

### Glycyrrhiza Glabra Extract Modulates Type 1 T Helper (TH1) and Regulatory T Cell-Related Immune Responses in an Animal Model of Breast Cancer

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#### ABSTRACT

**Background:** It is well-known that TH1 and Treg cells exert anti- and pro-tumorigenic activity, respectively. Thus, TH1 cell suppression together with Treg cell hyperactivation contribute to tumor development. Glycyrrhiza glabra (G. glabra) has various immunomodulatory and anti-tumorigenic properties.

**Objective:** To explore the impacts of G. glabra extract on different parameters related to TH1 and Treg cells using a breast cancer (BC) model.

**Methods:** Four groups of Balb/C mice bearing 4T1 cell-induced BC were treated intraperitoneally with either saline or G. glabra extract at dosages of 50, 100 and 150 mg/kg (G. glabra-50, G. glabra-100, and G. glabra-150, respectively). After sacrificing animals on day 26, the frequency of splenic TH1 and Treg cells, the levels of serum IFN- $\gamma$ , TGF- $\beta$ , and IL-12, and intra-tumoral expressions of granzyme-B, T-bet, and FOXP3 were assessed.

**Results:** Compared to untreated tumor control (UTC) group, treatment with G. glabra-50, G. glabra-100, or G. glabra-150 increased the survival rate, percentage of TH1 cells, and T-bet expression. Conversely, they reduced the percentage of Treg cells, and serum TGF- $\beta$  levels. In comparison to the UTC group, treatment with G. glabra-50 and G. glabra-150 increased the serum IL-12 levels. Treatment with G. glabra-100 and G. glabra-150 boosted granzyme-B expression. Treatment with G. glabra-50 decreased the FOXP3 expression. IL-12 levels were higher in mice treated with G. glabra-150 compared to those treated with G. glabra-100.

**Conclusion:** Treatment of mice with BC using G. glabra extract improved survival rate, reduced tumor growth, and modulated T cell-mediated immune responses.

Keywords: Breast Cancer, Cytokines, Glycyrrhiza Glabra, Mice, Regulatory T Cells, TH1 Cells

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#### Cite this article as:

Yousefi S, Basirjafar P, Zandvakili R, Masoumi J, Zainodini N, Khorramdelazad H, Gheitasi M, Jafarzadeh A. Glycyrrhiza Glabra Extract Modulates Type 1 T Helper (TH1) and Regulatory T Cell-Related Immune Responses in an Animal Model of Breast Cancer. *Iran J Immunol.* 2024; 21(2):132-146, doi: 10.22034/iji.2024.101133.2735.

Received: 2023-12-24 Revised: 2024-01-27 Accepted: 2024-01-28

### INTRODUCTION

Breast cancer (BC) is the most common kind of malignancy in women, with an assessed 2,300,000 new-diagnosed cases (11.70% of all cancers in women) and 685,000 fatalities in 2020 (1). Development of BC is strongly associated with several risk factors, including family history, age, overweight, menopausal status, usage of oral contraceptives, alcohol consumption, smoking, lifestyle choices, and genetic parameters (2). The most popular cancer treatments include radiotherapy, chemotherapy, and surgery, however, they are frequently unsuccessful and have severe side effects in the majority of patients (3, 4). Therefore, it is vital to develop innovative therapeutic approaches (such as immunotherapy and targeted therapy) to achieve full remission without negative side effects. Given that both the progression-free survival time as well as overall survival rate are favorable, immunotherapy is a feasible alternative treatment option for individuals with metastatic or recurrent malignancy (5).

Within the immune system, effector TH1 cells exhibit strong anti-tumor capabilities by producing IL-2, TNF- $\alpha$  and IFN- $\gamma$ , activating CD8+ cytotoxic T lymphocytes (CTLs) as well as natural killer (NK) cells to eliminate cancerous cells (6). In contrast, regulatory T (Treg) cells secrete immunedepressive cytokines IL-35, TGF-B and, IL-10 supporting cancer development (7, 8). In addition to Treg cells, other populations of immunosuppressive cells as well as other immunosuppressive molecules contribute to tumor development and progression (9). Therefore, it is very important to restore the patient's immune activity as soon as possible. Natural products and herbal medicine can activate the immune system, exert direct suppressive effects on malignant cells, and reduce drug-related side effects (10).

