An In-vitro Study on Bacterial Susceptibility and Novel Resistance to Allicin

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Abstract— As one of the most popular vegetables, garlic has been used by many cultures as a natural cure for numerous health problems. Ranging from the ancient Israelites to our modern society, garlic's effect on human health has been studied intensely. Used as an anti-inflammatory, antidepressant, and an anti-parasitic medication by many civilizations millenniums ago, garlic is still used as a popular "folk remedy." (Petrovska and Cekovska 106-110) Studies have shown that a particular compound in garlic called allicin possesses antibacterial qualities and has shown to be highly effective against antibiotic-resistant bacteria, such as methicillin resistant *Staphylococcus aureus* (MRSA). The purpose of this study was to examine the antibacterial efficacy of allicin against *S. aureus, K. pneumoniae, S. typhirium, P. aeruginosa,* and *E. coli*, and examine whether bacteria undergoes mutagenesis to attain resistance to allicin to possibly elucidate the mode of action for allicin's antibacterial efficacy in the future. It was postulated that all bacteria tested would be sensitive to allicin, and that *S. aureus* would be the most inhibited, based on prior studies. Mutagenesis has never been recorded in response to allicin, but it was postulated that the bacteria would undergo mutagenesis to show resistance to allicin. The Kirby-Bauer disc diffusion method was used to determine antibacterial efficacy and extent of mutagenesis. Results were inconclusive about the antibacterial efficacy of allicin. Although some bacteria, including *P. aeruginosa*, were somewhat inhibited by allicin, others, including *S. aureus*, were not affected at all. Mutagenesis did occur in *P. aeruginosa*, as the zones of inhibition were remarkably different between control and mutated plates. Although allicin is undoubtedly an interesting compound with unique qualities, further studies must be done to truly examine its antibacterial efficacy.

Index Terms—allicin, antibiotic resistance, pseudomonas aeruginosa, mutagenesis, garlic, microbiology, naturopathy

1 Introduction

Ilium sativum, better known as garlic, is a plant that is commonly used in cooking and is renowned for its pungent taste and smell. Used by many cultures as both a "folk remedy" and a sustainable source of nutrition, garlic possesses a number of beneficial properties with effectiveness that has withstood the test of time. Garlic has been used as a natural panacea to treat numerous medical problems by many civilizations throughout history. For example, in ancient India, garlic was used to treat a variety of health problems ranging from a simple loss of appetite to rheumatisms. The ancient Chinese used garlic more as an emotional stimulant, using it to treat depression. More synchronous with modern research, the ancient Israelites used garlic as an anti-parasitic drug. These ancient civilizations began primitive research on a subject that was rigorously explored from the 16th century up until today. [1]

It was the famous French microbiologist Louis Pasteur who, in 1858, first officially noted the antiseptic qualities of garlic. [2] Since then, research has expanded to examine allicin, an organosulfur compound found in garlic. Perhaps the most "biologically active" component of garlic, allicin is garlic's protection against attacks from microorganisms such as bacteria and fungi. However, for defensive purposes, allicin is only formed when the inner membrane of a garlic clove is penetrated and completely destroyed. Therefore, extraction of allicin is a difficult process, as the garlic has to be completely crushed to form allicin. [3] Allicin reacts with a myriad of enzymes to create its medicinal qualities. The process of creating allicin begins with the compound alliin, which is the main amino acid found in garlic. Seen in Fig. 1, alliin reacts with the enzyme alliinase to create the new compound allicin. This process, although simple, provides a challenge to scientists due to the instability of allicin. Formed in about 10 seconds, allicin dissipates very quickly as well. [4] [5] Due to this characteristic, extracting pure allicin requires sophisticated technology and therefore, the quality of laboratory produced allicin can vary.

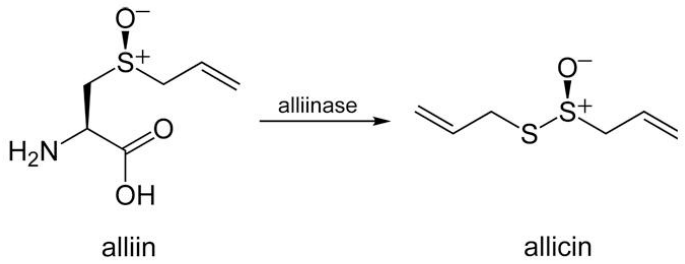

Fig. 1 Allicin is formed when the amino acid alliin is reacted with the enzyme alliinase to create the organosufur compound called allicin. Diagram modified from Wikimedia Commons – Image in Public Domain. [9]

