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ORIGINAL ARTICLE

Antitumor Immunostimulatory Effect of Sitosterol from *Salvia atropatana* on Tumor bearing mice

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ABSTRACT
Development of agents that specifically kill cancer cells and simultaneously elicit antitumor immune response is a step forward in cancer therapy. In the present study, we investigated whether the administration of sitosterol contributes to the augmentation of antitumor immunity and the regression of tumor tissues in a mouse model of breast cancer. This experimental and laboratory study has carried out on inbred female Balb/c tumor-bearing mice, aged from six to eight weeks. First, the examinations on Balb/c tumor-bearing mice were performed. Fifteen mice were classified into three groups (n=5). The optimized dose of sitosterol was injected intraperitoneally into the first group. In the second group (as positive control) and the third group (as negative control) cyclophosphamide anti-cancer drug and ethanol+PBS were applied, respectively. Tumor volume was assessed daily in the test and control groups with a digital caliper. When the tumor volume reached to the target size, the mice spine was cut and the spleen removed aseptically to isolate lymphocytes and proceed with the further tests. Spleen lymphocyte proliferation was assessed by Brdu test. To examine the influence of sitosterol on spleen lymphocyte cytokine production, IL-4 and IFN-γ were measured.In order to investigation of sitosterol adminstration effect , using flow cytometry, The percentage of CD4+CD25+Foxp3+ cells were measured. Based on the obtained data, decreased tumor volume in sitosterol -treated tumor mice were seen (P< 0.05). Also, the IFN-γ level in sitosterol -treated mice were statistically increased compared to the control group (P< 0.05), while the IL-4 level showed a significant reduction (P< 0.05). The increase in lymphocyte proliferation response in sitosterol -treated mice showed a significant difference compared to control (P< 0.05).Our finding indicated the significant reduction in splenic CD4+CD25+Foxp3+ T lymphocytes frequency in the sitosterol-treated mice toward control group. According to our findings we can suggested that sitosterol have immunostimulatory effect on immune system. The cytotoxicity and immunomodulatory properties of sitosterol were acknowledged in vivo. This herbal medication could be a potential treatment procedure in case of breast cancer.
Key words: *Sitosterol, Breast Cancer, IFN-γ, IL-4*

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INTRODUCTION

Breast cancer affects one in eight women during their lives. In the United States this common cancer kills more women than any cancer except lung cancer. Substantial support for breast cancer awareness and research funding could help to advances in the treatment of breast cancer. Recently Breast cancer survival rates have increased, and the number of deaths has been declining, because of some advances as earlier detection, and novel approaches to treatment and a better understanding of the complication. Among common treatment of breast cancer alternative therapy also could be used, that comprise of Works with patients body's immune system to help it to fight against cancer or to control side effects from other cancer treatments [1-6]. Indeed The use of complementary and alternative medicine among

patients with irredeemably disease such as cancer is increasing. The prevalence of this methods estimated Between 7 and 64percent, that patients with breast cancer have highest rate by 67 to 83percent allocated to usage these alternative Treatment methods [7-9].Herbal composition ranges of chemical agents, including isoflavnoids, phytosterols, alkaloids and other herbal agents, constitute deals which have immunomodulatory effects (10). The *Salvia genus* from Lamiaceae family comprises over 900species with different biological activities manifested by the different components, with pharmacological properties, in splay medicinal and therapeutically usage of their extracts. phytochemical analysis showed some salvia species have phenolic agents [11-13], that have anti-inflammatory, analgesic [14], antipyretic, haemostatic [15], hypoglycemic [16] and antitumor [17] effects. in addition to their *application* of these traditional herbal medicine as therapeutics agents, recently has been shown to have a significant antitumoral property [18].One of the compounds were isolated from the chloroform extract of the aerial part of *Salvia atropatana* phytosterol namely betasitosterol [19] in pharmacological applications of this sterols In addition to diverse application of this compounds, sterols has antioxidant properties [20].Phytosterols is a kind of sterols (common sterols included Beta-sitosterol, campsterol, stigmasterols) that are structurally similar to cholesterol. Indeed Epidemiological and laboratory findings showed that dietary phytosterols have protective effect against different kind of cancers, such as breast cancer. Cancer cells compared with other non-cancerous growths have higher state of oxidative stress, hence cancer cells have high levels of malonylealdehyde (indicator for increased level of oxidative damage to lipids) and low levels of coenzymeQ-10(potent antioxidant coenzyme) [21-23]. In addition, in cell culture studies , breast cancer patients with low levels of glutathione were on showed that Sitosterol modify the level of glutathione peroxidase and superoxide dismutase enzyme (SOD).Also beta Sitosterol is the main cause of FAS activation(cell surface death receptor that mediated extracellular apoptosis). FAS Activation led to recall of intracellular adapter proteins (FADD and TRADD). This pathway induces caspase-mediated apoptosis. Moreover phytosterols through effects on TRAIL ligand trigger apoptosis(major mediator of apoptosis via activation of caspase-8 with proteinase activity in many cancer cells) [23-25]. Considering these anti-tumor properties evidences we investigated the antitumor immunostimulatory effect of sitosterol on tumor tissues in a mice model of breast cancers as an effective cancer treatment.

