

Investigating Alternative Synthesis and Impregnation Methods for Silver Nanoparticles

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As more traditional antibacterials are used, the number and frequency of multi-drug resistant (MDR) bacteria increase. This rise in MDR bacteria is seen in many cases around the world, especially in developing countries. Many of these MDR bacterial strains originate and spread via hospitals and equipment. A possible solution is to sanitize the hospital environment frequently, utilizing silver nanoparticles (AgNPs), which have shown to be effective against MDR bacteria. Due to their cost and negative environmental impacts, AgNPs have not been viable for mass production. AgNPs can be attached onto surfaces for a prolonged effect through a process called impregnation; this would maintain sanitary environments. This study aims to investigate eco-friendly methods for AgNPs synthesis and impregnation. Two variables were tested; the stabilizing and reducing agent, NLE, OLE, and the impregnation type, heat via iron (HI) and microwave irradiation (MI). It was hypothesized that the *Azadirachta indica* leaf extract used to create the AgNPs would produce a more effective solution compared to *Olea europaea* leaf extract, due to differences in molecular makeup, and that the MI would produce a stronger attachment between the disc and AgNPs, due to a higher energy. A Kruskal Wallis Test H and an adjusted Dunn's post-hoc were conducted. The Kruskal-Wallis H test results, ($H=56.72$, 8 d.f., $p<0.01$), showed that differences among test groups existed, thus supporting the alternative hypothesis. It was concluded that NLE along with HI are possible alternatives to traditional methods, meaning that cheaper solutions to NP synthesis exist.

Introduction

Nanotechnology is said to be the future of innovation and technology, but it is not so glamorous. An important aspect of nanotechnology, and the most promising, is nanoparticles. Nanoparticles are small particles of elemental metal, usually permanently suspended in a solution. Nanoparticles are used for various purposes such as TiO_2 NPs used for blocking UVA and UVB Radiation. Ag, and Cu NPs are used for their antimicrobial properties. Metal based NPs have been used in HIV virus treatments and in the food industry as an antibacterial packing agent^[5]. AgNPs are also being used in the electrical sectors for microelectronics and medical imaging^[8]. Through impregnation AgNPs have been also used in the textile industry to create fabrics and clothing with advantages such as water repellency, increased washing durability, increased thermal qualities, and in medical clothing antimicrobial properties^[8]. Silver Nanoparticles (AgNPs) have shown great progress, but their costs for synthesis have kept them from advancing and being used in everyday life.

There are many different methods to synthesise AgNPs such as using physical synthesis, photochemical reduction, chemical reduction, electrochemical and biological synthesis^[7]. Many of these methods are very expensive, since they utilize chemicals and equipment such as UV Radiation, and X-Ray Radiation^[3]. Along with costs, there are other reasons such as a low product to reactant ratio which makes traditional methods unsustainable for long term use^[6]. A common process that is used to create AgNPs is chemical synthesis. In traditional chemical synthesis, silver nitrate (AgNO_3) is combined with a reducing agent and is stirred and agitated in a liquid solution. The reducing agent is used to reduce the AgNO_3 to silver particles. Once the color of the solution changes, indicating a chemical change and the production of AgNPs, a stabilizing agent is added, to prevent the AgNPs from agglomerating into larger nanoparticles^[3]. Synthesis of AgNPs using biological options such as bacteria, fungi, actinomycetes, yeast, algae, and plants have also been an ever growing option^[6].

Nanoparticles can also be impregnated (attached) into fabrics chemically. Attaching the nanoparticles to the fabrics allows for the nanoparticles to adhere to the fabrics for an extended period of time^[2]. This is a beneficial process, because adhering the nanoparticles to the fabrics allows the fabric to actually be antimicrobial. This allows for extended use of the fabric without the need for the fabric to be sanitized using harsh chemicals. Textiles and fabrics made using natural fibers such as wool and cotton are very good at hosting microbes and bacterial colonies^[9]. The textile industry and the military also utilize nanoparticle impregnated fabrics for their antimicrobial properties; they require less frequent cleaning. Antimicrobial clothing also reduces the chances of and the spread of infection^[2].

There has been a rise in the spread of infectious diseases due to ineffective sanitization methods and persistent bacterial strains^[5]. A possible solution is to eliminate the problem at the source. Bacteria is spread via humans throughout the world, so by inhibiting the growth of bacteria on clothing, the spread of bacteria is reduced and almost eliminated. Bacteria is also spread from person to person via public transport through contact with seats, carpets, etc. where bacteria and bacterial biofilms can grow unchecked. When an AgNPs molecule comes into contact with a bacterium, the smaller nanoparticles are drawn through the cell membrane of the cell due to the NP's affinity towards sulfur and phosphorus-containing molecules. The AgNPs have a sulfur and phosphorus affinity due to their charge. This nanoparticle then breaks all sulfur and phosphorus containing molecules inside the cell such as the DNA and proteins, eventually stopping the functioning of the cell. The larger nanoparticles, not able to pass through the cell membrane, agglomerate on the surface of the cell. The increased charge of the agglomerate causes it to eat away the cell membrane and change the morphology of the cell, thus allowing larger NPs into the cell^[6]. The purpose of this study is to find a greener and cheaper method of synthesizing AgNPs and an alternative method of impregnation. The effectiveness is based on the nanoparticle size and its surface to volume ratio, both of which can be measured using UV Spectro-vis and SEM respectively^[5]. This study aims to see if using greener methods affects the nanoparticles' antibacterial effect, along with the effect of the newly synthesized nanoparticles on alternative impregnation methods.

