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# Green Synthesis of Silver Nanoparticles and Their Effect on the Activity of Alkaline Phosphatase

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## ABSTRACT

Green synthesis of silver nanoparticles (AgNPs) is an eco-friendly, cost-effective, and less toxic approach. Silver nitrate is used as a reducing agent and is mixed with the lemon extracts via a top-down approach. AgNPs were synthesized using different parts of lemon extracts including lemon fiber, outer layer of lemon, and lemon seeds. The formation of Ag NPs was confirmed by the change in color from yellow to reddish brown. These AgNPs were then characterized via UV-VIS spectrophotometry and depicted maximum absorption between 420 to 440 nm. An alkaline phosphatase (ALP) assay was performed for these silver nanoparticles at an absorbance of 405 nm. The AgNPs enhanced the enzymatic activity of ALP.

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## INTRODUCTION

The demand for nanoparticles is continuously increasing day by day due to its vast circle of applications like chemistry, catalysis energy production, etc. For the very first time, Argemone Mexicana Leaf was being used for nanoparticle production in which $AgNO_3$ acted as both a reducing as well as capping agent to control the reaction, and the resulting nanoparticles were characterized using the UV Vis method, which proved to be very lethal against various bacterial species. In the end, it was concluded that the extracted material from Agremone Leaf was successful in carrying green synthesis of AgNPs but its capability to act as reducing and controlling agent was not fully proved. Silver nanoparticles synthesized from Mexicana leaves were very helpful for making different types of antibacterial agents. Most importantly the reaction of $AgNO_3$ and Agremone leaf extract was not complicated and easy to handle[1].

It is concluded that the silver nanoparticles synthesized by the reaction of liquid extract and silver nitrate through the biosynthesis method were very eco-friendly toxic against bacteria easily handled and a simple reaction with the resulting silver nanoparticles being covered by a faint layer having various metabolites and proteins embedded in it. Silver nanoparticles synthesized through biosynthesis were very cheap and easily affordable[2]. According to Kaviya, *et al.,* (2011), silver nanoparticles were very effective and stable as conducted from the studies of UV Vis, FTIR, and FESEM and proved to be effective agents against toxic bacteria. Silver nanoparticles of size varying between 16-28 nm of spherical shape made through the reaction of dried powder of the plant extract material and silver nitrate solution, which proved to have increased antimicrobial activity against different human pathogens[3]. Another study revealed that silver nanoparticles demonstrated potent antagonistic activity against bacteria and fungi, which possess potential applications in medicine and pharmaceutical fields[4].

In the past, the nanoparticles were produced only through physical and chemical methods, which proved to be expensive, and released various types of harmful chemicals that seem to affect their applications. As technology improved the nanoparticles were produced through the green synthesis approach which was very cheap, eco-friendly, and easily handleable due to the different biological compounds present in it. This green synthesis method also known as the biosynthetic method can be used for the synthesis of both silver and gold nanoparticles with different types of bacteria fungi and yeasts mostly used organisms with the the green synthesis approach strictly maintaining the size and shapes of the synthesized nanoparticles, which are used in almost every field of research[5].

It was concluded from the study, that silver nanoparticles produced from *Ficus benghalensis* were in a range of 16 nm and were spherical. It doesn't require any chemical for its synthesis method which is eco-friendly color changes due to the oscillation of conducting electrons at the interface between negative and positive permittivity material which is initiated by incident light[6]. According to Gondwal and Pant (2013), SEM showed different results making AgNPs to be spherical. These synthesized silver nanoparticles proved to have extraordinary antimicrobial properties against *K. pneumonia* and *S. typhi* and showed good antioxidant properties. Stable silver nanoparticles have been synthesized by using soluble starch as both the reducing and stabilizing agents; this reaction was carried out in an autoclave at 15 psi, 121 °C for 5 min[7]. Finally, we conclude, that AgNPs are important regarding future perspective[8]. Silver nanoparticles were synthesized using silver nitrate and the sample mixture was taken from the latex of *Jatropha Curcas*[9] via the green synthesis method.

