JIMMA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

COLLEGE OF NATURAL SCIENCES

DEPARTMENT OF CHEMISTRY

GREEN SYNTHESIS, CHARACTERIZATION AND ANTIMICROBIAL STUDY OF *PERSICARIA LAPATHIFOLIA* SEED EXTRACT SUPPORTED SILVER NANOPARTICLES

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JIMMA, ETHIOPIA

GREEN SYNTHESIS, CHARACTERIZATION AND ANTIMICROBIAL STUDY OF *PERSICARIA LAPATHIFOLIA* SEED EXTRACT SUPPORTED SILVER NANOPARTICLES.

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ABBREVIATIONS AND ACRONYMS

AgNPs	Silver nanoparticles
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
FCC	Face centered cubic
FT-IR	Fourier Transform Infra-red
NA	Nutrient agar
NPs	Nanoparticles
SPR	Surface Plasmon Resonance
TEM	Transmission Electron Microscope
UV-Vis	UV-Visible spectroscopy
XRD	X-ray Diffraction
ZOI	Zone of Inhibition

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ABSTRACT

Multi-drug resistance is a growing problem in the treatment of infectious diseases. The widespread use of antibiotics has produced resistance for many human bacterial pathogens. The advancement of nanotechnology, indicated bio-synthesis of nanoparticles supported on medicinal plants have improved therapeutic capacity Silver nanoparticles. Medicinal plants are parts of plant that have medicinal purposes. Preliminary phytochemical investigations of *Persicaria lapathiofolia* crude extract has showed the presence of different secondary metabolites as of flavonoids, Alkaloids and phenolic compounds. The main objectives of this study is to synthesize, characterize and evaluate antimicrobial study of *Persicaria lapathifolia* seed extract supported silver nanoparticles. The synthesized Silver nanoparticles were characterized by UV-Vis, FT-IR spectroscopy, Powder XRD analysis, and TEM images. The green synthesized Silver nanoparticles were characterized by color change pattern, and the broad peak at 413 nm in the UV–Vis region. The observed surface plasmon resonance suggested formation of small sized Silver nanoparticles. The color change observed during preliminary phytochemical investigation and the FT-IR spectroscopic analysis suggested flavonoids, phenols and Alkaloids are highly responsible for reduction and stabilization of the synthesized silver nanoparticles. From the crystallographic studies of XRD and images of TEM microscopic studies, the synthesized Silver nanoparticles were polycrystalline in nature owing to 18.9 nm and spherical in shape. Finally, the bio-assay test showed the highest antimicrobial activity of Silver nanoparticles than plant extract against *Escherichia coli* from rest of bacterial strains as a result of synergistic effect.

Keywords: *Nanoparticles, Persicaria lapathifolia, Antimicrobial activity.*

1. INTRODUCTION

In developing countries all over the world, large numbers of people die daily of preventable or curable diseases coming from microorganisms because of various reasons. One of the main reason for this could be the development of drug-resistant microorganisms. Moreover, the undesirable side effects of certain antibiotics by microorganisms have led to the design for new antimicrobial agents, mainly using medicinal plants [1]. A medicinal plant is any plant contains substances that can be used for therapeutic purposes or which are precursors for the synthesis of useful drugs. Several plants have been used in traditional medicine for many years to combat diseases coming from bacteria and fungi species. As an example, the plant *Persicaria lapathifolia* is antiseptic and astringent. So that it has been used by the local healer's [2, 3]. Some plants like *Persicaria lapathifolia* do seem to work although without sufficient scientific data to confirm their efficacy and have been used traditionally for the treatment of disease coming from a microorganism as of bacteria and fungi [4].

Nanoparticles are particles with their size ranging from 1-100 nm. They are synthesized by two approaches as top-down and bottom-up [5]. There are various methods for synthesis of nanoparticles (NPs) like physical, chemical and bio-synthesis methods. The physical and chemical methods of nanoparticles synthesis are expensive and not eco-friendly. The bio-synthesis methods for nanoparticles synthesis are called green technology [6]. Bio-synthesized metal nanoparticles based on green chemistry perspectives impose limited hazards to the environment and are relatively bio-compatible. Bio-synthesis method is an alternative to chemical and physical methods for the production of nanoparticles in an eco-friendly manner. This method for synthesis of nanoparticles includes plant-mediated bio-synthesis and microbial synthesis using bacteria, fungi, algae, and yeasts. The bacteria and fungi mediated synthesis of nanoparticles requires comparatively longer incubation time in the growth media for reducing a metal ion than a plant-mediated synthesis of nanoparticles. The synthesis of nanoparticles using plant extract has more advantages in comparison with bacteria and fungi since it is easy and require small time for synthesis [7]. Biosynthesis of nanoparticles using the extracts of plants like *Persicaria laphatifolia* is called green nanotechnology. This bio-synthetic method uses plants extract as stabilizers

and capping agents. The secondary metabolites or phytochemicals present in seed crude extracts like flavonoids, tannins, alkaloids, and others used as stabilizing and capping agents for nanoparticles synthesis [8]. There are various literature available in which many plant extracts were utilized for nanoparticles synthesis [9]. There were no literature reports on which *Persicaria laphatifolia* seed was utilized for the bio-synthesis. Using seed extracts of *Persicaria laphatifolia* as reducing and stabilizing agent the researcher was synthesized Silver nanoparticles and then evaluated the antimicrobial activity for the synthesized Silver nanoparticles and crude seed extracts alone. Due to unique chemical, physical properties and the high surface area to volume ratio of nanoparticles, they have an important role such as excellent antimicrobial agent compared the crude extract of different medicinal plants [10]. The picture of *Persicaria lapathifolia* seed revealed below (Figure 1).

Figure 1: The seed of *Persicaria lapathifolia.*

The synthesized nanoparticles were characterized by using UV-Visible spectroscopy, FT-IR spectroscopy, XRD analysis, and TEM images.

1.1 Statement of the problem

Drug resistance is a emerging problem in the treatment of infectious diseases. The widespread use of broad-spectrum antibiotics has produced resistance for many human bacterial pathogens. Advances in nanotechnology have opened new area in nano-medicine, allowing the synthesis of nanoparticles supported on medicinal plants to increase the therapeutic values of Silver nanoparticles, due to synergistic effect enhances the medicinal values of nanoparticles. This research work is a novel as there were no reports in the synthesis of nanoparticles using medicinal plant extracts of *Persicaria lapathifolia* seeds. Overall the researcher intends to answer the following questions.

1. Can *Persicaria lapathifolia* seed extract act as stabilizing and capping agent in the synthesis of silver nanoparticles?
2. Can the synthesized metal nanoparticles possess enhanced antimicrobial activity than the crude seed extracts?

1.2 Objectives of the study

1.2.1 General objective of the study

- To synthesize Silver nanoparticles supported on *Persicaria lapathifolia* seed extract, characterize and evaluate antibacterial activity.

1.2.2 Specific objectives of the study

- To prepare *Persicaria lapathifolia* seed extract.
- To synthesize Silver nanoparticles using the crude seed extracts of *Persicaria lapathifolia.*
- To characterize Silver nanoparticles using UV-Visible spectroscopy, FT-IR spectroscopy, Powder XRD and TEM images.
- To evaluate antimicrobial activity of the synthesized Silver nanoparticles and plant extract alone.

1.3 Significance of the study

This research work will have the following significance. The first one is it may be used as the reference by many stakeholders for further analysis and the obtained Nanocomposite may be scaled up and tested in vivo for the practical application tests.

2. REVIEW OF RELATED LITERATURE

2.1 Overview of Nanoparticles and Nanotechnology

Nanoparticles are particles between 1 and 100 nanometres (nm) in size. Nanotechnology is creating a growing sense of excitement in life sciences especially bio-medical devices and biotechnology. Nanoparticles can be made of materials of diverse chemical nature, the most common being metals, metal oxides, silicates, non-oxide ceramics, polymers, organics, and bio-molecules and they are designed with surface modifications to meet the needs of specific applications they are going to be used for [11].

Nanoparticles have different properties. Due to this, they are known to have a great scientific interest because of their sizes. A bulk material has constant physical properties regardless of its size, but at the nano-scale size-dependent properties are often observed. Nanoparticles are unique because of their large surface area and this dominates the contributions made by the small bulk of the material [12].

Nanoparticles with nano-scale in size possess inter-facial layer with a surrounding. The interfacial layer is an integral part of nanoscale matter, fundamentally affecting all of its properties. The interfacial layer typically consists of ions, inorganic and organic molecules. Organic molecules coating inorganic nanoparticles are known as stabilizers, capping and surface ligands [12, 13].

2.2 Synthesis of Nanoparticles

The synthesis method of Nanoparticles can be broadly classified as a bottom-up approach or the top-down approach. The bottom-up approach is one where smaller components of atomic or molecular dimensions self-assemble together. In the bottom to top approach, the chemical reduction is the most common scheme for syntheses of Silver nanoparticles. Different organic and inorganic reducing agents, such as Sodium Borohydride ($NaBH_4$), Sodium citrate, Ascorbate, elemental hydrogen, and Tollen's reagent can be used [14]. The top-down approach is where a process starts from a large piece and subsequently uses finer and finer tools for creating correspondingly smaller structures. In this top to bottom approach, suitable bulk material breaks down into fine particles by size reduction

with various lithographic techniques grinding, milling, sputtering and thermal/laser ablation [14].