In this study, the type of reducing/stabilizing agent was tested, which consisted of *Azadirachta indica* leaf extract (NLE) and *Olea europaea* leaf extract (OLE). The control test used trisodium citrate (TSC). The extraction method can work with any plant containing phenolic compounds, nitrogenous compounds, vitamins, reducing sugar, terpenoids and some other metabolites that are rich in antioxidant activity^[5]. *Azadirachta indica* and *Olea europaea* leaves contain many of these molecules, mainly terpenoids. The OLE and NLE both produce homogenous, spherical, small (same relative size) NPs. Some other plants that can be used to synthesise AgNPs are banana peels.

Another variable that was tested includes alternative impregnation methods such as heat via iron method and the microwave irradiation method. Other options to impregnate the silver nanoparticles onto fabric include the pad-dry-cure method, which is conducted through various sequences of dipping, drying, and curing^[8]. The heat via iron method requires the use of a clothing iron to align and attach the nanoparticles to the fabric while

the microwave irradiation method uses radiation. Despite their differences, both methods have shown to work best on organic fabrics such as wool and cotton compared to synthetics. This is because the nanoparticles bind and attach to the cellulose in the fabrics^[2]. Sterile paper discs were used in this study as the fabric onto which the nanoparticles were impregnated.

The antibacterial effects of each stabilizing/reducing agent and impregnation method were tested using a Kirby Bauer Disc Diffusion Test. The NP solution was added to sterile disc diffusion discs (0.6 cm diameter). The amount of bacterial and NP solution that was added to each antibacterial test remained constant in every test. The bacteria inoculated the nutrient broth, which was then used to inoculate the petri-dishes. *E. coli* is a gram-negative bacteria, commonly used for antimicrobial tests, making it a good test organism for this study. Since the purpose of this study was to reduce bacterial infections in clothes via different methods of synthesis, *E. coli* K-12 was the ideal organism to test as many deadly bacterial infections come from other strains of *E. coli*.

It was hypothesized for the primary test that if the *E. coli* K-12 broth were to be exposed to the NLE, OLE, and TSC, the bacterial broth would show the greatest response from the NLE AgNP solution. This would occur because *Azadirachta indica* compounds have shown to have stronger antibiotic properties compared to *Olea europaea* compounds. This is determined by the amount of terpenoids, flavonoids, phenolic compounds, and other molecules present in the plant. As previous studies have shown these molecules to help in the reduction and stabilization process, NLE would be more efficient in creating nanoparticles compared to OLE and TSC. It was also hypothesized for the second variable being tested that if AgNPs were to be impregnated on sterile discs, then the microwave irradiation method would produce a greater antibacterial effect due to the higher energy of the microwave causing more AgNPs to attach onto the cotton fabric. The iron used in the heat via iron method reaches temperatures up to 200-220°C, whereas the highest temperature of a microwave is about 100°C. This suggests that heat via iron would produce greater attachment^[2]. Based on heat energy, the heat via iron method would produce more energy and impregnate more nanoparticles, but the microwaves produced by the microwave are what attaches the nanoparticles to the fabric. The data that was collected for this test includes the bacterial inhibition data, zone of inhibition (cm).

Methods

In this study two variables were tested, the first being plant extractions, and the second being impregnation methods to impregnate silver nanoparticles. The entirety of this study aimed to see if the antibacterial ability of different impregnation techniques is affected by nanoparticles synthesized from different plant extracts.

To synthesize the silver nanoparticles using plant extracts, some preliminary steps were conducted. The nutrient broth was inoculated and incubated 24 hours in advance. Then the three nanoparticle solutions were created by first creating the extracts.

The OLE, and NLE extracts were created by adding 10 g of finely chopped leaves to 50 mL of deionized water. Then this solution boiled for 10 min, afterwards filtered and labeled as extract and set aside. This solution acted as the reducer and stabilizer for the synthesis of AgNPs.

The extract was added to the silver nitrate in a 1:4 ratio; 5 mL of the neem extract/olive extract to 20 mL of 1 M AgNO₃ solution. The 1 M AgNO₃ solution consisted of AgNO₃ and distilled water. This solution was stirred around at a temperature of 40-50°C until a color change (indicates the formation of AgNPs) was observed. This reaction yielded 25 mL of AgNPs via extract solution. The synthesis of AgNPs via the trisodium citrate, or vitamin C, was then conducted. A very similar process was used to create the AgNPs via TSC solution. The 1 M trisodium citrate was added to the 1 M AgNO₃ in a 1:4 ratio. Then the solution was kept under 50°C and stirred until the reaction was complete (indicated by the stopping of the solution's color change).

The antibacterial effect of the nanoparticles was measured using a disc diffusion test. A petri dish was filled with Mueller Hinton Agar and inoculated with *E. coli* K-12 by adding 1.5mL of the inoculated nutrient broth then spreading it using a cell spreader. Then 0.25 mL of nanoparticle solution was added to each 0.6 cm area of the diameter discs. A disc was placed in the center of each quadrant of the petri dish [Figure 3]. Then the petri dish was incubated for 24 hr at 38°C, afterwards the results were taken and the petri dishes were soaked in bleach for disinfection. The results were determined by the diameters of the disc and the ZOI.

The nanoparticle solutions created above were then subjected to impregnation. These solutions were impregnated into the discs via three different impregnation methods: heat via iron, microwave irradiation, and no impregnation. Using the heat via iron method, 0.25mL of the NP solution was added to each disc using a pipette. The discs were then wrapped in foil and ironed at 200-220°C for 5 min.

To make AgNPs impregnated fabric via the microwave irradiation method, the same method to saturate the disc with the nanoparticle solution were followed as the heat via iron. The discs were then placed in the microwave on 30 second intervals, for approximately 1-2 min.

For the discs that were not impregnated, 0.25 mL of the NP solution was added to the disc using a pipette without any further action.