Alkaline phosphatases (ALP) are mostly present in all types of organisms whether they lack membrane-bounded organelles (prokaryotes), or have membrane-bounded organelles (eukaryotes) because of their complexity, and diversity. Due to their substrate specificity, and chemical nature, they are broadly classified into four types. However, ALPs with non-specified substrate for a chemical reaction are classified according to variation in pH which can be seen from the fact that those enzymes that have a pH of 9 are termed as glycoprotein alkaline phosphatases that have a carbohydrate group attached to their polypeptide chain[10,11,12]. The ALPs play a vital role in the synthesis of proteins and nucleotide metabolism and nucleoprotein in cells[13,14]. Inorganic phosphate is given out by alkaline Phosphatase due to the hydrolysis of phosphor monoesters of R-O-$PO_3$ at the origin of the R group and at the same time serine formation is also observed due to reaction with water at alkaline pH.)[15].

## MATERIALS AND METHODS

### Reagents

Analytical grade silver nitrate ($AgNO_3$), distilled water, alkaline phosphatase (Thermo scientific, *Escherichia coli*, 5M *para*-nitrophenyl phosphate (PNP), diethanol amine buffer and $MgCl_2$ (9:1) as a buffer solution, NaOH (0.5 M) being used as a stock solution and HCl were utilized in this study.

### Synthesis of Silver Nanoparticles

#### *Preparation of Silver Nanoparticles Using Lemon Extracts*

Different parts of lemon were harvested from the nursery of International Islamic University, Islamabad. Six pieces of lemon crushed and added in 150 mL distilled water; boiled at 60-70°C for 20 min, filtered the extract through filter paper. Then clear extract was obtained via centrifuge at 4000 rpm for 20 min and used for NP synthesis. NP synthesis was performed at a standardized concentration such as 4 mL of each of the lemon extracts mixed with 4 mL of 5 mM silver nitrate solution in a dropwise manner at 50-60°C with constant stirring through a magnetic stirrer.

#### *Preparation of Silver Nanoparticles Using Different Parts of Lemon*

Silver nanoparticles are synthesized by using the different parts of lemon like its outer layer; inner fiber and seeds, which are then modified by a process known as plant extract formation. First of all the sample was prepared by cutting 5 to 6 lemons and then separating and purifying its different parts. The outer layer of lemon after properly separating then air-dried for about 2 h. The samples of lemon seeds, lemon fibers, and outer layers of lemon are being collected and air dried and are ready for further experiments to be carried out.

Lemon seeds, lemon fiber, and the outer layer of lemon were separated, placed in a petri dish, and washed with both tap and distilled water. After this, the seeds dried for

2 to 3 hours. As all lemon parts are dried, they are crushed by pestle and mortar and when all seeds are properly crushed, they are mixed with 150 mL of distilled water in a 250-mL flask. The mixture was then, heated for 20 min and when enough extract was made, it cooled at room temperature. The extract was then filtered. Process of filtration carried out for lemon fiber, lemon seeds, and outer layers of lemon.

After filtration, all three lemon extracts were centrifuged at 4000 rpm for 20 min then the supernatant was collected in a 250-mL flask with the pellet left discarded and followed by nanoparticle synthesis.

### *Silver Nanoparticles Synthesis Using Seed Extract*

After centrifugation lemon seed, fiber, and outer layer extract were treated with $AgNO_3$ as a reducing agent. 4 mL of $AgNO_3$ was taken in a flask and placed on a stirrer, and inside the flask, a magnetic stirrer was placed. Added 4 mL of lemon extracts through burette dropwise. Stopped the reaction as seen change in colour and allowed to cool at room temperature**.** A mixture of centrifuged extracts was mixed dropwise with the 1 mM silver nitrate solution via a top-down approach and mixed with the help of a magnetic stirrer. Changed in color from yellowish brown to reddish brown after a full mixing of extracts with the silver nitrate solution.

### *UV-Vis Spectrophotometer Analysis*

For UV-Vis analysis, 1 mL of reduced $AgNO_3$ solution was taken from stock solution via pipetting and transferred to 1.5 mL of a cuvette having 1 cm path length, and scanned the solution 300-800 nm range with a scanning speed of one nm/s. A control solution of diluted lemon extract without $AgNO_3$ was used.

