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## Studies on the Antimicrobial Effect of Allicin on the Intra Macrophages *Brucella*

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**Abstract:** *Brucella* sp. are facultative intracellular bacteria that capable of surviving inside professional and nonprofessional phagocytes. The most important virulence factors of *Brucella* are related to its capability of intra macrophage survival. Allicin the major component of garlic (*Allium sativum*) has antimicrobial activity and was examined for the ability to induce immune responses in murine peritoneal macrophages. In this study, garlic chloroformic extract prepared and quantity of allicin calculated with HPLC, then effect of extract on intramacrophage survival of *B. melitensis* Rev.1 and *B. abortus* S19 on cell culture of mouse peritoneal macrophages studied. Results indicated that extract was effective and eradicated intramacrophage brucellae in 1:40 (equal with 439  $\mu\text{g mL}^{-1}$  allicin), 1:80 (218  $\mu\text{g mL}^{-1}$  allicin) and 1:160 (128  $\mu\text{g mL}^{-1}$  allicin) extract dilutions after 24 h. To give attention to antimicrobial effect of garlic on intramacrophages *Brucella*, it seems to be useful in treatment of brucellosis.

**Key words:** *Brucella*, macrophage, allicin, anti microbial agents and garlic

### INTRODUCTION

*Brucella* spp. nonmotile, nonsporulating, gram-negative short rods, is important facultative intracellular pathogens of human and livestock (Baldwin *et al.*, 1994). The major site of residence of the *Brucella* in both the natural host and humans is the macrophage. Following entry into the host, the *Brucella* are taken up by macrophages, where they display a remarkable resistance to killing by these phagocytes. Indeed, the capacity of these bacteria to maintain long-term residence within macrophages serves as the basis for their ability to establish and maintain chronic infection. Numerous experimental studies using cultured macrophages from a variety of hosts including cattle, humans and mice have shown a clear correlation between the ability of the *Brucella* to resist killing by these host phagocytes and virulence (Drazek *et al.*, 1995; Harmon *et al.*, 1988; Jiang and Baldwin, 1993).

Brucellosis in humans, also known as undulant fever, is a chronic, debilitating febrile illness that can last for months and is characteristically difficult to treat with antimicrobial therapy. Typically, prolonged treatment (4 weeks or more) with two antibiotics is employed for treating brucellosis in humans (Young, 1989, 2000) and similar multiple antibiotics regimens for extended periods

also appear to be required in other hosts (Nicoletti *et al.*, 1985; Nicoletti and Chase, 1987). Even with the prolonged, combined antibiotic treatment regimen, relapses are not uncommon and the fact that no safe and effective vaccine exists that can be used to prevent human brucellosis (United States Department of Health and Human Services, 1996).

Garlic (*Allium sativum*), a member of the lily family, is a perennial plant that is cultivated worldwide. The garlic bulb is composed of individual cloves enclosed in a white skin. It is the bulb, either fresh or dehydrated, that is used as a spice or medicinal herb. Many beneficial healths related biological effects of garlic are attributed to its characteristic organosulfur compounds (Koch and Lawson, 1996). The best known and most extensively studied is allicin (diallyl thiosulfinate), the principal active substance of garlic extract, which is responsible for garlic's typical pungent smell. Allicin is produced during the crushing of garlic cloves by the action of the enzyme alliinase on the compound alliin (Agarwal, 1996). Allicin possesses various biological activities among which antibacterial, antifungal and antiparasitic effects are included (Koch and Lawson, 1996; Agarwal, 1996).

Since allicin is a major component of garlic and macrophages have been shown to be an important component of host defense against *Brucella*, we have

investigated the effect of chloroform extract of garlic (allicin) as a antimicrobial agent on intramacrophage *Brucella* by cell culture method.

## MATERIALS AND METHODS

**Preparation of garlic extract:** A weighed amount of store-purchased garlic was crushed for 1 min using a blender mixer in the presence of distilled water (2 times in weight). The aqueous extract was filtered, then chloroform was added (2 times in aqueous extract) and organic fraction (chloroformic phase) was collected. Chloroform separated from garlic extract in vacuum pump at 40°C and concentrated extract stored at -20°C until further use (Ahmad and Arina, 2001; Bocchini *et al.*, 2001). The purity and concentration of the allicin in the extract was determined by high performance liquid chromatography (HPLC). Quantitative determination of allicin was obtained using a LKB HPLC system with an SP 4290 integrator (Spectraphysics) was base on a previously published method (Rabinkov *et al.*, 1998; Miron *et al.*, 2000; Bocchini *et al.*, 2001).