As a medicinal plant, *Glycyrrhiza* glabra (G. glabra, also known as licorice) encompasses various biologically active components, such as glycyrrhizic acid,

glabridin, liquiritin, and liquiritigenin as well as flavonoids (11-13). G. glabra possesses a broad range of pharmacological impacts, such as immunoregulatory, anti-inflammatory, antioxidant, antitumor, and antiproliferative effects (14, 15). Various immunomodulatory properties have been attributed to G. glabra or its components. Glycyrrhizin and glycyrrhetinic acid induces interferon (IFN) which activates macrophages and augments natural killer cell activity (16). Glycyrrhizin promotes IL-2 production in activated T cells as well as supports IL-12 production in LPSactivated macrophages (17, 18). Bordbar et al. demonstrated that Glycyrrhizin-treated DCs promoted T cell proliferation accompanied by IFN-y production, while reducing IL-4 production, which indicates the potentiation of TH1 responses (19). Bhattacharjee et al. found that the glycyrrhizic acid promoted the expression of IL-12, IFN- $\gamma$ , and TNF- $\alpha$ , while blocking the expression of IL-10, and TGF- $\beta$ in mouse splenocytes (20). In an animal model of allergic asthma, it was found that treatment with glycyrrhizic acid diminished the TH2derived cytokines (IL-4, IL-5, and IL-13), while enhancing TH1 cytokine (IFN-y), therefore regulating TH1/TH2 cells (21).

The anti-tumorigenic effects of G. glabra or its components were also reported in various types of malignant cells, such as gastric cancer, breast cancer, melanoma, cervical cancer, hepatoma, and prostate cancer (13). G. glabra and its components exhibit anticancer properties by controlling pro-tumorigenesis inflammatory responses, inducing cell cycle arrest, inhibiting cancer invasion and metastasis, inducing cell apoptosis as well as suppressing various pro-tumorigenic signaling pathways (22). Licorice can decrease the adverse effects of chemotherapy and radiotherapy (such as fatigue, gastrointestinal toxicity, appetite loss mucositis, and anemia) as well as enhance the quality of life and decrease fatality (22). Although licorice is regarded as a safe, natural, and potent culinary remedy, various side effects have been reported due to its excessive consumption

(13). Despite the aforementioned beneficial effects of G. glabra, its immunomodulatory and immunotherapeutic potential was not investigated enough in cancers. Thus, the aim of this research was to investigate the impacts of G. glabra extract on some TH1 and Treg cell-related parameters in an animal model of BC.

#### MATERIALS AND METHODS

#### Animals and BC Induction

The BALB/c mice (female, aged between six and eight weeks) were purchased from Royan Institute (Tehran, Iran). The mice were kept in normal circumstances with 12 h light/dark cycles, a temperature ranging from 23 to 25 °C, and a humidity level between 45% and 60%. The mice were allowed free access to the usual chow pellet and water. The research procedure underwent ethical verification by the Ethics Committee of Rafsanjan University of Medical Sciences and was officially registered with the code: IR.RUMS.REC.1399.218.

To establish the BC in the mice,  $7 \times 10^5$ 4T1 cells obtained from Iran Pasteur Institute (Tehran, Iran), were subcutaneously administered into the right flank of the animals (23). Using a digital caliper, the tumors' growth was assessed every other day during the research. To accurately assess the tumor size, a specific formula was applied (24, 25):

Tumor volume  $(mm^3)$ =length of tumor  $(mm)\times$ width of tumor<sup>2</sup>  $(mm)\times$ 0.52.

Preparation of the Hydro-alcoholic Extract of G. glabra

The *G. glabra* roots were bought from a local herbal market in Kerman (Kerman, Iran). The surface of the roots was carefully washed with distilled water, then dried in the shade and ground into powder using a grinder. The crushed roots were macerated by immersing them in 70.0% ethanol for 72 h while stirring them frequently in a shaker. Then, Whatman filter paper No. 1 was used to filter the mixture. Finally, licorice hydroalcoholic extract was semi-air-dried and kept at 4 °C till use (26).

### Treatment Program with Extract of G. glabra

The mice with BC were classified into four groups, each containing 10 mice and treated with normal saline and G. glabra extracts [at doses of 50 mg/kg (G. glabra 50), 100 mg/ kg (G. glabra 100) and 150 mg/kg (G. glabra 150)]. The treatment schedule was commenced when the tumor size had reached a range of 115-120 mm<sup>3</sup>, which occurred approximately on the twelfth day after the tumor induction. The hydroalcoholic extract of G. glabra was dissolved in saline and then administered intraperitoneally (i.p) on a daily basis from day 12 to 26 following tumor induction, as illustrated in Fig. 1. For comparison purposes, a healthy group of animals devoid of tumor cell injection or G. glabra extract administration was also included. On day 26 after the tumor induction, five randomly selected mice from each group were sacrificed, and their serum, spleen, tumor, and lungs were collected for



Fig. 1: Treatment program of breast cancer mice with the G. glabra extract.

further analysis. To calculate the survival rate, another set of five animals from each group was tracked until day 100.

