolic extract ($p<0.0001$), *C. gileadensis* acetone ($p<0.05$), or sap extract ($p<0.05$) as compared with the control group. Among all extracts, the *C. radians* methanolic extract was the most potent one (Figure No. 1). Furthermore, the $CD3^+$ cell levels were lower in all groups treated with *C. radians* acetone ($p<0.0001$), methanolic extract ($p<0.0001$), *C. gileadensis* acetone ($p<0.0001$), or sap extract ($p<0.01$) than in the control group with untreated mice. The *C. gileadensis* acetone extract had the most potent effect (Figure No. 2). The data revealed a reduction in the percentage of $CD4^+$ cells in mice treated with *C. radians* acetone ($p<0.01$), methanolic extract ($p<0.0001$), *C. gileadensis* acetone ($p<0.0001$), or sap extract ($p<0.01$) compared with the mice in controls; the *C. radians* methanolic extract being the most potent one (Figure No. 3A). In addition, the percentage of $CD8^+$ cells decreased in mice given *C. radians* acetone ($p<0.001$), methanolic extract ($p<0.0001$), *C. gileadensis* acetone ($p<0.0001$), or sap extract ($p<0.001$); the *C. gileadensis* acetone extract being the most potent one (Figure No. 3B). The data shows that all extracts improved the CD4/CD8 ratio; however, only mice treated with *C. gileadensis* acetone extract recorded a significant difference in CD4/CD8 ratios ($p<0.05$, Figure No. 3C). Furthermore, there was an increase in $CD4^+/CD25^+$ cells in all groups treated with *C. radians* acetone ($p<0.05$), methanolic extract ($p<0.0001$), *C. gileadensis* acetone ($p<0.05$), or sap extract ($p<0.05$), compared with the controls (Figure No. 4A). Finally, the mice in the experimental groups had higher levels of $CD8^+/CD25^+$ cells than the controls after treatment with *C. radians* acetone ($p<0.01$), methanolic extract ($p<0.001$), *C. gileadensis* acetone ($p<0.05$), or sap extract ($p<0.05$, Figure No. 4B). The *C. radians* methanolic extract was the most potent extract on both $CD4^+/CD25^+$ and $CD8^+/CD25^+$.

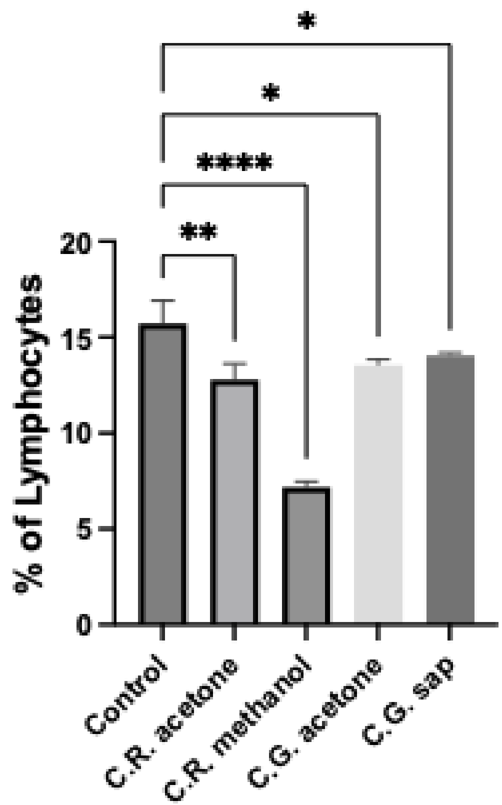

Figure No. 1
Levels of total lymphocytes
Mice were treated with different extracts of *C. gileadensis* and *C. radians*. The levels of total lymphocytes were measured in the blood using flow cytometry. Data are presented as mean ± standard deviation

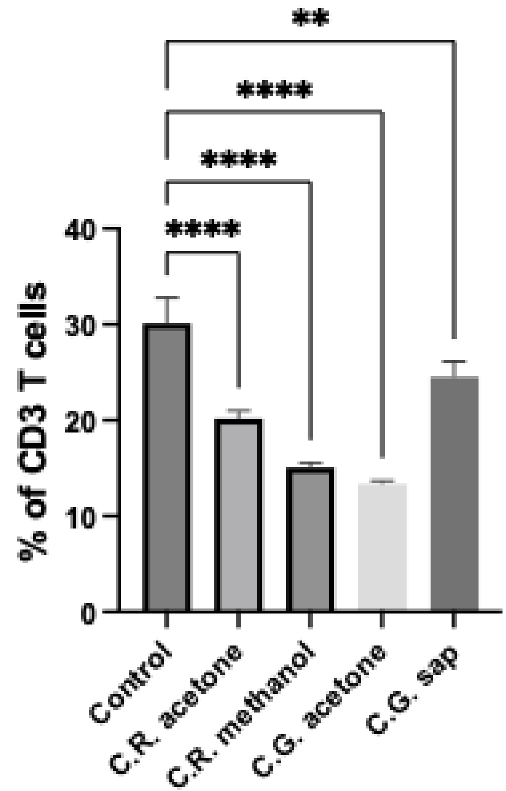

Figure No. 2
Percentage of CD3+ T cells
Mice were treated with different extracts of *C. gileadensis* and *C. radians* and the percentage of CD3 T cells was measured in the blood using flow cytometry. Data are presented as mean ± standard deviation

