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**Original Research Article**

# Garlic Extract Enhances the Peripheral Blood Cellular Immune Responses in Male ICR Mice

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## Abstract

We investigated the effects of garlic extract on the peripheral blood immune cells in mice treated with the extract for twenty days. The study was carried out on ten male ICR mice. The mice were divided into two groups. The control group received a distilled water and garlic extract group in which the mice received garlic extract at a dose of 500 mg/kg. b.w, for twenty days. Blood samples were collected on day ten and day twenty post inoculation (p.i) for differential leucocytic count (WBCs count and Lymphocytes %) and flowcytometric analysis. In comparison to the control group, garlic extract treatment induced a significant increase in WBCs count and lymphocytes percentage on day 10 and day 20 p.i. Furthermore, garlic extract administration to the experimental mice caused significant increase in the expression percentage of CD4, CD8 and γ-δ T cells when analyzed by flowcytometry on day 10 and increased dramatically on day 20 p.i. The WBCs count and lymphocytes percentage as well as the percentage of the expressed cells increased on day 20 more than their levels on day 10 p.i with a fixed dose of garlic extract. In conclusion, inoculation of garlic extract induced an increase in the immune responses of the cellular immunity in the peripheral blood circulation of mice.

**Keywords:** Peripheral blood - Garlic extract- immune responses- Flowcytometry- cells expression.



## INTRODUCTION

Immunomodulation and proliferation are the main basics for synthetic drugs and chemicals effects. On the other hand, the high cost, anticipated toxicity, and adverse effects of synthetic drugs render them undesirable for patients. On the opposite side, using of herbal plants as health supporters has increased widely in both consumers and scientific sites. In the previous study reports, several herbal plants have been listed that have immunomodulatory actions such as modulation of cytokine secretion, phagocytosis promotion and macrophage activation, as well as immunoglobulin production for allergic reactions [1]. The research interest was focused on an herbal plant that is reported to have immune-stimulating and immunomodulation properties, namely Allium sativum (garlic). It was reported that whole extracts or isolated compounds of garlic species modulate the lymphocyte's proliferative responsiveness in rodents as well as humans. In human lymphocyte cultures, garlic induced proliferative enhancement to the lymphocyte number [2, 3]. Garlic (Allium sativum) is one of the oldest medicinal plants used by different ancient cultures. Literature reported that garlic in the old days was used for treatment and prevention of some diseases [4]. It formed part of the daily diet of many Ancient Egyptians. It was fed particularly to the working class involved in heavy labor, as in the building of the pyramids, presumably to maintain and increase their strength, thereby enabling them to work harder and be more productive [5]**.** The biological effects of Allium sativum are related to its characteristic organosulfur compounds [6]. Once garlic is exposed to cutting or crushing, compounds in the intact garlic are converted into hundreds of organosulfur compounds in a short period of time. Garlic and its organosulfur compounds have been shown to reduce risk factors for cardiovascular diseases [7, 8], and to suppress cancer cell growth in vitro [9] and in vivo [10, 11]. Furthermore, garlic has many medical effects where it acts as anti-cancer [12], antioxidant [13], anti-inflammatory [14], anti -bacterial [15], anti-fungal [16], antiviral [17] and anti-parasitic [18, 19]. Recently, garlic has been recommended as a hopeful candidate for keeping the homeostasis of the immune system [20]. Several scientific reports have been done on animal models to investigate the effect of the various garlic components and formulations on

immunomodulatory activities [21-23]. Moreover, garlic has been proven to be a practicable immune response transformer [20]. Garlic stimulates the natural killer (NK) activity [24] and T-lymphocyte proliferation [25]. Also, garlic extract was shown to the supplementary and provider of the oxidative burst in peritoneal macrophages of BALB/c mice. [20]. In Leishmania major infected BALB/c mice garlic extract induced modification in the cytokine pattern which in turn stimulates T helper 1 (Th1) immune responses (IFN-γ, IL-2) [26]. Aged garlic extract (AGE), a famous garlic extract, had many pharmaco-immunological effects including: immunomodulation [27], tumor cell growth inhibition [28], anti-allergic effects [29], and chemo-preventative effects [30]. The purpose of the experiment was to evaluate the impacts exerted on standardized ethanol extracts of garlic on cellular immune responses in healthy ICR mice.