METHOD AND MATERIALS

Extraction and purification of sitosterol

The leaves of *Salvia atropatana* were collected from north of Iran, then these Iranian Salvia species were identified. Dried and powdered parts of *Salvia atropatana* (1000 g) were pretreated with n-hexane, ethyle acetate, and methanol (1:1:1). The extract solutions were filtered and concentrated in Rotary vacuum via distillation procedure to remains concentrated extract (at 50°c), then treated with methanol solution (48 Hours) to obtaining crude extracts. In order to Separation of salvia extract components, column chromatography method performed. To further purification in each fraction, column chromatography repeated (with smaller columns) and then thin layer chromatography with TLC plates were used. After purification by chromatography, we isolated 35 fractions that fractions 27-25 with single band known as Sitosterol. Purity was determined Using NMR.

Cell survival rate and cytotoxicity assessment

We use the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5diphenyltetrazolium bromide (MTT) to assessment of cell survival rate and estimation of cytotoxicity criterion. for this procedure we prepared the human breast adenocarcinoma cell line (MCF-7),then these cell line Were placed in a 96-well with a total volume of 200 ll per well (1×10^4 cells/well)in tissue culture plate .in following sitosterol extract subjoin. After 24, 48 , 72 h incubation , Cell survival rate was assessed by ELISA reader scanning

Mice

Female BALB/c mice (pasture institute, Tehran, Iran)weighting 150-200 g (ranging from six to eight weeks of age)were housed one week in SPF facility and free access to food and water conditions. The study was approved by the local animal welfare committee of Shahid Beheshti University, Iran. Our laboratory animal care guidelines were according to DHEW Publication No. (NIH) 85-23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD20205.After the tumors volume reached to 3000mm3, tumor cell extraction and purification procedure from the breast cancer bearing Balb/c mice were performed and tissue homogenize(tumor suspension) prepared, then Freeze/thawing process was repeated five times. In this case Suspension was sonicated under condition of: 3w power in 210 s, 30 s incubation, then followed by filtration, then cell suspension Lysate dialyzed. Eventually Protein concentration (extracted antigen) determined. (Micro-Bradford method using BSA standards).

Delayed type hypersensitivity (DTH)

Footpad swelling was used as a measure of DTH. after primary subcutaneously injection 0f 1×10^9 sheep red blood cells (sRBC) i.p, At various days immunization with sitosterol16.35 ug/mouse/day (for five days)performed .Sensitized mice were challenged subcutaneously with 1×10^9sRBC injection on day 5. Swelling response in the left hind footpad measured during three times 24, 48, 72 h.

Tumor transplantation and experimental planning

We induce Invasive Ductal Carcinoma derived from Balb/c mice that shares many characteristics with naturally occurring human breast cancer. By the time the primary neoplastic masses is palpable (volume of approximately 3000 mm3), Surgical removal biopsy performed. Tumor cells histological characterized. Tumor tissue subcutaneously transplanted to a syngenic female Balb/c mice. For each experiment15 mice were subdivided into three groups .A total of Group 1 (consist of 5 mice)were receiving intraperitoneally sitosterol according to the results obtained from the DTH test, at beginning of the experiment with 16.35 ug/mouse/day , and 10 day later. Group 2 were similarly treated with 0.1 ml cyclophosphamide (20 mg/kg pellets), Group 3, included 5 mice that inoculated with PBS/ethanol (2:1) via IP injection for 10 days. indeed 5 mice were used in each experimental and treatment groups.15 mice surviving 5 to negative control and 5 who received cyclophosphamide as positive control and 5 were selected for the test group. The tumor volume in three groups also measured.