DISCUSSION

Saudi Arabia possesses a highly varied collection of plant species that contain a valuable assortment of genetic resources in the form of both agricultural and medicinal plants (Elaidarous *et al*., 2022). *C. gileadensis* and *C. radians* are plants that grow extensively in Saudi Arabia, and there has recently been growing interest in research exploring the biological applications of *C. gileadensis*, with limited attention paid on *C. radians.* *C. gileadensis* has been discovered to possess antibacterial, antioxidant, and antihypertensive properties (Alhazmi *et al*., 2022; Bin Mokaizh *et al*., 2023). However, only a limited amount of research has been conducted to shed light on the immunomodulatory mechanism of *C. gileadensis*. Therefore, the present study explores the immunomodulatory effects of both *C. gileadensis* and *C. radians* extracts on different T lymphocyte populations.

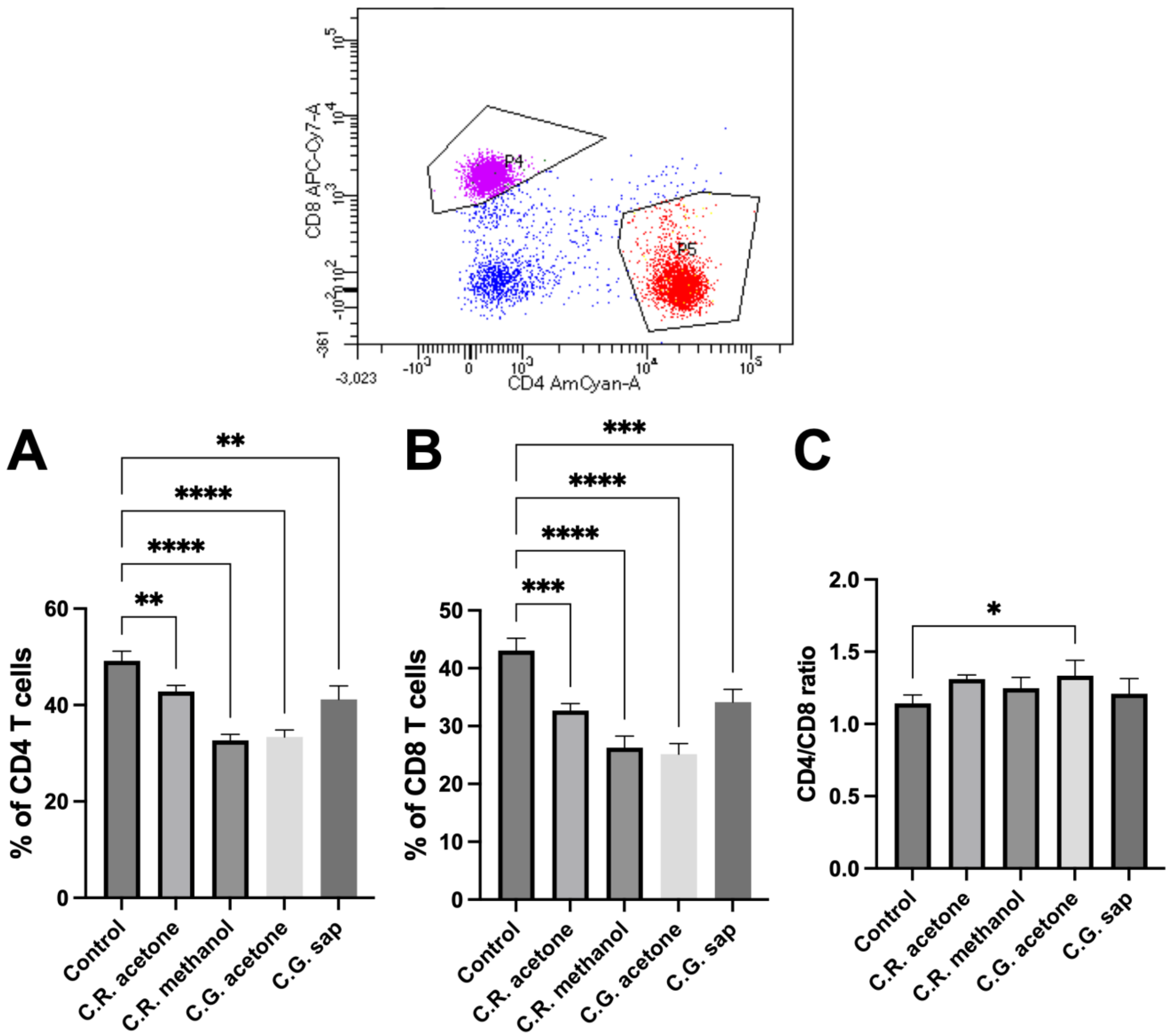

Figure No. 3
Levels of CD4+, CD8+ T cells, and CD4/CD8 ratio
Mice were treated with different extracts of *C. gileadensis* and *C. radians* and the percentage of CD4 and CD8 T cells (A and B) was measured in the blood using flow cytometry. CD4/CD8 ratio was also calculated (C). The top panel is a representative dot plot of CD4 and CD8 distribution.
Data are presented as mean ± standard deviation

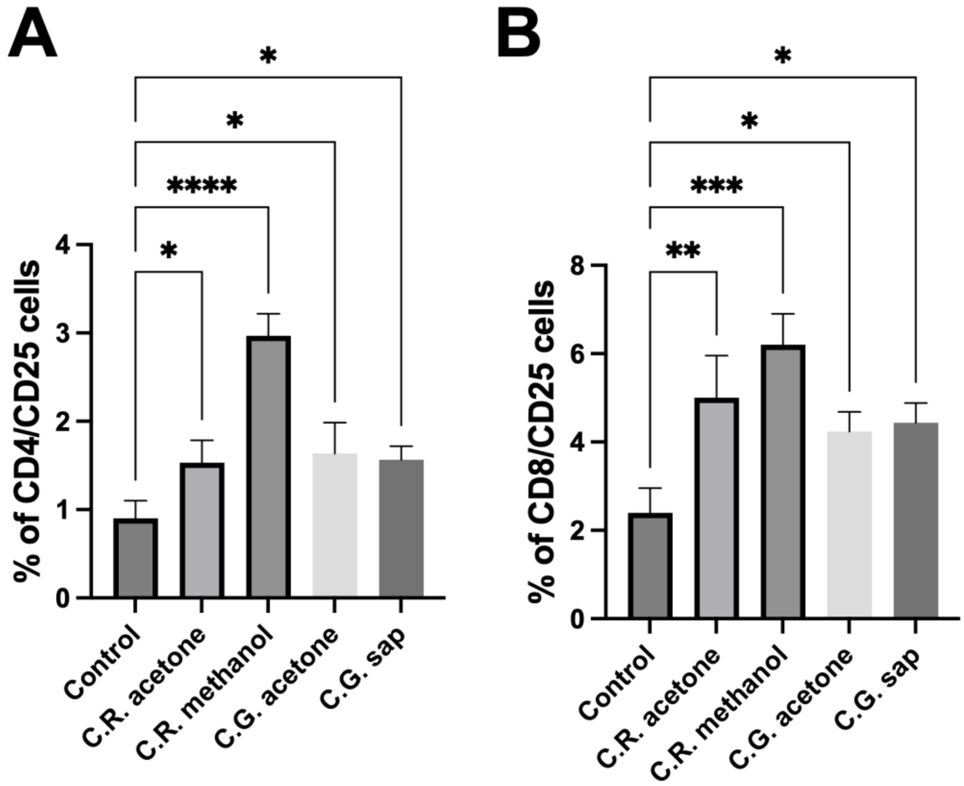

Figure No. 4
Percentage of $CD4^+/CD25^+$ and $CD8^+/CD25^+$ cells
