

RESEARCH ARTICLE

# Effect of 14-kDa and 47-kDa protein molecules of aged garlic extract on peritoneal macrophages

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## Abstract

**Introduction:** Garlic (*Allium sativum*), traditionally being used as a spice worldwide, has different applications and is claimed to possess beneficial effects in several health ailments such as tumor and atherosclerosis. Garlic is also an immunomodulator and its different components are responsible for different properties. The present work aimed to assess the effect of protein fractions of garlic on peritoneal macrophages.

**Materials and methods:** 14-kDa and 47-kDa protein fractions of garlic were purified. Mice peritoneal macrophages were lavaged and cultured in a microtiter plate and exposed to different concentrations of garlic proteins. MTT assay was performed to evaluate the viability of macrophage. The amount of nitric oxide (NO) was detected in culture supernatants of macrophages by Griess reagent and furthermore, the cytotoxicity study of culture supernatants was carried out on WEHI-164 fibrosarcoma cell line as tumor necrosis factor- $\alpha$  bioassay.

**Results:** MTT assay results for both 14-kDa and 47-kDa protein fractions of stimulated macrophages were not significant ( $P > 0.05$ ). Both 14-kDa and 47-kDa fractions significantly suppressed production of NO from macrophages ( $P = 0.007$  and  $P = 0.003$ , respectively). Cytotoxicity of macrophages' supernatant on WEHI-164 fibrosarcoma cells was not affected by garlic protein fractions ( $P = 0.066$  for 14-kDa and  $P = 0.085$  for 47-kDa fractions).

**Conclusion:** according to our finding, 14-kDa and 47-kDa fractions of aged garlic extract are able to suppress NO production from macrophages, which can be used as a biological advantage. These molecules had no cytotoxic effect on macrophages and do not increase tumoricidal property of macrophages.

**Keywords:** *Allium sativum*; nitric oxide; MTT; TNF- $\alpha$

## Introduction

Garlic (*Allium sativum*) is one of the oldest medicinal plants used by different cultures. Already in antiquity it was used for treatment and prevention of some diseases<sup>(1)</sup>. It has been shown that garlic has different applications as an antimicrobial,<sup>(2)</sup> antitumor,<sup>(3)</sup> antithrombotic, hypolipidemic, antiarthritic, and hypoglycemic agent.<sup>(4,5)</sup> A wide range of mechanisms has been suggested for different effects of garlic. Its antitumor effect would be attributable to its antiproliferative action on carcinoma cells and its inhibitory activity on angiogenesis.<sup>(6)</sup> Also, aged garlic extract (AGE) can regulate immune cells as

macrophages. AGE and its constituents are claimed to possess beneficial effects for prevention of atherosclerotic vascular diseases<sup>(7)</sup> which are thought to be at least partly due to its antioxidant properties,<sup>(8)</sup> suppression of CD36 expression and oxidized low-density lipoprotein (OxLDL) uptake into macrophages.<sup>(7)</sup> Our previous studies also demonstrated that garlic enhances natural killer (NK) cell activity<sup>(9)</sup> and T-lymphocyte function<sup>(10)</sup> and suppresses indoleamine 2,3-dioxygenase (IDO) expression.<sup>(11)</sup> A variety of compounds isolated from various preparations have been identified as being responsible for biological effects of garlic. The garlic oil derivatives, diallyl sulfide, diallyl disulfide, and allyl methyl sulfide have

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been shown to have significant effects on macrophage functions,<sup>(12)</sup> whereas allicin influences the apoptosis of macrophages.<sup>(13)</sup> In this study, we examined the protein fractions of AGE on peritoneal macrophages to evaluate its cytotoxicity effect, nitric oxide (NO) production as antioxidant, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) production as an antitumor activity of macrophages.

## Materials and methods

### Animals

Eight- to ten-week-old female inbred Balb/c mice were purchased from Pasteur Institute of Iran (Tehran, Iran). They were kept in animal house of Tarbiat Modares University, given standard mouse chow sterilized water throughout the study.