## MATERIAL AND METHODS

### Animals

Ten male ICR mice 5-7 weeks' age and weighing 31-37 g were purchased from Charles River Japan, Inc., Yokohama, Japan. They were kept under optimal conditions of hygiene, temperature, humidity, and light (cycles of 12 hr dark/light). All protocols were approved by the Institutional Review Board for Animal Experiments of Miyazaki University, Japan (approval No. 2011-007-7), in agreement with the laws of Japan and the act on welfare and management of animals. All animal procedures were conducted in a BSL1 room.

### Garlic Extract Preparation

Fresh garlic bulbs were obtained from a local market in Miyazaki, Japan. Garlic bulbs were peeled and grinded. Methanolic extract of garlic was as the following. Briefly, garlic samples were homogenized with absolute methanol in a two-liter flask, and then kept in the refrigerator at 5℃ overnight. The homogenized garlic was incubated in an oven at 75℃ for 8 hours to evaporate the excess methanol. The mixture is collected and filtered through Wattman filter paper (number 1) and the filtrate subjected to Soxhlet extraction according to the prescribed method of Eidi *et al*., [31]. After extraction, the solvent was filtered and lyophilized. The collected powder extract was dissolved in sterile water and used for the experiment.

### Experimental design

Ten male ICR mice were randomly grouped into two groups (n=5/group). The control group received 150 microlitters of distilled water. Garlic extract treated group, the mice inoculated with garlic extract at a dose of 500 mg/kg b.w, daily for twenty days. Whole blood samples were collected in heparinized tubes on days 10 and 20 post inoculation. The blood samples were used for WBCs count and lymphocyte % estimation, in addition, the flowcytometry analysis.

### Flowcytometry analysis

The focused cell populations were analyzed by flowcytometry. CD8, CD4 and γδ+ are specific for mice to recognize the surface epitope on the mouse CD8+, CD4+ and γδ+ T cells. The cells were stained at 37°C for 60 min with the following fluoro-chrome conjugated antibodies: FITC-anti-CD8 (YTs105.18), APC-Cytm7anti-CD4 (GK 1.5) and PE-anti-γδ T (GL3) (all reagents, BD-Pharmingen U.S.A). By using FACScan flow cytometer. The flowcytometry was up-regulated by Cytec Development software (Fremont, CA, United States) to allow for 1-color analysis for the cells separately. The software adjusted to express the percentage (%) of expressed T cells in the field of the expression graph to summarize the results. The flowcytometric preparations for analysis were done briefly as the following: Lymphocytes were isolated and evaluated according to Cruvinel *et al*., [32]. The isolated lymphocytes from peripheral blood were transported carefully into 96 wells plates which incubated with the stained antibodies for one hour. After, the prepared cells are passed to Facs for sorting. Obtained data was analyzed with the adjusted CELLQUEST Software (BD Biosciences CA, United States).

### Correlation Coefficient

Correlation a coefficient was computed in the garlic treated group to assess the linear relationship between day 10 and day 20 p.i regarding the tested parameters and the fixed dose of garlic extract as shown in table 1:

**Table 1: The Correlation Coefficient between variables in garlic extract group**

| **Garlic extract treated group** | | | | |
|---|---|---|---|---|
| Tested parameters | | Time post inoculation (p.i) | | |
| 1 | WBCs count × $10^3$ µ/L | Day 10 | Versus | Day 20 |
| 2 | Lymphocytes % | Day 10 | Versus | Day 20 |
| 3 | CD4 T cells % | Day 10 | Versus | Day 20 |
| 4 | CD8 T cells % | Day 10 | Versus | Day 20 |
| 5 | γ-δ T cells % | Day 10 | Versus | Day 20 |

**Statistical analysis**

Statistical analysis was performed with the statistical software package SPSS for Windows (version 20.0; SPSS Inc., Chicago, Ill.). Student's t test was used to compare data from the control and garlic extract groups. Results were expressed as M ± SE of the mean (S.E.M.). The p value which less than 0.05 was considered as significant result.