Nanoparticles are prepared by different methods like chemical, physical and biological methods. Most of the methods reported in the literature on chemical and physical methods are extremely expensive and also involve the use of toxic, hazardous chemicals such as stabilizers which may pose potential environmental and biological risks. The use of plant extracts to synthesize nanoparticles is receiving attention in recent times because of its simplicity, readily scalable and less expensive [15].

Literature has reported the synthesis of nanoparticles using green methods as a simple, economical and eco-friendly method in the synthesis route [15, 16]. Bio-synthesis of nanoparticles can be done by using bacteria, fungi and plant extracts [17, 18]. Synthesis of nanoparticles using fungi and bacteria require a comparatively longer incubation time in the growth media for reducing a metal ion. In comparison with the plant-mediated synthesis of nanoparticles, bacteria and fungi based nanoparticles, are less advantageous [7]. Bio-molecules found in the plant including secondary metabolites act as reducing and capping agents in the green synthesis of nanoparticles. This method appears to be alternative to the conventional chemical and physical method of nanoparticles synthesis and it is easy to develop a biological process for large scale production. In recent times, plant extracts of different plants have a dual advantage by acting as reducing and capping agents for the synthesis of nanoparticles without the requirement for another stabilizer as of chemical methods. In this method there is no need to use high pressure, energy, temperature, toxic chemicals and longer time of incubation due to this they have more advantages than chemical, physical and even bio-synthesis approaches based on bacteria and fungi [7, 19].

In the synthesis of nanoparticles, different parameters have to be taken into account and the synthesis depends upon various variables, including, amount of plant extract, pH, time, and temperature [20]. The amount of plant material was found to play a critical role in controlling the size and size disparity of nanoparticles. Synthesis of Gold and Silver nanostructures by using green tea *Camellia sinensis* extract was done [21]. In this nanoparticles synthesis, it was investigated green tea *Camellia sinensis* when the amount

of *Camellia sinensis* extract is increased; the resulted nanoparticles are slightly bigger and more spherical. In another study, synthesis of nanoparticles using plant extracts of *C. zeylanicum* bark showed that as more plant extract is added smaller metallic nanoparticles and narrow size distribution occur [22]. Regarding, the influence of pH on the bio-synthesis it was suggested that different values of pH affect nanoparticle size and shape. The Silver and Gold nanoparticles synthesized from fruit extract of *Tanacetum vulgare*, larger particle size could be obtained by decreasing the pH [23]. The UV-Visible spectroscopy used to study the effect of pH on the synthesized nanoparticles based on the observed SPR bands [24]. Temperature effects showed an increase in temperature levels leads to nanoparticle growth at a faster rate and reducing their average particles size. This is due to as the reaction temperature increases, the reaction rate increases. For instance, synthesis rate Silver nanoparticles became faster when the temperature increases [25].

Nanoparticles synthesis in aqueous phase leads to particle-particle aggregation since they are unstable by nature they tend to decrease total surface energy. This is caused by attractive Van der Waals forces between crystals, should be repressed to limit the final particle size at the nanometric scale [26]. The plant extract serves as a reducing and dispersing agent to separate metal ions from each other and it provides better size control of nanoparticles. After synthesis, they also remain on the nanoparticles as the capping agent and improve the biological activity. These bio-molecules act as reducing agents for metal ion reduction and also remain on the nanoparticles as the capping agents which help to minimize the aggregation of nanoparticles thereby controlling the morphology and also helping to protect/stabilize the nanoparticles, thus improving the biological potential [27].

2.3 Application and limitations of nanoparticles

Nanoparticles have many applications. The diverse applications of metal nanoparticles have been explored in biomedical, agricultural, environmental, and physiochemical areas. Recently, due to their unique physical, chemical properties, and low-cost preparation, nanoparticles have gotten great interest [28].

Metallic nanoparticles are being explored and extensively investigated as potential antimicrobial activities. Silver has always been used against various diseases used as an

antiseptic and antimicrobial against Gram-positive and Gram-negative bacteria low cytotoxicity [29]. Silver nanoparticles were considered, in recent years, particularly attractive for the production of a new class of antimicrobial and opening up a completely new way to combat a wide range of bacterial pathogens [30-32]. High antimicrobial activity of nanoparticles depends on the particle size that allows greater surface contact and direct interaction with the membranes of pathogenic microorganisms.

The antimicrobial activity of the nanoparticles is known to be a function of the surface area in contact with the microorganisms. Several studies propose that nanoparticles attach to the surface of the cell membrane disturbing the permeability and respiration function of the cell [33, 34]. The damage to the cell may be caused by the interaction of nanoparticles with Sulfur or Phosphorus-containing biomolecules in the cell such as DNA. Therefore, Sulfur-containing proteins in the membrane or inside cells and Phosphorus-containing elements like DNA are likely to be preferential sites for binding for like Silver nanoparticles [35, 36]. In this research work, the Silver nanoparticles were synthesized using bio-synthesis methods. This method represents a clean, non-toxic as well as eco-friendly procedure for synthesizing Silver nanoparticles using the seed extracts of *Persicaria lapathifolia.*

In spite of these advantages, nanoparticles do have limitations. For example, their small size and large surface area can lead to particle-particle aggregation, making physical handling of nanoparticles difficult in liquid and dry forms. Also, small particles size and large surface area readily result in limited drug loading and burst release. These practical problems have to be overcome before nanoparticles can be made commercially available [26]. To prevent this state of aggregation in synthesized nanoparticles, by suspending the nanoparticles in the right solution with the right amount of nanoparticles, also it requires standardization. However, it can be prevented by adding an emulsifying agent like detergent or surfactant. Also, functionalization or coating the surface of the nanoparticles with some molecule helps reduce the state of aggregation.

2.4 Plant extracts and their uses

Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, flavonoids quinones, and others. Those secondary metabolites serve in plant defense mechanisms against predation by microorganisms. As it is shown in (figure 2) secondary metabolites which have been found in some plant extract have antimicrobial activities. Plant seeds of *Persicaria lapathifolia* are rich in flavonoids and other secondar y metabolities. There are many classes of flavonoid like anthocynin, proanthocyanidins, fl avonols, flavones, glycoflavones, biflavonyls, flavanones, and isoflavones [37]. Different classes of secondary metabolites act as stabilizing and capping agent for nanoparticles synthesis. An example of the structure for simple phenols (phenolic acids), includes caffeic acids, catechol, and eugenol. The structures classes of flavonoids (flavones), such as flavone, catechin, and chysin were revealed below. Moreover, structures for classes alkaloids, terpenoids, and quinones exampled as harmane, menthol, quinone respectively also shown as the following.

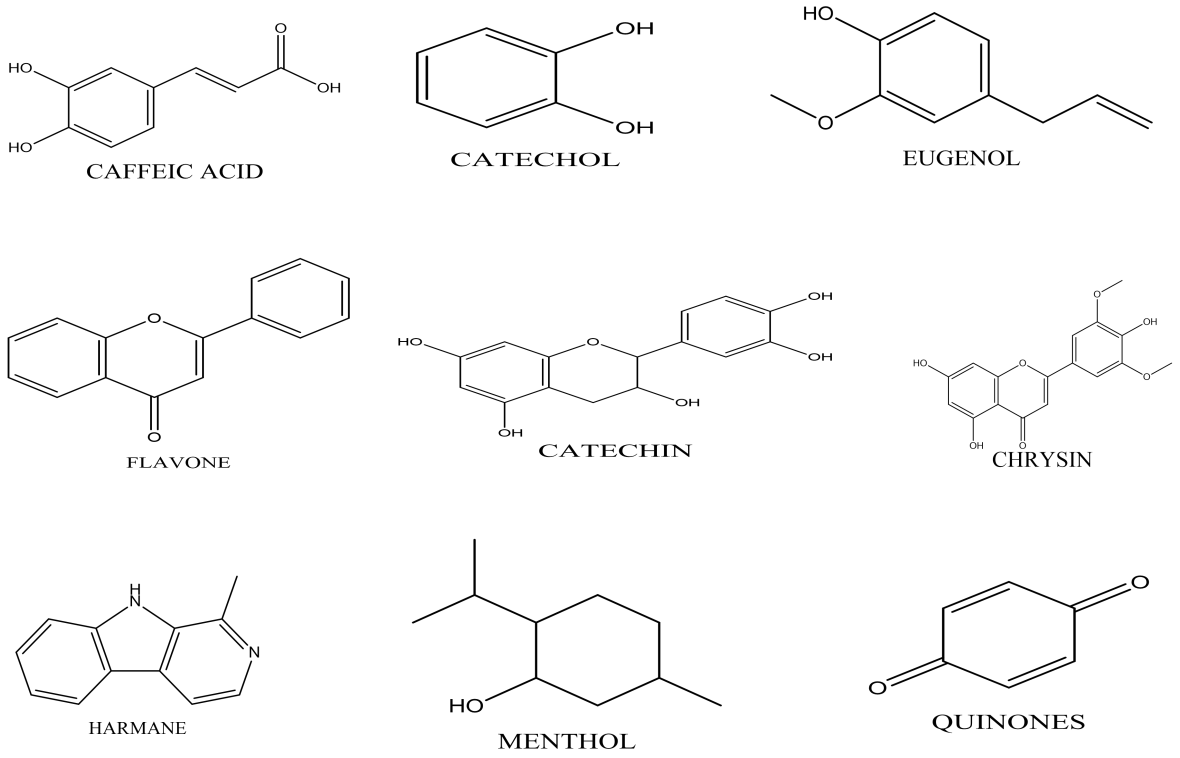


Figure 2: Examples of chemical compounds which is found in plants as secondary metabolites.