### Isolation of 14-kDa and 47-kDa fraction from garlic

Fresh garlic bulbs were obtained from Hamadan, Iran. Dry garlic bulbs were peeled and kept in freezer for twelve months in  $-20^{\circ}\text{C}$ . The obtained garlic bulbs were homogenized with two parts of distilled water in a varying blender. The homogenized blend was then filtered under vacuum through Whatman paper (number 1) and the filtrate was centrifuged at 3400g for 30 min. The clear supernatant was collected. Twenty seven grams of  $\text{NH}_4\text{SO}_4$  was added to 1 L of the supernatant and centrifuged at 3400g for 30 min. The residue was resuspended in saline and dialyzed against buffer saline. The isolated fractions were subsequently run on G50 gel chromatography for further isolation and then measured via Bradford assay and evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). L.A.L test (Limulus Amebocyte Lysate) was done for confirm of absence lipopolysaccharide (LPS) in garlic fractions.

### SDS-PAGE electrophoresis

12% (w/v) polyacrylamide gel was used in order to evaluate the purity of molecules and to estimate the molecular mass by the standard protein. After electrophoresis, the gel was fixed by methanol and acetic acid formaldehyde for 60 min and stained with coomassie blue.

### Macrophage culture and stimulation

Macrophages were harvested from BALB/c mice peritoneal cavity with 10mL of Roswell Park Memorial Institute (RPMI) 1640 (Sigmee Chemical Co. UK). The cells were centrifuged at 200g and washed, then adjusted to  $1.5 \times 10^6$  cells/mL in RPMI medium supplemented with 11 mM sodium bicarbonate, 2 mM L-glutamine, 100 U/mL

penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin, and 5% fetal bovine serum. The  $3 \times 10^5$  cell suspensions were plated (200  $\mu\text{L}/\text{well}$ ) onto 96-well flat-bottomed plates (Nunc) and incubated for 4 h for adherence in a humidified atmosphere with 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ . The nonadherent cells were removed by washing the wells with PBS (pH=7.2) three times. Adherent cells contained macrophages incubated for 48 h in RPMI medium. For stimulation, different concentrations of 14-kDa (5, 10, and 20  $\mu\text{g}/\text{mL}$ ) and 47-kDa (5, 10, and 20  $\mu\text{g}/\text{mL}$ ) were added to the cultures as triplicated wells. Nonstimulated macrophages were considered as negative control and the stimulated ones with 50 IU/mL interferon- $\gamma$  (IFN- $\gamma$ ) were considered as positive control.

### Measurement of nitrite concentration

NO was measured using the method of Stuehr and Nathan.<sup>(14)</sup> NO was released into the supernatants of cultured macrophages. NO is unstable and rapidly converts into nitrite and nitrate. Detection of nitrite amount for the purpose of estimating NO level synthesis in the cultures was determined by the standard Griess reagent (1% sulfanilamide/0.1% N-(1-naphthyl) ethylenediamine dihydrochloride/2.5%  $\text{H}_3\text{PO}_4$ ) (Merck). In brief, 50  $\mu\text{L}$  of test solution (supernatants of macrophage culture) was mixed with 50  $\mu\text{L}$  of Griess reagent in a 96-well flat-bottomed plate in triplicate order. After 15 min, absorbance was measured in a Multiskan MS microplate reader at wavelength of 540 nm. Nitrite concentration was determined from the standard curve of sodium nitrite.<sup>(15)</sup>

### MTT reduction assay

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5-Diphenyl-tetrazolium Bromide) reduction assay is widely used to evaluate cell viability. MTT reduction is interpreted to be indicative of cellular metabolic activity.<sup>(16)</sup> Briefly, after 48 h of stimulation, MTT (5 mg/mL in PBS) in one tenth of total volume was added to wells and incubated for 4 h at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$ . Later, supernatants were gently removed and 100  $\mu\text{L}$  of acidic isopropanol (0.04 M HCl in isopropanol) was added in order to dissolve the formazan crystals generated by MTT reduction in living cells. Absorbance was read at wavelength of 540 nm. The test result was expressed as Stimulation Index (STI), which is optical density at 540 nm (OD540) of the test samples/OD540 of negative control.