## RESULTS

**The effect of garlic extract on peripheral leukocytes and lymphocytes %**

In comparison to the control group, treatment of garlic extract induced significant increase in the WBCs count × $10^3$ µ/L at P<0.02 and lymphocytes % at day 10 p.i. The continued administration of garlic extract induced further elevation at day 20 p.i. as shown in Fig 1. In the garlic treated group, WBCs count and lymphocytes % dramatically increased from day 10 to day 20 p.i as shown in Fig 5.

**The effect of garlic extract administration on peripheral blood immune cell expression**

Administration of the experimental mice with garlic extract at a dose of 500 mg/kg.b.w caused significant elevation in the percentage of expressed immune cells in the circulatory blood. At day 10 p.i, the percentage of CD4, CD8 and γδ T cells were significantly increased in comparison to the control group. Furthermore, the percentage of expressed cells in the peripheral blood exhibited more elevation regarding continuous administration of garlic extract to day 20 p.i. as shown in Fig (3, 4, and 5) the increase in the expressed immune cells in garlic extract treated group in time dependent manner from day 10 to day 20 p.i as shown in Fig 5.

**Linear correlation of the tested parameters in the garlic extract group between day 10 and day 20 p.i**

A Pearson product-moment correlation coefficient was computed to assess the linear relationship between tested parameters in the garlic extract group in a time-dependent manner. There was a strong positive correlation between day 10 and day 20 p.i with the fixed dose of garlic extract in all the tested parameters. The leukocyte count and lymphocytes % on day 10 exhibited positive correlation with their values on day 20 p.i. The correlation coefficient was r = 0.5501, 0.0850 respectively and the relationship was significant p<0.0001. In this correlation, the increase in the leukocyte count and lymphocytes % at day 20 p/I was associated with the increase in the days of garlic extract administration as shown in Fig 6. Also, between day 10 and day 20 p.i there was a strong positive correlation in the expressed Cd4, CD8 and γδ T cells percentage at the fixed dose of garlic extract. The correlation coefficient was r = 0.0402, 0.1623, 0.2308 respectively, and the relationship was significant p<0.0001. Regarding this correlation, the increase in the expressed immune cells was associated with the increase in the time of administration of the extract when the dose of garlic extract was fixed, as shown in Fig 7.

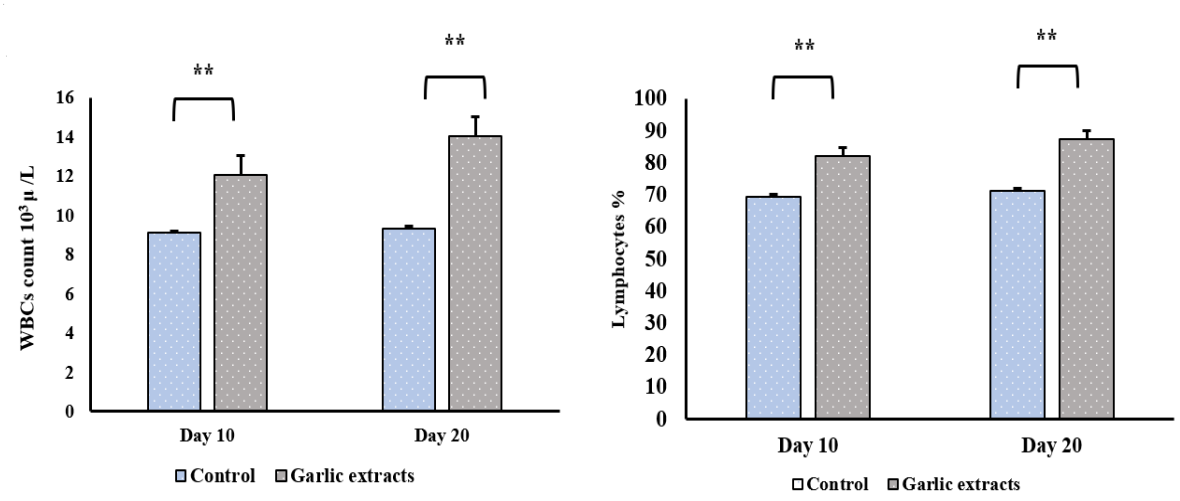


**Figure 1: Effect of garlic extract on the WBCs count and Lymphocytes % in the peripheral blood of mice inoculated with the extract for 20 days. Garlic extract treatment induced significant increase in the WBCs count and lymphocytes % on day 10 and exhibited more increase on day 20 p.i when compared with control group. Bars are means ± SE. stars means significant difference at P< 0. 05.**