#### Spleen Index Measurement

One of the main features of inflammation associated with cancer that results from myeloid cell proliferation is splenomegaly (27). To assess the impact of the therapy on the immunological alterations in the spleen, the index of this lymphoid organ was computed for each group of animals. After animal sacrifice on day 26, the spleen index in all the investigated mice was determined using the specified formula: (spleen weight/ body weight)×100.

#### *Measurement of the Frequencies of TH1 and Treg Cells in the Spleen*

After the animal sacrifice on day 26, the frequencies of TH1 and Treg cells in the mouse spleen were determined. Each spleen was flushed with RPMI-1640 to prepare a single-cell suspension, followed by the elimination of RBCs using lysis buffer. After washing the cells,  $5 \times 10^5$  splenocytes were placed in a 2.5 milliliter test tube in a volume of 100 µl. The cells expressing CD4+ IFN- $\gamma$  were designated as TH1 cells, while those expressing CD4+ FOXP3 were identified as Treg cells. The cellular surface marker CD4 was stained using the anti-mouse CD4-FITC antibody. Subsequently, the cells were fixed and permeabilized using relevant buffer (Biolegend USA) and the intracellular IFN-y and FOXP3 were stained using the anti-mouse IFN-y-PE and anti-mouse FOXP3-PE antibodies, respectively. Finally, the cells were gently rinsed and suspended in 0.5 milliliter of PBS solution. The cell

suspensions were subjected to evaluation using a flow cytometer system (Partec GmbH, Germany). The data obtained from the flow cytometer instrument were carefully analyzed using the FlowJo v7.6.1 software (Tree Star Inc., Ashland, USA).

# Assessment of the Levels of IL-12, TGF- $\beta$ , and IFN- $\gamma$ in the Serum

After the blood sample collection from the retro-orbital sinus on day 26, the serum amounts of IL-12, IFN- $\gamma$  and TGF- $\beta$  were detected by ELISA kits (BioLegend, USA) following the manufacturer's protocol. Principally, the cytokine kits were based on sandwich ELISA. Briefly, specified amounts of the serum samples and standards (in duplicate) were added to the wells of plates coated with specific capture monoclonal antibodies against mouse IL-12, IFN-γ, and TGF-β. After incubation and washing, a biotinylated monoclonal antibody against mouse IL-12, IFN- $\gamma$ , and TGF- $\beta$  was added to the related wells. After that avidinhorseradish peroxidase was added, followed by the addition of TMB substrate producing a blue color. Finally, the optical density of the produced color was determined at 450 nm using an ELISA reader system. The serum quantities of the cytokines were determined using standard curves and expressed as Pg/mL.

#### Assessment of Intra-tumoral Gene Expression

After the separation of the total RNA samples from tumor specimens, they were transcribed to complementary DNA (cDNA) using commercial kits (Bionner, Korea). The real time-PCR reactions were prepared by mixing proper amounts of SYBR Green master mix (PCR-Biosystems, UK), cDNA, as well as specific primers (Table 1) and

Table 1. Forward and reverse primers for assessment of the expression of target genes

Target	Forward	Reverse	Product
genes	(5' 3')	(5' 3')	size (bp)
T-bet	TTCCCATTCCTGTCCTTCAC	CCACATCCACAAACATCCTG	70
FOXP3	GGCCCTTCTCCAGGACAGA	GGCATGGGCATCCACAGT	61
Granzyme-B	GGGGCCCACAACATCAAAGA	GCCTTACTCTTCAGCTTTAGCA	131
β-actin	CGATGCCCTGAGGCTCTTT	TGGATGCCACAGGATTCCA	59

amplification was done using a relevant equipment (Applied Biosystems, USA). The temperature program of the real time-PCR was set to95 °C for 1 min.; then 40 consecutive cycles each including 95 °C for 5 seconds, 60 °C for 30 seconds; and lastly 72 °C for 30 seconds. The expression of the interested genes (granzyme B, T-bet, and FOXP3) to the housekeeping control ( $\beta$ -actin gene) was calculated by the 2<sup>- $\Delta\Delta$ ct</sup> formula. The amplification efficiency of the used primers was about 94.0%.