Spleen cell proliferation index

After animal sacrifice, spleen cancer cell under sterile conditions using the needle perfusion method and sterile RPMI-1640 containing 10% fetal bovine serum were isolated for this study**.**

The spleen cells were cultured at three groups

For each mice as negative control (without stimulation), test (stimulation with tumor Lyzat) and positive control (stimulation by phytohemaglutinin) that were prepared in three wells. Cells were incubated for 4 days at 37 ° C and 5% co2. For each well, 20 microliter of Bromodeoxyuridine (BRDU) Labeling solution added and then incubated for 2 h at 37 ° C with 5% co2 .with the BrdU cell proliferation kit , BrdU uptake were determined. (Roche Diagnostic GmbH, Mannheim, Germany).

Spleen cell isolation and cytokines (IFN-γ, IL-4) profile measurment

In order to investigation of impact of *Salvia atropatana* crude extract (sitosterol) on the cytokine profile pattern of mice splenic tumor cell , isolation of spleen cells in optimal condition with preparation of cell suspensions, in RPMI 1640 and density centrifugation performed. IFN-γ, IL-4levels by ELISA sandwich method measured. (Mice Capture monoclonal antibody against IFN-γ and IL4) With biotin-conjugated mouse monoclonal antibody (IFN-γ antibody, IL-4 antibody).

Flowcytometric Determination of spleen T CD4 + CD25 + FoxP3 +cells

The spleens cell suspension sprepared and after washing and labeling with monoclonal antibodies (anti-CD4, anti-CD25 and anti-Foxp3), direct immunofluorescence staining performed. Using trypan blue exclusion procedure livability of test determined. In following after washing and antibody immunostaining, to determination of T-lymphocytes subpopulation Flow cytometric analysis were used. (Flowcytometery EPICS (Coulter).

Statistical analysis

Statistical analysis was performed using SPSS software (Version 16, USA). Comparisons between groups were performed by using the one-way ANOVA and independent t-test and. $P<0.05$was considered significant.

RESULTS

Purity fractions of *Salvia atropatana* was determined by thin layer chromatography. Purified material (sitosterol) confirmed by H NMR spectroscopy that presented in figure 1.

To investigation of sitosterol effect on tumor volume in breast cancer

Bearing mice, each subject of group 1 receive a dose 16.35 (µg / mouse / day) of sitosterol . The second group as a positive control, received cyclophosphamide. The third group as a negative control, received phosphate buffered saline / ethanol injection. The results showed that the progression of tumor growth between groups were significantly different. (figure2).Our findings revealed that sitosterol administration significantly decreased tumor growth rate compared to control group. ($P <0.05$)