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The goals of this study were to ascertain whether freshly extracted allicin – not produced with sophisticated or patented technology [6] – has antibacterial efficacy, and if so, to see whether mutagenesis can be induced to cause resistance. The 5 bacteria used in this experiment, *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus aureus* (*S. aureus*), *Salmonella typhirium* (*S. typhirium*), *Klebsiella pneumoniae* (*K. pneumoniae*), and *Escherichia coli* (*E. coli*), were chosen due to their pathogenic nature and their prevalence throughout the human microbiome. It was postulated that allicin would have a significant impact on the growth of *S. aureus*, as noted in the study conducted by Leng et al. [7], and a noticeable impact on the other strains of bacteria. In a world where severe antibiotic-resistant illnesses such as methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug-resistant tuberculosis occur on a regular basis, a non-antibiotic treatment protocol is vital to ensure the safety of not only individuals but populations as well. Allicin has shown to be effective against these two severely pathogenic diseases. [4] However, perhaps the bacteria can become resistant to allicin too. To test this, a specific bacterial species, *P. aeruginosa* PAO1, was chosen and mutated in an attempt to become resistant to allicin. Mutagenesis in a bacterial species to induce resistance to allicin has never been recorded previously. It was postulated that mutagenesis would occur, and the *P. aeruginosa* sample would become resistant to allicin.

2 Materials and Methods

2.1 Extraction of Allicin from Freshly Crushed Garlic

Garlic, bought at a local supermarket, was crushed into a paste using a mortar and pistle and doused in ethyl alcohol. This fluid dissolved the necessary substances in the garlic and was transferred into numerous 1.7ml tubes. These tubes were centrifuged and rehydrated repeatedly until a concentrated extract was formed. This extract was placed in a vacuum concentrator until it solidified. After final rehydration, this liquid extract was used to extricate allicin.

Thin-layer Chromatography (TLC) was used to extract allicin from this garlic extract. The solvent system used was Ethyl Acetate and Isopropanol in a 3:1 ratio. Multiple drops of the extract were pipetted onto the TLC plate, which was then placed in a chamber for approximately 30 minutes. It was then removed and examined for separation. 3 distinct ares were formed on the plate: the lighter color at the bottom (lowest concentration of allicin), the darker color at the bottom (low concentration of allicin) and the light color at the top, which contained the higher concentration of the newly separated allicin, as seen in Fig. 2. These areas were scored off using a razor blade and placed into 3 tubes hydrated with ethyl alchol. This was the allicin used for experimentation.

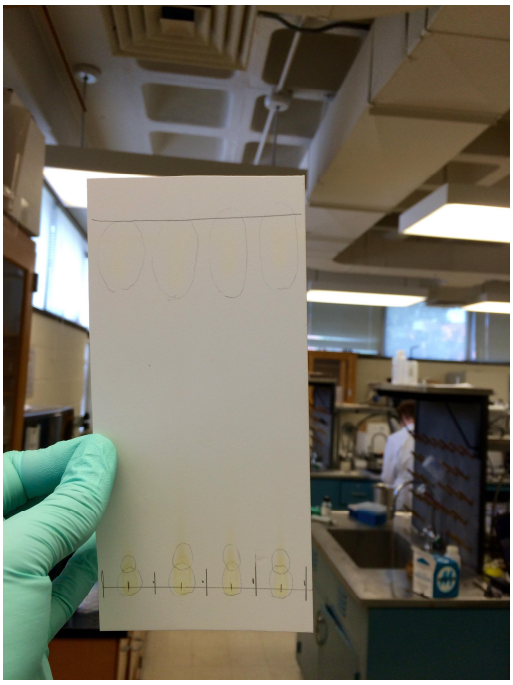

Fig. 2 The plastic-backed TLC plate used in the experiment – the areas of allicin concentration have been circled in pencil. Going in order from bottom to top, the lowest and low concentrations of allicin are at the bottom and the high concentration is at the top.

2.2 Testing Antibacterial Efficacy of Allicin

Cultures of the five aforementioned bacterial species were spread onto standard LB Agar plates. 15 plates were plated total – 3 concentrations with 5 different bacterial species. After the bacteria cultures were spread and dried, the Kirby-Bauer disc diffusion method was used. The purpose of this test is to determine the antibacterial efficacy of a substance by creating zones of inhibition (ZOI). A sample disc was placed in the center of each plate and infused with 8µl of the various allicin samples extracted above. After overnight incubation, ZOI were measured and examined with the various concentrations and species. Due to the asymmetrical nature of the ZOI, three measures of the diameter were taken and averaged. These averages were used to determine whether or not a bacteria species seemed to be sensitive to allicin.