### Measurement of TNF- $\alpha$ bioactivity

The WEHI-164 clone 13 mouse fibrosarcoma cell line displays dose-dependent cytotoxicity in response to TNF- $\alpha$ .<sup>(17,18)</sup> 100  $\mu\text{L}$  of the cell suspension WEHI-164 clone 13 (20,000 cell/mL) in RPMI complete medium was cultured in 96-well tissue culture microtiter plates (Gibco-Nunc,

Paisley, UK) and 100  $\mu$ L of cell-free supernatants from cultures of stimulated and control macrophages were added. WEHI cultures were then incubated for 20 h at 37°C, 5% CO<sub>2</sub> in a humidified incubator. After incubation, 20  $\mu$ L of a 5 mg/mL MTT solution (Sigma) was added and after 4 h of incubation in 37°C, the pellets were dissolved in acidic isopropanol (0.04 M HCl in isopropanol). OD was read at wavelength of 540 nm. Each sample dilution was assayed in triplicate and Suppression Index (SPI) was calculated.

### Statistical analysis

Results are expressed as mean  $\pm$  SD. Statistical analysis was performed using the Kruskal-Wallis and Mann-Whitney *U*-test. The *P* values < 0.05 were considered to indicate significant differences.

## Results

### Isolation of 14-kDa and 47-kDa fractions of garlic

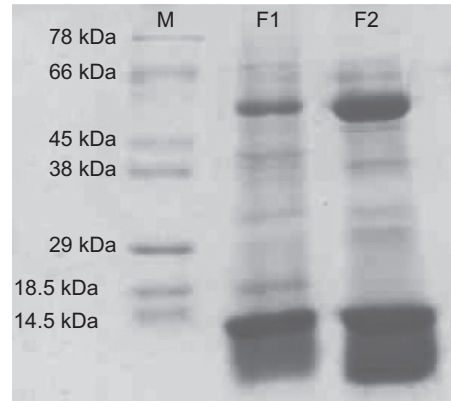
Immunomodulatory molecules were purified from the garlic extract by means of ammonium sulfate and the fractions were collected by centrifugation. L.A.L test reaction was showed that protein fractions has no any LPS, because LPS induce gel coagulation and produce blue color. The isolated fractions were next run on G50 gel chromatography for further isolation of the 14-kDa and 47-kDa proteins. In order to evaluate the purity, these protein molecules were run on the SDS-PAGE electrophoresis and the results indicated the presence of one 14-kDa band along with a band of 47-kDa molecule (Figure 1).

### The effect of 14-kDa protein on MTT assay and NO production

After purification, concentration of 14-kDa protein fraction was determined by Bradford method. 5, 10, and 20  $\mu$ g/mL of 14-kDa proteins was added to each well of microtiter plate of macrophage cultures. As shown in Figures 2 and 3, all three concentrations of the 14-kDa protein significantly decreased NO production from macrophages (*P*=0.007) and it was higher in IFN- $\gamma$  stimulated group compared to control (*P*<0.001). Results of MTT assay indicate that viability of stimulated macrophages with different concentrations was the same as control group (*P*=0.768).

### The Effect of 47-kDa protein on MTT assay and NO production

For the case of 47-kDa proteins purified from AGE, NO production and macrophage viability was evaluated



**Figure 1.** Garlic 14-kDa and 47-kDa fraction bands evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis. In order to evaluate the purity of proteins, the protein molecules were run on the SDS-PAGE electrophoresis and the results indicated the presence of 95% purified bands of 14-kDa and 47-kDa molecules. M, Marker; F1, 27  $\mu$ g/1  $\mu$ L of garlic extract; F2, 55  $\mu$ g/1  $\mu$ L of garlic extract. (See colour version of this figure online at [www.informahealthcare.com/ipi](http://www.informahealthcare.com/ipi))