### *Kinetic Study of Alkaline Phosphatase (ALP)*

Kinetic assay of ALP performed by mixing enzyme into substrate buffered solutions having pH 9 at 37$^{o}$C for 10 min in a water bath. The amount of product (*PPP*) formed was measured every minute at 405 nm against their respective blanks. Comparative enzyme assay for lemon fibers, lemon seeds, and outer layers of lemon was carried out in a water bath at room temperature for 10 min.

### *Kinetic Assay of ALP without NPs*

ALP assay was performed in the absence of NPs at standardized conditions as mentioned above by adding a fixed amount of ALP (200 µL) in different substrate concentrations. Such as 0.2 mL of enzyme ALP, 2.5 mL of diethanolamine and $MgCl_2$ (9:1) as a buffer solution, 0.5 M NaOH as a stop solution, and 0.3 mL of 5 mM solution of (*p*NPP) as a stock solution which was the substrate for ALP at 405 nm of each substrate concentration in time interval established. A similar experiment was performed for a blank solution in which 0.2 mL of distilled water was used instead of ALP.

### *Kinetic Assay of ALP with Ag-NPs*

Similarly, ALP assay with studied NPs performed by repeating the experiment as stated above, a fixed amount of NP solution (0.5 mL) was used in all experiments. The NP solutions added to the substrate buffer solution before the addition of enzyme (0.2 mL) at a given substrate solution that is 0.3 mL of substrate (*p*Npp 5 mM) at 405 nm of each substrate concentration in time intervals were established.

### *Unit Calculation Formula for ALP*

$$\Delta A\ 405nm\ min\ Test - \Delta A\ 405nm\ min\ Blank\ (Vf)/\ (18.5)\ (VE)$$

Vf = Total volume of the assay used which is 3 mL

VE = Volume of the enzyme used that is 0.2 mL for all experiments[16].

**Table 1. Kinetic Assay for Test and Blank Solutions at 405 nm Absorbance Having ALP, Distilled Water, And Silver Nanoparticles.**

| **Test (Reaction mixture of Enzyme)** | **Blank (Reaction mixture without enzyme)** |
|---|---|
| Substrate = 0.3 mL<br>Enzyme = 0.2mL<br>Buffer = 2.5 mL | Substrate = 0.3 mL<br>Water = 0.2 mL<br>Buffer = 2.5 mL |
| **Test NPs (Reaction of the enzyme with NPs)** | **Blank NPs (Reaction mixture without enzyme containing NPs)** |
| Substrate = 0.3 mL<br>Enzyme = 0.2 mL<br>Buffer. = 2.0 mL<br>NPs = 0.5 mL | Substrate = 0.3 mL<br>Water = 0.2 mL<br>Buffer = 2.0 mL<br>NPs = 0.5 mL |

## RESULTS

Three experiments were carried out for extracts of lemon seed, fiber, and outer layer. The results are shown in Tables **1**, **2**, and **3**. The enzyme assay was performed 3 times, in which the test solution was compared with blank

solutions. In the test solutions enzyme was used in 0.2 mL while in blank solutions distilled water was taken in the same amount. After this, the blank solutions are subtracted from the test solutions. In the end, it was deduced from the assay that the absorbance of the test solution was higher as compared to blank solutions, which meant that the nanoparticles enhanced the activity of the ALP.

UV analysis of lemon seeds, fiber, and outer layer extracts are shown in Figure **1**. The below-mentioned figure demonstrated the peak of lemon seeds (A) extract at 420 nm, and fiber extract (B) at 430 nm whereas, the peak of outer layer (C) extract at 425 nm. This means that silver nanoparticles are present in all three extracts of lemon because silver nanoparticles lie in the range of 417-444 nm[16]. The comparative peaks for lemon fiber, lemon seeds, and outer layers of lemon extract vary between 410 and 450 nm and 425 nm respectively in Figure **1(D)**.

