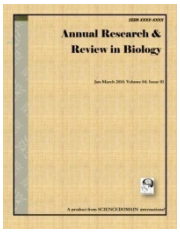

Annual Research & Review in Biology
4(20): 3087-3095, 2014

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Antibacterial Effect of *Allium akaka* Herbal Extract on Planktonic and Biofilm Cells of Pathogen Bacteria in Laboratory Conditions

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MN and HZ designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. All authors managed the analyses of the study, literature searches, read and approved the final manuscript.

Original Research Article

Received 5th March 2014
Accepted 8th April 2014
Published 31st May 2014

ABSTRACT

Aims: Antimicrobial effect of *Allium akaka* herbal extracton the formation of biofilm and colonization of clinical and food-born pathogens are important in this research.
Study Design: In the recent years, traditional medicine and identification of herbs have been highly addressed due to the problems arising from consumption of chemical drugs and bacterial resistance to the antibiotics in planktonic and biofilm modes. *Allium* (with the scientific name *Allium akaka*) contains sulfide and sulfur compounds with strong antimicrobial effects.
Place and Duration of Study: Islamic Azad University of North Tehran Branch Laboratory, between February 2012 and May 2013.
Methodology: Efforts have been made in this research to study the inhibitory effect of *Allium* ethanol extract on *Staphylococcus aureus* and *Salmonella typhimurium* bacteria using disk diffusion and macro dilution methods (concentrations 62.50, 125, 250 and 500 mg lit^{-1}). Also in this experimental research, the effects of different concentrations of *Allium* extract on biofilm formation bacterial and *S. aureus* and *S. typhimurium* pathogenic bacteria were studied using micro-titer method.
Results: The best effect of extract was for *S. typhimurium* bacterium which was estimated

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at 125mg lit^{-1}. The lowest dilution for *Allium* herbal extracts that impeded the formation of biofilm in both *S. aureus* and *S. typhimurium* bacteria included a dilution of the 0.55 gr ml^{-1} within the first 24 hours and had lower effects on non-formation of biofilm within the next 48 and 72 hours.
Conclusion: The present study shows that ethanol extract of *A. akaka* has antibacterial activity against *S. aureus* and *S. typhimurium* bacteria also the ability to diminish the biofilm formation.

Keywords: Allium akaka; extract; antibacterial effect; biofilm; micro-titer plate.

1. INTRODUCTION

In the present age, extensive studies have been conducted on herbs. Presentation of medicines with natural effective materials has opened new horizons to the community of physicians and pharmacists [1,2] in the manner that currently about one-third of the medicines used in human communities is constituted by medicines with natural and herbal origins [3]. Herbs and natural products are the origin of 60 to 80% of current antibiotics [4] and use of herbal extracts and essences are highly addressed by researchers due to presence of antimicrobial combinations [1,2]. *Allium* (with the scientific name *Allium akaka*) is of *Liliaceae* species and among one of the field vegetables [5]. *Allium* is a self-fertilizing plant grown in the foothills of Alborz Mountain and Yaylaks (summer highland pastures) around Tehran like Fasham and Lashkarak. This plant is a kind of wild garlic with garlic and onion properties. In traditional medicine, *Allium* has been used for blood purification, digestion problems, blood pressure reduction, and also for treatment of headache, liver diseases, diarrhea and arthritis [6,7]. In most reports, antimicrobial effects of *Allium* kind are attributed to its sulfur compounds which are highly reactive. Therefore, plants such as *Allium sphaerocephalon* and *Allium sativum* have strong antimicrobial effects [8]. In this research therefore, the inhibitory effect of ethanol extract of *Allium* leaves on gram positive and gram negative bacterial in planktonic and biofilm modes was studied.

2. MATERIALS AND METHODS

2.1 Preparation of Plant Material

Allium was collected during May 2012 from Fasham and Lashkarak heights in the suburbs of Tehran and was identified in the herbarium of the faculty of biological science in north Tehran Branch. Its leaves were then dried and ground in suitable ventilated conditions and far from sunlight.

2.2 Preparation of Extracts

The extraction of plant leaves were conducted by using ethanol solvent. For this purpose, plant powder was mixed after weighing (100g) with 500ml of the intended solvent and was placed in Soxhlet extractor. Extracting was conducted within 12 hours. Finally, the solution obtained from extraction which contained dissolved compounds of vegetable material was placed in Rotary Evaporator system at the temperature of 40ºC for concentration. The extract was then kept in darkness in a temperature of 4ºC until the time of tests. To prepare different dilutions from the obtained extract, 2g of the extract was mixed with 1 ml of ethanol solvent and continuous dilutions of 125, 250, 500, and 1000 mg lit^{-1} were prepared [9].

