

Therapeutic Efficacy of Garlic (*Allium sativa*) Against Burn Wound Infection by *Pseudomonas aeruginosa*

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Abstract: Treatment of multi-drug resistant *Pseudomonas aeruginosa*, which causes burn infection is a big challenge in clinics and needs novel strategies. Garlic extract has potent *in vitro* antibacterial activities against number of gram negative and gram positive bacteria including *P. aeruginosa*. The aim of this study, was to evaluate *in vivo* therapeutic efficacy of garlic extract in treating burn wound infection caused by *P. aeruginosa* in burned mouse model. Burn was induced on the back of anesthetized animals by hot water, after the hair removal. Bacterial infection was established by topical applying of highly pathogenic clinical isolate of *P. aeruginosa*. Potential of garlic extract on reduction of mortality was evaluated by topical application of 10% (v/v) garlic extract on burned and infected animals (treatment group 1) and was compared with two control groups: Burned and infected animals either treated with topical Silver Sulphadiazine (SSD) (1%) (treatment group 2) or left untreated (treatment group 3). The same groups were subjected to evaluate bacterial counts in organs (blood, liver, spleen and skin). Our results indicated that topical administration of garlic extract (10%) extended the survival of mice for 3-6 days, compared with survival of the untreated group. Both garlic extract and SSD treatments reduced the microbial loads in vital organs (blood, liver, spleen), compared to that of untreated control group ($p < 0.05$). These results demonstrate the potential of garlic extract in the treatment of burn wound infection caused by *P. aeruginosa*.

Key words: *Pseudomonas aeruginosa*, burn infection, silver sulphadiazine, garlic extract

INTRODUCTION

Burns are among the main causes of death of humans in the world, especially in developing countries. The world's death record for fire related burns in 2002 was about 322000 (WHO, 2006). Despite advances in medical cares, infections are the major causes of death in patients with sever burn (Church *et al.*, 2006). Burn injury by itself and subsequent suppression of immune system predispose burn patients to infections with wide variety of microorganisms including bacteria (Revathi *et al.*, 1998), fungi (Spebar and Lindberg, 1970; Enoch *et al.*, 2006) and viruses (Sheridan *et al.*, 2000) gram negative rod, *P. aeruginosa*, is among the leading causes of burn wound infection (Rastgar Lari *et al.*, 1998; Japoni *et al.*, 2006; Tredgat *et al.*, 2004) causing tissue necrosis and sepsis which is often fatal (Wurts *et al.*, 1995). The situation for patients with *P. aeruginosa* infections is particularly problematic, because this organism rapidly evolves to multi-drug resistant forms and spreads through hospital wards and burn units (Douglas *et al.*,

2001; Hsueh *et al.*, 1998) so it could not be eradicated readily with common antibiotic therapies (Livermore, 2002). To combat this critical situation and reduce risk of antibiotic resistance development there is a compelling need for novel strategies in treatment of *P. aeruginosa* infections.

Garlic (*Allium sativum*) has been used as a medicine since ancient times and has long been proven to have excellent antimicrobial activity against wide variety of microorganisms *in vitro* condition (Harris *et al.*, 2001). Garlic extract contains small molecules (Allicin and its breakdown products) that cross the cell membranes and combine with sulfur-containing groups in amino acids and proteins, thus interfering with cell metabolism (Ankri and Mirelman, 1999). Indeed, allicin and garlic extract have been shown to have a wide spectrum of antibacterial activity, including effects on enteric and oral bacteria, *Staphylococcus*, *Clostridium*, *Mycobacterium* and *Helicobacter* sp. *in vitro* condition (Harris *et al.*, 2001; Ross *et al.*, 2001; Bakri and Douglas, 2005). It has also been shown that garlic extract has good antibacterial

activity against *P. aeruginosa* (Tsao and Yin, 2005). This has further been demonstrated by finding of a novel mode of action for garlic extract against *P. aeruginosa*; it involves in inhibiting of biofilm formation, a process takes place in pathogenesis of the bacterium in lung infection (Rasmussen *et al.*, 2005).