Results

Data collection was completed by measuring (cm) the zone of inhibition from the Kirby Bauer Disc Diffusion test. There were two variables to each data point, impregnation type and the solution type of the disc. There were three solution types (NLE AgNPs, OLE AgNPs, and TSC AgNPs) and three impregnation types (none, heat via iron (HI), and microwave irradiation (MI)), creating 9 distinct test types. Descriptive statistics and an ANOVA were planned to be conducted on the ZOI data set separated by test type [Appendix A: Figure 4]. After data collection, due to non-parametric data the parametric ANOVA was replaced with its non-parametric equal, the Kruskal Wallis H Test.

The descriptive statistics, which were calculated using the ZOI, separated the data by the test type consisting of parameters such as mean, standard deviation, median, maximum, and skewness [Figure 5]. Heat via Iron TSC displayed the highest mean, quartile 1, median, and quartile 3 with the values 1.97, 1.70, 2.20, and 2.38 respectively. Microwave irradiation NLE displayed the lowest mean and median with the values 1.11 and 0.90 respectively. These observations can be seen in Figure 5 and are complemented by the observation of Figure 6. Within the TSC solution tests the highest median was yielded when TSC was used with heat via iron impregnation type, 2.20 cm, and without an impregnation type, 1.40 cm.

The standard deviations calculated displayed interesting properties. It seems that the heat via iron: NLE, OLE, and TSC tests displayed higher standard deviations of 0.75, 0.79, and 0.61 respectively. While the microwave irradiation: NLE, OLE, and TSC tests displayed value of 0.44, 0.32, and 0.37 respectively. The microwave irradiation standard deviations are similar to the no impregnation: NLE, OLE, and TSC standard deviations which were 0.42, 0.53, and 0.35 respectively. The standard deviations seen in the descriptive statistics are also supported by the ZOI boxplots

[Figure 6]. By observing the box plots it is seen that the heat via iron impregnation type has the highest variability since the boxplot displayed the highest interquartile range. Another descriptive statistic calculated is skewness, as the normal probability plots generated displayed that the data presented is not normal. It seems that most of the test type data sets displayed a positive skew, except the heat via iron: TSC with a skewness calculation of -0.93. Microwave irradiation displayed the highest skewness value of 2.09. Heat via iron displayed the least amount of skewness, with a calculation of 0.21.

Randomization was incorporated into the experiment by the use of a random number generator. As seen in the petri-dish set up diagram [Figure 3], there were four tests in each petri-dish; a random number generator was used to decide which four of the test types were to go into the petri-dish. This was done for all tests, and petri dishes to ensure randomization in the data set. An ANOVA was set to be conducted on the data set, but the test type data was not normal. Non-parametric data meant that the data is skewed, which is supported by the descriptive statistics, thus an ANOVA would not provide accurate results. Though some of the test type data is normal, since the data sets needed to be compared to each other, an ANOVA was not conducted. Kruskal Wallis H test is conducted, as it does not require data to be normal and accepts outliers, which is why no outliers were removed from the data set.

The results of the Kruskal Wallis H test displayed that there were significant differences between test types. The test is conducted at an α -value = 0.05, and χ^2 value = 15.507, and the computed p-value = <0.01. The test showed that the overall mean rank is 144, while the mean ranks of heat via iron NLE, and heat via iron TSC were 181.1, and 213.5 respectively. The results of the Kruskal Wallis H test, (H = 56.72, 8 d.f., p = <0.01), rejected the null hypothesis and suggested the alternative hypothesis, NLE or microwave irradiation would produce the greatest zone of inhibition, was supported. To find where the differences lie within the test types, a Post-Hoc Dunn's test was conducted at an α -value = 0.05. The Dunn's test is adjusted using the Benjamini-Hochberg FDR method to reduce the possibility of type I errors. The None NLE is shown to be significant against None TSC (p-value = 0.0017), heat via iron NLE (0.0017), and heat via iron TSC (<0.001). The None OLE is significant against heat via iron TSC (0.0032), and microwave irradiation (0.0462), similarly the heat via iron OLE is also significant against heat via iron TSC (0.0021), and the microwave irradiation NLE (0.0423). The None TSC is significantly different against the microwave irradiation NLE, OLE, and TSC with p-values of <0.01, <0.01, and <0.01 respectively. This also applies to heat via iron NLE and heat via iron TSC which are both significant against the microwave irradiation NLE, OLE, and TSC all with p-values of <0.01. [Figure 8]

Discussion

Silver nanoparticles have been the center of many studies around the world, but due to the expensive process and materials required to create AgNPs they are not widely used. In this study, alternative methods and their effectiveness were tested. Two alternative silver nanoparticle synthesis methods were tested: neem leaf extract and olive leaf extract. The two impregnation methods used were heat via iron and microwave irradiation, with no impregnation method as the control. It was hypothesized that the microwave irradiation would yield higher antimicrobial properties than the heat via iron without any impregnation. It was also hypothesized that the NLE would exhibit higher antimicrobial properties than the OLE, and TSC test solutions. Based on the hypothesis it can be said that AgNPs produced using NLE and impregnated using microwave irradiation would display the highest antimicrobial properties, thus higher zones of inhibition (ZOI). The results of this study did not support the hypothesis. The results of the study suggested that the optimal impregnation type is heat via iron, while the optimal solution type is silver nanoparticle synthesized using NLE or TSC. The descriptive statistics display the trend while the inferential Kruskal Wallis H Test confirms these differences.

The descriptive statistics that were calculated displayed trends, which were then confirmed by the inferential tests. The 3 heat via iron tests displayed highest means, standard deviations, and maxes compared to the other two impregnation groups [Figure 5]. On the contrary, the 3 microwave irradiation tests show the lowest means and medians compared to the other two impregnation groups. This shows that the preferred method is heat via iron, as it displayed the higher antimicrobial effect. The OLE solution median is always less or comparable than the TSC solution median [Figure 5]. The OLE and the TSC solution displayed controlled results throughout all of their tests, as this is supported by their IQR and standard deviation [Figure 5]. Unlike the TSC and OLE solutions NLE displayed higher variability [Figure 6]. The boxplot [Figure 6] displays NLE and TSC as comparable in its antimicrobial effect, but if its variability and means are accounted for TSC seems to be the better option. TSC exhibits a higher mean and median than NLE in all scenarios, also TSC is more reliable as it has lower variability.