# Histological Analyses of the Tumor and Lung Tissues

The lung and tumor tissue specimens were collected from the mice after the sacrifice of the animals on day 26. The tissue samples were then fixed and embedded using formalin and paraffin, respectively. After tissue sectioning and staining (with hematoxylin and eosin), lung and tumor sections were examined under a light microscope to identify local metastasis and angiogenesis, respectively.

#### Statistical Analyses

One-way ANOVA was applied to compare the data between the two groups, while Student's T test was employed for comparing variables between the multiple groups. Furthermore, the log-rank test and Kaplan-Meier curve were utilized to compare the survival rates between the different groups.

The two-way ANOVA test was also employed to compare the body weight gaining between the different groups. All statistical analyses of the collected data were performed using the widely used Graph Pad Prism 8.0.2 software. A threshold of p < 0.05 was specified as the statistical significance level.

#### RESULTS

#### *G. glabra Extract's Impacts on the Survival Rate, and the Growth of Tumors*

There were 5 mice in each group to check the survival percentage, followed

up to day 100. In all the groups except the healthy control, all the mice died before day 100. The median survival rates for the UTC, G. glabra-50, G. glabra-100, and G. glabra-150 groups were 38, 46, 52, and 59 days, respectively. The healthy control, G. glabra-50, G. glabra-100, and G. glabra-150 groups showed a significantly greater survival rate in comparison with the UTC group (p=0.001, p=0.006, p=0.003 and p=0.001, respectively). The survival rates in the UTC groups, G. glabra-50, G. glabra-100, and G. glabra-150 groups were remarkably lower than in the healthy group (p=0.001). The survival rate in the G. glabra-50 group was lower than the G. glabra-100 and G. glabra-150 groups (p=0.02, p=0.004, respectively), (Fig. 2A).

The tumor sizes in different groups from day 12 following tumor induction to the end of the study were displayed in Fig. 2B, Table 2. The G. glabra-50 group displayed significantly decreased tumor sizes on days 18, 20, 22, 24, and 26 compared with the UTC group (*p*=0.006, *p*=0.003, *p*<0.0001, *p*<0.0001 and p=0.001, respectively). Compared with the UTC group, tumor sizes reduced in the G. glabra-100 group on days 22, 24, and 26 (p=0.003, p=0.02, and p < 0.0001, respectively). The tumor sizes also diminished in the G. glabra-150 group on days 22, 24, and 26 (p=0.0001, p=0.0001, and p < 0.0001, respectively). The tumor volumes on days 18, 20, 22, 24, and 26 after tumor induction did not significantly differ between G. glabra-treated groups (Fig. 2B). At the end of the study, the mean weight of tumors in the G. glabra-50, G. glabra-100, and G. glabra-150 groups significantly reduced in comparison with the UTC group ( $p \le 0.05$ ,  $p \le 0.05$ , and  $p \le 0.001$ , respectively) (Figs. 2C, 2D).

#### *G. glabra Extract's Impacts on the Weight of Body and Splenic Index*

The mean percentage of body weight gain was calculated for different groups from the beginning to the end of the study, without incorporating tumor and spleen weights. The mean of body weight gain in



**Fig. 2.** Illustrates the comparisons related to the survival rate, tumor volume, tumor weight, and tumor images. A): The survival rate is compared between the healthy control group and the mice with BC that received different doses of G. glabra or normal saline (the untreated tumor control, UTC). B): The tumor volume is compared between the UTC group and the mice with BC that received different doses of G. glabra, on different days following tumor induction. C): The tumor weight is compared between the UTC group and the mice with BC that received after the scarification of mice on the last day. D): Tumor images in the UTC group and G. glabra-treated groups taken after the scarification of the mice on the last day. \*Represents the differences compared with the healthy control group; #Represents the differences compared with the UTC group.

the G. glabra-50 and G. glabra-150 groups significantly increased compared with the UTC group (p=0.003 and p=0.005, respectively), the mean of body weight gain in the G. glabra-100 group was remarkably lower than the G. glabra-50, G. glabra-150 groups (p=0.002 and p=0.006). The mean of body weight gain in the G. glabra-100 group was remarkably lower than in the healthy control group (p=0.0005). The body weight gain did not substantially differ between the G. glabra groups (Fig. 3A).