The objective of present study, was to evaluate the *in vivo* efficacy of garlic extract in the experimental treatment of burn wound infection by *P. aeruginosa* in burned mouse model.

MATERIALS AND METHODS

Bacteria: *P. aeruginosa* was provided by clinical laboratory of Imam Hospital, Medical University of Ardabil. The bacterium had been isolated from a burn patient with *P. aeruginosa* septicemia. The isolate was a multi-drug resistant organism. It was resistant against 7 antibiotics including: Cephalothin, cefaclor, ampicillin, cotrimoxazole, ceftizoxime, imipenem and meropenem; susceptible against ciprofloxacin, amikasin, gentamicin. The susceptibility pattern against ceftriaxone was intermediate.

The bacterium was cultured in trypticase soy broth medium, stored at 4°C for daily uses and 70°C along with 15% glycerol for subsequent uses.

Burned mouse model wound infection: Eight week old Swiss albino female mice were obtained from the mouse breeding colony at institute Pasture of Iran. Burn was induced as described previous (Rumbaugh *et al.*, 1999). Briefly, groups of mice were anesthetized with ketamin-xylazin (50 mg kg⁻¹ intramuscularly) and their back were shaved; to induce burn in the back of animals, the mice were securely placed into a template with an opening specifically designed to expose 15% of their body surface. Thermal injury was induced by placing the exposed area of the shaved skin into 90°C water for 10 s. A subcutaneous injection of 0.8 mL of 0.9% NaCl solution was administered as fluid replacement. To establish the lethal dose of bacteria for burn wound infection, the mice were preprimed with cyclophosphamide (200 mg kg⁻¹ of body weight) given intraperitoneally. Three day later, the mice were shaved and burns induced as described above. The number of 10¹¹ freshly prepared bacteria (100 µL inoculums in PBS), were then topically applied on the site of the burned area.

To optimize the survival duration time of mice after burn induced infection, two groups of 5 mice (burned and infected group and burned but non-infected group) were used. The mortality rate was monitored for several days

compared with non-infected control group and the day that animals in infected group were died was recorded. In later experiments, to determine the bacterial load in animal organs, the mice were sacrificed the day before death.

Treatment procedures: Groups of 5 mice were burned and challenged with *P. aeruginosa*. At 2 and 8 h post challenges on day 1 and twice daily on days 2 and 3, the burn site was treated topically as follows:

First group of mice were treated with SSD (1%) as standard therapy for burned skins (Klasen, 2000). The second group was treated with garlic extract (10%) and the last group was treated with PBS as control. On day 5 after treatment, these mice were sacrificed and quantitative bacterial counts of the skins, livers, spleens and blood were performed.

Additional groups of treated and untreated mice were used for assessment of mortality or survival rates. This experiment was followed-up for 12 days.

Quantitative tissue culture: To determine the bacterial counts in the blood and organs of experimental animals, blood, spleens, livers and the burnt skins were aseptically removed. The blood (200 µL) was serially diluted in sterile PBS and 100 µL of the diluted blood was cultured on trypticase soy agar plates for growth. For the tissues, samples were homogenized in 2 mL of sterile PBS by hand-mead tissue grinder. The tissue homogenates were serially diluted in sterile PBS and then cultured on trypticase soy agar plates. The inoculated plates were incubated at 37°C overnight. The number of colonies that grew on plates were counted and presented per milliliter for blood and gram for tissues.

Extraction of garlic: Garlic cloves were obtained from a local farmer. Prior to extraction, the stem and dead leaves were removed from the garlic bulbs. A total of 500 g of garlic cloves were crushed using a food blender along with 200 mL of sterile distilled water and the obtained mash was squeezed and the suspension was filtered through Whatman no. 1 filter paper. The filtrate was sterilized by passing through 0.45 µm nitro cellulose membrane and stored at 4°C until use.