**Isolation of peritoneal macrophages:** Thioglycollate-elicited peritoneal exudates cells were obtained from 6 to 8-week-old female BALB/c mice following Intraperitoneal injection of 1 mL Brewer Thioglycollate Broth (4.05 g/100 mL) (Difco) and lavage of peritoneal cavity with 5 mL of medium (RPMI 1540 with 10% heat-inactivated fetal calf serum) 4 days later. The cells were washed twice and re suspended in RPMI 1640 with 10% FCS and plated in 96-well polystyrene micro titer plats (Costar, Cambridge, Mass) at  $0.27 \times 10^4$  macrophage per well. The macrophages were incubated for 2 h at 37°C and 5% CO<sub>2</sub> allowed adhering. Then supernatant was aspirated and the adherent macrophage monolayer was washed three times with medium (Sathiyascelan *et al.*, 2000).

**Treatment of peritoneal macrophages with garlic extract:** Peritoneal exudates cells were counted for *in vitro* viability by dye exclusion test with trypan blue (Hudson and Hay, 1989), before and 24 h after incubation of macrophages with various dilutions of extract (1:20, 1:40, 1:80 and 1:160) (Kang *et al.*, 2001). Heated extract (80°C for 30 min) and Salin Normal were used as negative controls

**Effect of extract on intramacrophages *Brucella* sp.:** *B. melitensis* Rv1 and *B. abortus* S19, were grown at 37°C in *Brucella* agar (Mereck) with 10% CO<sub>2</sub> for 48 h to stationary phase, resuspended in Phosphate-Buffered Saline (PBS), washed and resuspended in the same buffer. Bacterial numbers were determined by

comparing the optical density at 600 nm with a standard curve (Arenas *et al.*, 2000). Then bacterial suspension ( $5 \times 10^5$  cfu/mL) was added to peritoneal seeded macrophages and incubated for 2 h at 37°C with 5% CO<sub>2</sub> allowed to phagocytosis of brucellae by macrophages. After, gentamicin at a concentration of 50 µg mL<sup>-1</sup> was added and cultures incubated for 1 h (37°C with 5% CO<sub>2</sub>) for killing of extracellular brucellae. Then the monolayer was washed three times with RPMI 1640 with 10% FCS and various dilutions of extract (1:40, 1:80, 1:160, 1:320 and 1:640) were added. After 24 h incubation (37°C with 5% CO<sub>2</sub>) Triton X-100 was added to lyse the macrophages. The number of cfu in lysates was determined by serial dilutions and plating on *Brucella* agar as previously described (Drazek *et al.*, 1995; Arenas *et al.*, 2000; Eze *et al.*, 2000). Heated extract (80°C for 30 Min) and salin Normal were used as negative controls.

**Data analysis:** Viability counting of macrophages and bacterial CFU analysis were determined independently for each well. Data are expressed as means±standard errors of the mean (SEM) for each treatment group. The significance of differences between groups was determined by one-way analysis of variance (ANOVA) and the p<0.01.

## RESULTS

**Purity of purified garlic extract and concentration of allicin in the extract:** Garlic extract was crushed and mixed distilled water. The aqueous extract was filtered, then chloroform was added and the organic fraction was collected. The purity of garlic extract was evaluated by Nuclear Magnetic Resonance (NMR) as shown in Fig. 1. The concentration of the allicin in the extract was determined by high performance liquid chromatography (HPLC) (Table 1).