The UTC mice exhibited a greater splenic index in comparison with the healthy group (p < 0.0001). Treatment of BC mice with G. glabra-50 and G. glabra-150 significantly decreased splenic index compared with the UTC group (p=0.001, and p=0.04, respectively). No remarkable difference was found between the G. glabra-100 group and the UTC group regarding the splenic index. However, the G. glabra-50, G. glabra-100, and G. glabra-150 groups showed a significantly larger spleen index in comparison with the healthy group (p<0.0001, p=0.0003 and p=0.0004, respectively) (Figs. 3B and 3C).

#### *G. glabra Extract's Impacts on the Frequency* of Splenic TH1 and Treg Cells

The mean percentage of splenic TH1 cells in G. glabra-50, G. glabra-100, and G. glabra-150 groups was substantially greater than in the UTC group (p=0.01, p=0.02, and p=0.002, respectively). No significant difference was found regarding the mean percentage of TH1 cells between the groups receiving different doses of G. glabra (Figs. 4A & 4C). The mean percentage of splenic Treg cells in G. glabra-50, G. glabra-100, and G. glabra-150 groups decreased compared with the UTC group (p=0.03, p=0.005, and p=0.008, respectively). The mean percentage

Table 2. Statistical comparison of the differences of tumor sizes between UTC group and G.
glabra treated groups (G. glabra-50, G. glabra-100, and G. glabra-150) on different days after
tumor induction

Groups	UTC	G. glabra-50	G. glabra-100	G. glabra-150		
Day 18 after tumor induction						
UTC	-	0.006	0.094	0.279		
G. glabra-50	0.006	-	0.751	0.414		
G. glabra-100	0.094	0.751	-	0.947		
G. glabra-150	0.279	0.414	0.947	-		
Day 20 after tumor induction						
UTC	-	0.003	0.060	0.138		
G. glabra-50	0.003	-	0.749	0.521		
G. glabra-100	0.060	0.749	-	0.982		
G. glabra-150	0.138	0.521	0.982	-		
Day 22 after tumor induction						
UTC	-	< 0.0001	0.0036	0.0001		
G. glabra-50	< 0.0001	-	0.219	0.781		
G. glabra-100	0.0036	0.219	-	0.754		
G. glabra-150	0.0001	0.781	0.754	-		
Day 24 after tumor induction						
UTC	-	< 0.0001	0.0226	0.0001		
G. glabra-50	< 0.0001	-	0.242	0.990		
G. glabra-100	0.0226	0.242	-	0.396		
G. glabra-150	0.0001	0.990	0.396	-		
Day 26 after tumor induction						
UTC	-	0.0016	< 0.0001	< 0.0001		
G. glabra-50	0.0016	-	0.711	0.424		
G. glabra-100	< 0.0001	0.711	-	0.967		
G. glabra-150	< 0.0001	0.424	0.967	-		

Untreated tumor control (UTC)



**Fig. 3.** Illustrates the comparisons related to the body weight gain, splenic index, and spleen images. A): The body weight gain is compared between the healthy control group and the mice with BC that received different doses of G. glabra or normal saline (the untreated tumor control, UTC). B): The index of spleen is compared between the healthy mice, the UTC group and the mice with BC that received different doses of G. glabra, measured after the scarification of the mice on the last day. C): Spleen images in the UTC group and G. glabra treated groups taken after the scarification of the mice on the last day. \*Represents the differences compared with the healthy control group; \*Represents the differences compared with the healthy control group; \*Represents the differences compared with the healthy control group; \*Represents the differences compared with the healthy control group; \*Represents the differences compared with the healthy control group; \*Represents the differences compared with the healthy control group; \*Represents the differences compared with the utc group.



**Fig. 4.** Illustrates the dot blots of the flow cytometry regarding the splenic TH1, and Treg cells as well as the comparisons related to the splenic TH1, and Treg cell frequencies. A): The dot blots of TH1 cells and in B), the dot blots of Treg cells in the healthy group and the mice with BC that received different doses of G. glabra or normal saline (the untreated tumor control, UTC). C): The frequency of TH1 cells and in D): the frequency of Treg cells are compared between the healthy mice, the UTC group and the mice with BC that received different doses of G. glabra. \*Represents the differences compared with the healthy control group; #Represents the differences compared with the UTC group.

of splenic Treg cells was not significantly different between the groups receiving different doses of G. glabra (Figs. 4B & 4D).