Statistical analysis: The Wilcoxon rank sum test and the Fisher's exact test (Statview; Abacus Concepts, Inc., Berkeley, CA) were used to determine significant differences between the numbers of CFU g⁻¹ tissue in bacterial colonization experiments and between groups for the mortality experiments, respectively. A p value less than 0.05 was considered statistically significant.

RESULTS

Establishment of burn infection: The results showed that topical administration of 10^{11} CFU of *P. aeruginosa* on burn sites of mice resulted in eventual death of all animals on day 6 post challenges. Whereas all burned but non-infected control group animals survived in the same period of the time.

Effect of garlic extract treatment on survival rate: To determine whether garlic extract treatment of wound infection would further enhance survival and decrease mortality rate of burned and infected mice, infection was induced by topical application of the bacteria into burn site on the animal's backs and treated the same manner. The results indicated that topical administration of garlic extract (10%) extended the survival of mice for 3-6 days in comparison with survival rate of the untreated group during 12 days follow-up (Table 1). On day 6 all of the 5 mice of untreated control group were died while 100% of garlic extract treated group were survived during the same time.

The efficacy of garlic extract for the treatment of burn wound infection was compared to that of SSD (1%), a standard therapy that is used in treatment of burn wound infection. Garlic extract was found to be effective as SSD (1%) resulted in 100% survival until the day 8 post challenges and treatment ($p > 0.05$). But after the day 9, survival rate decreased significantly and reached 0% on day 12. Whilst SSD treated group survived during 12 days follow-up.

Quantitative culture data: To determine the ability of garlic extract to inhibit the spread of bacteria from infected burn wound to other organs, quantitative bacterial counts of the skins, livers, spleens and blood were performed compared to untreated control and SSD treated groups

Table 1: Effects of topical uses of garlic extract and SSD on mortality in burned mice infected with the *p. aeruginosa*

| Treatment | No. of dead mice/total no. of mice on day: | | | | | | | | | | | |
|----------------------|--|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| Untreated control | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 5/5 | 5/5 | - | - | - | - | - |
| SSD (1%) | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |
| Garlic extract (10%) | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 1/5 | 1/5 | 4/5 | 5/5 |

Table 2: Quantitative culture data from treated and untreated mice

| Treatment | Median CFU g ⁻¹ or mL of indicated tissues | | | |
|-------------------|---|---------------------------|----------------------------|----------------------------|
| | Skin | Liver | Spleen | Blood |
| Untreated control | 4.07×10 ⁴ ±114.1 | 2.14×10 ³ ±37 | 1.63×10 ³ ±19.2 | 3.45×10 ⁴ ±28.1 |
| SSD (1%) | 8.0×10 ³ ±95.6 | 4.2×10 ³ ±22 | 1.6×10 ³ ±17 | 2.3×10 ⁴ ±15.9 |
| Garlic extract | 3.9×10 ³ ±102.3 | 2.2×10 ³ ±18.3 | 1.4×10 ³ ±20 | 1.5×10 ³ ±20.7 |

(Table 2). The results showed the both garlic extract and SSD treatments reduced the microbial loads in indicated organs compared to that of untreated control group ($p < 0.05$). A significant reduction of bacterial counts was not observed in garlic extract treated group skins compared to that of SSD treated group but about three other organs the count were significantly lower in SSD treated group ($p < 0.05$).

DISCUSSION

Several studies showed garlic extract and its derivatives were used successfully against multi-drug resistant *P. aeruginosa in vitro* (Bakri and Douglas, 2005; Tsao and Yin, 2001; Iwalokun *et al.*, 2004). *In vivo* application of garlic extract in treatment of bacterial infections is limited to *p. aeruginosa* lung infection in murine model that its result is consistent with our findings (Bjamsholt *et al.*, 2005).

