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## Original Article

### The Effect of Garlic Extract on Expression of IFN $\gamma$ And Inos Genes in Macrophages Infected with *Leishmania major*

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## ABSTRACT

**Background:** The study was aimed to show the effect of molecular mechanism of Aqueous Garlic Extract (AGE) on expression of IFN $\gamma$  and iNOS genes in *Leishmania major*.

**Methods:** *Leishmania major* promastigotes (MRHO/IR/75/ER) were added to the in-vitro cultured J774 cell line, the cells were incubated for 72 hours. Various concentrations of garlic extract (9.25, 18.5, 37, 74, 148 mg/ml) were added to the infected cells. MTT assay was applied for cellular proliferation. After 72 hours of incubation, supernatants were collected and total RNA was extracted from the infected cells. The express of IFN $\gamma$  and iNOS genes were studied by RT-PCR method.

**Results:** The colorimetric MTT assay after 3 days of incubation showed cytotoxic effect of garlic extract with an IC50 of 37 mg/ml. In addition, IFN $\gamma$  and iNOS genes expression by RT-PCR indicated that garlic extract lead to over expression of these genes in J774 cell line infected with *L. major*.

**Conclusion:** Garlic extract exerts cytotoxic effect on infected J774 cell line. In addition, the hypothesis that garlic can improve cellular immunity with raising the expression of IFN $\gamma$  and of iNOS genes confirmed.

**Keywords:** Aqueous Garlic Extract (AGE), *Leishmania major*, Macrophage, Gene Expression

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## Introduction

**L**eishmaniasis is an infectious disease caused by different species of *Leishmania* protozoa with about 12 million people currently infected and 350 million people at risk of infection (1, 2).

There are two major strategies to treat leishmaniasis: chemotherapy and immunotherapy. Chemotherapy with antimonials are not very effective, instead, a number of immunotherapeutic approaches have been tested with encouraging results. Several cytokines such as IFN- $\gamma$  or GM-CSF, alone or in combination with chemotherapy, have been effective in the progression of the disease treatment (3).

Garlic belongs to the Liliaceae family and is scientifically named as *Allium sativum*. The antibiotic properties of garlic have been known for a long time. For example, in World War II, garlic was used as an antibacterial agent for prevention of gas gangrene. The antibacterial effect of garlic on Gram positive and negative bacteria has been defined for a long time (4). Several studies have been performed on antimycotic properties of garlic including its effects on clinically important dermatophytes *Aspergillus niger* and *Candida albicans* (5). In recent years, a few studies have been performed on the effects of garlic extract for treatment of cutaneous leishmaniasis. It has been suggested that garlic modulates progression of leishmaniasis by augmentation of immune system. Identification of specific component of garlic extract, which is effective in treatment of cutaneous leishmaniasis, is important for confirmation of treatment protocol. Current drugs, such as glucantime, have several side effects and lead to direct cellular damage. If an herbal-based drug eliminates *Leishmania* by augmentation of immune system, it has the potential of possession a wider margin of safety (6).

This project aimed to study the effects of garlic extract on expression of IFN- $\gamma$  and iNOS in macrophages infected with *Leishmania major*.

## Materials and Methods

### *Preparation of aqueous garlic extract (AGE)*

Two hundred g of aged garlic bulbs (*Allium sativum*) were divided into separate cloves. The cloves were peeled and then ground in a small pieces. The garlic aqueous extract was collected by Fromtling and Bulmer modified method (7, 8). Briefly, the minced garlic was shaken with 200 ml of sterile distilled water on a shaker for 48 hours at 37 °C. This mixture was centrifuged at 2300 rpm for 25 min. Then, the supernatant was filtered through Whatman no.1 filter paper (Whatman Corp., Bedford, Mass). The supernatant was sterilized by passing through a 0.2  $\mu$ m Nalgene filter (Nalgene Labware Div., Nalge/Sybron Corp., and Rochester, N.Y.). The sterile lyophilized extract was kept frozen at -80 °C until used.