The trends shown by descriptive statistics are proven by the inferential test the Kruskal Wallis H test and post-hoc Dunn's test adjusted using the Benjamini-Hochberg FDR method. The descriptive statistics showed that the preferred impregnation method was heat via iron, and the inferential statistics displayed that heat via iron tests groups do significantly vary from other test groups. The inferential tests also unveiled new trends. The NLE was shown to be significantly different from the heat via iron NLE and TSC and the None TSC. The descriptive statistics showed that the NLE has a lower mean and median than the None TSC and the heat via iron NLE and TSC, thus heat via iron NLE and TSC or None TSC are better than using only NLE as an antibacterial agent. Furthermore heat via iron NLE, TSC, and None TSC were all significantly different from all the microwave irradiation tests. This further supports the idea that microwave irradiation results in the lowest antibacterial properties. The None OLE and the heat via iron OLE were significantly different from the heat via iron TSC and microwave irradiation NLE. The descriptive statistics showed that the heat via iron OLE and None OLE have higher antibacterial properties than microwave irradiation NLE, due to higher means and medians, but less than heat via iron TSC. This goes on to support that heat via iron or None is a better impregnation option than the microwave irradiation. This also supports that TSC solution is a better solution to use than the OLE silver nanoparticle solution. It should also be noted that based on the descriptive and inferential statistics, None TSC, Heat via Iron NLE and TSC do not significantly differ from each other. Since these three test groups seem to display superior antibacterial properties when compared to any OLE, and microwave irradiation tests or the None NLE test. Though the results of this study do not support the proposed hypothesis, they do suggest that some of the tests display better antimicrobial properties than others. These suggested test types are the TSC AgNPs with no impregnation, and the heat via iron impregnated NLE AgNPs, and the heat via iron impregnated TSC AgNPs.

Many sources did not clearly align to agree or to disagree with these results. This was due to the specificity of this study. Many studies have individually investigated the production of AgNPs using NLE and OLE and the impregnation using heat via iron and microwave irradiation, but they have not been studied together. It has been found through comparative research that the results seen in this study align with some studies but not all, and even their differences in methodology and materials could explain the differences. The results of this study showed that NLE and TSC displayed higher ZOI's, thus higher antimicrobial capabilities in comparison to OLE. These results are complemented by results from different studies, which state that the NLE produces a smaller nanoparticle size than OLE. Verma and Mehata's (2015) study showed that NLE produces an NP with the absorption spectra of 400 nm, and this is supported by Ahmed, Saifullah, Ahmad, Swami, and Ikram's (2016) recorded value of 441 nm^[10] [1]. These values compared to Khalil, Ismail, El-Bagdady, and Mohamed's (2013) OLE NP absorption spectra value of 449 nm^[4]. A smaller absorption spectra means a smaller nanoparticle size, this is important as a smaller nanoparticle yields higher antibacterial properties as it is able to easily pass through the cell membrane.

Smitha's (2017) results coincide with the results of this study, as they both state that NLE produces nanoparticles with high variability in nanoparticle size thus high variability in the nanoparticle's antibacterial effect^[9]. Though NLE has a higher variability than OLE extract nanoparticles, NLE nanoparticle production was fast and convenient, as the OLE took longer to produce^[9]. Rashidipour and Heydari (2014) stated that OLE AgNPs displayed higher anticancer activities compared to AgNPs synthesized using traditional methods^[7]. This implies that OLE produced smaller nanoparticles than traditional methods, contradicting the results of this study, which found that TSC displayed higher antibacterial properties, thus smaller nanoparticles than OLE nanoparticles. This difference in results could possibly be due to differences in methodology. Rashidipour and Heydari (2014) synthesized their nanoparticles in a four hour time frame, whereas the nanoparticles in this study were synthesized in a 30-45 minute time frame.

Impregnating nanoparticles using the microwave irradiation method, have shown to increase the antibacterial effect of AgNPs, even over multiple washes^[9]. This contradicts the results of this study, as this study found that OLE decreased the antibacterial ability of AgNPs, due to microwave irradiation showing lower ZOI's in general. The study conducted by Eremenko, Petrik, Simirnova, Rudenko, and Marikvas (2016) stated that heat via iron is one of the most effective impregnation methods out there, especially against multidrug resistant bacteria. Their study investigated multiple bacteria, and they found that heat via iron was not effective to some bacterial colonies, but it was effective against others such as *E. coli*.

Though the results of this study and many others state that NLE is a better option than OLE and that heat via iron is a better option than microwave irradiation, opposing results can be explained whether it be through methodology or materials. Though the inferential tests and descriptive analysis show that TSC, NLE, no impregnation, and heat via iron have greater antibacterial properties than OLE and microwave irradiation, some of these variables are controlled. Another fact to consider is if the impregnated fabric is going to be used once or over time, because if used over time, then any form of impregnation is required for the fabric to retain its antibacterial properties. When considering the synthesis of the nanoparticles, NLE and OLE were explored as options due to their low cost. With TSC being more costly than neem and olive leaves, the equally high antibacterial impact produced is overridden.