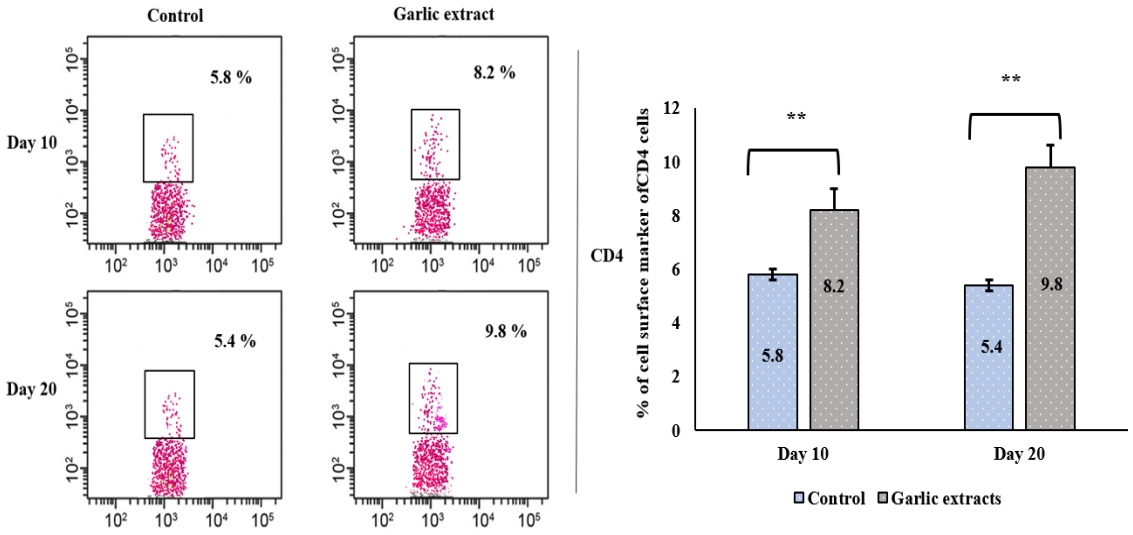


**Figure 2: Effect of garlic extract on the expression of CD4 surface cell marker in the peripheral blood of mice inoculated with the extract for 20 days. Garlic extract treatment induced significant increase in the CD4 expression % on day 10 and exhibited more elevation on day 20 p.i when compared with control group. Bars are means ± SE. stars means significant difference at P< 0. 05**

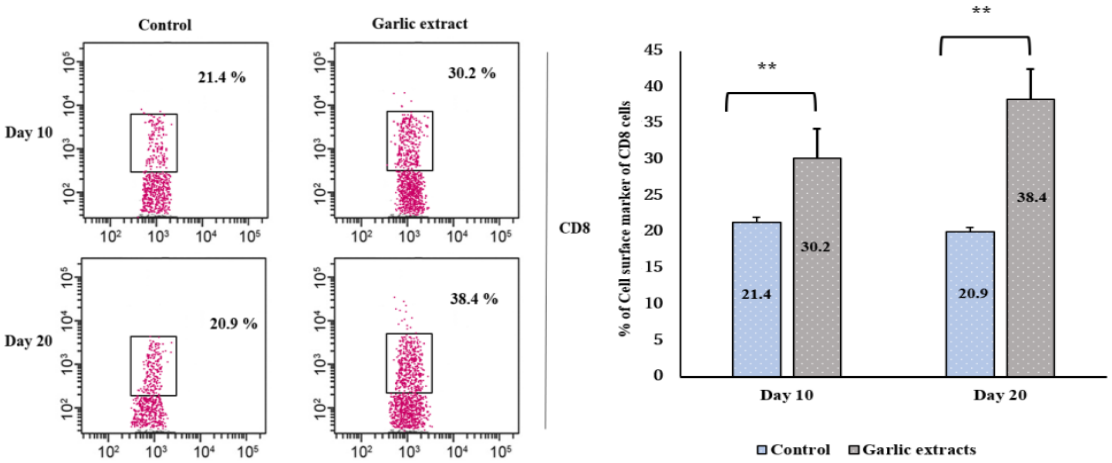


**Figure 3: Effect of garlic extract on the expression of CD8 surface cell marker in the peripheral blood of mice inoculated with the extract for 20 days. Garlic extract treatment induced significant increase in the CD8 expression % on day 10 and exhibited more elevation on day 20 p.i when compared with control group. Bars are means ± SE. stars means significant difference at P< 0. 05**