Plants are major renewable resources whose extracts used for various biological applications such as anticancer activity, antidiabetic, antimicrobial activity, protection of the liver, antioxidant and, the cardio-protective effect [38]. From potential uses of plant extracts, the antimicrobial activity against pathogenic microorganism is known. Due to this, antibiotics that show low efficacy in treating human and animal diseases through antibiotic resistance must be replaced with new drugs to combat the burden of these pathogens [39]. Hence, medicinal plants are expected to be the best source of obtaining a variety of drugs as [40]. Also medicinal plants act as reducing agents for metal ion reduction and also remain on the nanoparticles as the capping agents which help to minimize the aggregation of nanoparticles [27].

Persicaria lapathifolia (family Polygonaceae) is among the medicinal plant that have been commonly used by traditional healers in some parts of Ethiopia. Despite the wide usages of this plant in the traditional circles for the treatment of various diseases, the phytochemical information of the seeds of this plant and its microbial activity has not been addressed. There are report for the isolation of three flavonoid compounds along with their antibacterial and antifungal activities from the seeds of *Persicaria lapathifolia* [41]. The whole plant part of *Persicaria lapathifolia* is antiseptic and astringent [2]. Moreover, the infusion of this plant part has been used in the treatment of stomach complaints, VD and fevers [3, 42].

In Ethiopia, different communities have extensively been using medicinal plants such as *Persicaria lapathifolia* [43-45]. Such plants, however, need to be investigated for a better understanding of their properties, safety, and efficiency [46].

A considerable effort has been made by researchers to find efficient extraction methods to get high efficiency from various plants as such exampled in (figure 3) below [1].

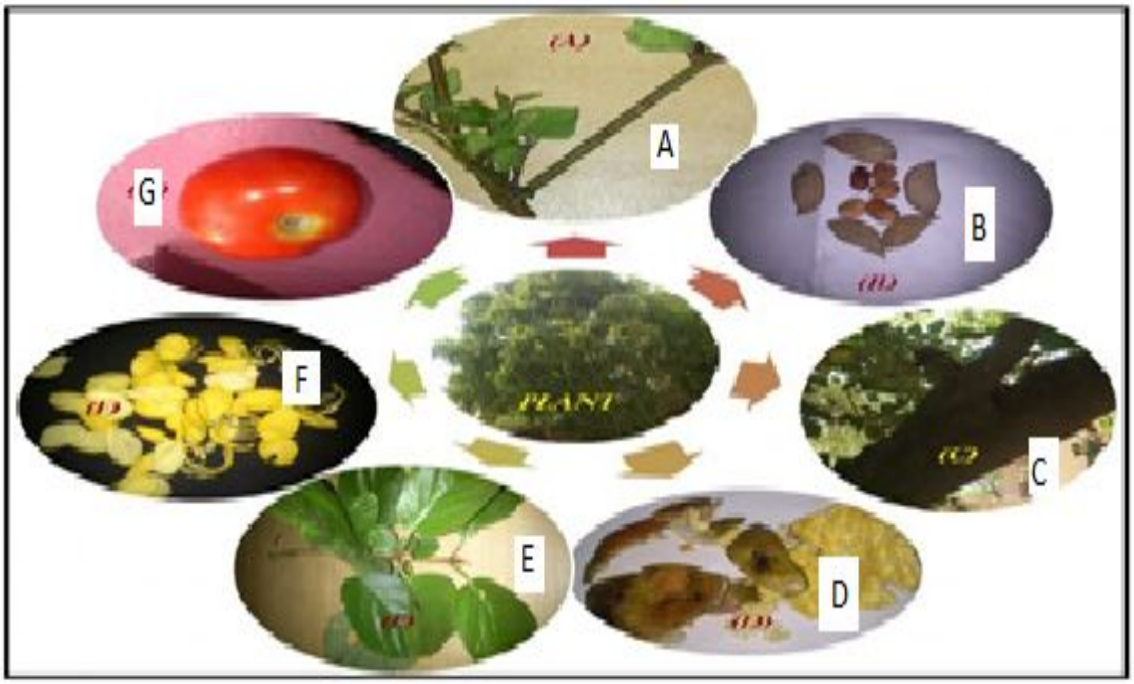


Figure 3: Synthesis of nanoparticles using various parts of plants (A) Stem, (B) Seeds, C) Bark, (D) Peel, (E) Leaves, (F) Flowers and (G) Fruits.

Plant extracts are often environmentally and economically friendly materials and have been explored in the synthesis of nanoparticles [14-18]. All parts of a plant bearing antioxidants or sugars, including leaves, fruits, roots, seeds, and stems, can be used as shown in (figure 3) above. Moreover used in the synthesis process, replacing potentially hazardous chemicals. Several plant extract mediated synthesis of silver nanoparticles have been reported in the literature [47]. For instance use of red Apple *Malus Domestica* [48], *Brucea antidysenterica* leaves [49], *Geranium* leaf [50], *and Soybean* seed extract [51] was reported.

3. MATERIALS AND METHODS

3.1 Chemicals and reagents

Laboratory reagent Silver Nitrate ($AgNO_3$) as chemical precursor (HiMedia Laboratories, PVt. Ltd.), extra pure (98.5%), MW = 169.68 g/mol; Potassium bromide (KBr, 99.5%, BDH chemicals Ltd Poole, England); Methanol (CH_3OH, 99.8%, HPLC grade); Chloroform ($CHCl_3$, 99.8%, HPLC grade); 10% Sodium hydroxide (NaOH), Mercury chloride (HgCl), Potassium Iodide (KI), Ferric Chloride ($FeCl_3$), 0.1 M Hydrochloric acid (HCl), 0.1 M Sodium Hydroxide (NaOH), DMSO (Dimethyl sulfoxide), potassium bromide (KBr, 99.5%, BDH chemicals Ltd Poole, England), and Mueller Hinton agar were used for the present study.

3.2 Apparatus

Magnetic stirrer, Filter paper, Mortar & Pestle, Test tubes, Beakers, Erlenmeyer flasks, Volumetric flasks, Pipettes, Petriplates, Petridish, Forceps, Aluminum foils, Quartz, swab and centrifuge tubes were used for the present study.

3.3 Instruments

UV-Visible spectrometer (JENWAY, BILBBY SCIENTIFIC Ltd, England); FT-IR spectrometer (SHIMADZU 1730, JAPAN); XRD (BRUKER D8 ADVANCED XRD, West Germany); Transmission electron microscopy (HITACHI 600 TOKYO, China); pH meter (pH meter-016, England); Rotary evaporator (LABOROTA 4000- EFFICIENT, HEIDOLPH, Germany); Analytical balance (AEADAM-DCT302, CHINA); Centrifuge (PLC-02, GEMMY INDUSTRIAL CORP, England); Hot plate (STUART-CB162); Sterilizer; Autoclave; Incubator; Oven (OV150C, England), Refrigerator (PENTANE LR, 1602, Japan); were used for the present study.

3.4 Experimental Methods and Procedures

3.4.1 Plant collection and processing

The seed of *Persicaria lapathifolia* was collected from Ajip condominium area, Jimma twon, Oromia regional state, Ethiopia in January, 2018. The plant material was deposited

in Jimma university Inorganic laboratory. Then the collected plant material was chopped into smaller pieces and shade dried at room temperature for 21 days.

3.4.2 Preparation of plant Seed extracts

The air-dried seed was ground to a small size to facilitate easy solvent penetration using Mortar and Pestle. Then Maceration or soaking was used as extraction methods [52]. By using Maceration (1:10 w/v) sample to solvent ratio the 20 g of powdered seeds of *Persicaria lapathifolia* was added to 200 mL of Methanol Chloroform (50:50) solution separately into 1000 mL Erlenmeyer flasks and allowed to stand at room temperature for a period 3 days with frequent agitation [53]. Then the solution was filtered off using Whatman No.1 filter paper and the obtained filtrate was concentrated using rotary evaporator. The resulting solid was weighed and stored at 4^0C.