Due to the size of the experiment and the number of trials there is a possibility for errors in the experiment. A possible error that could have affected the experimentation is the amount of bacteria applied to each petri-dish. *E. coli* K-12 was used in this study, the solution that was applied onto the petri-dish was a nutrient broth inoculated with the *E. coli* K-12. An error could have occurred if the nutrient broth was inoculated with different amounts of the liquid bacterial solution, or if the amount of the broth was added to the petri-dish differed from dish to dish. Though the amount of bacterial solution was added to the broth and the amount of the broth was added to the petri-dish was controlled, human error could have occurred. But this should not be a large source of error in the data, as the trials were randomized. Due to the fact that the nanoparticle solutions had different consistencies, another possible source of error in the experimentation could be the amount of nanoparticle solution added to each test. Though it was predetermined that 1/20 of a mL of the solution would be added to each test, human error could have introduced some variability.

This study has shown that alternative synthesis methods and impregnation methods are viable and comparable to traditional synthesis methods. Despite these facts, more research is required on the economics and environmental impact of these nanoparticles. A possible future study that is needed to ensure the effectibility of these impregnation methods is to test some impregnation methods. Some methods might be designed for short term, but a higher antibacterial effect, while others are designed for long term lower antibacterial effect. A study on the worldwide distribution of neem trees, and their role in their local and global ecosystems needs to be investigated. This study is needed to assess how mass production of silver nanoparticles using neem leaf extracts would affect ecosystems around the world. This would be to ensure that solving the issue of multidrug resistance bacteria will not create another environmental issue.

Acknowledgements

I would like to give a special thanks to my peers who assisted in data collection and experimentation especially Ridha Fatima, Paulina Trifinova, Rachael Hernandez, Srestha Samaddar, and Sydni Moore. This study would not have been possible without the help from my teachers, especially Mrs. Michelle Spigner, and faculty at Spring Valley High School. I would also like to acknowledge my family for providing emotional and financial support when it was most needed.

Notes and References

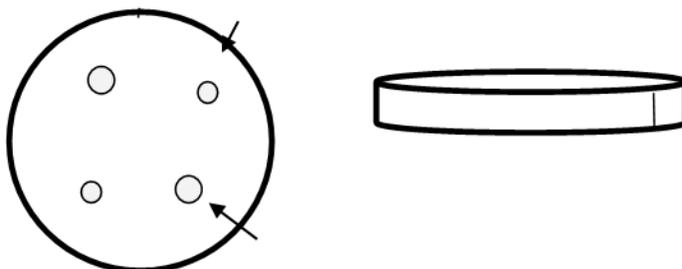
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[Figure 1] Alternative AgNPs Synthesis Experimental Design Diagram

<p>Investigating the effectiveness of different silver nanoparticles made using neem leaf extract, and olive leaf extract on <i>E. Coli</i> K-12 over a 24 hour period.</p> <p>Hypothesis- It was hypothesized that the silver nanoparticles made using the neem leaf extract would provide the highest zone of inhibition throughout the 24 hour exposure time, this was predicted due to the fact that neem leaves produce, on average, a smaller silver nanoparticle size, compared to the other variables being tested.IV- AgNPs made from different reducing and stabilizing agents.</p>			
<p>Antimicrobial Effectiveness on <i>E. Coli</i> K-12: Kirby Bauer Disc Diffusion Test</p> <p>*For each impregnation type</p>	Neem Leaf Extract	Olive Leaf Extract	Trisodium Citrate
	30	30	30
<p>DV The dependent variable was the inhibition of <i>E. coli</i> K-12, measured via a Kirby-Bauer Disc Diffusion Test, in centimeters.</p> <p>Control The control in this experiment was trisodium citrate, it is a commonly used reducer and stabilizer for the creation of AgNPs. The same test, along with the appropriate method will be used.</p> <p>Constants Some constants can include the temperature with which the bacterial tests are incubated at which would be 37°C, also the amount of solution applied to each bacterial broth (measured in mL). Also the pH (13), temperature (50C), and concentration (1-4 ratio) of the extract/trisodium citrate in the reaction to create the silver nanoparticles will remain constant throughout the experiment.</p>			

[Figure 2] Alternative AgNPs Impregnation Methods Experimental Design Diagram

[Figure 3] Diagram of the Petri Dish Set-Up



This diagram displays how the sterile discs were implemented into the petri-dish. Each petri-dish was divided into four quadrants, and a test was conducted in each at random. The petri-dishes were numbered to keep track of them, they were also dated and time to ensure that they were incubated for the full 24 hr period.

[Figure 5] Descriptive Statistics of ZOI (cm) by Test Solution and Impregnation Type

Test Types	Count	Mean	SE Mean	StDev	Min	Q1	Median	Q3	Max	Skewness
Heat via Iron: NLE	32	1.7530	0.1330	0.752	0.8	0.90	1.80	2.40	3.1	0.21
Heat via Iron: OLE	40	1.4820	0.1250	0.791	0.7	0.90	1.20	1.80	3.9	1.44
Heat via Iron: TSC	28	1.9710	0.1160	0.612	0.7	1.70	2.20	2.38	2.9	-0.93
Microwave Irradiation: NLE	29	1.1069	0.0824	0.444	0.8	0.80	0.90	1.30	2.7	2.09
Microwave Irradiation: OLE	23	1.1283	0.0663	0.318	0.8	0.80	1.00	1.30	1.7	0.7
Microwave Irradiation: TSC	36	1.1778	0.0619	0.3712	0.8	0.90	1.00	1.28	2.1	1.46
None: NLE	32	1.1406	0.0747	0.4226	0.7	0.90	1.05	1.35	2.7	1.9
None: OLE	34	1.3559	0.0915	0.5333	0.7	1.00	1.20	1.60	2.9	1.46
None: TSC	33	1.5288	0.0619	0.3555	0.8	1.30	1.40	1.80	2.4	0.57

Figure 5 shows the descriptive statistics for the zone of inhibition data. The data is split into nine groups, based on type of impregnation and silver nanoparticle solution type. The table displays the mean, median, standard deviation (StDev), maximums (Max), and skewness of each test type. Heat via iron TSC has the highest mean median and max. The other Heat via Iron tests also have the highest means and medians. On the other hand, microwave irradiation NLE has the lower mean and median. While all the trials without an impregnation type do not have any outstanding results. The heat via iron tests, NLE, OLE, and TSC, show the highest standard deviations, while microwave irradiation has the lowest values.