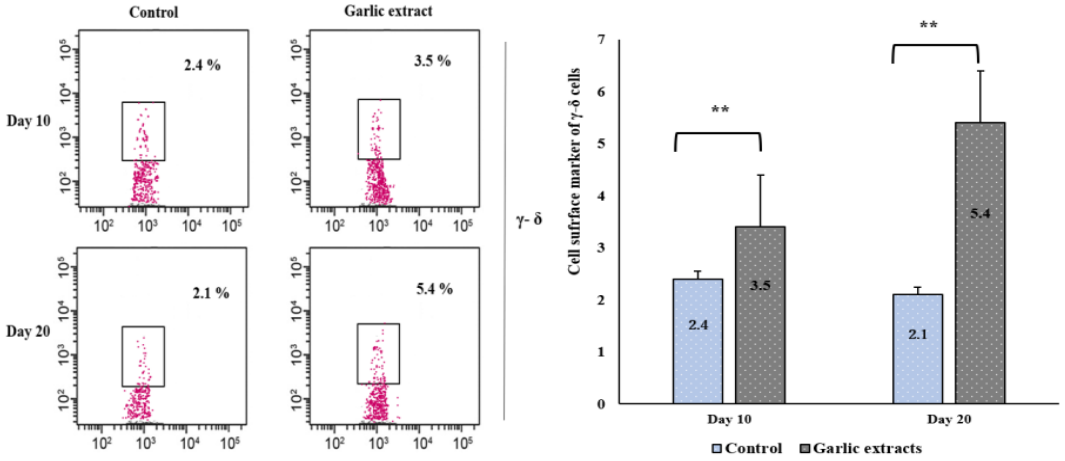


**Figure 4: Effect of garlic extract on the expression of γ-δ surface cell marker in the peripheral blood of mice inoculated with the extract for 20 days. Garlic extract treatment induced significant increase in the γ-δ expression % on day 10 and exhibited more elevation on day 20 p.i when compared with control group. Bars are means ± SE. stars means significant difference at P< 0. 05**

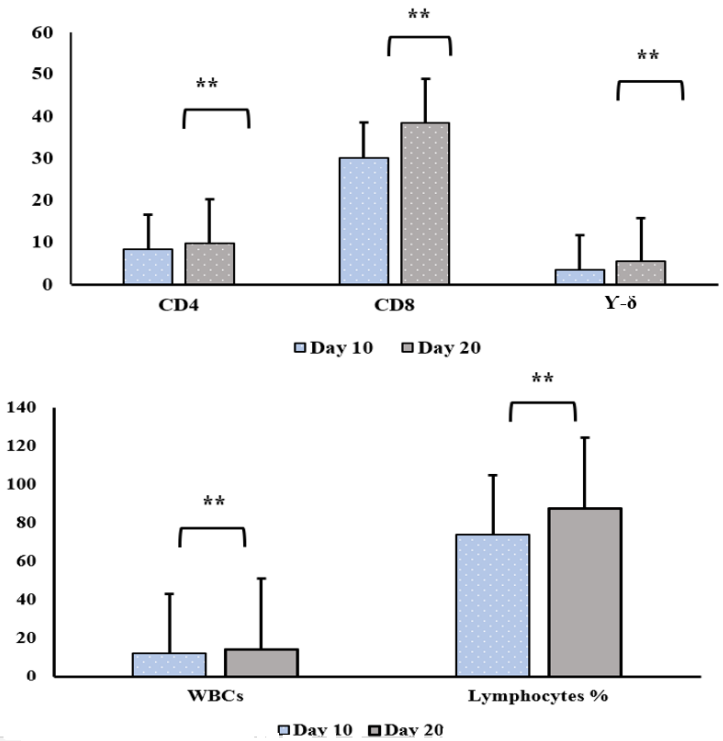


**Figure 5: Showing the kinetic of the expression of CD4, CD8 and γ-δ T cells as well as WBCs count and lymphocytes % in garlic extract group from in days 10 and 20 p.i. the surface cell markers and WBCs count and lymphocytes % were increased significantly in day 20 p.i when compared with day 10 p.i. Bars are means ± SE. Stars means significant difference at P< 0. 05**