Mice were treated with different extracts of *C. gileadensis* and *C. radians* and the percentage of $CD4^+/CD25^+$ (A) and $CD8^+/CD25^+$ (B) cells was measured in the blood using flow cytometry.
Data are presented as mean ± standard deviation

In this study, we first evaluated the percentage of total lymphocytes and $CD3^+$ T cells in mice treated with *C. gileadensis* and *C. radians*. We observed a reduced prevalence of these cells, and more specifically, the number of $CD4^+$ and $CD8^+$ cells diminished following treatment with extracts from these plants. Many studies have been conducted that have confirmed the immunomodulatory properties of natural products. For instance, a study found that aqueous *Prosopis strombulifera* extract decreases $CD4^+$ and $CD8^+$ cell levels and T cell response (Persia *et al*., 2023). $CD8^+$ secretes many pro-inflammatory cytokines, such as tumor necrosis factor (TNF) and IFN-γ. Several herbal medications improve immunity by regulating processes linked to T cells (Li *et al*., 2022). The adaptive immune system functions as a secondary barrier against pathogen-specific invasion, predominantly by utilizing T and B cells. In brief, T cells play a significant role in facilitating cellular immunity and in regulation of the immune response (Li *et al*., 2022). CD4 helper cells and CD8 cytotoxic cells are the two main types of T cells in the mammalian immune system (Karimi *et al*., 2021), and these T cells could develop into several types of Th helper cells, such as Th1, Th2, Th17, or Treg cells, which are capable of mediating diverse types of immune responses (Moudgil & Venkatesha, 2023).

