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Qualitative Evaluation and Biological Effects of the Leaf and Bulb Heat-stable Proteins in Two Garlic Clones (*Allium sativum*)

Farzaneh Ghanbari[1], Hojatallah Mazaherilaghab[*1], Gholam Khodakaramian[2]

[1]*Department of Plant Breeding, Bu-Ali Sina University, Hamedan, Iran*
[2]*Department of Plant Protection, Bu-Ali Sina University, Hamedan, Iran*

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ABSTRACT

Due to pathogen resistance as well as the high costs and adverse environmental effects of the use of chemical pesticides, researchers are looking for other ways to control pests and diseases such as biological control. Many studies prove the antibacterial effects of the biochemical compounds of the garlic plant, but there is no report on the antimicrobial activities of the heat-stable protein of the garlic plant. Considering the proven role of these proteins in response to stresses, this study was conducted to investigate the antibacterial role of these proteins on *Ralstonia solanacearum* and *Streptomyces scabies* bacteria. The antimicrobial properties of each bacterium were tested in separate experiments using a completely randomized factorial design with three factors and three repeats. Heat-stable protein from Clones, tissues, and various concentrations applied to *R. solanacearum* bacteria expressed a highly significant difference in the diameter of the inhibition zone and The highest inhibition zone was related to the leaf of Hamadan Clone. The lowest minimum inhibitory concentration MIC and minimal bactericidal concentration MBC were related to the leaf HSP of the Hamadan clone. As a result, Hamadan leaves with smaller MIC and MBC and the larger inhibitory zone in relation to the other treatments showed the highest inhibitory effect. In SDS-PAGE electrophoresis the leaves heat-stable protein electrophoresis banding only the HSP40 family was observed, while, on the garlic cloves families, small HSP (sHSP), HSP40, HSP70, HSP90, and HSP100 were seen. The results indicate that heat-stable protein from garlic could be used as a major antimicrobial agent against pathogenic *R. solanacearum* bacteria but had no biological role as an antimicrobial on *S. scabies* bacteria. The results of the present research show that the HSP of garlic plants can be used to create resistance to *R. solanacearum* bacteria.

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1. Introduction

In recent years, there has been an increase in the number of diseases caused by bacterial, fungal, and viral infections. Infections affect plants at different stages of agricultural production. Depending on weather conditions and the phytosanitary condition of crops, the prevalence of diseases can reach 70-80% of the total plant population, and the yield can decrease in some cases down to 80–98% (Nazarov *et al*., 2020).

Today, the main problem in modern agriculture is not to use chemical poisons, because the use of chemical poisons, in addition to the resistance of pathogenic agents to these poisons, brings high costs and destructive environmental effects (Singhai *et al*., 2011). Currently, the attention of many researchers has been drawn to certain classes of plant polypeptides that are capable of exerting antimicrobial effects (Gan *et al*., 2021).

Garlic, with the scientific name *Allium sativum*, is an herbaceous and biennial plant from the *Alliaceae* family (Saif *et al*., 2020). Garlic extract inhibits the growth of Gram-positive and Gram-negative bacteria, such as *Staphylococcus*, *Streptococcus*, *Micrococcus*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Lactobacillus*, *Pseudomonas*, *Shigella*, *Salmonella*, *Proteus*, and *Helicobacter pylori* (Bin *et al*., 2020).

Vascular wilt disease is caused by *Ralstonia solanacearum* bacteria and is one of the important

* Corresponding author.
E-mail address: hojat.mazahery@basu.ac.ir

bacterial diseases of potatoes (Moussa *et al*., 2022). Among the existing control methods, the use of resistant cultivars and biological control methods are safe and effective methods in controlling this disease (Mirzaei-Najafgholi *et al*., 2015). *Streptomyces scabies* are a group of bacteria that cause an important and economic disease of common potato scab. This bacterium attacks the root and crown of potatoes and other tuberous vegetables (Liu *et al*., 2021).

Antimicrobial peptides are small proteins that are constituents of the innate immune system in every kind of living organism. They can act by disrupting the microbial membrane or without affecting membrane stability. Through membrane or metabolic disruption, they defend an organism against invading bacteria, viruses, protozoa, and fungi. High efficacy and specificity, low drug interaction and toxicity, thermostability, solubility in water, and biological diversity suggest their applications in food, medicine, agriculture, animal husbandry, and aquaculture (Dini *et al*., 2022). The fact that the development of resistance by the microbes against the AMPs is relatively slower or delayed compared to that against conventional antibiotics makes them prospective alternative therapeutics of the future (Sarkar *et al*., 2021).