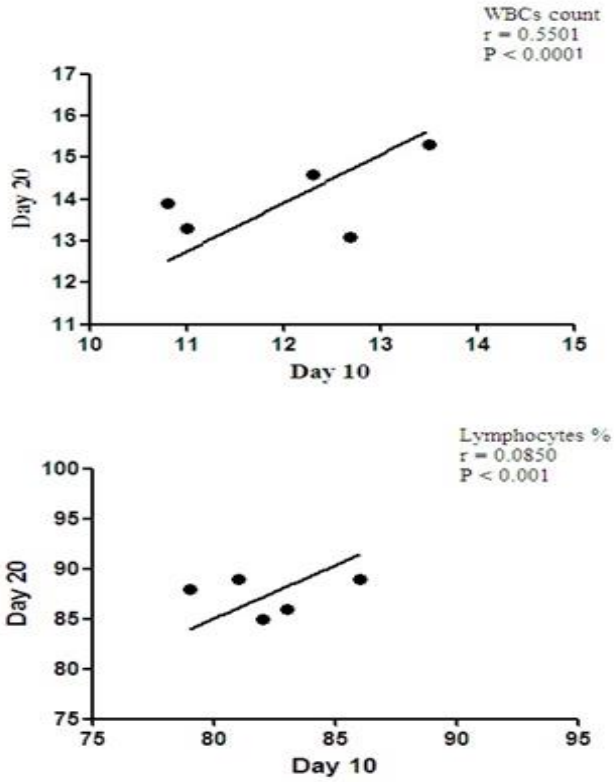


**Figure 6: Linear correlation coefficient between day 10 and day 20 to the tested WBCs count and lymphocytes % with the fixed dose of garlic extract in garlic extract treated group. The WBCS count and lymphocytes % exhibited at day 10 versus day 20 p.i.**