2.3 Induction and Measurement of Mutagenesis

Mutagenesis was induced in *P. aeruginosa* by using a potent mutagen, 30-percent hydrogen peroxide solution. Using the same disc diffusion method, new sample discs were placed next to the original discs from above in the three *P. aeruginosa* plates. This discs were infused with 2µl of the mutagen and incubated overnight. In these plates, ZOI were examined to

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see if a mutation occurred by seeking colonies that grew within the ZOI, as this showed that the colonies were resistant to allicin. Only one instance was found, and it was extracted using an inoculating loop and resuspended in Phosphate Buffered Saline (PBS) solution. This would be the mutant used in the experiment. Then, unmutated *P. aeruginosa* was extracted in the same method and placed in PBS. This would be the control used in the experiment.

Allicin was extracted again using a similar but not the same procedure. TLC was not utilized; instead, the garlic extract extricated directly before TLC was used. 3 plates of control and 3 plates of mutant *P. aeruginosa* were plated to create a comparative study on the ZOI between the control and the mutant plates, as seen in Fig. 3. Disc diffusion was again used, and a sample disc was placed in the middle of each disc infused with 8µl of the garlic extract. The ZOI were measured and compared.

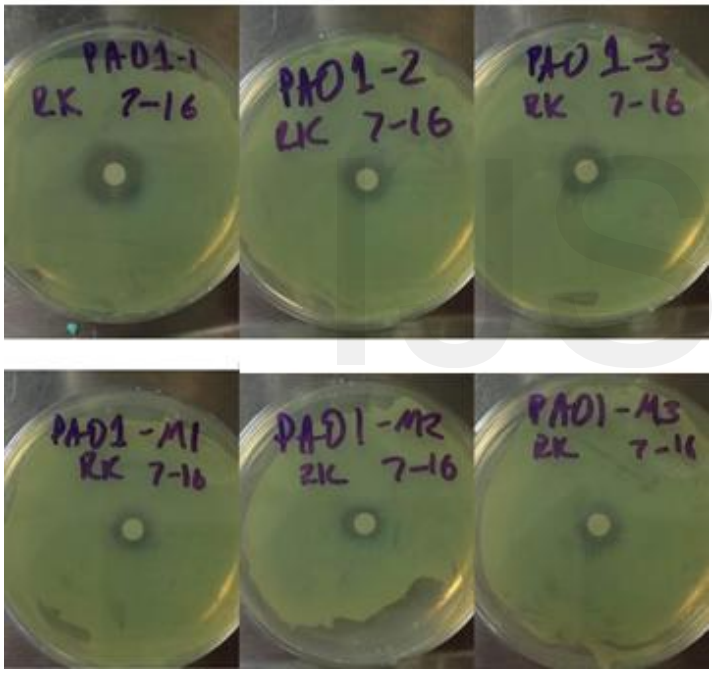

Fig. 3. Comparative ZOI between normal (top) and mutated (bottom) P. aeruginosa.

3 Results

In general, the results of this study were far from conclusive. The lower concentrations of allicin had little effect on the bacteria, and the higher concentration was only effective on two species. The mutagenesis, however, possibly succeeded in creating an allicin-resistant strain of *P. aeruginosa*.

3.1 Efficacy of Allicin on Various Bacteria

The freshly extracted allicin did not conform to the postulations made about its antibacterial efficacy. It was expected that allicin would produce large or significant ZOI to clearly show that it was antibacterial. However, as seen in Table 1, the ZOI, although visible, were clearly subpar and contrary to the results expected. The diameter of the disc was 6 mm. The ZOI of every bacteria species tested was at least 0.5 millimeters and at most 4.2 millimeters from the sample disc. As seen below, the lowest, low, and high concentrations of allicin had little effect on most of the bacteria, surprisingly including *S. aureus*. However, the highest concentration had some effect on *K. pneumoniae* and *P. aeruginosa*. The bacteria species that was most affected by the allicin was *P. aeruginosa,* which was therefore chosen for mutagenesis.

Table 1. Mean Zones of Inhibition based on concentration (in mm.) to the nearest significant figure.