[Figure 6] Boxplot of ZOI (cm) Categorized by Impregnation Type

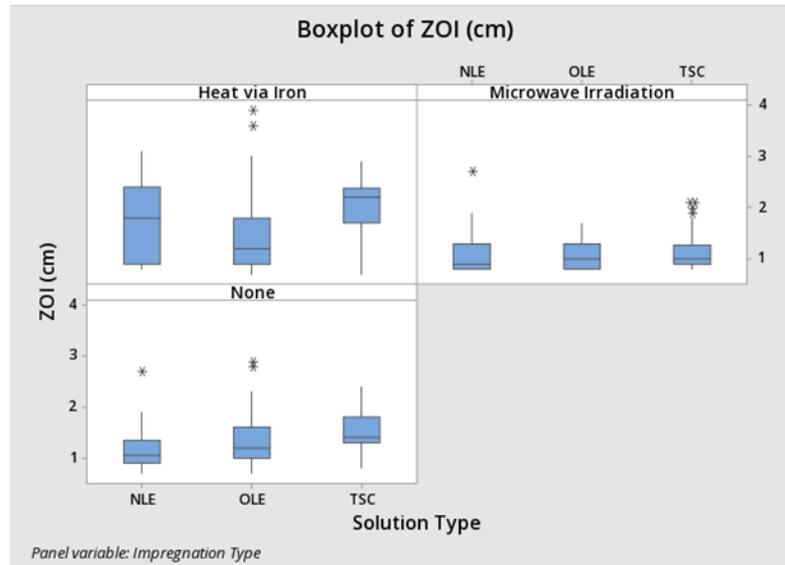


Figure 6 contains boxplots of the zone of inhibition (cm) of each impregnation type. Within each impregnation type boxplot, the boxplot compares its solution type data. As seen in the figure, the TSC seems to have the highest means in each impregnation type, while also maintaining a relatively small IQR. The OLE constantly has the lowest or one of the lowest medians.

[Figure 7] Kruskal Wallis H Test

Test Types	N	Median	Mean Rank	Z-Value
Heat via Iron NLE	32	1.80	181.1	2.68
Heat via Iron OLE	40	1.20	142.8	-0.10
Heat via Iron TSC	28	2.20	213.5	4.67
Microwave Irradiation NLE	29	0.90	94.9	-3.36
Microwave Irradiation OLE	23	1.00	109.1	-2.10
Microwave Irradiation TSC	36	1.00	119.1	-1.93
None NLE	32	1.05	107.5	-2.64
None OLE	34	1.20	143.4	-0.05
None TSC	33	1.40	181.3	2.74
Overall	287		144	
Null hypothesis	H ₀ : All medians are equal			
Alternative hypothesis	H ₁ : At least one median is different			
Method	DF	H-Value	P-Value	
Not adjusted for ties	8	56.36	<0.01	
Adjusted for ties	8	56.72	<0.01	

Figure 7 displays the results of the Kruskal Wallis H Test, of the nine different test types. The test is conducted at a $\alpha = 0.05$ level. The test produced a p-value of <0.01 for both adjusted and not adjusted for ties, and the H-value produced was much greater than their corresponding χ^2 values.

Since the calculated p-value is less than the set α -value, the null hypothesis is rejected thus pointing to the alternative hypothesis. The Kruskal Wallis Test H results, (H=56.72, 8 d.f., $p < 0.01$), indicates that there are significant differences between test types.

[Figure 8] Post-Hoc Dunn's Test: Adjusted using Benjamini-Hochberg FDR

	NLE	OLE	TSC	Heat via Iron NLE	Heat via Iron OLE	Heat via Iron TSC	Microwave Irradiation NLE	Microwave Irradiation OLE
OLE	0.1282							
TSC	<i>0.0017</i>	0.1148						
Heat via Iron NLE	<i>0.0017</i>	0.1156	0.9912					
Heat via Iron OLE	0.1232	0.9912	0.1014	0.1023				
Heat via Iron TSC	<i><0.001</i>	<i>0.0032</i>	0.1793	0.1793	<i>0.0021</i>			
Microwave Irradiation NLE	0.6354	<i>0.0462</i>	<i>0.0003</i>	<i>0.0003</i>	<i>0.0423</i>	<i><0.001</i>		
Microwave Irradiation OLE	0.9912	0.1793	<i>0.0043</i>	<i>0.0044</i>	0.1793	<i>0.0001</i>	0.6354	
Microwave Irradiation TSC	0.6354	0.2821	<i>0.0050</i>	<i>0.0052</i>	0.2820	<i>0.0001</i>	0.3006	0.7109

The results of the Post-Hoc Dunn's test of the test types is displayed in Figure 8. The Post-Hoc Dunn's Test, conducted at an α -value = 0.05, is adjusted by the Benjamini-Hochberg FDR method, to reduce the possibility of type 1 errors. Heat via iron TSC and heat via iron NLE were significantly different from the microwave irradiation solution groups. The results of the Dunn's test shows that more differences lie between the heat via iron solution groups and no impregnation solution groups, then with the microwave irradiation solution groups.