**Table 2. Readings of Lemon Fibre. outer Layer of Lemon, and Lemon Seeds.**

| | | Experiment 1. | Experiment 2 | Experiment 3. | Average |
|---|---|---|---|---|---|
| Test | Lemon Fibre | 0.679 | 0.184 | 0.322 | 0.395 |
| Blank | | 0.531 | 0.132 | 0.246 | 0.303 |
| Test NP | | 1.092 | 0.448 | 0.662 | 0.734 |
| Blank NP | | 0.795 | 0.10 | 0.365 | 0.420 |
| Test | Outer Layer of Lemon | 0.680 | 0.199 | 0.321 | 0.4 |
| Blank | | 0.510 | 0.122 | 0.220 | 0.284 |
| Test NP | | 0.596 | 0.855 | 0.765 | 0.73 |
| Blank NP | | 0.498 | 0.410 | 0.399 | 0.43 |
| Test | Lemon Seeds | 0.634 | 0.186 | 0.354 | 0.391 |
| Blank | | 0.598 | 0.134 | 0.279 | 0.337 |
| Test NP | | 0.520 | 0.534 | 0.544 | 0.532 |
| Blank NP | | 0.364 | 0.370 | 0.392 | 0.375 |

**Table 3. Synthesis of Silver Nanoparticles from Lemon Fibre, Outer Layer of Lemon.**

| ***Enzyme Assay*** | | |
|---|---|---|
| | ***Without Nanoparticles*** | ***With Nanoparticles*** |
| Lemon Fibre | = (0.395 – 0.303) (3)/18.5(0.2)<br>= 0.092(3)/3.7<br>= 0.276/3.7<br>= 0.074µ/ml | = (0.734 – 0.420) (3)/18.5(0.2)<br>= (0.314) (3)/3.7<br>= 0.942/3.7<br>= 0.254µ/ml |
| Outer Layer of Lemon | = (0.4-0.284) (3)/18.5(0.2)<br>= 0.116(3)/3.7<br>= 0.348/3.7<br>= 0.094 | = 0.73 – 0.43) (3)/18.5(0.2)<br>= 0.3(3)/3.7<br>= 0.9/3.7<br>=0.2432 |
| Seeds of Lemon | = (0.391 -0.337) (3)/18.5(0.2)<br>= 0.054(3)/3.7<br>=0.162/3.7<br>= 0.043 | = 0.532 – 0.375) (3)/18.5(0.2)<br>= 0.151(3)/3.7<br>= 0.471/3.7<br>= 0.127 |