Heat-stable proteins or heat-shock proteins (HSP) are key components of helping cell homeostasis in both favorable and unfavorable conditions of cell growth. They are responsible for the folding, assembly, transport, and degradation of proteins in a wide range of normal cellular processes. They also function in stabilizing proteins and membranes and can help in protein refolding under stress conditions (Wang *et al*., 2004; Ahmad *et al*., 2020). In addition to heat shock conditions, these proteins are activated by other stresses such as ultraviolet rays, oxidative stress, chemicals, various metals, bacterial and viral infections, etc. (Nourbakhsh and Rezaei, 2012). Heat-stable proteins are very hydrophilic and resistant to the thermal precipitation of solution. Plant heat shock proteins (HSPs), as chaperones, play a pivotal role in conferring biotic and abiotic stress tolerance (ul Haq *et al*., 2019). These proteins can tolerate temperatures that can be much higher than can be tolerated by their host organisms (Ahmad *et al*., 2020).

Heat-stable proteins of *Arabidopsis thaliana* exhibited antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* (Park and Seo, 2015). The antimicrobial activity of heat-stable proteins of the Costus pictus plant from the ginger family against human pathogenic bacteria, namely, *Escherichia coli*, *Pseudomonas*, *Bacillus subtilis*, and *Staphylococcus aureus* has been reported (Manjula and Shubha, 2011). Heat-stable proteins of mulberry can be used as a strong antimicrobial agent against Escherichia coli, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus subtilis* (Manjula and Shubha, 2011).

Considering the proven role of heat-stable proteins in response to biotic and abiotic stresses and the increasing need for environmentally friendly antibacterial compounds, this study aims to investigate the potential of producing these proteins in Garlic plants to prevent the severe effects of biological stresses of *R. solanacearum* and *S. scabies* bacteria.

2. Materials and methods

The present research was carried out in the Faculty of Agriculture Research Farm, Bu-Ali Sina University, Hamadan, Iran. Garlic plants, which included two clones of Hamadan and Sari, were collected from the farms of Hamadan province and Mazandaran province, respectively. In this research, Gram-negative bacteria *R. solanacearum* and Gram-positive *S. scabies* were used. Both mentioned bacteria were obtained from the Department of Plant Medicine, Faculty of Agriculture, Bu-Ali Sina University.

To extract the total soluble protein (TSP), one gram of the target sample was beaten well with two milliliters of one molar ammonium sulfate solution as a buffer. Then they were transferred to 2 ml micro tubes and after being placed in liquid nitrogen, they were placed in a -20°C freezer for one day and night. After this time, the microtubes were centrifuged at 8000 rpm and 4 degrees Celsius for 10 minutes. The clear supernatant phase inside the micro tubes, which was the soluble protein isolated from these tissues, was removed (Dong *et al*., 2019).

To isolate heat-stable proteins, microtubes containing soluble proteins were placed at 70°C for 10 minutes and then placed in ice for 20 minutes. Then, they were centrifuged for 20 minutes at 12,000 rpm with a temperature of 4 degrees Celsius. After centrifugation, thermally unstable proteins were deposited at the end of the microtubes, and the clear supernatant phase, which was heat-stable proteins, was kept (Park *et al*., 2022).

Evaluation of the antibacterial activity of proteins was done by the Agar well diffusion method with 3 repeats (Gajic *et al*., 2022). For this purpose, bacterial suspension was cultured on a nutrient agar medium. Then, wells with an approximate diameter of 6 mm were drilled at regular intervals and a suitable distance from the plate wall. In each well, 25 microliters of dilutions of 156 mg/ml, 15.6 mg/ml, 0.156 mg/ml, and 0.156 mg/ml protein and ammonium sulfate were poured into one of the wells as a control. The results after 24 hours were shown as the Zone of inhibition (Fig. 1). The evaluation of the results of the Agar well diffusion method was done as a three-factor factorial in the form of a completely randomized design with three repetitions and the Comparison of means was also done by the LSD method at a 1% probability level.

The serial dilution method in the tube with 3 repetitions (Fig. 2) was performed to measure the minimum inhibitory concentration (MIC) and the minimum lethal concentration (MBC) of heat-stable proteins (Kowalska-Krochmal and Dudek-Wicher, 2021). The Kruskal-Wallis test was used to analyze the results of the serial dilution method of heat-stable proteins of two clones. This test is equivalent to the non-parametric independent F test in the one-way analysis of variance method. When the basic assumptions of variance analysis such as the normality of data distribution and equality of variance between groups are not established, the Kruskal-Wallis test is used (Asghari Jafarabadi and Mohammadi, 2015).