**Effect of purified garlic extract on macrophages:** To determine the effect of the purified garlic extract on viability of macrophages, murine peritoneal macrophages were treated *in vitro* with various dilutions of purified garlic extract for 24 h. Viability of macrophages before and

Table 1: Concentration of allicin in the extract dilutions

Dilution of extract cells	Allicin concentration (µg mL <sup>-1</sup> )
1:20	1071±SD
1:40	439±SD
1:80	218±SD
1:160	128±SD
1:320	64±SD
1:640	22±SD

p<0.05 indicates a significant difference in comparison with pre training.  
SD: Standard Deviation. Heated extract (80°C for 30 Min) and salin normal were used as negative controls

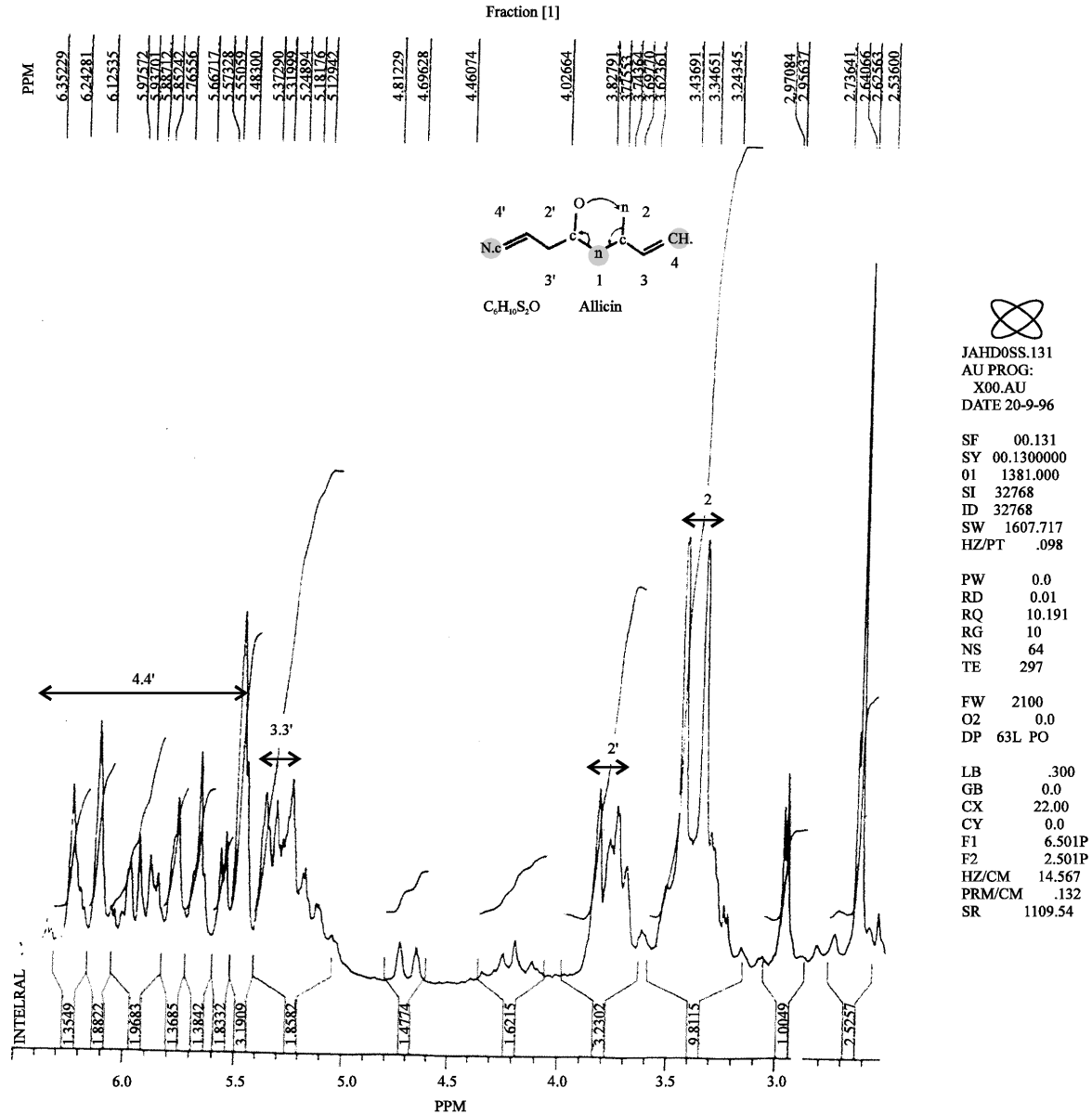


Fig 1: NMR spectrum of allicin

Table 2: Means of viable macrophages at 0 and 24 h after treatment with purified garlic extract