In this study, we have shown that the two plants (*C. gileadensis* and *C. radians*) enhance the immune system by increasing the CD4/CD8 ratio. Consistent with the findings from our data, several natural products have been proven to enhance the immune system via various mechanisms (Shin *et al*., 2020; Wang *et al*., 2020; Al Mahmud *et al*., 2023; Bakir, 2023). For example, administering

Ophiopogon japonicus extract to mice produces an elevation in the CD4+/CD8+ ratio and an improvement in the functions of humoral and cellular immune (Li *et al*., 2022). Previous studies have suggested that natural compounds improve the immune system in several ways, including equilibrating pro- and anti-inflammatory cytokines (Alanazi *et al*., 2023). We measured the pre-treatment and post-treatment levels of $CD4^+/CD25^+$ and $CD8^+/CD25^+$ regulatory T cells in our study subjects, and both the *C. gileadensis* and *C. radians* extracts induced the proliferation of these cells. Interestingly, these cells release cytokines that reduce inflammation, such as IL-10 and transforming growth factor (TGF)-β (Murakami *et al*., 2022). An inverse relationship between the $CD4^+/CD25^+$ and $CD8^+$ cells has been reported-interestingly, $CD4^+/CD25^+$ cells inhibit the expression of $CD8^+$ cells (Murakami *et al*., 2022).

Limited data were found in the literature regarding the biological significance of *C. radians*, but some related examples of *C. radians* with immunomodulatory activities within the same family were reported. For example, several compounds and flavonoids derived from the extract of the *Cynanchum acutum* plant demonstrate anti-inflammatory effects in a rat model of type 2 diabetes mellitus (T2DM). The examined substances decreased the levels of inflammatory indicators and controlled the expression of NF-κB and miR-146a (Abdelhameed *et al*., 2021). NF-κB is a group of transcription factors that can control the activity of several genes related to immunological and inflammatory responses. Studies have shown that *Cynanchum paniculatum* increases the expression of NF-κB p65 subunit mRNA, and affects the downstream genes involved in the production of inflammatory mediators COX-2 and interleukin-1 beta (IL-1β) (Chen *et al*., 2020). The root of *Cynanchum wilfordii* has been ascribed with anti-inflammatory properties (Jang *et al*., 2016). Consistent with our results, $CD4^+$ and $CD8^+$ cells were reduced in mice treated with *Cynanchum wilfordii* (Jang *et al*., 2016).

The observed immunomodulatory properties of *C. gileadensis* are currently better understood than those of *C. radians* (Mokaizh *et al*., 2023; Mokaizh *et al*., 2024a). Enzymatic bioassays have been used to evaluate the anti-inflammatory properties of *C. gileadensis* (Mokaizh *et al*., 2023), revealing that this herb significantly reduces lipoxygenase activity. Previous research findings have revealed that lipoxygenase plays a significant role in the inflammatory process (Mokaizh *et al*., 2023). In addition, *C. gileadensis* has been reported to play a profound role in wound healing and the inflammatory process (Alhazmi *et al*., 2022). The antimicrobial and anti-inflammatory properties of *C. gileadensis* promote wound healing (Alhazmi *et al*., 2022). Furthermore, it was reported that flavonoids, lignans, and steroids are among the components produced by *C. gileadensis* with anti-inflammatory activities (Al-Abdallat *et al*., 2023; Shadid *et al*., 2023; Ahmed *et al*., 2024). Another study found that *C. gileadensis* contains heptasiloxane, hexadecamethyl, which has been documented to possess anti-inflammatory properties (Mokaizh *et al*., 2024b). *C. gileadensis* suppresses the activation of transcription factors, particularly NF-κB, which is involved in the growth and proliferation of cancer cells. NF-κB is also implicated in the control of several factors linked to inflammation, including cytokines, chemokines, and inflammation-associated fibroblasts (Bouslama *et al*., 2019).

CONCLUSION

C. gileadensis and *C. radians* reduce T cell response and $CD4^+$ and $CD8^+$ cell levels. Furthermore, both herbs increase the prevalence of Treg cells (i.e., $CD4^+/CD25^+$ and $CD8^+/CD25^+$). The data generated in this study indicate that these plants possess immunomodulatory properties in mice, but further research is needed to confirm these findings.

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ETHICAL APPROVAL

The animal study protocol was accredited by the National Committee for Bioethics at Taif University and the Committee considered that the proposal fulfills the requirements (approval number: 45-226).

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