As indicated in results, topical treatment of burned and infected mice with garlic extract, partially extended the survival time of animals over untreated group but SSD treated group survived during follow-up period. Prolonged survival rate of SSD treated group versus garlic extract treated group may be attributed to mechanism of action of two substances. SSD is bactericidal agent (Klasen *et al.*, 2000) whereas in spite of pure derivatives of garlic (Bakri *et al.*, 2005; Tsao *et al.*, 2001), garlic extract depends on its biologically active ingredients content, may acts as a bacteriostatic agent (Leuschner and Ielsch, 2003). On the other hand, treatments were used in two different formulations that affect the maintenance time of drug on burn site and so the results. SSD could be present prolonged time on burn site because it was used as commercial cream but garlic extract used in simply aqueous form.

The treatment results showed topical administration of garlic extract was effective ($p < 0.05$ versus untreated control group) in preventing systemic spread of local infection of the burned site into vital organs such as liver, spleen and blood. It showed that beside antimicrobial effects, garlic extract well diffused depth of the wound site and prevents the spread of bacteria which is a very important aspect of the use of the topical agents for burns. Previously the ability of allicin, the main biologically active antimicrobial ingredient in garlic, to cross through membranes (artificial and biological) was studied. The researchers observed that phospholipids bilayers do not constitute a barrier for allicin penetration. They concluded that the biological effectiveness of allicin is because of its high intracellular reactivity with both low and high molecular weight thiols, as well as its accessibility resulting from high membrane permeability

(Miron *et al.*, 2000). Garlic is commonly used in traditional dietary and medicinal applications (Ellen, 2005). Reports about the safe and successful intravenous apply of garlic derivatives in china against invasive bacterial and fungal infections have been made (Davis, 2004). It has been suggested, that the selective toxicity of garlic derivatives is due to that human cells contain glutathione, a sulfur-containing amino acid that combines with allicin, thus preventing cell damage (Robinko *et al.*, 2000). This is a superiority for garlic extract versus the other topically used antimicrobials that well diffuse but are toxic when enter inside the body such as Mafenide acetate and povidone-iodine (Monafo and Ayvazian, 1978) or has poor activity against *P. aeruginosa* as nitrofurazone (Pirnay *et al.*, 2003). On the other hand, there are documented evidences that *P. aeruginosa* develops resistance against SSD that makes it ineffective in clinic (Pirnay *et al.*, 2003) whereas it has been suggested that development of resistance to allicin arises 1000-fold less easily than it dose to certain antibiotics (Gupta and Viswanthan, 1995).

Garlic extract act synergistically in combination with common antibiotics against *P. aeruginosa*. In an experiment Tsao and Yin (2001) observed additive and synergistic effects of garlic extract in combination of ceftazidime, gentamicine, imipenem and meropenem with garlic derivatives (Tsao *et al.*, 2001). In another study Bjamsholt *et al.* showed garlic extract treated *P. aeruginosa* is more sensitive against tobramycin (Leuschner *et al.*, 2003).

About the burn infection treatment the ideal agent would be broad spectrum and unlikely to induce resistance in the infective organism and it would be to stimulate immune responses and also to reduce oxidative damages.

Garlic with its wide range antimicrobial activists, has been subject of many studies and could be a potential alternative in treatment of burn patients. Beside its antimicrobial effects, it has approved antioxidant activity that scavenges damaging free radicals release during burn process (Sener *et al.*, 2003; Leelarungrayub *et al.*, 2006; Haycock *et al.*, 1997) and induces suppressed immune system of burn patients (O'Sullivan and O' Conner, 1997; Salman *et al.*, 1999; Colic and Savic, 2000).

CONCLUSION

The preliminary results presented in this study, suggest that garlic extract may offer a promising and novel means for the treatment of *P. aeruginosa* burn wound infection and open new insight into further investigation on this area.

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