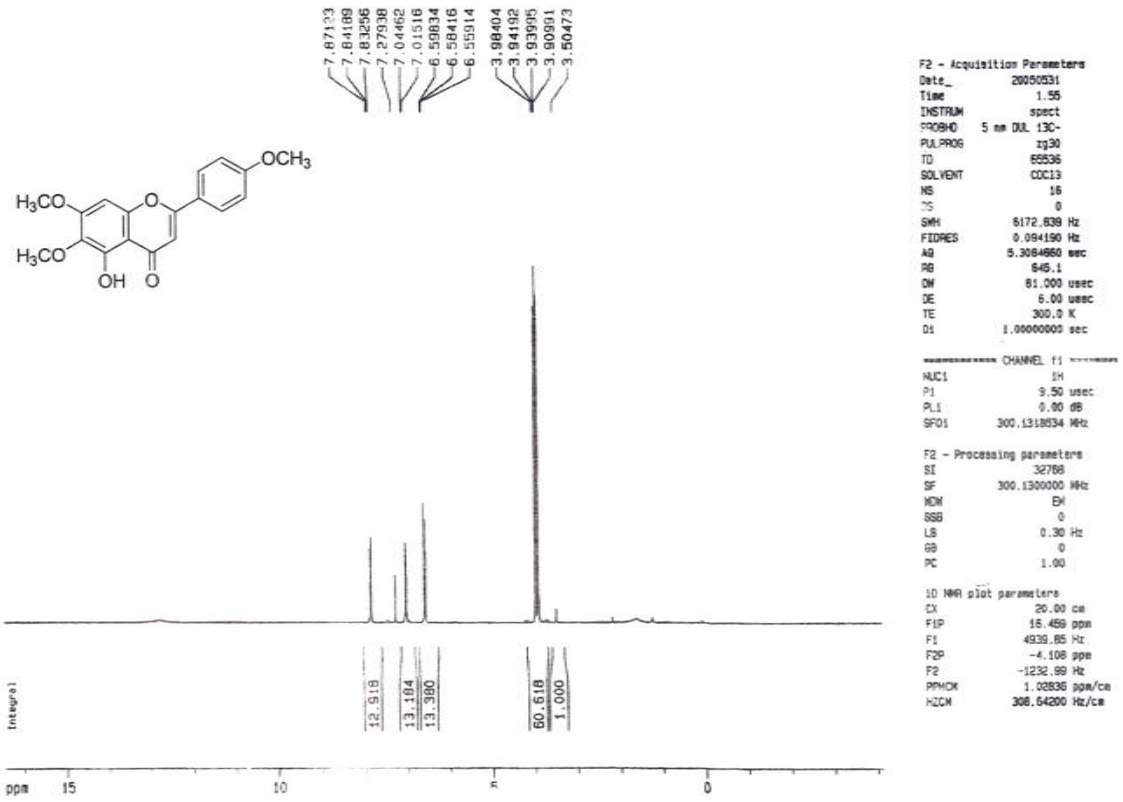

Fig. 1. H NMR spectroscopy of *Salvia atropatana* (sitosterol)

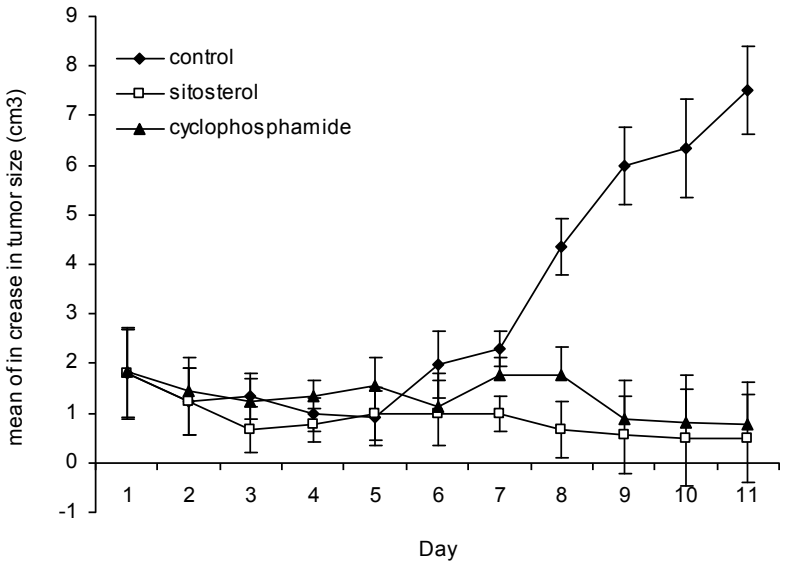

Figure2: The mean tumor volume (mean± SD) in the negative control group (with PBS / EtoH injection), positive control (with cyclophosphamide treatment) and sitosterol treated group.

Effect of intraperitoneally injected sitosterol on lymphocyte proliferation index

Results of BRDU test (splenic lymphocytes stimulation by tumor antigen) as stimulated cell proliferation index presented in Figure 3. Our Finding revealed sitosterol treated splenocytes have higher proliferative response toward control group ($P < 0.05$).

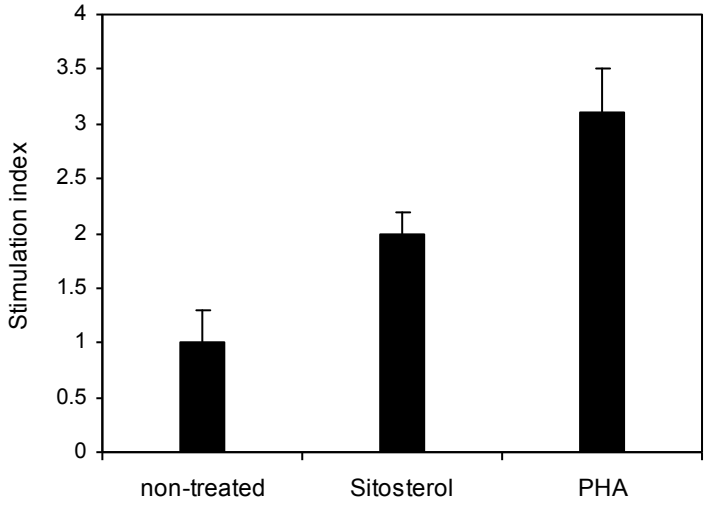

Figure 3: lymphocyte stimulation index (five mice/group).sitosterol-treated splenocytes, significantly have higher proliferation response toward controls. ($p< 0.05$)
Data presented as mean ± S.D.