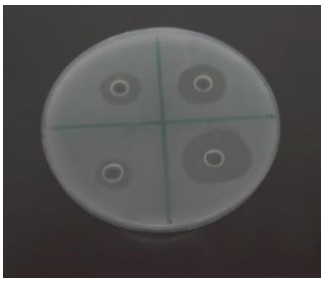

Figure 1. An example of the zone of inhibition of *R. solanacearum*

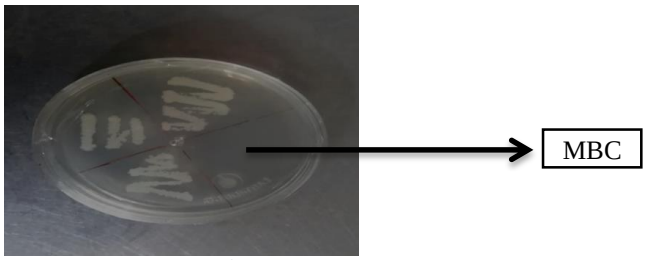

Figure 2. An example of the Minimum Lethal Concentration (MBC) test

Protein electrophoresis was performed by the SDS-PAGE method (electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulfate). This experiment was performed in 12% separating gel and 5% thickening gel (Matsumoto *et al*., 2019). The materials and tools used in this experiment include a small electrophoresis tank (Paya Research Company), glass mold, spacer, power supply, Hamilton syringe, and acrylamide materials including bis-acrylamide, ammonium persulfate, TEMED, Tris base, glycine, sodium dodecyl sulfate (SDS), Bromophenol blue, low molecular weight (LMW) markers, hydrochloric acid and glycerol, beta 2-mercaptoethanol, ethanol and vaseline. The names of the markers used in this research are myosin (200 kDa), beta-galactosidase (116 kDa), phosphorylase (97 kDa), bovine serum albumin (66 kDa), o-albumin (45 kDa), carbonic anhydrase (31 kDa), inactivating trypsin (21 kDa), lysozyme (14 kDa) and aprotinin (6.5 kDa).

Statistical data analysis including analysis of the variance and comparison of means was done by SAS 9.1 software and comparison of means was done through LSD test. SPSS 16 software and the Kruskal-Wallis method were used to perform non-parametric analysis of variance of the tube dilution series test data. All experiments were performed with 3 repetitions.

3. Results and discussion

In testing the inhibitory effects of the heat-stable protein of the garlic plant on *R. solanacearum* bacteria, all simple effects and double interaction effects were significant at a 1% probability level (Table 1). The significance of each of the simple effects of clone, texture, and concentration means that the size of the Zone of inhibition for two clones of Hamadan and Sari, two tissues of bulb and leaf as well as four different concentrations of protein applied are very significantly different. The significance of the double interaction effect of the clone in the tissue indicates that the pair of clone and tissue factors did not act independently of each other in the effect on the Zone of inhibition and the different tissues used were able to differ creating a very significant difference in the size of the Zone of inhibition for the two types of investigated clones, and the largest diameter of the Zone of inhibition is related to the leaves of the Hamadan clone. The significance of the double interaction effect of the clone in the concentration means that different clones in different

concentrations have created a very significant difference in the size of the zones of inhibition of bacterial growth and the largest diameter of the Zone of inhibition is related to the concentration of 156 mg/ml Hamadan clone. The significance of the double interaction effect of the tissue in the concentration indicates that different tissues in different concentrations have created a very significant difference in the size of the halos of inhibiting the growth of bacteria, and the largest diameter of the halo of non-growth is related The concentration of Hamadan leaves is 156 mg/ml with the amount of halo is 27.6 mm. The non-significance of the triple interaction effect of the clone in the tissue in the concentration indicates that there is no significant and significant effect between the growth inhibition of different concentrations of heat-stable protein in the different tissues of the two clones (Table 2).

The zone of growth inhibition was not observed in the test of the inhibitory properties of the heat-stable protein of bulb and leaves of two clones of Sari and Hamadan garlic plants on *S. scabies* bacteria. Therefore, our experiments did not show any inhibitory activity of the heat-stable protein of the garlic plant against gram-positive bacteria *S. scabies*. This result is consistent with the result (Mortazavi *et al*., 2014) that the effect of the antimicrobial compounds of the ethanolic extract of the shell and pistachio kernel on gram-negative bacteria was greater than on gram-positive bacteria. Mortazavi et al. (2014) considered the possible cause of this result to be the resistance of the peptidoglycan layer of the cell wall of gram-positive bacteria.