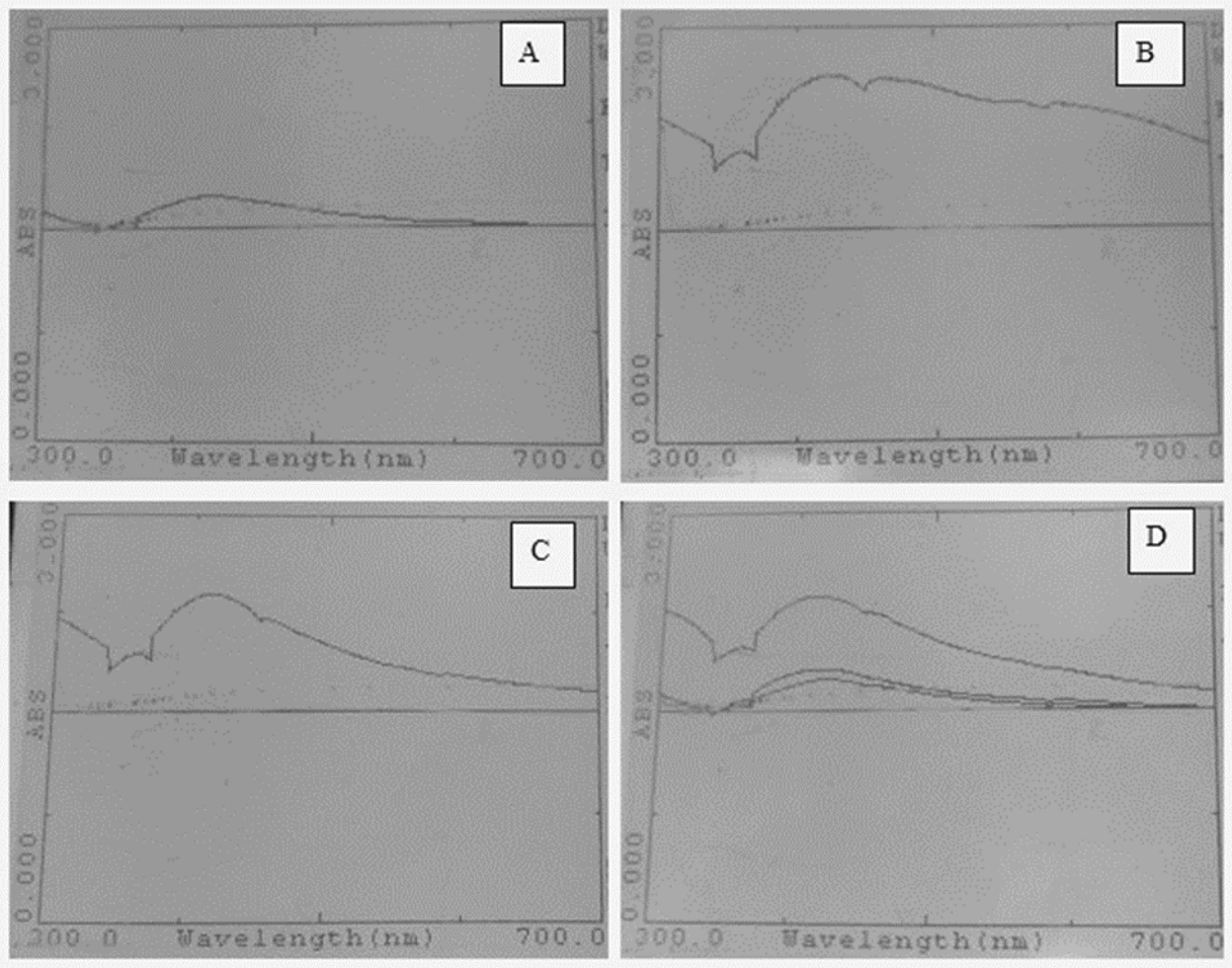


**Figure 1.** UV Spectrophotometry of lemon seeds extract (A), lemon fiber extract (B), lemon outer layer extract (C), and their comparison (D). Wavelength range 300-700 nm.

## DISCUSSION

In this study silver nanoparticles were synthesized by using the different parts of lemon and its extracts through green synthesis method which is cost effective less toxic easily scalable and eco-friendly approach. $AgNO_3$ was used as a capping agent that was used to control the reaction and the reaction stopped as the color changed from yellow to reddish brown. Synthesized AgNPs were characterized using UV vis spectrophotometry which showed peaks varying between 420 to 450 nm for the different parts of lemon[16,24].

There has been a lot of research on synthesizing AgNPs through the green synthesis of different plants and their extracts as green synthesis is cost cost-effective less toxic easily scalable method. As is the case for Malik Arjuna *et al*., 2011 who used *Ocimum* leaf extract for synthesizing AgNPs in which they used $AgNO_3$ (1mM) as a capping agent and the presence of silver nanoparticles was identified by the change in color to reddish yellow and after doing its UV vis spectrophotometry he got the peak at 436 nm[2,24]. Similarly, Allafchian *et al.,* (2016) used *phlomis* leaf extracts to synthesize silver nanoparticles using a green synthesis approach. The capping agent used was silver nitrate, which is mixed with the extract of *phlomis* leaves, and as a result the color changes in a few seconds from light yellow to dark brown, which confirms the presence of silver nanoparticles which are characterized by using UV vis absorption spectrophotometry which gives a peak of around 440 nm[17,20].

Similarly, Gondwal *et al.,* 2013 used the aqueous extract of *Calotropis Procera* to make silver nanoparticles through a

green synthesis approach[18,21]. Silver nitrate was used as a reducing agent, which mixed with the *Calotropis procera* extract thereby indicating the change in colour from yellowish brown to dark brown, which gives us an indication of the presence of silver nanoparticles that further characterized by UV Vis spectrophotometry giving peaks in the range of 449 to 452 nm[19,22,23].

## CONCLUSION

This study concluded that silver nanoparticles boosted the activity of alkaline phosphatase. This may help to benefit from silver nanoparticles in processes regulated by alkaline phosphatase in the future.

## CONFLICT OF INTEREST

None.

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None.

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