Effect of sitosterol injection on IFN- γ and IL-4 production pattern

In order to evaluation of sitosterol intraperitoneally injection effect on cytokine pattern in Splenic mononuclear cell culture using the ELISA method.The concentrations of cytokines IFN-γ and IL-4 were measured. Various levels of the cytokines was observed among all groups ($P <0.05$). Sitosterol treated mice significantly have higher level of IFN-γ ($P <0.05$) in compared with the control group; While in contrast there are significant reduction in IL-4 level in sitosterol treated subjects toward controls($P <0.05$) .Results shown in Figure 4.

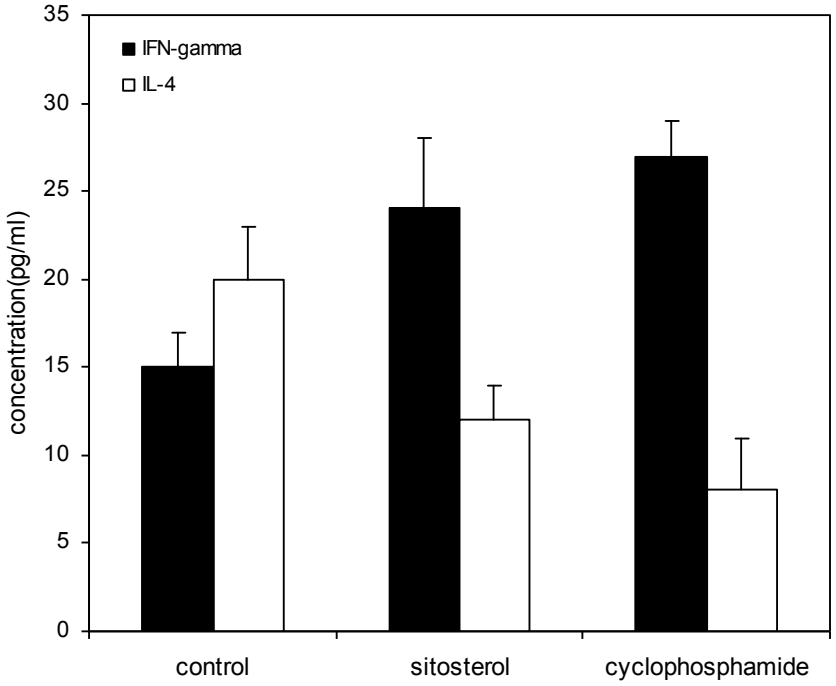

Figure 4: cytokines response (IFN-γand IL-4) in sitosterol treated mice and negative control (PBS / EtoHinjection) and cyclophosphamide treated mice as positive control. IFN-c has higher level in the sitosterol treated mice, ($p < 0.05$) toward control group, while IL-4 has lower level ($p< 0.05$).
Results presented as the mean ± S.D

Effect of sitosterol administration on the CD4+CD25+Foxp3+ regulatory T cells percentage (level)
To examination of sitosterol treatment effect on the number of splenic CD4+CD25+Foxp3+ regulatory T lymphocytes, The percentage of CD4+CD25+Foxp3+ cells using flow cytometry were measured .our finding shown there are significant reduction in splenic CD4+CD25+Foxp3+T lymphocytesin the sitosterol-treated mice toward control group. Data presented in figure 5.