3.5 Preliminary phytochemical screening

Preliminary phytochemical screening were done to check the presence of important phytochemical groups that are used for reduction and stabilization of the Silver nanoparticles. 0.1 g crude extracts were dissolved in 50 mL of its Methanol Chloroform (50:50) solvents. The obtained concentrated solution was used for phytochemical screening according to Harborne and Kokate [54, 55].

3.5.1 Test for Phenols

A small amount of extract was taken and a few drops of methanol and few drops of Ferric chloride were added. The solution was shaken well till the appearance of greenish-yellow color appeared suggesting the presence of phenol [54, 55].

3.5.2 Test for Flavonoids

A small amount of extract was taken and mixed with 10 % Sodium hydroxide which results in greenish-brown color which indicates the presence of flavonoids [54, 55].

3.5.3 Test for Alkaloids

A small amount of extract was taken and mixed with Mayer's reagent which is the mixture of Mercuric chloride and Potassium iodide. The formation of a reddish-brown precipitate indicates the presence of alkaloids [54, 55].

3.6 Bio-synthesis of Silver nanoparticles

30 mL of concentrated solution was prepared by dissolving 1 g seed extract in to in 30 mL of Methanol Chloroform (50:50). The 4 mL of the seed extract was taken from the prepared concentrated solution and added to 100 mL of 0.01 M of $AgNO_3$ in solution. The reaction mixture was stirred using magnetic stirrer for 30 min & allowed to stand in the dark for 24 h. Then the reaction solution was centrifuged at 10,000 rpm for 20 minutes. The supernatant liquid was decanted off and the residue was repeatedly washed to remove impurities at the surface of the Silver nanoparticles [56]. The obtained deep brown precipitate was air-dried. The synthesized silver nanoparticles were then kept for characterization by UV-Vis, FT-IR, XRD, TEM images, and antibacterial studies.

3.7 Methods of Characterization for the synthesized Silver nanoparticles

3.7.1 UV-Visible (UV-Vis) spectroscopic studies

This technique is used to quantify the light that is absorbed or scattered by a sample in the wavelength range of 200-800 nm. Moreover, the size and shape of nanoparticles in aqueous suspensions controlled and examined using this technique [57, 58]. From the SPR bands, information about particle size can be obtained. The shift to longer wavelength suggests increasing in the particle size, where as the shift to shorter wavelength suggests the decreasing in the particle size of nanoparticles [59]. According to Mie's theory, only a single SPR band is expected in the absorption spectra of spherical nanoparticles**,** but if two or more SPR bands appear they are considered as anisotropic particles [60-65]. The color appearing is due to excitation of Surface plasmons in metallic nanoparticles [24].

3.7.2 Fourier transform infrared (FT-IR) spectroscopic studies

Fourier transform infrared spectroscopy was done to know the bio molecules found at the surafce of the synthesized Silver nanoparticles. The dried precipitate of Silver nanoparticles were grounded with KBr and cast into a pellet and made ready for analysis on FT-IR spectrophotometer in the diffuse reflectance mode operating at a resolution of 4 cm^{-1} [66].

3.7.3 X-ray diffraction (XRD) studies

The XRD pattern of the synthesized nanomaterial was then recorded using an X-ray diffractometer [64]. A thin film of the sample of Silver was made by dipping a glass plate for XRD studies. The diffraction pattern was recorded with Cu and Ag targeted K radiation at a wavelength of 1.5405 Å. The scanning was done over 2θ value range of 0^0 to 90^0 at 0.02 min^{-1} and at 1 second time constant. The instrument was operated at a current of 30 mA and voltage of 40 kV. The crystalline domain size was calculated using the Scherer formula.

$$D = 0.94\lambda/ \beta \cos\theta$$

Where D = Average crystallite size, λ = X-ray wavelength,= Full width at half maxima (FWHM) of XRD spectral peak (in radians) and θ = Bragg's angle.

3.7.4 Transmission Electron Microscopy (TEM) studies

Transmission Electron Microscope used to know the morphology and particle size distribution of silver nanoparticles. The grid for TEM analysis was prepared by placing a drop of the nanoparticle suspension on a carbon-coated copper grid and allowing the water to evaporate inside a vacuum dryer. The grid containing silver nanoparticles was scanned by a Transmission Electron Microscope [34].

3.8 Antibacterial Activity Studies

The antibacterial assays of the Silver nanoparticles were done for two-gram positive *Staphylococcus aureus, Bacillus* and gram-negative *Escherichia coli, Salmonella typhi* using paper disc diffusion method. Mueller Hinton agar media was used to cultivate the bacterial strains.

3.8.1 Preparation of inoculums

Test bacterial strains were transferred from the stock cultures as streaked on Mueller Hinton agar plates and incubated for 24 h at 37 ^{0}C. Then well separated bacterial colonies were used as inoculums. The medium was then poured to sterile Petriplates, allowed to solidify and used for the bio-test [67]. Bacteria were transferred using a bacteriological loop to autoclaved nutrient agar that was cooled to about 45^{0}C in a water bath mixed by gently swirling the flasks. A fresh culture of inoculums of each culture was streaked on nutrient agar media using sterile swab and allowed to grow for 24 h. The Sterile Whatman filter paper discs (6 mm) were soaked in the prepared solution then placed on to each Petridish. Then plates were then incubated at 37 for 24 h.

3.8.2 Preparation of test solutions

Five test solutions were prepared for the bacterial test and labeled as 1, 2, 3, 4 and 5 for test solution. Among five samples three samples were from Silver nanoparticles at the concentration of 1 mg/mL, 3 mg/mL and 5 mg/mL and one sample was from plant extract at concentration levels of 10 mg/mL in DMSO (Dimethyl sulfoxide). The 0.01M of $AgNO_3$ test solution was also prepared. Zones of inhibition were measured after 24 h of incubation. The magnitude of antibacterial effect against, gram-positive bacteria *Staphylococcus aureus, Bacillus subtilis* and gram-negative *Escherichia coli, Salmonella typhi* was determined based on the inhibition zone measured in the disk diffusion method. The clear zones formed around each disk were measured in millimeter.

3.9 Methods of Data Analysis

Origin version six software was used to analyze the data collected from UV-Visible, XRD, and FT-IR spectroscopy. Image J software was used to calculate the size of nanoparticles from TEM images.

4. RESULT AND DISCUSSION

4.1 PHYSICOCHEMICAL PROPERTIES

4.1.1 Color change observed during phytochemical investigations

The preliminary phytochemical investigations showed the following colors.

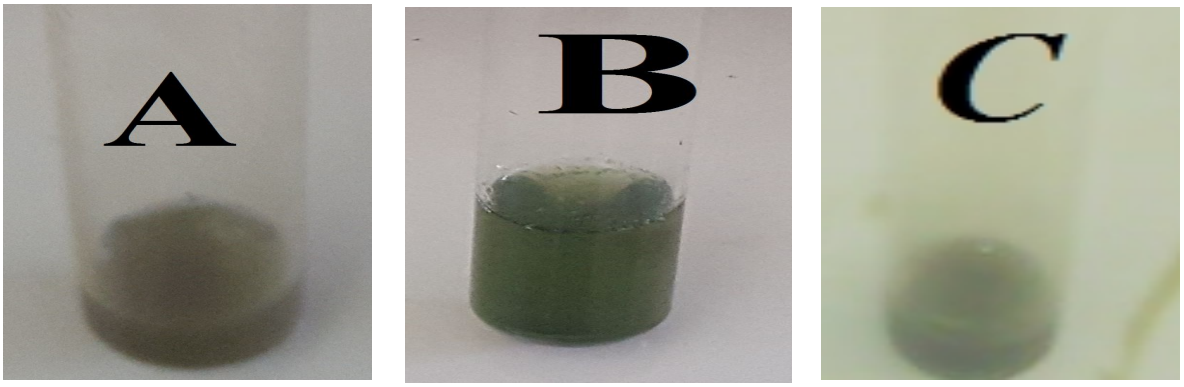


Figure 4: Color change observed during phytochemical investigations. A) Phenols B) Flavonoids C) Alkaloids.

Table 1: Lists of phytochemical groups and indications '+' shows presence.

Labels	Phytochemicals	Test	Observations	Inferences
A	Phenols	few drops of methanol and ferric chloride	Greenish yellow	+
B	Flavonoids	10 % sodium hydroxide	Greenish brown	+
C	Alkaloids	mercuric chloride and potassium iodide	Reddish brown	+

4.2 UV-Visible (UV-Vis) spectroscopic studies

Reduction of Silver ions to Silver nanoparticles was visually identified by a color change of yellow to deep brown in the aqueous reaction medium after addition of plant extract. The color change suggested formation of Silver nanoparticles due to the reduction of Silver ions [68]. As indicated in (Figure 5) below the formation of silver nanoparticles was due to splitting in the ground state of Silver from ^2D electronic configuration into $^2Eg_2$ and $^2T_{2g.}$ There is only one possible electronic transition from the lower $^2Eg_2$ to $^2T_{2g}$ in the cases of d^9. So one strong absorption band was observed at 413 nm in the UV-Visible region. This absorption band were the characteristics absorption band for the formation of Silver nanoparticles [69].