### G. glabra Extract's Impacts on the Serum Levels of IL-12, IFN- $\gamma$ , and TGF- $\beta$

The serum IFN- $\gamma$  level was enhanced in the G. glabra-150 group as compared with the healthy control and the UTC groups (p=0.02and p=0.03, respectively). No significant difference was found regarding the serum IFN- $\gamma$  levels between the groups receiving different doses of G. glabra (p=0.1) (Fig. 5A). The serum IL-12 levels in G. glabra-50 and G. Glabra-150 groups significantly increased in comparison with the UTC group (p=0.008 and p=0.007, respectively). Also, the serum level of IL-12 in the G. glabra-150 group displayed a significant elevation in comparison with the G. glabra-100 (p=0.03) (Fig. 5B). The mean serum levels of TGF- $\beta$  in G. glabra-50, G. glabra-100, and G. glabra-150 groups substantially reduced in comparison with the UTC group (with p=0.01, p=0.001, and p=0.001, respectively). The mean serum amounts of TGF- $\beta$  in the G. glabra-150 group decreased as compared with the G. glabra-50 and G. glabra-100 groups (p=0.02 and p=0.04, respectively) (Fig. 5C).

![](_page_8_Figure_1.jpeg)

**Fig. 5.** Comparison of the cytokine quantities in the serum from the healthy control group, tumor-bearing untreated mice (the UTC group), and those treated with different doses of G. glabra. A): The levels of IFN- $\gamma$ , B): The levels of IL-12, C): The levels of TGF- $\beta$ , D): The ratio of IFN- $\gamma$ /TGF- $\beta$ , E): The ratio of IL-12/TGF- $\beta$ . \*Represents the differences compared with the healthy control group; \*Represents the differences compared with the UTC group.

The serum IFN- $\gamma$ /TGF- $\beta$  ratio remarkably enhanced in G. glabra-50, G. glabra-100, and G. glabra-150 groups compared with the UTC group (p=0.007, p=0.006, p=0.002, respectively). Also, a significant increase was found regarding the IFN- $\gamma$ /TGF- $\beta$  ratio in G. glabra-150 group compared with the G. glabra-50 group (p= 0.013) (Fig. 5D). The serum IL-12/TGF- $\beta$  ratio significantly increased in G. glabra-50, G. glabra-100, and G. glabra-150 groups compared with the UTC group (*p*=0.009, *p*=0.01, *p*=0.0001, respectively). Also, there was a significant increase in the G. glabra-150 group compared with the G. glabra-50, and G. glabra-100 groups regarding the IL-12/TGF- $\beta$  ratio (p=0.012, and, p=0.033, respectively) (Fig. 5E).

#### G. glabra Extract's Impacts on the Expression Amounts of Granzyme B, T-bet and FOXP3

T-bet and FOXP3 are designated as the specific transcription factors of TH1 and Treg cells, respectively. Granzyme-B is also expressed in CTLs contributing to their cytotoxicity. The intra-tumor expression of the T-bet transcription factor gene in G. glabra-50, G. glabra-100, G. glabra-150 groups significantly enhanced in comparison with the UTC group (p=0.015, p=0.013, and p=0.001, respectively). The expression level of T-bet considerably enhanced in G. glabra-150 group compared with G. glabra-100-treated mice (p=0.02) (Fig. 6A).

The gene expression of the Granzyme-B substantially elevated in G. glabra-100 and G. glabra-150 groups compared with the UTC group, (p=0.001, and p=0.003 respectively). There was also a significantly increased Granzyme-B gene expression in the G. glabra-100, and G. glabra-150 groups compared with G. glabra-50 group (p=0.001 and p=0.003, respectively) (Fig. 6B).

The FOXP3 gene expression declined in G. glabra-treated mice compared with the UTC group with a remarkable reduction in G. glabra-50 group (p=0.01). No substantial differences were detected between the G.

![](_page_9_Figure_1.jpeg)

**Fig. 6.** Illustrates the comparisons related to the expression of transcription factors within the tumor tissues between the mice with BC that received different doses of G. glabra or normal saline (the untreated tumor control, UTC). A): mRNA expression of T-bet, B): mRNA expression of Granzyme-B. C): mRNA expression of FOXP3. Represents the differences compared with the healthy control group; #Represents the differences compared with the UTC group.

glabra-treated groups considering FOXP3 expression (Fig. 6C).