S. scabies bacteria may have shown resistance to the heat-stable protein of garlic due to the peptidoglycan present in the cell wall. The peptidoglycan layer of gram-positive bacteria is much thicker (20-80 nm) than that of gram-negative bacteria (7-8 nm). Gram-positive bacteria have a single lipid membrane surrounded by a cell wall consisting of a thick layer of peptidoglycan and lipoteichoic acid. However, the cell wall of Gram-negative bacteria includes a thin layer of peptidoglycan in the periplasmic space between the inner and outer lipid membrane (Brown *et al*., 2015; Pasquina-Lemonche *et al*., 2020). It is possible that, during the process of extracting the thermally stable protein of the garlic plant, fat was combined with proteins. For this reason, it is easier to pass through the external lipid membrane of gram-negative bacteria *R. solanacearum* and inhibit this bacterium, but they are not able to pass through the thick layer of peptidoglycan of gram-positive bacteria S. *scabies*. In addition to these, the most developed secretory system is seen in Gram-negative bacteria. This situation has increased absorption and excretion in these bacteria. Also, the cell wall of Gram-negative bacteria has a lot of phospholipid and electric charge, which gives these bacteria the potential to absorb many substances (Bernstein, 2019). Probably, these characteristics caused more heat-stable protein absorption in the gram-negative bacterium *R. solanacearum* than the gram-positive bacterium *S. scabies*.

To fight the infection of microbial pathogens, plants have evolved various defense reactions that include the production of antimicrobial proteins, lytic enzymes, secondary metabolites, membrane proteins, and ribosomal proteins (Kaur *et al*., 2022). Among the molecules with antibacterial activity that have been isolated from plants, proteins, and peptides with antimicrobial activity have recently been reported. These peptides have shown broad activities against a wide range of microorganisms, including Gram-positive and Gram-negative bacteria, protozoa, yeasts, fungi, and viruses (Chung *et al*., 2020; Al Akeel *et al*., 2014). Heat-stable proteins, water-soluble and temperature-resistant (non-enzymatic) are important antimicrobial agents to prevent the growth of pathogens (Farha *et al*., 2012). Antibacterial assays revealed that *Opuntia ficus-indica* heat-stable protein fraction of seeds exhibits moderate antibacterial activities against gram-negative and gram-positive bacteria, with a minimum inhibition concentration ranging from 0.25 to 1 mg/ml (Drira *et al*., 2023). Several roles were determined for HSPs in the immune system including intracellular roles (e.g., antigen presentation and expression of innate receptors) as well as extracellular roles (e.g., tumor immunosurveillance and autoimmunity). It was observed that exogenously administered HSPs induced various immune responses in immunotherapy of cancer, infectious diseases, and autoimmunity (Bolhassani and Agi, 2019).

According to reviews, no research has been done on the biological effects of heat-stable protein in garlic plants, but the research results (Manjula and Shubha, 2011) showed that the antimicrobial effect of heat-stable protein increases with increasing protein

concentration. In another research (Manjula and Shubha, 2011), they observed that with the increase in the concentration of heat-stable protein, there is a constant increase in the Zone of inhibition for the studied bacteria. According to the results obtained in this research, the heat-stable proteins of garlic inhibited the growth of *R. solanacearum* bacteria. In all cases, there was a direct relationship between the diameter of the Zone of inhibition and the concentration of these proteins against this bacterium. In this way, with the increase in the concentration of heat-stable protein, in all cases, the diameter of the Zone of inhibition has also increased. This process of effect on bacteria indicates that the heat-stable protein of this plant has a specific antibacterial effect, which increases with the increase in the concentration of these proteins (Table 2).

Table 1. Analysis of the variance of the Zone of inhibition (mm), different tissues, and concentrations of heat-stable protein of two clones of Hamadan and Sari of garlic plant on *R. solanacearum*

Sources of variation	Degrees of freedom	means of squares
clone	1	48^{**}
tissue	1	161.33^{**}
clone × tissue	1	8.67^{**}
concentration	3	289.09388^{**}
clone × concentration	3	4.84722^{**}
tissue × concentration	3	7.66388^{**}
clone × tissue × concentration	3	1.07722^{ns}
error	32	1.0239
Coefficient of variation		6.19

ns and ** are respectively non-significant and significant differences at the 1% probability level.