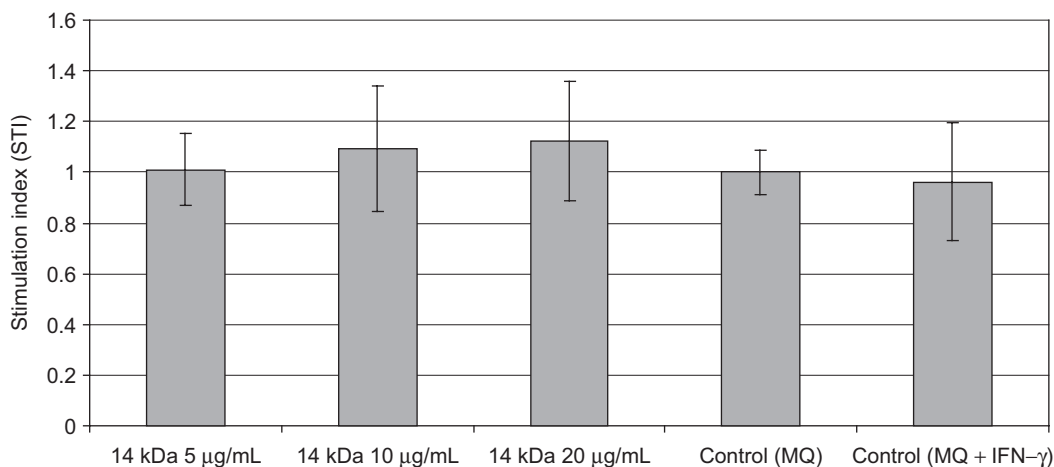
and the results illustrated in Figures 4 and 5 indicate that the amount of NO release from macrophages in all examined concentrations of 47-kDa protein are significantly lower than control group (*P*=0.003) and IFN- $\gamma$  stimulated macrophages produce higher NO levels than nonstimulated controls (*P*<0.001). Macrophages in all groups show the same viability as resulted from MTT assay (*P*=0.739).

### The effect of 14-kDa and 47-kDa proteins on TNF- $\alpha$ bioactivity

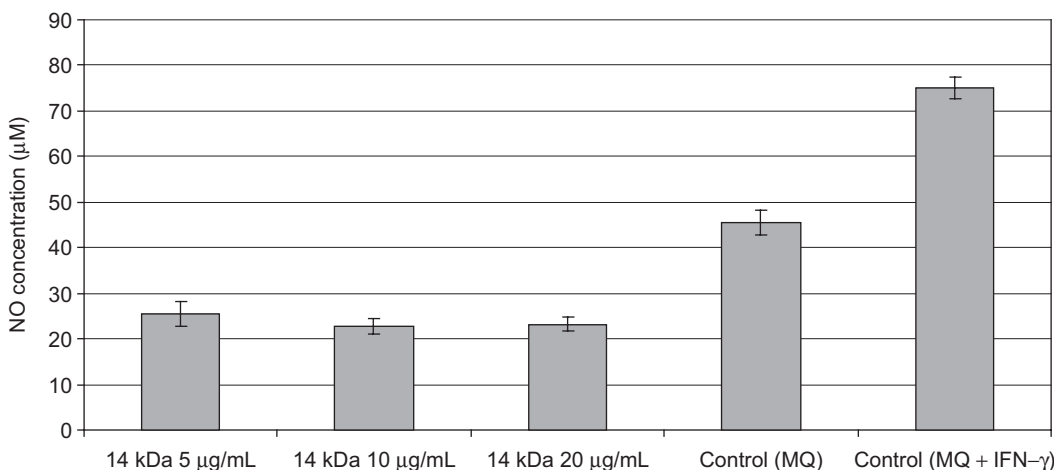
Analysis of suppression index for cytotoxic effect of macrophage supernatant TNF- $\alpha$  on WEHI-164 (Figure 6) showed that both of 14-kDa and 47-kDa proteins could stimulate macrophages to produce and release TNF- $\alpha$  as it resulted in decreased viability of WEHI cells as lower SPI. The results are not nevertheless statistically significant in both of the cases (*P*=0.066 for 14-kDa and *P*=0.085 for 47-kDa proteins).