2.3 Microorganisms

The bacteria under study including *Staphylococcus aureus* ATCC1113 and *Salmonella typhimurium* were prepared from the microbiological laboratory of the Islamic Azad University, north branch of Tehran.

2.4 Antibacterial Effect of Extracts on Planktonic Cells

The effect of extract in preventing from the growth of bacteria was studied through disk diffusion and macro dilution methods.

2.4.1 Disk diffusion method

In disk diffusion method (10), a microbial suspension equal to McFarland 0.5 standard opacity (approximate concentration of CFU/ml108 ×1/5) was first prepared from each 16-hour strain in growth phase (in BHI agar medium at the temperature of 37ºC) and was superficially cultured in Mueller-Hinton agar medium in swab sterile conditions.Then, 30µl of each extract concentration (125, 250, 500, 1,000, and 2,000mg ml^{-1}) was placed on a blank paper disc (PadtanTeb). The discs were then placed on the bacterium-containing medium with appropriate spacing after drying and complete evaporation of solvent. In each test, the witness disc was considered to contain solvent and to lack any extraction. In this method, the plates containing extract and bacterium medium were kept inside an incubator in a temperature of 37ºC in equal conditions for 24 hours. After that period, inhibitory level was measured and recorded based on the diameter of the formed no-growth halos. The tests were repeated for three times and the average diameter of no-growth halos was calculated.

2.4.2 Minimum Inhibitory Concentration (MIC) and Minimum Bactericide Concentration (MBC)

After reviewing the results of disk diffusion, Minimum Inhibitory Concentration (MIC) and Minimum Bactericide Concentration (MBC) were measured according to microbiology standards and by using Broth micro-dilution method [11]. After preparation of ethanol extract and passing that through a 0.2µm filter, the dilutions were prepared in Muller Hinton Broth medium. Then, 100µl of bacterium suspension with 0.5 McFarland concentrations was added to each of the tubes containing medium and extract. The tube containing medium and bacterium was considered for a positive control and the tube containing medium and extract was considered for a negative control. The tubes were kept in an incubator at a temperature of 37ºC for 24 hours. After that period, opacity of the pipes was evaluated by considering their comparison to the witness and based on the growth/no-growth of the bacteria. Concentration of the first tube which lacked any growth was considered as the minimum inhibitory concentration or extract bacteriostatic (MIC). Then, one ml of the pipes with no opacity was inseminated into the solid mediums and was kept in incubator at the temperature of 37ºC for 24 hours. After re-incubation, the plates with no bacterial growth were considered as Minimum Bactericide Concentration (MBC) or extract bactericide on bacterium.

2.5 Review of Biofilm Formation

For the formation of biofilm, bacterial suspension with OD=0.01 was prepared from a 24-hour medium of pure bacterium by spectrophotometry [12]. At first, the intended

concentrations were prepared from the extract in sterile micro-tubes with 2 ml volume. For this purpose, 0.2-0.6gr ml^{-1} of the extracts was prepared in Muller Hinton Broth and 190µl of them was poured into 96-cell micro-plate walls. In this test, walls 1 and 2 were considered as witness, in the manner that wall 1 included medium and wall 2 included medium and bacterium. Wall 3 and the next walls were filled with different dilutions of extract. 190µl of the extract and medium was poured into the walls and 20µl of the intended bacterium which included *Staphylococcus* and *Salmonella* in this research was added to the wall. After performing these operations, we accurately place the micro-plate inside an incubator with a temperature of 37ºC so that the conditions for biofilm formation are provided. The effect of extract on the formation of bacterial biofilm was investigated within 24, 48 and 72hours. In the fourth day, micro plate was taken out of the incubator and its contents were discharged and it was washed with distilled water for 3 times. In order to paint 200 µl of safranin solution, 2% of which was poured into the walls and was washed with distilled water after 2 minutes. Finally, 200ml of ethanol-acetone solution was added to each wall and the amount of OD was read after 15 minutes with ELISA Reader system in a wavelength of 492 nm. Data was reviewed and studied by using ANOVA and TUKEY-KRAMER tests in EXEL and MINITAB software packages.

3. RESULTS AND DISCUSSION

The analyses of no-growth halos in disc diffusion method indicates the effect of herbal extract in preventing from the growth of studied bacteria, but this effect was more observed on *S. typhimurium*, in the manner that in the highest concentration (2000mg ml^{-1}) and lowest concentration (125mg ml^{-1}) the average no-growth halos were estimated 25mm and 8mm, respectively (Table 1). The results of MIC (Bacteriostatic) and MBC (Bactericide) amounts of extract on each of the bacteria are shown in Table 2. The lowest dilution of *Allium* extracts within the first 24 hours which hindered biofilm formation in *S. aureus* and *S. typhimurium* was 0.55gr ml^{-1}. In the next 48 and 72 hours, a lower effect on non-formation of biofilm was observed. It seems that the effect of extract on non-formation of biofilm is reduced after 24 hours.