	SA	KP	PA	ST	EC
Lowest (light)	7.0	8.0	7.8	7.8	7.7
Low (dark)	7.5	7.7	8.7	6.7	7.0
High (top)	7.0	8.7	10.2	6.5	7.3
Average ZOI	7.2	8.1	8.9	7.0	7.3

3.2 Mutagenesis in *P. aeruginosa*

One aspect of this study compared the ZOI between the two plates with and without mutagenesis. The results are shown in Table 2. This aspect of the study was very conclusive. It showed that mutagenesis actually occurred in *P. aeruginosa*. Although there was not a large difference in ZOI between the control and mutated plates, there was still a visible and significant difference ($p<0.05$). The average ZOI for the control sample was 15.4 mm and the average ZOI for the mutated sample was 12.0 mm. This significant difference between the control and mutated plates showed that bacteria, if mutated, can most likely become resistant to allicin as well as antibiotics.

Table 2. Comparative ZOI between control and mutated *P. aeruginosa* (in mm.) to the nearest significant figure.

	Plate 1	Plate 2	Plate 3	Average
Control	17.7	14.0	14.5	15.4
	Plate M1	Plate M2	Plate M3	Average
Mutant	11.3	11.8	12.8	12.0

4 Discussion

This study was undertaken to determine whether manually extracted allicin had an antibacterial effect, and if so, to see whether mutagenesis can be achieved to cause allicin resistance in a particular strain of bacteria (in this case, *P. aeruginosa*). Compared to the outlined expectations, this study proved to be inconclusive about the antibacterial properties of freshly extracted allicin, but showed that mutagenesis can be achieved in a strain of bacteria to cause resistance to allicin.

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4.1 Ineffectiveness of freshly extracted allicin against majority of tested bacteria species

The freshly extracted allicin did not live up to the postulations made at the beginning of this study. Although it undoubtedly has some antibacterial effect, it was not to the extent expected. There could be many possible reasons for this result. As stated above, allicin freshly extracted in a laboratory has varying quality. The quality depends on the extraction method; if mistakes were made during extraction, then naturally the quality will suffer. If the concentration of allicin compared to other compounds is low, then the purity of allicin will decrease exponentially. As mentioned previously, allicin has a short half-life; therefore, some may have dissipated before being introduced into the bacteria. Lastly, when infusing the sample discs, some of the allicin may have spilled out of the disc. This could possibly lead to false results regarding ZOI. To prevent some of these issues in the future, many improvements can be implemented. Instead of crushing the garlic with a mortar and pistle to make a garlic paste, it can be blended to ensure complete obliteration of the cellular structure of garlic. The purity of allicin can be checked using mass spectroscopy. By using this technique, the chances of using allicin of a weak concentration is reduced. A last step would be to buy pure allicin that was extracted through a patented and sophisticated extraction procedure. This step should be used as a last resort, as an objective of this study was to examine the antibacterial properties of freshly crushed garlic – however, in trials for future medical applications, this may be a better option due to the importance of the potency of allicin.

4.2 Mutagenesis in *P. aeruginosa*

As predicted, mutagenesis was most probably induced in a sample of *P. aeruginosa*. The disparity between the control and the mutated samples was significant enough to reach the conclusion that *P. aeruginosa* mutated to become resistant to allicin, although which specific genes caused this mutation is yet to be determined. Samples from this have been preserved, so that an in-depth examination of the genetic structure of *P. aeruginosa* can be done in the future. An interesting occurrence during this portion of the study was that the bacteria had a much higher sensitivity to allicin in this experiment than in the first trials at the beginning of the study. A possible reason for this is that even though allicin was extracted in a similar method, some variable changed when TLC was not used to create a more pure or potent form of allicin. Nevertheless, the results showed strong evidence that allicin had an inhibitory effect on *P. aeruginosa* and that the bacteria can be mutated to form resistance

4.3 Future directions

In the future, as antibiotic-resistance increases, allicin will become an important part in the treatment of bacterial infections and diseases. Although not truly effective in its freshly extracted form, pure allicin will perhaps be effective against many bacterial species. In fact, studies have been done in which allicin and an antibiotic have been combined to form a very potent antibacterial medication. [8] Studies such as this show that there is room for improvement and innovation regarding allicin in the field of biotechnology. In terms of this study, by examining the mutated strain of *P. aeruginosa*, the mode of action for the antibacterial effect of allicin may be elucidated. In the future, allicin can hopefully be used against both antibiotic-receptive and antibiotic-resistant bacterial illnesses. By creating a new pharmaceutical product made out of a natural source, the human microbiome will be a healthier and better functioning body.

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