#### *G. glabra Extract's Impacts on Cancerous Tissue and Metastasis in Lung*

Histopathological evaluation of tumor tissues showed necrotic areas, blood vessels, and lymphocyte infiltration (Fig. 7). In the UTC group, the blood vessels were shown with a black arrow. In tumor tissues from the UTC group, a large number of blood vessels were observed. The blood vessels at the periphery of the tumor reduced in groups G. glabra-50, G. glabra-100, and G. glabra-150, especially in the G. glabra-150 one. The white arrow indicated necrosis in tumor tissues, absent in the UTC group, whereas in the G. glabra-50, G. glabra-100, and G. glabra-150 groups, the necrosis regions increased with increasing doses. As shown in Fig. 7 with the lightning sign, in the G. glabra-150 group, lymphocyte

![](_page_9_Figure_7.jpeg)

**Fig. 7.** Illustrates the histopathology of the tumor tissues in the mice with BC that received different doses of G. glabra or normal saline (the untreated tumor control, UTC). Necrosis areas, blood vessels and leukocyte infiltration were indicated by white arrow, black arrow and lightning bolt, respectively. Histopathologic patterns of the tumor tissues from A): UTC group, B): G. glabra-50 treated mice, C): G. glabra-100 treated mice, D): G. glabra-150 treated mice.

![](_page_10_Figure_1.jpeg)

**Fig. 8.** Histopathologic patterns from A): The healthy control group, B): The UTC group, C): G. glabra-50 treated mice, D): G. glabra-100 treated mice, E): G. glabra-150 treated mice.

infiltration into the tumor tissue was observed.

Histopathological examination of lung tissues from the healthy group showed normal alveoli with thin walls. As indicated in Fig. 8, in the UTC group, the alveoli exhibited a smaller size and a thicker inter-alveolar wall. Numerous areas of metastasis were visible around which there was inflammation. Metastasis was also noted in the lung tissue specimens from the G. glabra-50 group, which was much less than in the UTC group. The alveoli did not yet return to normal and their walls were slightly wider than those in the UTC group. In the G. glabra-100 and G. glabra-150 groups, the alveoli almost returned to a normal state the inter-alveolar wall became thinner, and no lung metastasis was observed. The inflammation around bronchial branches was visible in lung tissues from the G. glabra-100 group.

#### DISCUSSION

Medicinal compounds derived from herbal sources can be an appropriate alternative therapy for cancer patients among all available therapeutic choices. The data of the present study revealed that the BC mice treatment with the G. glabra-50, G. glabra-100, and G. glabra-150 increased the survival rate, while reducing the growth of tumor compared with the UTC group. Treatment with G. glabra-50 and G. glabra-150 prevented body weight loss in BC mice. 4T1 cells generate a highly metastatic tumor that can invade other tissues, including the lungs (28). In our study, we showed that in the G. glabra-treated groups, the amount of metastasis in lung tissue reduced. Especially in G. glabra100 and G. glabra-150 groups, lung metastasis was largely prevented. Treatment with G. glabra-100 and G. glabra-150 had

also the greatest impacts on inhibiting tumor growth and declining its volume. The finding presented here also elucidated the tumor size in the G. glabra 50 group was smaller than that in the G. glabra-100 and G. glabra-150 groups on days 18-22 after the tumor induction, but the differences were not remarkable. However, opposite results were observed between G. Glabra-treated groups regarding tumor weights. Thus, it seems that the weight and size of the tumors were not completely compatible between G. Glabra-treated groups. Factors such as intra-tumor necrosis can cause tumor weight loss. On days 26 after the tumor induction, the tumor size was slightly smaller in G. glabra-100 and G. glabra-150 groups compared with the glabra-50 group, indicating possible longer persistence of greater doses of G. glabra extract.