Table 1. Average diameter of no-growth halos (mm) caused by *A. akaka* extracts using disc diffusion

Extract	**Extract concentration mg ml^{-1}**				
Bacteria	2000	1000	500	250	125
S. aureus	23	20	12	5	2
S. typhimurium	25	23	15	13	8

Table 2. MIC and MBC amounts of ethanol extract of *A. akaka* (mg ml^{-1})

Bacteria	**MIC**	**MBC**
S. aureus	250	500
S. typhimurium	150	250

Table 3. Comparison of average effect of different concentrations of *Allium* extract on *S. aureus* biofilm

Time Extract concentration	24 hours	48 hours	72 hours
Control	1.358a	1.36a	1.41
30%	1.34a	1.35a	1.39
35%	1.24a	1.28a	1.38
40%	1.25a	1.3a	1.23
45%	1.20a	1.27a	1.18
50%	1.19a	1.24a	1.11
55%	0.04b	0.015b	1.02
60%	0.03b	1.35a	1.02

Figures with uncommon letters have significant differences

The results of ANOVA table related to concentrations of *Allium* extract on the growth of *S. aureus* biofilm. Table number indicates high significance of concentrations 0.55gr ml^{-1} and 0.6gr ml^{-1}. Comparison of concentrations in different time intervals shows that the extract was quite effective in non-formation of biofilm in 0.55gr ml^{-1} and 0.6gr ml^{-1} concentration within the first 24 hours, within 48 hours it prevented from biofilm formation only in 0.6gr ml^{-1} concentration and within 72 hours neither of the concentrations had any effect on non-formation of biofilm. Upon increase of time interval, the effect of extract on non-formation of biofilm was reduced.

Table 4. Comparison of average effect of different concentrations of *Allium* extract on *S. typhimurium* biofilm

Time Extract concentration	24 hours	48 hours	72 hours
Control	1.3583a	1.456a	1.1507
30%	1.35a	1.35a	0.9917
35%	1.3a	1.26a	0.696
40%	1.2333a	1.3a	0.6373
45%	1.2a	1.3a	0.51
50%	0.05b	1.19a	0.3787
55%	0.033b	1.035b	0.317
60%	0.01b	0.01b	0.2593

Figures with uncommon letters have significant differences

The results of ANOVA table for concentrations of *Allium* extract on the growth of *S. typhimurium* biofilm indicate high significance of 0.6gr ml^{-1}, 0.55gr ml^{-1} and 0.50gr ml^{-1} concentrations. Comparison of average concentrations in different time intervals shows that the extract prevented from biofilm formation within the first 24 hours; however, in other concentrations it had no effect on non-formation of biofilm. Moreover, the extract prevented from biofilm formation within 48 hours in 0.6gr ml^{-1} and 0.55gr ml^{-1} concentrations. It seems that as the time increases, the effect of extract on non-formation of biofilm is reduced, in the manner that comparison of average 72-hour treatments has no effect on non-formation of biofilm.