### *Macrophage*

A BALB/c-derived macrophage-like cell line (J774) was purchased from Pasteur Institute (Tehran, Iran). Cells were grown in Dulbecco's modified minimum essential medium (DMEM) containing heat inactivated 10% FCS. All media were supplemented with 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 250 ng/ml amphotericin B.

### *Leishmania*

A clone of *L. major* strain (MRHO/IR/75/ER) was kindly provided by Dr. Mohebbi (Tehran University of Medical

Sciences,) for establishing experimental infection.

Briefly,  $5 \times 10^5$  cells/ml *L. major* promastigotes, were cultured in RPMI<sub>1640</sub> medium (pH 7.2, containing 25 mM HEPES) supplemented with 10% heat inactivated fetal bovine serum (FBS) and antibiotics at 24 °C for 96 h and subcultured at cell densities of  $2 \times 10^7$  to  $2.5 \times 10^7$  cells/ml.

#### ***L. major promastigotes culture and in vitro assay of parasite growth in macrophage***

Briefly, promastigotes of *L. major* were cultured in RPMI 1640 media containing 10 % fetal calf serum (FCS) for 72 h at 24 °C. Promastigotes in the stationary growth phase were used to infect cultures of adherent macrophages at a final ratio of five parasites per macrophage. Parasites were pelleted at 1000 rpm in a rotor for 10 min at room temperature and then resuspended in RPMI 1640 containing 20 % FCS at a concentration of  $10^6$  parasite/ml. Parasites were washed with RPMI 1640 and immediately prior to addition of parasites, the macrophages were washed with medium. To initiate infection, promastigotes were added to  $1 \times 10^6$  macrophages. After addition of parasites, the macrophages were incubated at 33°C in 5 % CO<sub>2</sub>. Infection was allowed to proceed for 24 h and then unphagocytosed parasites were removed by washing with medium, and the remaining cells were incubated for 72 h at 33°C. Macrophages were then fixed in methanol and stained with Giemsa stain for determination of intracellular parasite numbers. The mean percentages of survival in the culture medium were calculated based on the number of *Leishmania* in untreated culture medium as 100 %.

#### ***MTT assay for cell viability and garlic treatment***

Supernatants were collected after initial stimulation at 24, 48, and 72 hours. Cells were reinsulated with various concentrations

of garlic extract in 9.25, 18.5, 37, 74 and 148 mg/ml. The supernatants were treated with absence of either parasite or garlic, presence of parasite and without extract and with presence of either parasite or garlic. All supernatants were then stored at -20°C until they were assessed for their of cytokines. Cell viability was examined based on the MTT assay (9). The absorbance was measured at 450 nm using ELISA reader (Awareness, Statface 3100).

All values were expressed as the mean  $\pm$  S.D of three independent measurements Results were expressed as the concentration that inhibited parasite growth by 50% (IC<sub>50</sub>: half-maximal inhibitory concentration).

#### ***RNA extraction and cDNA synthesis from L. major infected J774 cell***

RNA was extracted base on the manufacturer protocol. Briefly,  $10^6$  infected J774cells were collected and treated with IC<sub>50</sub> dose of AGE. By using RNA FAST kit, 1 mL of RNA extractor was added to cells. 200 $\mu$ L of chloroform was added to the solution and shaken gently. Finally, 100 $\mu$ L Isopropanol was added to the supernatant. All the solutions samples were centrifuged at 12000 RPM. Supernatant was discarded, and to the precipitates was added 20  $\mu$ L of dH<sub>2</sub>O including DEPC and was frozen in -20°. cDNA library was prepared by using Accu-power RT. Premix kit 1 $\mu$ g RNA was added to 30 pMol of Revers primer and the mixture was incubated at 70°C for 5 minutes and immediately put on ice, and then lyophilized in various vials.

#### ***RT- PCR Reform for iNOS and IFN $\gamma$ Genes***

The mRNA expression levels of iNOS and IFN $\gamma$  genes were analyzed by semi-quantitative reverse transcriptase PCR (RT-PCR)

method after 4 hours of exposure of infected cells to garlic extract.

All the primers used in this study were designed by Gene Runner & Primer Premier Software.

For iNOS and *IFN* $\gamma$  genes the sequence of the forward and reverse primers were respectively.