The anti-tumorigenic impacts of G. glabra extract and some of its active compounds were also indicated in other studies. Accumulating evidence indicates that G. glabra has different anti-cancer impacts, such as inducing apoptosis, cell cycle arrest, and cytotoxic effects (29). It was revealed that G. glabra extracts exerted cytotoxicity effects and induced morphological alterations in 4T1 mouse BC cells in time and dosedependent manners (30). Licoricidin-treated 4T1 cells also displayed reduced migration and matrix metallopeptidase-9 (MMP-9) secretion (31). Furthermore, the treatment of 4T1 tumor-bearing mice with licoricidin reduced lung metastasis and diminished the expression of VEGF receptors in tumor tissues (31). G. glabra-derived flavonoid, namely isoangustone A (IAA) decreased DNA synthesis, caused cycle cell arrest and reduced the expression levels of antiapoptotic factors (such as cyclin A and cyclin D1) in 4T1 cells (32). Furthermore, treatment of 4T1 tumor-bearing mice with the G. glabra-derived licochalcone E (LicE) reduced tumor growth, decreased lung metastasis, and reduced the expression of various antiapoptotic factors (such as Bcl-2, cyclins and cyclin-dependent kinases), while increasing the expression of pro-apoptotic factor Bax (33). LicE also exerted inhibitory effects on the expression of angiogenesis-related factors (such as VEGF and VEGF receptors) and effectively decreased the movement and invasion of 4T1 cells in vitro (33). Anti-proliferative effects of glycyrrhizin, glycyrrhetinic acid and liquiritigenin on human BC cell lines were also indicated (34, 35). Moreover, some G. glabra-derived components such as glycyrrhizic acid, and glabridin can reduce the viability of human BC cells (36, 37). It has been also demonstrated that glycyrrhetinic acid effectively inhibited M2 macrophage-induced cell expansion and migration in 4T1 cells. In mice bearing 4T1 tumor, glycyrrhetinic acid remarkably suppressed tumor growth, lung metastasis, and angiogenesis. In tumor tissues, glycyrrhetinic acid reduced the number of M2 macrophages but promoted the number of M1 macrophages (38). In addition, glabridin inhibits cancer stem celllike characteristics in human BC cells (39). Saeedifar et al. showed that treatment with G. glabra only or in combination with ginger suppressed tumor growth in a colorectal mouse model (40). Wu et al. showed that glycyrrhizin inhibited the growth of lung tumors in mice (41).

The findings displayed here indicated that treatment of BC mice with the G. glabra extract improved the TH1-related parameters including the number of splenic TH1 cells, serum levels of IFN-y cytokine (especially, G. glabra-150), serum levels of IL-12 cytokine (especially, G. glabra-50 and G. glabra-150), and promoted T-bet and Granzyme B gene expression (especially, G. glabra-100 and G. glabra-150). In a mouse colorectal cancer model, Ayeka et al. showed that Licorice polysaccharides prevented tumor growth, prompted immunity and organ index, and enhanced the activation of CD4<sup>+</sup> T and CD8<sup>+</sup> T cells (42). Ma et al. showed that Glycyrrhizic acid has an anti-asthmatic effect by enhancing IFN-y and suppressing IL-4, IL-5, and IL-13 (43).

The data displayed here indicated that BC

mice treatment with the G. glabra extract modulated the Treg-related parameters including the splenic Treg cell numbers, the serum levels of TGF- $\beta$ , and FOXP3 expression (especially in G. glabra-50). Zhang et al. showed that Glycyrrhizic acid weakened the expression of TGF- $\beta$  and the phosphorylation of its downstream targets (44). The results presented here indicated that the lowest TGF-B levels were observed in G. glabra-150 group. The FOXP3 expression declined in G. glabratreated groups compared with the UTC group with a remarkable reduction in G. glabra-50 group. The reasons for the inconsistency of FOXP3 expression with TGF-β levels and the number of splenic Treg cells remain to be explained in future studies. One explanation may be that the greater doses of G. glabra may differentially influence the gene and protein expression of FOXP3. Thereby, despite of FOXP3 gene expression, minimum amounts of TGF- $\beta$  and a lower number of Treg cells were observed in G. glabra-150 group.

#### CONCLUSION

The findings of the present investigation exhibited that the treatment of BC mice with G. glabra extract promoted survival rates, prevented tumor growth and metastasis, enhanced the splenic TH1 cell frequency, upregulated the TH1-related parameters such as IL-12, T-bet, and Granzyme-B expression. However, splenic Treg cell frequency and the expression levels of the Treg cell-related molecules, such as TGF- $\beta$ and FOXP3 expression reduced in G. glabratreated mice. In the G. glabra-150 group, the greatest amounts of Th1-related factors as well as the longest survival rate and the lowest tumor weight were observed.

#### ACKNOWLEDGMENT

This research was supported by a grant [PN: 97000410] from the Rafsanjan University of Medical Sciences, Rafsanjan, Iran.

#### AUTHORS' CONTRIBUTION

AJ designed and conceptualized the study. SY, PB, RZ, JM, NZ, HK, SS, MG, and MN conducted all the experiments. SY, PB, RZ, JM, and NZ collected the data. SY, PB, RZ, and JM analyzed the data. AJ, SY, and JM wrote the manuscript. AJ reviewed the scientific content. All authors read and approved the final manuscript

#### **CONFLICTS OF INTEREST**

The authors declare no conflict of interest.

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