## Discussion

Garlic (*Allium sativum*) holds a unique position in history and was recognized for its therapeutic potential.<sup>(19)</sup> A wide range of biological activities of garlic *in vitro* and *in vivo* have been verified. Garlic extract modulates immune responses and has been widely used as a flavoring agent and also as a traditional medicine to treat diseases including microbial infections, hyperlipidemia, cancer, and heart diseases.<sup>(20)</sup> Different compositions of garlic are responsible for different effects.



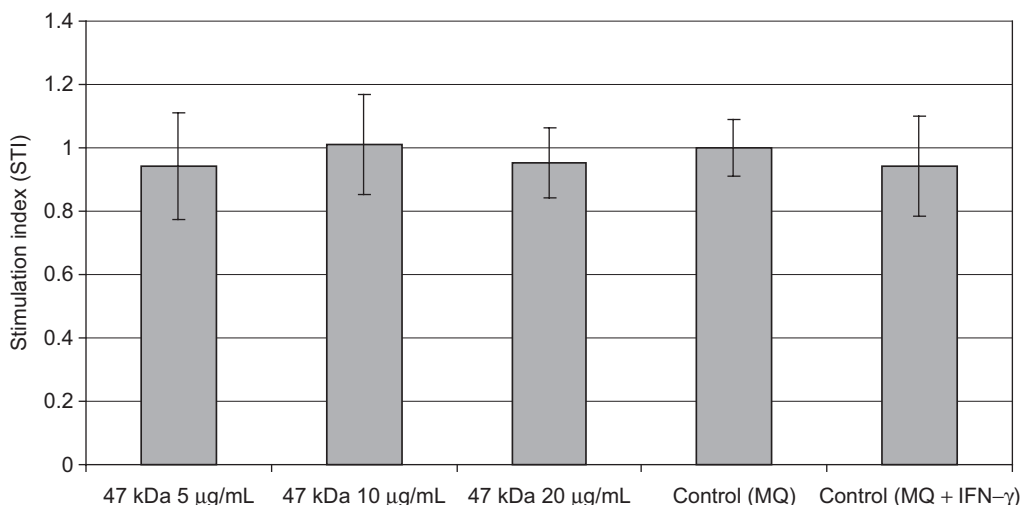
**Figure 2.** The values (mean  $\pm$  SD) of MTT reduction by peritoneal macrophages treated with various concentrations of 14-kDa fraction of garlic and 50 IU/mL interferon- $\gamma$ . MTT reduction capability was the same in all groups ( $P > 0.05$ ). (See colour version of this figure online at [www.informahealthcare.com/ipi](http://www.informahealthcare.com/ipi))



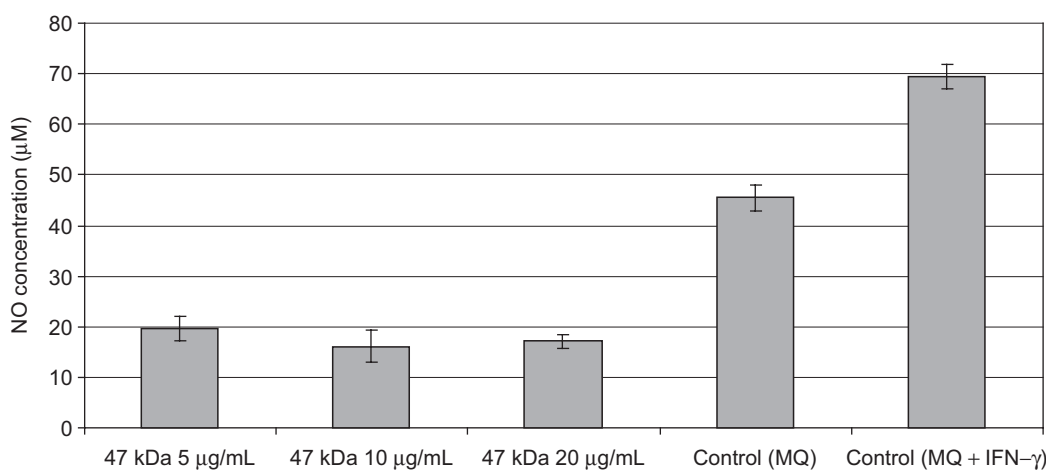
**Figure 3.** The values (mean  $\pm$  SD) of NO production by peritoneal macrophages treated with various concentrations of 14-kDa fraction of garlic and 50 IU/mL interferon- $\gamma$  (IFN- $\gamma$ ). IFN- $\gamma$  increased NO production ( $P < 0.001$ ) and all 14-kDa concentrations decreased NO release from macrophages ( $P < 0.05$ ). (See colour version of this figure online at [www.informahealthcare.com/ipi](http://www.informahealthcare.com/ipi))