For iNOS:

Forward primer 5'-

TGCCGGAAGGCGGCTCATTC-3'

Reverse primer

5'CGCAGTGCGTTGCGCATACC-3'

For *IFN* $\gamma$ :

Forward primer 5'-

TGCCGGAAGGCGGCTCATTC

Reverse primer 5'-

CGCAGTGCGTTGCGCATACC-3'

$\beta$ -actin mRNA was used as internal control to adjust the amount of mRNA in each sample.

Briefly, Tag polymeras kit of SinaGene Company was used 30pmol of the forward primer and the reverse primer with 200 $\mu$ M dNTP and 2 $\mu$ M MgCl<sub>2</sub> were added. After standard incubation, All PCR products were run on 1.5% agarose gel for 2 hours and stained with ethidium bromide then recorded using a transluminator. Quantification of the PCR band intensities was accomplished by Kodak 1D image analysis software (Eastman Kodak Co). The values were normalized by the value obtained from the  $\beta$ -actin mRNA.

Data were analyzed by Wilcoxon signed rank test using GraphPad-Prism-5 software.

### **Statistical analysis**

All the experiments were repeated at least three times, and representative results were analyzed. Statistical significance ( $P < 0.05$ ) was analyzed by Student's *t*-test using SPSS version16.

## **Results**

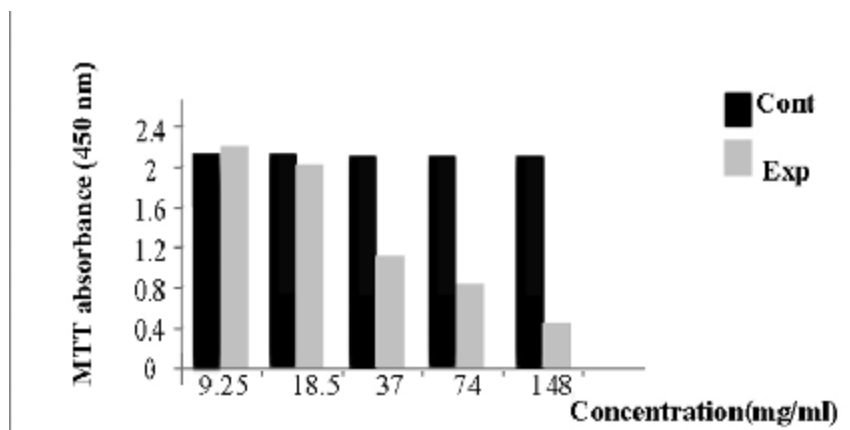
### **Measurement of effective AGE concentration on Leishmania infected macrophage by MTT assay**

The results of MTT assay complied from three experiments in different times 18, 24, and 48 hours are shown in Fig. 1. The viability of the cells was decreased after 48 hours. The result indicated that 37 mg/ml was the best concentration for cells lyses during 48 hours (Fig.1).

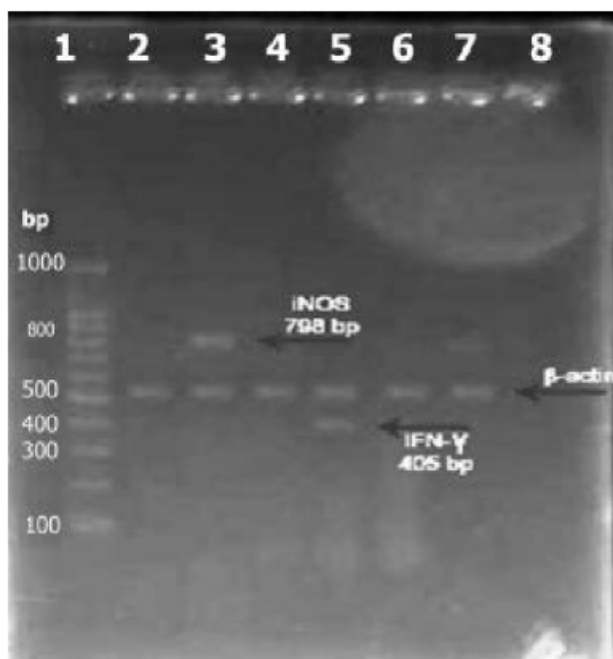
### **Expression of genes (iNOS & *IFN* $\gamma$ ) in J774cell infected with Leishmania major**

The results show that garlic extract with IC50 dose influence the expression iNOS & *IFN* $\gamma$  genes in J774cells. The amount of mRNA of these genes increase to high level. In contrast, this effect was not revealed with the uninfected cells.