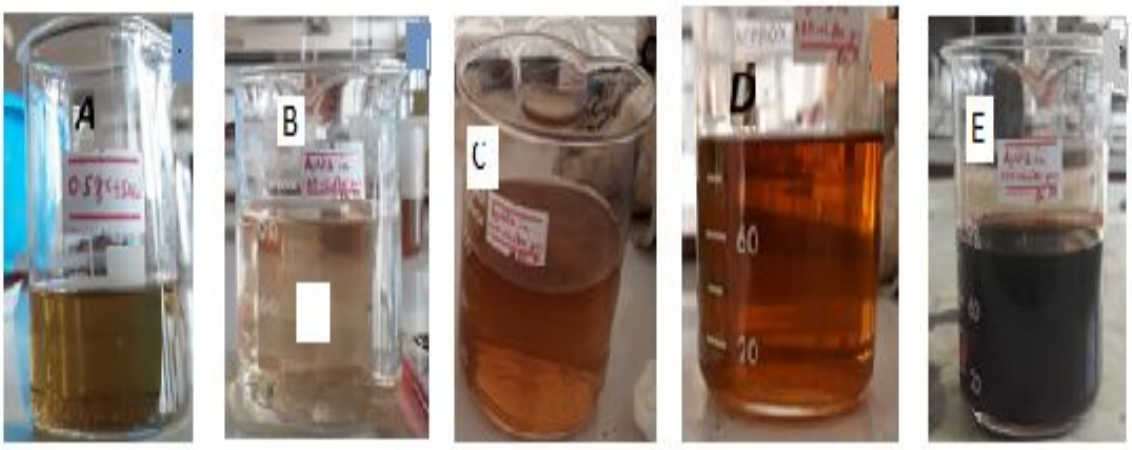


Figure 5: Plant extracts solution and Silver Nitrate solution A) Seed extract B) Silver nanoparticles solution having plant extract after 10 minutes C) after 30 minutes D) after 90 minutes E) after 1440 minutes (1 day).

The absorption maximum appeared at 413 nm by exhibiting red shift as discussed in later section of factors affecting nanoparticles formation. Generally, nanoparticle formation increases band gap, as a result energy difference between the conduction band and valance band increases. The band gap for Silver nanoparticles are calculated to be 3.01 eV. The obtained result are very higher than reported values of 2.6 eV in larger sized silver nanoparticles [70]. As the particle decrease, the energy required to excite the

surface plasmon electrons increases [71]. Based on the spectrum data the band gap of Silver nanoparticles were determined using equation below.

$$E_g^* = \frac{hc}{\lambda_c}$$

Where E_g^* = Band gap energy of the nanoparticle,

λ = Wavelength absorbed by the sample,

h = Planck's Constant, 6.625×10^{-34} J·s

c = the speed of light, Also, 1eV= $1.6*10^{-19}$ Joules (Conversion factor)

$E^*_g = (6.626*10^{-34}\ J.s)(2.779*10^{8}\ m/s) / 413*10^{-9}\ m$

$E^*_g = 4.809*10^{-19}$ J = 3.01 eV

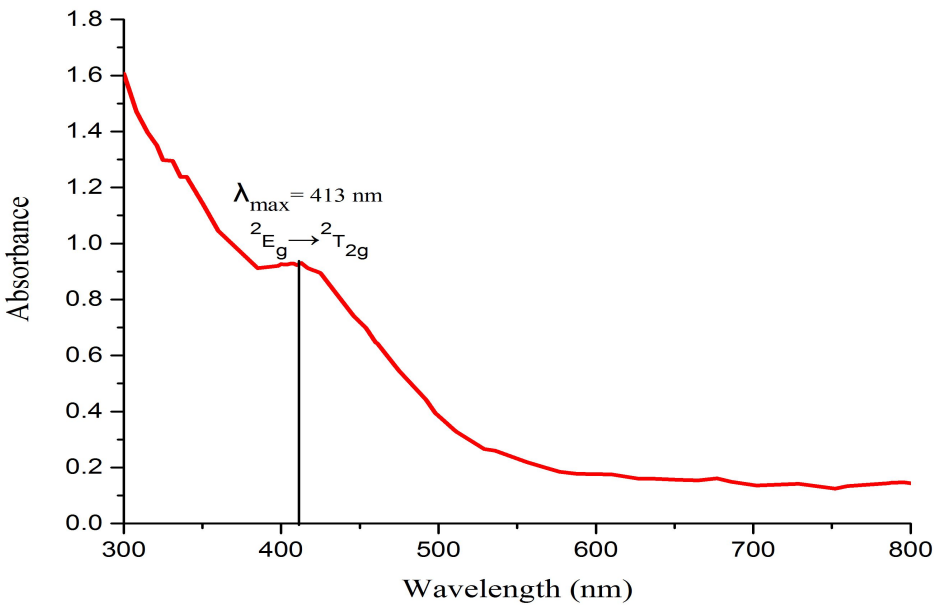


Figure 6: The characteristic Silver single SPR band showing around 413 nm.

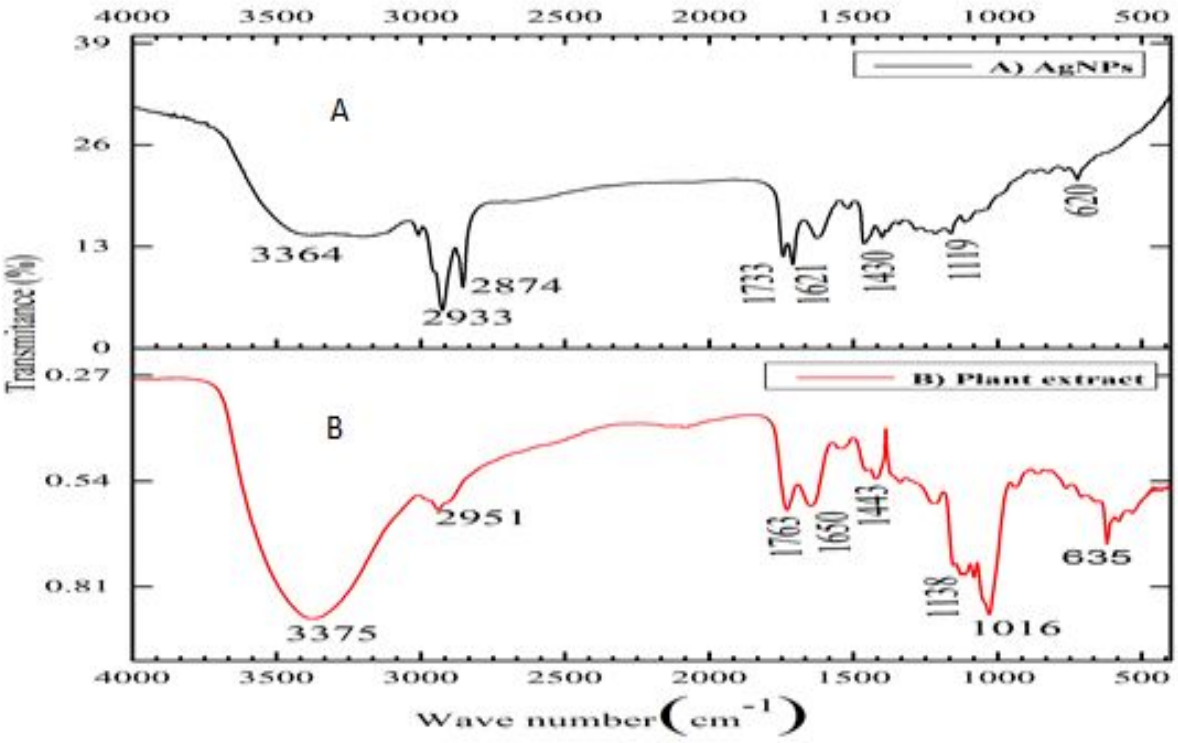
UV-Visible spectroscopy technique is commonly used to characterize the surface plasmon resonance properties of synthesized nanoparticles. Literature has revealed that Silver nanoparticles absorb the spectrum in the UV-Visible region of around 400 nm due

to the excitation of the localized surface plasmon resonance [72, 73]. Silver nanoparticles may show surface plasmon band of around higher wavelength range up to 500 nm [74]. Several research works have been written the relation between UV-Visible spectrum and nanoparticle properties [75–77]. As an example, Silver nanoparticles of the uniform spherical size expected to show a single peak in the UV-Visible spectrum around near to 400 nm. The Silver nanoparticles synthesized from plant extracts of *Cyperus rotundus* suggests that smaller sizes of nanoparticles produced and have shown a single peak at 446 nm [76]. The presence of a broad and weak band at 480 nm for Silver nanoparticles was due to surface plasmon resonance band of large-sized Silver nanoparticles [75, 77]. Silver nanoparticles synthesized from *Eleusin indica* plant extract showed higher at maximum absorbance at a wavelength of 452 nm range [77]. Silver nanoparticles of irregular shapes have two or more peaks depending on their symmetry. For instance, Silver nanoparticles synthesized using *Melastoma malabathricum* plant extract have more than one peak in the wavelength range of 550 to 780 nm, implying irregularity in their nanoparticle shapes [75]. The synthesized silver nanoparticles from seed extracts of *Persicaria lapathiofolia* have shown strong plasmon resonance band at 413 nm and it was comparable as spherical and small-sized Silver nanoparticles [76].