Table 2. Comparison of mean and standard error of different concentrations of heat-stable protein of two garlic tissues and leaves of two Sari and Hamadan clones of garlic plant on the diameter of the zone of inhibition of *R. solanacearum*

clone	tissue	0.156 mg ml^{-1}	1.56 mg ml^{-1}	15.6 mg ml^{-1}	156 mg ml^{-1}	control
Hamadan	leaf	13.1 ± 0.84^{fg}	16.4 ± 1.28^{c}	21.3 ± 0.48^{b}	27.6 ± 0.84^{a}	0 ± 0.0^{i}
	bulb	9.7 ± 0.33^{h}	14 ± 1.58^{efg}	15.3 ± 0.33^{de}	21.3 ± 0.33^{b}	0 ± 0.0^{i}
Sari	leaf	12 ± 0.0^{g}	14.5 ± 0.0^{ef}	17.5 ± 0.5^{b}	23 ± 0.5^{b}	0 ± 0.0^{i}
	bulb	8.88 ± 0.34^{h}	13.67 ± 0.34^{efg}	14.7 ± 0.34^{de}	18.5 ± 0.59^{c}	0 ± 0.0^{i}

Averages with non-common letters have statistically significant differences based on Duncan's method at a 1% probability level. The witness in this experiment was one molar ammonium sulfate solution.

The result of the Kruskal-Wallis test data of the minimum inhibitory concentration and the minimum lethal concentration test shows that the applied treatments with 3 degrees of freedom and P-value equal to 0.031 and 0.034 respectively at the five percent probability level have a statistically significant difference. As a result, in this classification, Hamadan leaf and Sari leaf were placed in the first category and Hamadan and bulb Sari were placed in the second category (Table 3).

The comparison of MIC and MBC results is similar to the results of the bacterial growth inhibition zone, which was consistent with the results of the study (Mortazavi *et al*., 2014). So that it is similar to the results of the halo of growth inhibition, which has the most inhibitory effect of HSP on the leaves of the Hamadan clone, in the test of the tube dilution series, the effect of the HSP on the leaves of this clone is greater than that of other treatments, and the lowest concentration that can be 90% of inhibiting the growth of bacteria (MIC) is related to HSP of Hamadan leaf at the rate of 0.76 mg/microliter. The lowest concentration of heat-stable proteins that inhibited the growth of 99.9% of bacteria (MBC) is related to Hamadan leaf HSP at the rate of 7.56 mg/microliter (Table 3). As a result, Hamadan leaf with smaller MIC and MBC and larger growth inhibition halo than other treatments showed the most inhibitor effect compared to other treatments.

Table 3. Analysis of the minimum inhibitory concentration (MIC) and minimum lethal concentration (MBC) of the heat-stable protein of two Sari and Hamadan garlic clones on *Ralstonia solanacearum*

Sources of variation	Average MIC	Average MBC	class
Hamadan leaf	0.76	7.56	one
Sari leaf	1.04	10.4	one
Hamadan bulb	10.92	62.4	two
Sari bulb	15.22	106.41	two

The result of protein electrophoresis is shown in Fig. 3. According to the data related to the quantitative amounts of heat-stable proteins obtained by the Bradford method, it was tried to pour equal amounts of protein (50 microliters) into the wells. In this way, the protein concentration in different columns will be equal and the gel columns will have a uniform color.

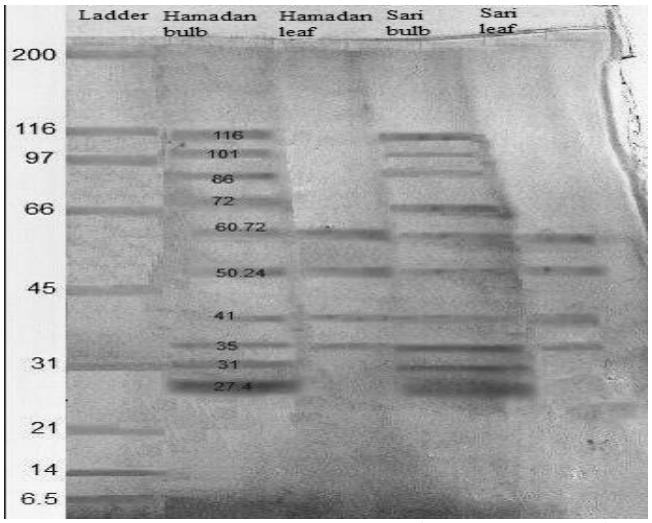

Figure 3. Heat-stable protein band pattern of leaves and bulbs of two garlic clones