Dilution of extract	Percentage of viable macrophages $\pm$ SD
Lack of extract at 0 time	83.0 $\pm$ 7
Normal saline (control) after 24 h	78.6 $\pm$ 6
1:20 after 24 h	26.4 $\pm$ 4*
1:40 after 24 h	69.0 $\pm$ 6
1:80 after 24 h	78.2 $\pm$ 8
1:160 after 24 h	85.5 $\pm$ 3

\*p<0.05 indicates a significant differences comparing with the control group. SD: standard Deviation. Heated extract (80°C for 30 Min) and salin Normal were used as negative controls

after incubation with dilutions of extract were determined and expressed (Table 2 and Fig. 1). Results indicated that

dilution 1:20 of extract shown inhibitory effect on peritoneal macrophages while this effect were not seen by other dilutions.

**Effect of purified garlic extract on intramacrophages *Brucella* spp.:** In order to assess the number of cfu of *B. abortus* S19 and *B. melitensis* Rev.1 in lysates macrophages for both treated and untreated macrophages, both lysate were cultured in Brucella agar. The results in Table 3 showed a significant decrease in the number of colony forming in the Brucella agar plates comparing with control group. We also noticed that at the

Table 3: Means number of cfu of intramacrophages *Brucella* per mL in lysates by plating (primary inoculation was  $5 \times 10^5$  bacteria)

Dilution/strain	S19±SD	Rev±SD
Normal saline (control)	$(106 \pm 5) \times 10^3$	$(227 \pm 3) \times 10^3$
1:40	Not growth*	Not growth*
1:80	Not growth*	Not growth*
1:160	$1.2 \pm 1^*$	$1.8 \pm 1^*$
1:320	$176 \pm 100^*$	$187 \pm 100^*$
1:640	$(64 \pm 7) \times 10^2^*$	$(95 \pm 2) \times 10^2^*$

\*p<0.01 indicates a significant differences comparing with the control group. SD: standard Deviation. Heated extract (80°C for 30 Min) and saline Normal were used as negative controls

dilutions of 1:40, 1:80 and 1:160 causes complete elimination of intracellular brucella at 24 h.

## DISCUSSION

Macrophages are particularly important for the survival and spreading of *Brucella* during infection (Arenas *et al.*, 2000). Garlic extract have long been established to exhibit wide spectrum antimicrobial activity and immune enhancing effects. Since allicin is one of the active principles in garlic, the effect of allicin on various functions of murine peritoneal macrophages was investigated (Koch and Lawson, 1996; Agarwal, 1996; Kang *et al.*, 2001).

In present studies and after quantitative determination of allicin in the extract by HPLC (Table 1), the effect of extract dilutions on viability of macrophages was determined (Table 2). Results indicated that dilution of 1:20 of extract shown inhibitory effect on peritoneal macrophages while this effect was not seen by other dilutions. Therefore we not use from dilution 1:20 for next study (antimicrobial effect of extract on intramacrophage brucellae).

The effect of purified garlic extract on antimicrobial intramacrophage brucellae, the number of cfu of *B. abortus* S19 and *B. melitensis* Rev.1 in lysates for each treatment group by plating (Table 3) shown significance differences with control group. Dilutions 1:40, 1:80 and 1:160 causes complete elimination of intracellular brucellae at 24 h. With due attention to results of Table 2 and 3, it is apparent that allicin not only had strong antimicrobial activity on brucellae, but also activated macrophages and these activated cells exhibited the significant increase in killing of intracellular brucellae.

In conclusion, the data presented here clearly show that garlic extract was effective on intramacrophages *Brucella* spp. *in vitro*. Also other investigators had shown the immune enhancing effect of garlic extract such as activation of macrophages and T-lymphocytes (Koch and Lawson, 1996; Agarwal, 1996; Kang *et al.*, 2001). Garlic has been used different preparation of its which included, dried powder, juice and oil. The majority

of studies used dried garlic powder (Elkayam *et al.*, 2001) and we suggest using of garlic and its compounds in treatment of brucellosis, especially for animal brucellosis, there is no effective antibiotic therapy or multiple antibiotics regimens for extended periods also appear to be required.

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