4.3 Fourier transform infrared (FT-IR) spectroscopic studies

Fourier transform infrared spectroscopy analysis was done to identify biomolecules that are responsible for Ag^{+} ions reduction and capping of biologically reduced Silver nanoparticles. Figure 10 (B) shows the FT-IR spectrum *Persicaria laphathiofolia* seed extract showing peaks at 3375, 2951, 1763, 1650, 1138, 1016 and 635 cm^{-1}. A majority the of observed peaks also exist in the FT-IR spectrum of Silver nanoparticles with few marginal shifts to lower wavenumber due to reduction of electron density of phytochemicals as a result of sharing of electron density to Silver nanopartcles and capping the surfaces of nanoparticles. For example, all peaks marginally shifted to 3364, 2933, 2874, 1733, 1621, 1119, and 620 cm^{-1} as indicated in (figure 10) below. A broad peak at 3375 cm^{-1} indicated shows the O–H bond stretching of alcohols and phenolic compounds [78]. The absorption peaks at 2951 cm^{-1} could be due to stretching of –CH in an alkane. The peak at 1763 and 1650 cm^{-1} corresponds to C–O stretching in the carboxyl

attached to the amide linkage in amide (–NH–C=O) so indicating N–H bent primary amines. The absorption band at 1443 cm^{-1} indicated the presence of C–N stretching in amide. The two bands present at 1016 and 635 cm^{-1} could be indicative of the –O– stretching vibrations of aromatic and aliphatic amines respectively [79].



Figures 7 (A): FT-IR spectra for AgNPs **B)** FT-IR spectra for seed extract of *Persicaria laphathiofolia*

As above Figure 10 (A) shows the FT-IR spectrum of Silver nanoparticles displayed bands at 3364 cm^{-1} (OH of alcohol) and 1621 cm^{-1} (–NH–C=O) and suggested flavonoid or phenols and Alkaloids inside *Persicaria laphathiofolia* might be actively involved so responsible for the reduction of Ag^{+} to Ag^{0} [80]. The involvement of flavonoid in the reduction of metal ions using plant extracts is also evidenced by another study [81].

4.4 X-ray diffraction (XRD) studies

The crystalline nature of the Silver nanoparticles synthesized from *Persicaria lapathiofolia* seed have been studied by XRD analysis. The XRD diffractogram clearly

shows the main peaks at (2θ) range from 10º to 80º (Figure 11). The XRD spectrum of the Silver nanoparticles showed four diffraction peaks at 38.03°, 44.20°, 64.40° and 77.37° corresponding to the (hkl) values of (111), (200), (220) and (311) planes respectively. So, it was confirmed that the Silver nanoparticles are crystalline in nature and had face centered cubic (FCC) crystal structures. There were unassigned peaks appeared at 18.25°, 21.09° and 29.21°. This unpredicted crystalline structures suggested the presence of bio-organic compounds occurring on the surface of the Silver nanoparticles as a result of crystallization of bio-organic phase on the surface of Silver nanoparticles [82]. Indeed this is clearly seen from TEM images and FT-IR spectrum. These peaks was weaker than those of Silver. A similar result was observed by [83, 84], who identified crystalline peaks (32.28°, 46.28°, 54.83°, 67.47°, and 76.69°) which were also obvious in a lot of works in which the XRD pattern included the relevant 2θ range. The average crystalline size of the Silver nanoparticles was estimated using the Debye–Scherrer's equation [85].

$$D = 0.9\lambda / \beta \cos\theta$$

Where D = Average crystallite size, λ = X-ray wavelength = 0.15406, β = Full width at half maxima (FWHM) of XRD peak (in radians) and θ = Bragg's angle (in radians).

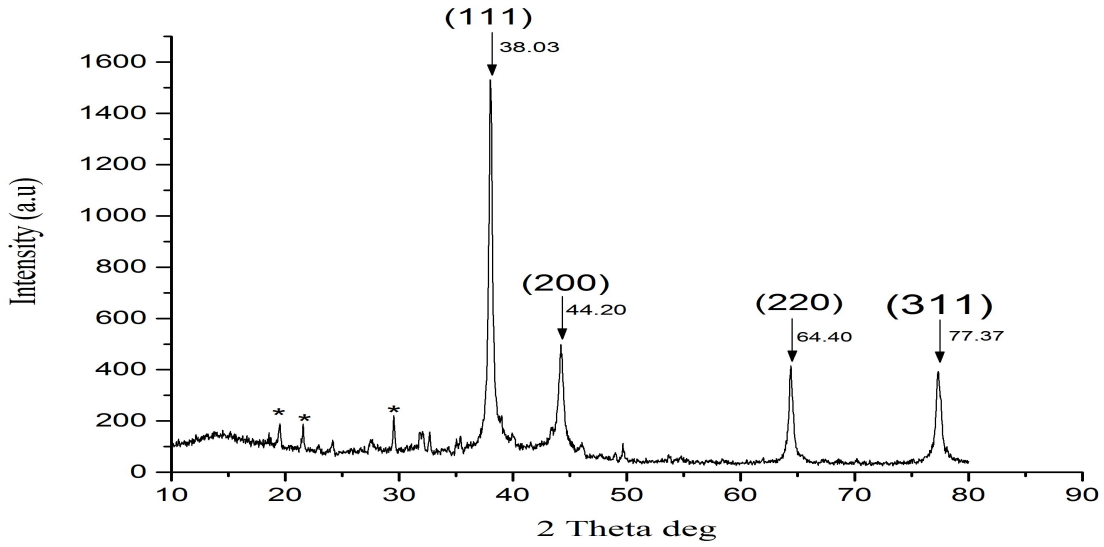


Figure 8: XRD of Silver nanoparticles showing four peaks at are given 2θ values.

Table 2: The variation of the crystalline size of biosynthesized Silver nanoparticles.

2 θ of intense peak in degree	hkl	FWHM of Intense peak (β) in degree	FWHM in (radian)	Cos Θ in (radian)	Size of the particles (D) nm	Average particles size (D) nm
38.03611	111	0.4660	0.0082	0.945	18.83	18.9
44.20281	200	0.71389	0.0124	0.927	12.54	18.9
64.40753	220	0.42201	0.0073	0.847	23.24	18.9
77.37166	311	0.50615	0.0088	0.780	21.00	18.9

The calculated size of Silver nanoparticles was between 12 nm and 24 nm. The average particles size was found to be 18.9 nm as it is shown below in (table 2).

4.5 Transmission Electron Microscopy (TEM) studies

The size and morphology of *Persicaria laphatiofolia* seed extract mediated Silver nanoparticles synthesis was determined by TEM analysis. Majority of the synthesized Silver nanoparticles were predominantly spherical and some are ellipsoidal in the form of agglomerates due to the high surface area of the synthesized AgNPs. Notably majority of particles in TEM image are not in physical contact with each other and appeared separated by the organic layer due to the presence of some organic layers surrounding AgNPs as indicated in (figure 11). The thin organic layers important to prevent physical contact between nanoparticles, as a result, stabilized nanoparticles. Similarly,[86]. The result from TEM image is comparable with FT-IR data that revealed the presence of polyphenolic components of flavonoids and Nitrogen containing functional groups of Alkaloids at the surface of the synthesized Silver nanoparticles [78, 79].

The degree of crystallinity is evaluated through comparison or division of crystallite size obtained by TEM image to the XRD pattern particle sizes. The particles size of the

samples estimated from the TEM image is larger than XRD analysis. The calculated sizes of nanoparticles from TEM image was found to be 24.1 nm. Since the crystallinity index value more than 1, then the obtained nanoparticles assumed to be polycrystalline [86].