Appendix A

[Figure 4] Zone of Inhibition (cm) of different solution and impregnation types

Test Number	Impregnation Type	AgNPs Solution	ZOI (cm)	Test Number	Impregnation Type	AgNPs Solution	ZOI (cm)
1	None	TSC	0.7	145	Heat via Iron	OLE	2.2
2	None	OLE	0.8	146	Microwave Irradiation	TSC	2.1
3	Heat via Iron	OLE	0.9	147	Microwave Irradiation	TSC	1.7
4	Microwave Irradiation	TSC	0.9	148	Microwave Irradiation	TSC	1.9
5	Microwave Irradiation	TSC	0.7	149	None	TSC	1.2
6	Heat via Iron	TSC	1.3	150	Microwave Irradiation	OLE	0.95
7	Heat via Iron	TSC	0.9	151	None	TSC	1
8	Microwave Irradiation	OLE	1.4	152	Microwave Irradiation	OLE	1
9	Microwave Irradiation	TSC	1.6	153	None	OLE	0.9
10	Heat via Iron	TSC	1.9	154	None	OLE	0.8
11	Microwave Irradiation	TSC	2.1	155	Microwave Irradiation	NLE	0.8
12	Heat via Iron	TSC	2.1	156	Heat via Iron	NLE	0.9
13	Microwave Irradiation	TSC	2.7	157	None	NLE	1.9
14	Heat via Iron	OLE	1.7	158	Heat via Iron	NLE	1
15	Microwave Irradiation	OLE	1.8	159	None	OLE	1.3
16	None	TSC	0.9	160	Microwave Irradiation	TSC	2.1
17	Heat via Iron	NLE	0.8	161	Heat via Iron	TSC	0.9
18	Heat via Iron	NLE	0.9	162	Heat via Iron	NLE	0.9
19	None	TSC	0.9	163	Heat via Iron	NLE	0.8
20	None	OLE	0.9	164	Microwave Irradiation	TSC	0.8
21	None	NLE	0.8	165	None	OLE	0.8

22	Heat via Iron	NLE	0.8	166	Microwave Irradiation	NLE	0.9
23	Microwave Irradiation	NLE	1.4	167	None	NLE	2.4
24	Microwave Irradiation	NLE	1.3	168	None	TSC	1.1
25	Heat via Iron	NLE	0.9	169	Microwave Irradiation	TSC	0.9
26	None	TSC	0.8	170	None	TSC	1
27	Heat via Iron	NLE	0.9	171	Heat via Iron	NLE	0.9
28	Microwave Irradiation	OLE	0.8	172	Microwave Irradiation	OLE	1.1
29	Microwave Irradiation	TSC	0.9	173	Heat via Iron	TSC	2.1
30	Microwave Irradiation	OLE	0.9	174	Heat via Iron	TSC	0.9
31	Heat via Iron	NLE	1.8	175	Microwave Irradiation	OLE	1.4
32	Heat via Iron	OLE	2.6	176	Microwave Irradiation	NLE	1.7
33	None	OLE	1.7	177	Microwave Irradiation	TSC	2.7
34	Heat via Iron	TSC	2.2	178	None	NLE	2.8
35	Heat via Iron	OLE	0.7	179	Heat via Iron	TSC	2.5
36	None	OLE	1.6	180	Microwave Irradiation	NLE	2.7
37	Microwave Irradiation	OLE	1.7	181	Heat via Iron	TSC	2.4
38	Microwave Irradiation	TSC	1.1	182	Heat via Iron	TSC	1
39	Heat via Iron	OLE	1.7	183	Microwave Irradiation	OLE	0.7
40	None	TSC	0.9	184	None	TSC	1
41	Heat via Iron	OLE	3.6	185	Microwave Irradiation	TSC	2.4
42	Heat via Iron	NLE	1.4	186	Microwave Irradiation	TSC	1
43	None	NLE	1.6	187	Microwave Irradiation	OLE	0.7
44	None	TSC	2.2	188	None	OLE	0.9
45	Heat via Iron	OLE	0.8	189	None	NLE	1.1
46	None	OLE	2.1	190	Heat via Iron	TSC	1.15

47	None	NLE	0.8	191	None	TSC	1.4
48	Microwave Irradiation	NLE	0.8	192	Microwave Irradiation	TSC	1.2
49	None	OLE	2.2	193	None	OLE	1.5
50	Heat via Iron	NLE	1.3	194	None	OLE	1
51	None	NLE	0.9	195	Heat via Iron	NLE	0.8
52	None	NLE	0.8	196	Heat via Iron	NLE	1.5
53	None	TSC	1.2	197	Microwave Irradiation	OLE	1.6
54	Microwave Irradiation	OLE	1	198	None	TSC	2.3
55	Heat via Iron	NLE	0.9	199	None	NLE	1.9
56	Heat via Iron	OLE	0.8	200	None	TSC	1.7
57	Heat via Iron	OLE	1.4	201	Heat via Iron	NLE	1.6
58	Microwave Irradiation	OLE	1.6	202	Heat via Iron	OLE	1.4
59	Heat via Iron	NLE	1.3	203	Heat via Iron	TSC	2.1
60	Heat via Iron	NLE	1.7	204	None	OLE	1.4
61	Microwave Irradiation	OLE	2.6	205	Heat via Iron	TSC	2.4
62	None	OLE	1.3	206	None	OLE	1.2
63	None	OLE	3	207	Heat via Iron	NLE	1
64	Heat via Iron	NLE	2	208	Heat via Iron	NLE	1.2
65	Heat via Iron	NLE	0.9	209	Heat via Iron	OLE	2.8
66	Heat via Iron	TSC	1.7	210	None	TSC	1.3
67	Microwave Irradiation	TSC	1	211	Microwave Irradiation	TSC	1.4
68	Microwave Irradiation	TSC	1.4	212	Heat via Iron	TSC	1.6
69	None	NLE	1.6	213	None	OLE	1.6
70	Microwave Irradiation	TSC	2	214	Heat via Iron	NLE	2.7
71	Heat via Iron	TSC	1.1	215	Heat via Iron	OLE	1.3
72	Microwave Irradiation	TSC	1.2	216	Microwave Irradiation	NLE	1.2