$\beta$ -actin was used as the internal control for genes expression study.



**Fig.1:** Results from MTT assay as indicated in macrophages on various AGE concentrations compare with control group



**Fig. 2:** RT-PCR results from iNOS and IFN $\gamma$

**PCR Bonds products:** From left to right

**Line 1:** marker indicate M.W from 100 bp to 1000 bp

**Line 2:** Lack of iNOS expression in infected macrophages without AGE.

**Line 3:** Lack of iNOS expression in non-infected macrophage without AGE.

**Line 4:** Lack of IFN $\gamma$  expression in infected macrophages without AGE.

**Line 5:** iNOS expression in infected macrophages with AGE.(798 bp)

**Line 6:** IFN $\gamma$  expression in infected macrophages with AGE. (405 bp)

**Line 7:** Lack of IFN $\gamma$  expression in non-infected macrophages without AGE.

**Line 8:** Bond 538 bp as a control.

## Discussion

There is no vaccine against leishmaniasis, and treatment options are limited. Pentavalent antimonial still remains the first line therapy for cutaneous and visceral disease but with known side effects in most treated patients. An alternative treatment is amphotericin B, but this drug is also highly toxic and expensive. Consequently, there is need for new and less toxic drugs for treatment of the diseases (10,11). Previous studies has shown that garlic induces shift in cytokine pattern in *L. major*- infected Balb/c mice we were interested in investigating whether it could stimulate leishmanicidal activity in infected macrophages and to determine its mechanism of action (8). We examined the ability of garlic extract to stimulate iNOS and IFN- $\gamma$  gene expression in infected J774 cell. Our results showed the effective dose of AGE for induction the *L. major* infected macrophage gene expression at 37 mg/ml after 48 hours treating.

Garlic extract is more effective than glucantime, and a combination of garlic and glucantime is much more effective in decreasing lesion size than either glucantime or garlic alone, besides, combined therapy leads to a Th1-type cytokine pattern similar to that induced by garlic alone (8). Our finding confirms Ghazanfari's data suggesting that garlic extract contains an immunomodulator, which modulates cytokine patterns towards a Th1-type response and the development of an effective cell-mediated response (8).

Treatment with AGE has several advantages. Preparation of AGE is very easy and even efficient at lower dose of AGE for stimulation of macrophage (12-14), AGE induces IL-12 production and subsequent NO production in macrophage, providing the best stimulation for induction of production of NO. as a lethal molecule for *L. major*

(15). It may provide us with a highly effective approach for the treatment of advanced leishmaniasis. Gurunathan et al. reported that vaccination with DNA encoding the immunodominant single parasite *Leishmania* homolog of receptors for activated C kinase parasite antigen could elicit prolonged protective immunity to *Leishmania major* in an IL-12- and IFN- $\gamma$ -dependent manner (16, 17).

Nitric oxide production is most important effector to eradicate the parasite. Probably, the increasing of iNOS that stimulated from AGE has the major role. Some studies showed IFN $\gamma$  over expression in cellular immunity has an important role, too. These reports investigated the R10 fraction of AGE, and demonstrated immunomodulatory mechanism. The MW of the glycoprotein is 14 K<sub>D</sub> (18).

The study presented here has provided interesting preliminary data, which support roles of AGE effect to the outcome of *Leishmania major* infection via macrophage activation. Our results showed that AGE concentration of 37 mg/ml over 48 hours incubation would destroy amastigotes by over expression of iNOS & IFN $\gamma$  genes in macrophages. Therefore, iNOS & IFN $\gamma$  is crucial for defense against parasitic pathogens, which could affect the immune response to invading *Leishmania* parasites.

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