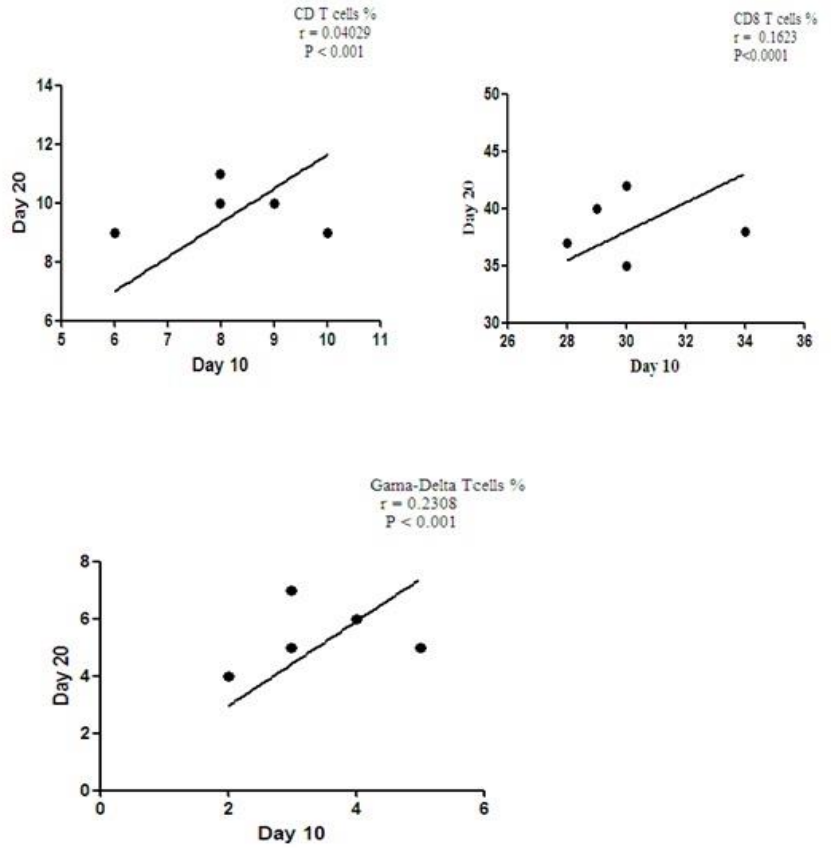


**Figure 7: Linear correlation coefficient between day 10 and day 20 to the expressed CD4, CD8 and γ-δ T cell surface markers with the fixed dose of garlic extract in garlic extract treated group. The % of expressed cells exhibited positive correlation on day 10 versus their % on day 20 p.i.**

## DISCUSSION

There are many beneficial biological effects of garlic that have been reported. There are different health related biological impacts of garlic, including lowering serum cholesterol, triglycerides and blood pressure. In addition, it has anticancer activity, immunomodulatory effects, anti- bacterial and anti-parasitic effects [6]. The widely different effects of garlic preparations and extracts as well as the beneficial and useful properties may be due to the organosulfur and other compositions contained in different concentrations. In this study, inoculation of garlic extract for twenty days to the experimental mice increased the leukocyte count and lymphocytes' % in the peripheral circulation. The same results were observed by Iranolye, [34], who reported that the increase in total white blood cell count was associated with an increase in neutrophils, lymphocytes and monocytes percentage following garlic feeding for 30 days. Our results confirm that garlic extract enhances the immune system against pathogens and infections related to the body. In accordance, leucocytes were increased sharply when infection occurs [35], that confirms the anti-infection role of garlic extract. The immune system stimulation and anti-infection role of garlic extract was attributed to the improvement and the activation of natural killer cells, T-lymphocytes and other immune functions. This suggestion is in agreement with Tang *et al*., and Sumiyoshi *et al*., [36, 37].

It was important in this work to specify and sort the type of immune cells belonging to the lymphocyte's percentage so that the percentage of expression of CD8+, CD4+ and γδ+ T cells was evaluated via fllowcytometeric analysis. Our findings revealed that administration of garlic extract induced a significant increase in the percentage of expressed cells on day 10 and day 20 p.i. our data comes in agreement with results observed by Khalil *et al*., [38]. Our obtained results confirm the immune stimulatory and immune modulatory effects of garlic extract were reported before by many scientific studies. In general, the adaptive immune system role in the body defense depends on lymphocytes, which are present in three main forms: B cells, CD8+ T cells, and CD4+ T cells [39]. Garlic extract enhances the proliferation of CD4 T cells which belonging to TH1 cells and are needed to give activating signals for macrophages. The macrophage activation and is the principal effector action of TH1 cells. Furthermore, CD4 T cells, in addition to activation of macrophage cells, have a critical role in the host defense mechanism against the intracellular and extracellular pathogens that resist killing after being phagocytosis by macrophages [40]. Also, garlic extract increased the expression of CD8 T

cells, which confirms that garlic enhances the cytotoxicity of intracellular pathogens such as coccidian parasites. In a study reported by Khalil *et al*., [41] revealed that garlic extract administration reduced the number of intestinal oocysts in mice infected with *Eimeria vermiformis* due to an increase in the number of intraepithelial Cd8 T lymphocytes. In addition to the cytotoxic effect of CD8 T cells, they also produce a potent macrophage-activating cytokine named IFN-γ, which is secreted by activated Cd8 T cells [40]. Finally, Gamma delta (γδ) T cells proliferated by garlic extract administration in the experimental mice. It was demonstrated that (γ-δ) T cells stimulate the rapid activation and proliferation of other lymphoid cells and the transportable role as anti -pathogen effect in αβ T-cell-deficient mice infected by *Eimeria vermiformis* oocysts [42]. Furthermore, γδT cells caused activation of immunoglobulin A (IgA) responses in the mucosal immunity [43]. This immunoglobulin plays a critical role against mucosal pathogens and has a fast effectiveness [44].

## CONCLUSION

Garlic extracts possess stimulatory and immune modulatory effects on the peripheral cellular immune responses, which allows enhancement of the body's immune defense against various intracellular and extracellular pathogens.

## ACKNOWLEDGMENT

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**Conflict of Interest:** No conflict of interest

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