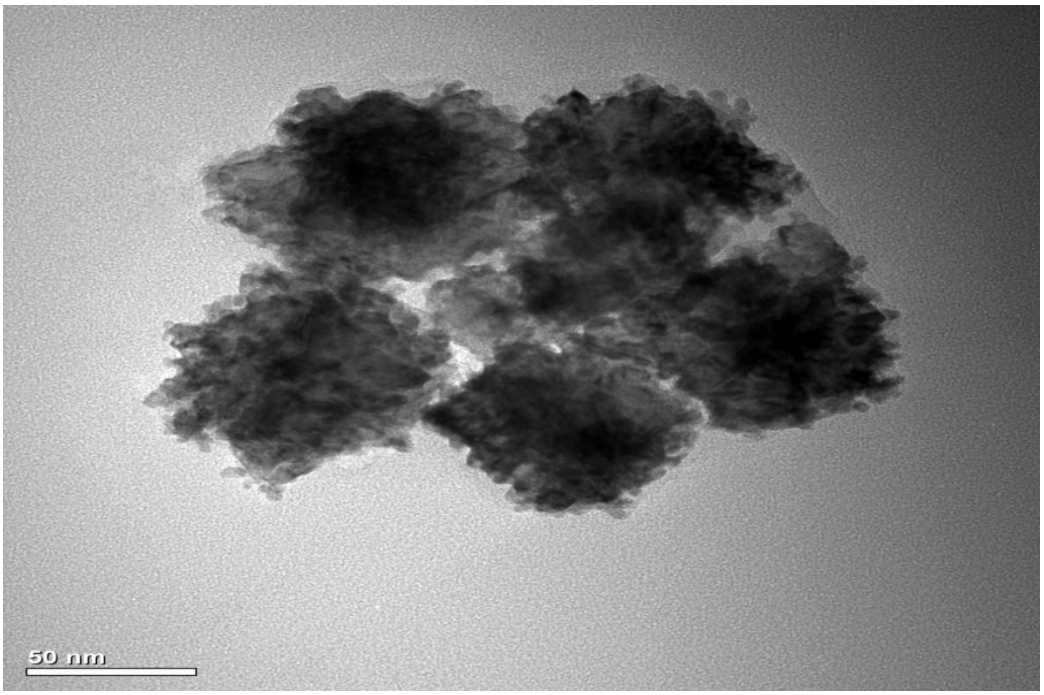


Figure 9: A 50 nm resolution studies of synthesized Silver nanoparticles with TEM analysis.

4.6 Effects of different parameters on the synthesized Silver nanoparticles

4.6.1 Effect of time on the synthesis of nanoparticles

The effect of time on the synthesis of Silver nanoparticles was studied. There was no color change noticed in the Silver Nitrate solution in the absence of plant extract, But with other solution having Silver Nitrate and plant extract color change observed. The kinetics of the reaction monitored by UV–Visible spectroscopy analysis revealed that the reaction was slow in the beginning indeed, up to 30 min from the start of the reaction, the formation of Silver nanoparticles was very much slow. After 30 min, the nucleation was initiated very rapidly and the formation of Silver nanoparticles occurred very fast after 30 min had passed. This is reflected by the sudden appearance of the characteristic band of

Silver nanoparticles at ~413 nm after 90 min of reaction time as indicated by green color in (figure 7). The reaction was allowed to continue further and no considerable change was observed which points towards the completion of the reaction after ~90 min.

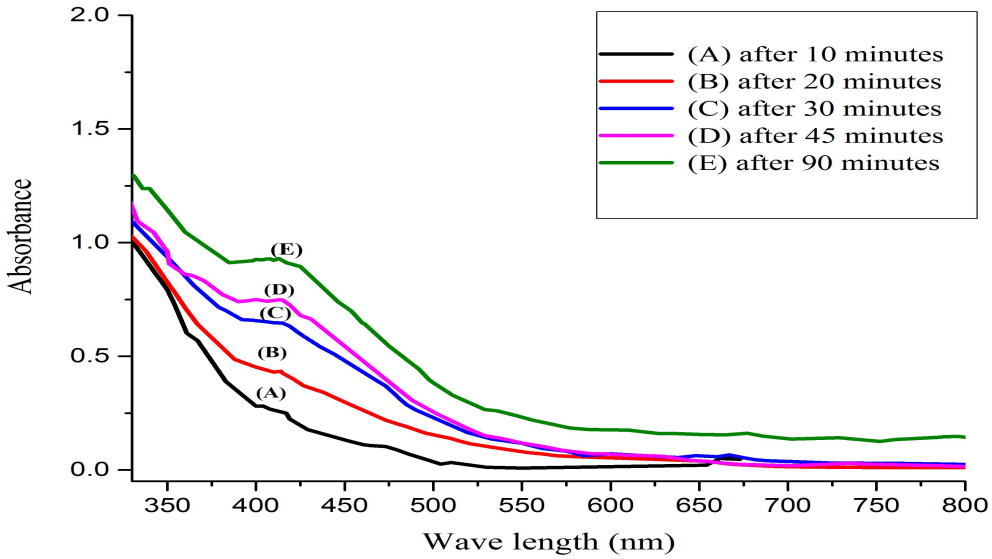


Figure 10: UV–Vis absorption spectra kinetic reaction study of the as-synthesized Silver nanoparticles at different time intervals.

4.6.2 Effect of plant extract concentration

Plant extract concentration has a significant effect on the quality and size of synthesized Silver nanoparticles [87]. To assess the effect of concentration of plant extract on the synthesis Silver nanoparticles different samples were prepared. 1 g of seed extract was dissolved in 30 ml of Methanol Chloroform (50:50) solution. Three beakers having 100 ml of 0.01 M Silver Nitrate solution were prepared. Into all three beakers a 2 ml, 4 ml, and 6 ml of the prepared seed extract were added to observe the change SPR of the colored solution in the UV–Vis region. The concentration of Silver nitrate and time are given for reduction was kept fixed in all these tests. As (Figure 1: in appendix section) demonstrates the UV–Vis spectra of Silver nanoparticles prepared using different concentrations of plant extract. The previous study showed that the concentration of plant extract had a significant effect on the quality of Silver nanoparticles [87].

The characteristic UV–Vis absorption peak of Silver nanoparticles showed a significant shift towards the red end (higher wavelength) or the blue end (lower wavelength) depending upon the particle size, shape, state of aggregation [88]. Similarly, the amount of *Persicaria lapathiofolia* plant extract did considerable effect on the size of synthesized Silver nanoparticles, which was effectively monitored by the UV–Vis analysis. The solution 0.01 M Silver Nitrate with 4 ml of seed extract of *Persicaria lapathiofolia* has shown blue shift and showed sharp SPR peak around 413 nm suggesting the formation of small-sized silver nanoparticles with higher band gap than the rest concentration levels. Moreover, as there was no additional peak suggesting the absence of a state of aggregations and so selected as optimum concentration to obtain small-sized nanoparticles. The Silver Nitrate solution with 6 ml of seed extract as showed red shift by exhibiting broad peak around 450 nm and additionally appearance of another peak around 600–700 nm confirmed the presence of aggregation of Silver nanoparticles [89]. The Silver Nitrate solution with 2 ml seed extract had no significant peak around 400 nm and suggesting there was very small or no formation of AgNPs. Generally, a broad peak around higher wavelength indicates an increase in particle size, while sharp peak at a shorter wavelength represents a smaller particle size [88]. Additionally, literature reports also revealed that by increasing the concentration of plant extract, the size and the size distribution of the NPs can be increased [89].

4.6.3 Effect of pH

The pH of the reaction mixture also plays an important role in the nanoparticles synthesis. 0.1 M of HCl and 0.1 M of NaOH were used to adjust the pH of nanoparticles solution. At lower pH 2.9 and 4.3, the color change of aqueous mixture was slower than that at higher pH at 8.1 and 9.3. The color intensity of the reduction process increased with the increase of the pH (Figure 2: in appendix section). At lower pH of 2.9 and 4.3, the observed SPR band were broad in the wavelength range between 430 and 420 nm respectively. This was indicative of the formation of polydispersed nanoparticles. At lower pH peak broadening occurred due to the excitation of longitudinal plasmon vibrations [90]. Moreover, the observed broadband at lower pH is due to the formation of larger-sized Silver nanoparticles [91]. Lower pH suppresses nanoparticles formation and

higher pH enhances the nanoparticles synthesis process. While a relatively narrow peak and maximum absorbance at higher pH that occurred is due to the formation of well dispersed and small-sized Silver nanoparticles [92].

The higher absorbance and relatively narrow band were formed around 400 nm at higher pH of 8.1 and 9.3. Similarly, Literature showed that pH plays an important role in size and shape control synthesis process of Silver nanoparticles as basic environment increases the nucleophilic nature of plant extract solution, as a result, facilitated reduction Silver Nitrate solution to Silver ion. So the synthesized Silver nanoparticles are also in agreement with earlier reports that the addition of an alkaline ion in the reaction mixture is necessary to carry out the reduction of metal ions [93].

4.7 Antimicrobial activity of silver nanoparticles

In recent times, due to the advancement of nanotechnology Silver nanoparticles are of great interest because of its better antibacterial activities [94]. The highest antimicrobial activity of nanoparticles comes from the ability to be attached to the cell membrane surface of pathogens. Thus by disturbing their permeability and cause structural changes in bacteria and fungi. So eventually leads to cell death [95, 96]. Silver nanoparticles penetrate inside the bacteria and cause damage by interacting with the electron Phosphorous and Sulfur-containing compounds such as DNA and proteins, resulting in cell death [97]. Small size, spherical shape and high surface area to volume ratio to interact with cell walls of pathogens give them better antimicrobial activity for Silver nanoparticles.