Immunomodulatory actions of garlic have been well studied using different purified compounds. In spite of this, garlic's immunomodulatory effects exerted by protein(s) have been rarely reported.<sup>(21)</sup> It has been shown that garlic contains two major proteins constituting 96% of the total garlic proteins.<sup>(10,22)</sup> Ghazanfari et al. isolated two major proteins in their studies, i.e., 14-kDa and 47-kDa proteins.<sup>(10)</sup> Our previous studies demonstrated that 14-kDa molecule isolated from garlic enhanced delayed type hypersensitivity response,<sup>(10)</sup> T-cell proliferation<sup>(23)</sup> and NK cell activity.<sup>(9)</sup> We had in addition shown that it could suppress IDO induction<sup>(11)</sup> and attenuate inflammatory features of allergic airway inflammation in murine model.<sup>(24)</sup> The 47-kDa protein is less studied and is mentioned as a subunit of 110-kDa garlic agglutinin (ASA110).<sup>(21)</sup> In current study, we have shown that both 14-kDa and 47-kDa fractions of AGE

are capable of preventing NO production from macrophages. Suppression of NO production and antioxidant effect of 14-kDa protein of garlic would be beneficial in several health circumstances such as reducing the risk of cardiovascular disease, stroke, cancer and aging, including the oxidant-mediated brain cell damage which is implicated in Alzheimer's disease.<sup>(25)</sup> Inducible NO synthase (iNOS) has recently been shown to be present in human atherosclerotic lesions and promote the formation of deleterious peroxynitrite. These findings suppose that as garlic compounds, the 14-kDa protein along with allicin and ajoene have reduced iNOS mRNA and protein expression in activated macrophages.<sup>(26)</sup> The 47-kDa protein of garlic as well as other compartment of garlic, i.e., allicin and ajoene, has beneficial effects in oxidative diseases such as atherosclerosis. These antiatherosclerosis effects of garlic proteins would be



**Figure 4.** The values (mean  $\pm$  SD) of MTT reduction by peritoneal macrophages treated with various concentrations of 47-kDa fraction of garlic and 50 IU/mL interferon- $\gamma$ . MTT reduction capability was the same in all groups ( $P > 0.05$ ). (See colour version of this figure online at [www.informahealthcare.com/ipi](http://www.informahealthcare.com/ipi))