73	None	NLE	0.9	217	Heat via Iron	NLE	1.1
74	None	NLE	1	218	Microwave Irradiation	OLE	2.3
75	Heat via Iron	NLE	2.9	219	Microwave Irradiation	OLE	1.5
76	Heat via Iron	TSC	1	220	Heat via Iron	TSC	1.8
77	Heat via Iron	OLE	1.1	221	None	NLE	1.4
78	Heat via Iron	NLE	0.8	222	None	OLE	1
79	Heat via Iron	NLE	0.8	223	Heat via Iron	NLE	0.9
80	Heat via Iron	NLE	0.9	224	None	TSC	1.8
81	Microwave Irradiation	OLE	1.2	225	None	NLE	1.8
82	Microwave Irradiation	OLE	1.2	226	None	OLE	1.4
83	Microwave Irradiation	TSC	2.3	227	Microwave Irradiation	NLE	1.1
84	None	OLE	1.9	228	Heat via Iron	OLE	1.4
85	None	TSC	1.4	229	None	NLE	1.4
86	Heat via Iron	OLE	1	230	Microwave Irradiation	NLE	0.9
87	Microwave Irradiation	OLE	1.2	231	None	NLE	1
88	Microwave Irradiation	TSC	1	232	None	TSC	1.1
89	None	NLE	0.9	233	Microwave Irradiation	OLE	1.1
90	None	OLE	0.8	234	None	NLE	1.2
91	None	NLE	0.8	235	Microwave Irradiation	OLE	2.9
92	Heat via Iron	NLE	2.1	236	Microwave Irradiation	TSC	1.8
93	Heat via Iron	OLE	0.8	237	None	OLE	0.8
94	None	OLE	0.8	238	Microwave Irradiation	NLE	1.2
95	Heat via Iron	OLE	0.8	239	None	OLE	1.2
96	Microwave Irradiation	OLE	0.9	240	Heat via Iron	NLE	0.7
97	None	OLE	3.9	241	None	NLE	1
98	None	OLE	1.3	242	None	TSC	1.3

99	Heat via Iron	OLE	1	243	Microwave Irradiation	OLE	2.1
100	Heat via Iron	NLE	1.1	244	Heat via Iron	OLE	0.9
101	None	OLE	1	245	None	TSC	1.4
102	Heat via Iron	NLE	2.2	246	Heat via Iron	OLE	1
103	Microwave Irradiation	TSC	2.2	247	Microwave Irradiation	NLE	0.7
104	Heat via Iron	OLE	1.8	248	Heat via Iron	TSC	1.6
105	None	OLE	1.2	249	Heat via Iron	OLE	1.7
106	None	NLE	2.4	250	None	TSC	1.9
107	Heat via Iron	NLE	1.3	251	None	OLE	0.9
108	Heat via Iron	TSC	2.9	252	None	OLE	0.9
109	Microwave Irradiation	NLE	1.9	253	None	TSC	1.2
110	Heat via Iron	NLE	2	254	Microwave Irradiation	OLE	1.1
111	None	OLE	1.3	255	Heat via Iron	TSC	1.3
112	Microwave Irradiation	NLE	2.4	256	Microwave Irradiation	TSC	1.3
113	Microwave Irradiation	OLE	1.4	257	Microwave Irradiation	NLE	0.9
114	None	OLE	1	258	Heat via Iron	OLE	1
115	Microwave Irradiation	NLE	1.2	259	Heat via Iron	NLE	0.9
116	None	NLE	3.1	260	Microwave Irradiation	TSC	1.6
117	Microwave Irradiation	OLE	1.2	261	Microwave Irradiation	OLE	0.9
118	None	TSC	1.1	262	None	NLE	0.7
119	Microwave Irradiation	NLE	3	263	Microwave Irradiation	NLE	0.7
120	Microwave Irradiation	TSC	1.8	264	None	OLE	1.9
121	Microwave Irradiation	TSC	2.1	265	Heat via Iron	OLE	0.7
122	None	TSC	1	266	None	NLE	1.1
123	None	NLE	1.1	267	None	TSC	1.3
124	Heat via Iron	TSC	0.8	268	Heat via Iron	TSC	1.8

125	Heat via Iron	TSC	1.2	269	None	OLE	0.8
126	Microwave Irradiation	TSC	2.2	270	None	OLE	1
127	None	OLE	1.8	271	Microwave Irradiation	OLE	1.1
128	Heat via Iron	TSC	2.3	272	Microwave Irradiation	TSC	1.6
129	None	TSC	2.4	273	Heat via Iron	TSC	1.2
130	None	OLE	1.7	274	Heat via Iron	TSC	0.8
131	None	NLE	2.8	275	Heat via Iron	NLE	0.8
132	None	TSC	2.2	276	Heat via Iron	TSC	1.9
133	Microwave Irradiation	NLE	2.4	277	Microwave Irradiation	TSC	1.2
134	None	TSC	2.2	278	None	TSC	1.8
135	None	TSC	1.1	279	None	OLE	1.1
136	Heat via Iron	NLE	1.2	280	Microwave Irradiation	NLE	0.9
137	Heat via Iron	NLE	2.2	281	Heat via Iron	NLE	0.8
138	Heat via Iron	TSC	1.3	282	Microwave Irradiation	OLE	1.6
139	Microwave Irradiation	NLE	1.9	283	Heat via Iron	NLE	1.4
140	Microwave Irradiation	OLE	0.9	284	Microwave Irradiation	TSC	1.5
141	Heat via Iron	OLE	0.8	285	None	NLE	0.9
142	Microwave Irradiation	NLE	0.8	286	Heat via Iron	TSC	1.4
143	Microwave Irradiation	NLE	0.8	287	None	TSC	1.3
144	Heat via Iron	OLE	0.9				