In this study, gram-negative bacteria showed more susceptibility towards Silver nanoparticles when compared to gram-positive bacteria due to lack of thick layers of peptidoglycans structure. The penetration of Silver nanoparticles through the cell membrane of gram-positive bacteria is not that much easy when compared to gram-negative bacteria. The antimicrobial activity of synthesized Silver nanoparticles against harmful pathogenic bacterial species at different concentrations levels of (1mg/mL, 3mg/mL, 5mg/mL) and seed extract at 10 mg/mL were done to evaluate dose effects on those bacterial species. As (table 3) above shows that upon increasing the

concentration of silver nanoparticles, the zone of inhibition was increased. So the zone of inhibition was directly proportional to the concentration of silver nanoparticles.

Table 3: Summary of antibacterial activity assay.

Inhibition zone diameter (mm)							Positive control (mm)
Bacterial strains	$AgNO_3$ (0.01M)	*Pers. Laphi* 10 mg/mL	Ag-Per (1) 2mg/mL	Ag-Per (2) 3mg/mL	Ag-Per (3) 5mg/mL	DMSO	Ciproflo-xacin
E.coli	14 ± 0.11	12.5 ± 0.12	14 ± 0.41	18 ± 0.38	24.5 ± 0.6	–	32.33 ± 0.46
Bacillus subtilis	12.3 ± 0.1	7.5 ± 0.43	8.3 ± 0.5	11.1± 0.29	12.3 ± 0.1	–	21.88 ± 0.28
St. aures	8.74 ± 0.22	12.5 ± 0.34	13.5 ± 0.1	14.4 ± 0.6	20.5 ± 0.9	–	25.17 ± 0.35
Salmonella	6 ± 0.49	–	–	8.3 ± 0.3	10.7 ± 0.2	–	31.2 ± 0.32

The present study also clearly revealed that the Silver nanoparticles in size 12–24 nm with spherical shape have shown high potential as an antimicrobial agent. The antibacterial activity of the synthesized silver nanoparticles has been investigated against gram-positive bacterial strains of *Bacillus, Staphylococcus aureus* and gram-negative strains of *Escherichia coli, Salmonella typhi*. The obtained result in this study showed improved antimicrobial activity than the Spherical-shaped Silver nanoparticles of 20–60 nm in size that has 13 mm ZOI against *Escherichia coli*. Moreover, the obtained result was comparable with spherical shaped Silver nanoparticles of 20–35 nm in size that has 19 mm ZOI against *Escherichia coli* [98]. From this green approach of Silver nanoparticles synthesis, the obtained nanoparticles of 18.9 nm average particle size have

revealed highest antimicrobial activity against *Escherichia coli* than the rest of bacterial strain and have shown 12.5-24.5 mm ZOI at different concentration levels. The bio assay summarized in table 3 above. The observed zones of inhibitions at 5mg/mL concentration level were 24.5, 20.5, 12.3 and 10.7 mm for *Escherichia coli, Staphylococcus aureus, Bacillus, and Salmonella typhi* respectively.

Figure 11: Antibacterial assay: Zone of inhibition seen well around Green Synthesized Silver Nanoparticles A) *E.coli B) Bacillus C) St. aures D) Salmonella* and *Ciprofloxacin* controls at the center of each Petriplates.

5. CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

A green method for the synthesis of Silver nanoparticles using seed extracts of *Persicaria lapathiofolia* has been demonstrated. The metal nanoparticles were characterized by UV-Vis, FT-IR, XRD, and TEM images. Synthesis of Silver nanoparticles can be affected by different parameters like pH, concentration, and time. Regarding the pH effect on the synthesis of Silver nanoparticles, increasing in pH of nanoparticles solution leads to rapid color change and formation of nanoparticles. At optimum concentration levels, the formation and change in color of nanoparticles solution were observed from yellow to deep blue after 24 h. That was indicated the formation of silver nanoparticles. Crystalline nature of the nanoparticles is evident from sharp peaks in the XRD pattern having average particles size of 18.9 nm. The FT-IR spectroscopy revealed different secondary phytochemicals like flavonoids, alkaloids, and others were around the synthesized Silver nanoparticles. The TEM images showed well dispersed nanoparticles having a size of 24.1 nm with a spherical shape having small agglomerates coming from the high surface area. Generally, the synthesized Silver nanoparticles of 18.9 nm have shown better antimicrobial activities than the crude seed extracts of *Persicaria laphtifolia.*

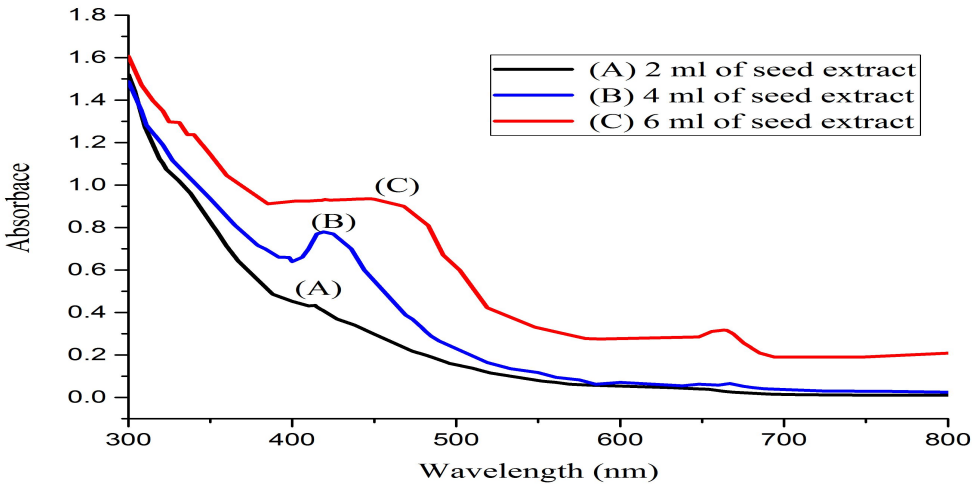
5.2 **RECOMMENDATION**

Using green synthesis methods, silver nanoparticles synthesized, characterized and evaluated the antimicrobial activities. So the recommendation to future can be indicated as follows:

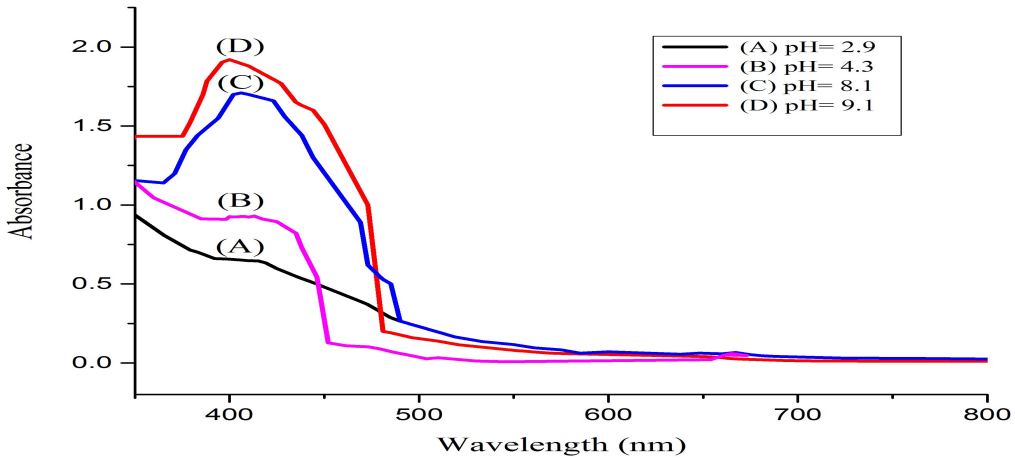
- This green approach of the nanoparticles synthesis has to be well adopted since it is simple, less expensive, rapid, and moreover its environmentally friendly.
- The synthesized silver nanoparticles could be tested in vivo for large scale production at industrial levels due to its promising results.
- As a whole part of *Persicaria laphatiofolia* has medicinal activities other researchers in this area may synthesize Silver nanoparticles from the root or leaf or other parts of the plants.
- As seed extracts of *Persicaria lapatiofolia* are rich in secondary phytochemicals like flavonoid and alkaloids. The researcher highly recommends that further work could be done to know which secondary metabolites are highly contributed to the formation of Silver nanoparticles by comparing with isolated compounds from crude extracts.
- Additional characterization techniques like TEM-EDX and XPS must be utilized to know the surface composition of the synthesized nanoparticles and more about the surface of nanoparticles.
- Generally, as a crude extracts of *Persicaria lapatiofolia* have been used by local healers it is worth to increase the medicinal values of the plant by using as a precursor for nanoparticles synthesis. Since the joint effect will increase the medicinal values of plant extract.

6. APPENDIX

6.1 Appendix Figures



Appendix Figure 1: UV – Vis absorption spectra of the as-synthesized Silver nanoparticles at different plant extract concentration a) 2 ml of plant extract b) 4 ml of seed extract c) 6 ml of seed extract



Appendix Figure 2: Effect of different pH levels on the synthesis of Silver nanoparticles shows that maximum synthesis occurred at high pH.

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