3.1 Antibacterial Properties of *A. akaka* Extract on the Planktonic Bacteria

Use of herbs for treatment of diseases has been common since a long time ago among different nations including Iran. Most of the essences and extracts were supplied from special and native plants of the zone. For this reason, the antibacterial effect of *A. akaka* which is one of the species of *Allium* grown in the suburbs of Tehran was studied in this research. Review and comparison of results indicate that the presence of *A. akaka* extract can be effective in preventing from the growth of bacteria. The results of the effect of ethanol extract in disc diffusion method showed that reduction of extract concentrations was directly related to the reduction of diameter of no-growth halos in the studied bacteria. Considering Table 2, the least growth prohibiting concentration was reported for *S. typhimurium* with a concentration of 125mg ml^{-1}. On the whole, sensitivity of the studied bacteria was observed in *S. typhimurium* and *S. aureus*, respectively. The properties of various species of *Allium* are referred in several reports as having treatment value in improvement of microbial infections and diseases [13,14]. Nelsone et al. [15] studied the effect of *Allium cepa* on *S. aureus* and *P.* aeruginosa clinical isolations and reported that ethanol extract of the aforesaid herb prohibited the growth of tested bacteria and that such capability was directly related to the increase of extract concentration. As for the extracts of six species of *Allium* collected from Hamedan, Chehregani et al. [14] concluded that the extracts had antibacterial properties. In all extracts, the best effect with the least MIC was observed for *Bacillus subtilis*. The studies made by Ivanova et al. [7] specified that methanol extract of *Allium ursinum* was ineffective for *Escherichia coli* but it had a high preventive effect on *S. aureus*. The study made by Deresse in 2010 concerning the effect of *A. sativum* aqueous extract as the recognized species of *Allium* on *S. aureus* isolations with different clinical resources indicated a relatively high sensitivity of bacterium at the presence of extract, in the manner that MIC reported the extract at 15mg ml-1 [16]. In the present study, ethanol effect of *A. akaka* as one of species of *Allium* could be observed in non-growth of the studied bacteria. For this reason, this study is aligned with other studies. Of course, it is very difficult to compare the reported results concerning antibacterial effects of extracts. Some of the reasons include difference in surveying methods, resources for preparation of extract and even bacterial strains under study [17]. As Marilena reported in his study, the combinations existing in herbal extracts are different based on growth geographical zone, age of the plant and even drying and extracting methods for extraction of herbal combinations [17]. The studies made by Chowdhuryshowed that *A. sativum* aqueous extract had prohibitory effect on the growth of *S. typhimurium*. He proposed that use of extract combinations of the plant would be useful in food preservative systems in order to prevent from microbial contaminations and the subsequent diseases [18]. Considering that the best effect of *A. akaka* ethanol extract was found to be useful for preventing from the growth of *S. typhimurium* with the least MIC as compared to *S. aureus*, this plant can be used in food industry in the future upon conducting more studies. Arzanlou et al. [19] studied the effect of *A. sativum* aqueous extract in vivo conditions and said that the aqueous extract of that plant could heal the injuries caused by burning and *Pseudomonas* infection. In the present study, the effect of ethanol extract of *Allium* on non-growth of bacterium in laboratory conditions was confirmed in the first step so that in the next steps its effect in vivo conditions may be studied as well [19]. Hanennajjaa et al. [20] studied on *Allium roseum* species and proved the antimicrobial properties of its aqueous extract *on S. typhimurium, S. aureus and P. aeruginosa* and announced the reason in sulfur-containing combinations existing in that species. On the other hand, the studies made by Balestra et al. [21] indicated the prohibitory effect of aqueous extract of *A. sativum* on pathogenic bacteria of tomato including *Pseudomonas syringae*.

3.2 Antibacterial Properties of *A. Akaka* Extract on the Bacterial Biofilm Formation

In this study, the antibacterial properties of *A. akaka* extract on the bacteria were studied in planktonic condition and researches in the field of the effect of plant extract on bacterial biofilm were conducted as well. The results (Tables 3 and 4)indicated the effect of herbal extract on non-formation of biofilm in both gram positive and gram negative bacteria. The obtained statistical results showed that the plant extract had a higher effect on non-formation of biofilm for gram negative *S typhimurium* bacterium. The best dilution of herbal extract within the first 24 hours that hindered biofilm formation in both *S. aureus* and *S. typhimurium* bacteria was a dilution of 0.55gr ml^{-1}. In the next 48 and 72 hours, less effect on non-formation of biofilm was observed. It seems that the effect of extract on non-formation of biofilm is reduced after 24 hours. In the studies made by Perez-giraldo et al. [22] on allicin as the most important element in garlic, its effect on non-growth of *Staphylococcus epidermidis* was reported and some studies were also conducted concerning the effect of allicin of that plant on the produced biofilm on the said bacterium using polystyrene micro-titer plates. Prohibitory effect of this extract was observed in 4 mg ml^{-1} dilution within 24 hours. He justified the reason by the presence of thiosulfate compounds in garlic. In his studies, Lazar [23] used the extract of *A.sativum* to remove the adhesive force and high power of the biofilm produced by the bacteria producing dental plaque including *Streptococcus mutans* and announced its anti-biofilm effects. The result of the present research indicating the effect of *A. akaka* as *Allium* species on non-formation of biofilm was observed within the first 24 hours which is consistent with the studies made by other researchers including Perez – giraldo and Lazar with the exception that in the present research a complete extract and a higher dilution was used but in the previous studies, net combination of the plant with more antibacterial properties and as a result higher effect was used with a lower dilution. The studies made by Sandasi et al. [24] on several species of herbs including *A. sativum* on the biofilm of Pathogen *Listeria monocytogenesis* proved the slowness property (less than 50%) of the biofilm produced by the said bacterium in a concentration of 1mg ml^{-1} from methanol and chloroform extract of the plant within eight hours.

4. CONCLUSION

Therefore, considering the results obtained from this research, the increasing constraints for using antibacterial chemicals such as side effects and resistance of bacteria in biofilm condition and medicinal resistance, the need to substitution of these materials with natural materials and herbal combinations is felt and this can provide the ground for future studies in order to substitute herbal materials in parallel with preservation of health and improvement of community.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=543&id=32&aid=4770