In the electrophoresed samples, protein bands with weights of 27.4 to 116 kDa were observed, among which proteins with molecular weights of 35, 41, 50.24, and 60.72 kDa were common among the four protein samples. Bulb bands are in the range of 27.4 to 116 and leaf bands are in the range of 35 to 60.72 kDa. The bands of the two clones are similar to each other, as well as the leaves of the two clones. Since proteins are part of the primary metabolites and are coded directly by genes, they are less affected by the environment. Therefore, the similarity of the bands can be attributed to the original genetic status or genome of these two clones. This similarity indicates that, although these two clones have been cultivated in different areas for years, they have maintained their genomic status.

Generally, HSPs are categorized into six families based on their molecular weight including small HSPs (sHSPs), HSP40, HSP60, HSP70, HSP90 and large HSPs. The range of HSPs varies from 10 to more than 100 kDa. Moreover, their specific sites and physiological roles change within the cell depending on their size (Bolhassani and Agi, 2019). Some types of heat-stable proteins with defense roles in plants based on molecular weight are listed in Table 4 (Park and Seo, 2015).

Table 4. Types of heat-stable defense proteins of plants based on molecular weight

Class	Molecular Weight (KD)	Function	position
HSP100	100-104	Protein refolding	Cytosol, Mitochondria, Chloroplast
HSP90	82-90	Protein turnover	Cytosol, nucleus, mitochondria, chloroplast
HSP70	68-75	Refolding and preventing protein aggregation	Cytosol, nucleus, mitochondria, chloroplast
HSP40	35-54	chaperone	Cytosol
sHSP	15-30	Aggregation barrier, chaperon	Cytosol, nucleus, mitochondria, chloroplast

Adapted from Park and Seo (2015).

According to the investigations, in the bands obtained from the electrophoresis of heat-stable proteins of the leaves, only two clones of the HSP40 family were observed, while in the circulating bands of two clones of the families, small HSP (sHSP), HSP40, HSP70, HSP90, and HSP100 were seen. based on this, the heat-stable protein bands obtained from the bulb were more, while the number of bands formed on the bands related to leaf protein was less. According to the results of antimicrobial tests, garlic leaf proteins have played a more effective biological role. Since only the HSP40 family is expressed in leaves and this family is also expressed in the leaves, it is concluded that the effective proteins found in the leaves are also expressed in the leaves. Despite the similarity in these proteins, we can expect that the leaves have the same biological role as the leaves. Nevertheless, when the difference in the biological role of the two tissues is observed, it can be concluded that the same 4 bands in both the tissues of the stem and the leaf (HSP40 family) can have the same biological role. However, due to the presence of more protein bands in sorghum, these additional proteins may neutralize the biological role of effective proteins (common bands). In such a case, it can be said that maybe other proteins have been able to play a probiotic role in the culture medium for *R. solanacearum* bacteria and reduce the antibiotic effects of 4 effective bands.

HSPs can be considered as therapeutic agents or therapeutic targets for a variety of infectious diseases and cancers. Several studies indicated the role of host HSPs in the defense response against invasion by a pathogen. Moreover, heat shock proteins are important mediators of cellular homeostasis by maintaining protein stability and functionality and activating potent immune responses (Bolhassani and Agi, 2019).

Several thousands of antimicrobial peptides have been isolated from various natural sources like

microorganisms, plants, insects, crustaceans, animals, humans, etc. to date. However, only a few of them have been translated commercially to the market so far. A lot of potential therapeutic molecules can be developed by taking inspiration from the vast repertoire of antimicrobial peptides that nature has to offer (Sarkar *et al*., 2021).

In general, the results of the present research prove that the heat-stable protein of the garlic plant has an effective role in inhibiting the growth of *R. solanacearum* bacteria. Therefore, it is possible to produce biological poisons from the heat-stable protein of garlic plants or to use the gene(s) encoding these proteins in breeding sensitive plants and creating transgenic plants resistant to *R. solanacearum* bacteria.

Conflict of interests

All authors declare no conflict of interest.

Ethics approval and consent to participate

No human or animals were used in the present research.

Consent for publications

All authors read and approved the final manuscript for publication.

Availability of data and material

All the data are embedded in the manuscript.

Authors' contributions

All authors had an equal role in study design, work, statistical analysis and manuscript writing.

Informed consent

The authors declare not to use any patients in this research.

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