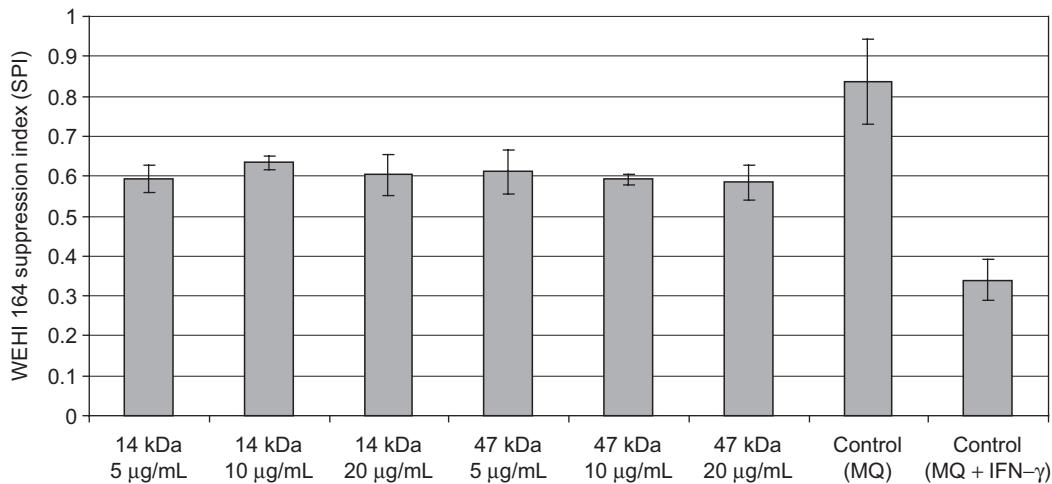
**Figure 5.** The values (mean  $\pm$  SD) of NO production by peritoneal macrophages treated with various concentrations of 47-kDa fraction of garlic and 50 IU/mL interferon- $\gamma$  (IFN- $\gamma$ ). All 47-kDa concentrations suppressed NO production compared to nonstimulated macrophages ( $P < 0.05$ ) and IFN- $\gamma$  induced NO release ( $P < 0.001$ ). (See colour version of this figure online at [www.informahealthcare.com/ipi](http://www.informahealthcare.com/ipi))

adjacent to other effect of garlic extract which inhibits CD36 expression and OxLDL uptake in macrophages.<sup>(7)</sup> Garlic extract has antitumor effects and can inhibit proliferation of tumor cells, but in case of macrophages as an antitumor immune cell, it has been shown that allicin as an important component of garlic inhibits the apoptosis of macrophages.<sup>(13)</sup> We showed that 14-kDa and 47-kDa protein fractions of garlic had no cytotoxic effect on peritoneal macrophages. Analysis of stimulated macrophages' supernatant culture with 14-kDa and 47-kDa fractions on WEHI fibrosarcoma cell line had no significant cytotoxic effect on these cells; therefore, these molecules could not enhance antitumor activity of macrophages. WEHI fibrosarcoma cells are sensitive to TNF- $\alpha$  which is released from macrophages<sup>(17,18)</sup> and these cells have been used for TNF- $\alpha$  bioassay. The

exact molecular mechanisms of these molecule proteins functions are not clear and further studies are required for more clarifications, whereas affect on cytoplasmic nuclear factor- $\kappa$ B factor and expression of mRNA are main intracellular mechanisms that are used by garlic components such as allicin.<sup>(27)</sup> In other studies, total proteins have been found to induce apoptosis through caspase 3 and 9.<sup>(28)</sup> Water and alcoholic extracts of garlic have been reported could affect calmodulin and subsequently calcium-dependent NO synthesis.<sup>(29)</sup>

## Conclusions

So it could be concluded that 14-kDa and 47-kDa garlic proteins do not influence TNF- $\alpha$  production from



**Figure 6.** Suppression index (SPI) mean  $\pm$  SD for cytotoxic effect of macrophage supernatant culture on WEHI-164 fibrosarcoma cells. Differences in all concentrations of 14-kDa and 47-kDa fractions were not significant compared to nonstimulated control ( $P > 0.05$ ). SI for 50 IU/mL interferon- $\gamma$  stimulated macrophages' supernatant was lower than control ( $P = 0.03$ ). (See colour version of this figure online at [www.informahealthcare.com/ipi](http://www.informahealthcare.com/ipi))

macrophages. In summary, this study showed that 14-kDa and 47-kDa fractions of AGE are able to reduce NO production from macrophages and this effect would be beneficial in several conditions, remarkably in atherosclerosis.

## Declaration of interest

This work was supported by a grant of Tarbiat Modares University. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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