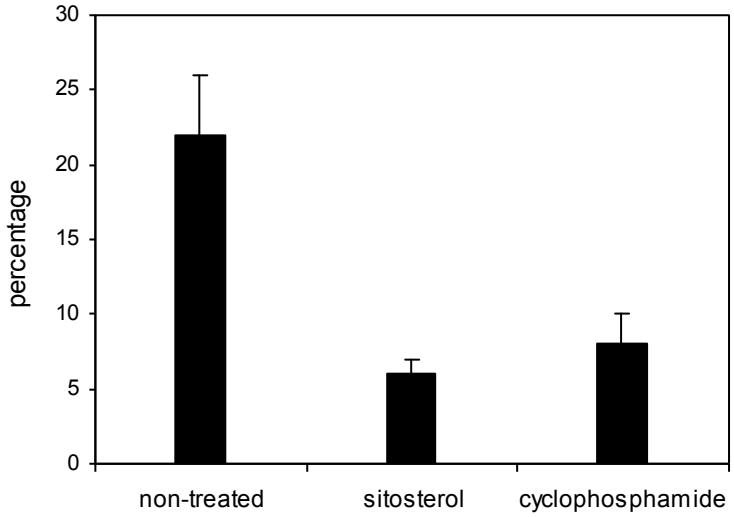

Figure 5: Splenic CD4+CD25+Foxp3+ regulatory T lymphocytes frequency. Sitosterol treated mice have lower number compared to control group. ($p< 0.05$)

DISCUSSION
Multidrug resistance is one of most common complication in cancer treatment, which, Resistance to therapy is a main reason in the failure of many kinds of cancer treatment. It affects patients with a variety of cancers and solid tumors, including breast cancers. The immune system is a key player in omission and control of early tumor progression. The immune response against tumors concluded a multistep phenomenon, and various components involving the innate and adaptive immune system. Focus on alternative therapeutic methods based on Reinforcement the immune response as preventer of initial tumor formation and in following tumor progression, have potential importance role in immunomudulation therapy [25-17]. According to experimental evidences could be suggested that medicinal plants and traditional medicine as following items that should be broken tumor cells resistance against therapeutic agents, and also have lower side effects. These complementary methods with different mechanisms can lead to reinforcement of immune response, improve the quality of life, repair and regeneration of the body strength and help to reduce the side effects of cancer treatments. In this study, we examined the cytotoxic and immunostimulatory properties of purified sitosterol extracted from *Salvia atropatana* plant on breast cancer bearing mice .as we design multistep procedure: after extraction and purification of sitosterol from *Salvia atropatana* , splenic cell DTH test carried out to obtain of optimal effective dose of sitosterol, that utmost cellular response. Immune responses dose 16.35µg / mouse / day have such as feature in following to evaluate of sitosterol intraperitoneally injection. Influence on tumor size during treatment, splenocytes proliferation, IFN-c and IL-4 secretion and CD4+ CD25+ FOXP3+ Treg cells frequency in different groups were measured and compared. There are significant differences in studied variables between Sitosterol treated groups compared to control group. Our finding indicated that injection with dose 16.35µg / mouse / day reduce the tumor growth ($p < 0.05$). In following to intraperitoneally injection of sitosterol then spleen cell isolation, these splenocye treated with lysate antigen to assessment of proliferative response. Our result shown that the splenocyte proliferation response in sitosterol treated subjects were significantly increased ($p < 0.05$) .This elevated immune response against tumor could be attributed to modulation of cytokine pattern by responsible immune cells. Indeed the injected dose of sitosterol decreased the rate of tumor growth. In lymphoid tissues dendritic cells and macrophagesas antigen presenter cells activate theTCD4 +, TCD8 +cell, to combat against tumor, but Treg cells and various types of myeloid cells have suppression effect on tumor specific Tcells(CD8+) and CD4+effector T cells. Achievement of effective therapeutic program depends on

reverse the inhibitory effect on TH1 function that actually enable to increased cytokines (TNF-α, IFN-γ, IL-2) secretion and progress these function by cell interaction contact with other immune cells (NK cells and CTLs) to activation of antitumor cytotoxic effects [26-31]. The results off low cytometry analysis revealed that , sitosterol treatment reduce the number of spleen $CD4^+$ $CD25^+$ $FoxP3^+$ cells($p<0.05$).
Based on the study, sitosterol shifts the cell immune response toward TH1 response. Based on our results we can suggested that because of our selective sitosterol therapeutic dose was much lower from cytotoxic level, it has been reported that this effects of regulatory T-cells is due to this immunomodulation effect on immune system .This research impress the use of herbal medicines to treat cancer with outside effects on healthy cells and show that longer treatment with the Sitosterol lead to Treg cells immunomodulation that is a potential treatment procedure in case of various type of cancers included breast cancer.

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