CHARACTERIZATION AND ANTIMICROBIAL STUDY OF SILVER NANOPARTICLES PREPARED USING *ARTEMISIA VULGARIS* LEAF EXTRACT

A DISSERTATION WORK SUBMITTED FOR THE PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE MASTER OF SCIENCE DEGREE IN CHEMISTRY

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BOARD OF EXAMINER AND CERTIFICATE OF APPROVAL

This dissertation entitled, **"Characterization and antimicrobial study of silver nanoparticles prepared using *Artemisia vulgaris* leaf extract"** by Sandhya Parajuli under the supervision of Assoc. Prof. Dr. Puspa Lal Homagai, Department of Chemistry, Amrit Campus, Tribhuvan University, Kathmandu, Nepal, and co-supervision of Asst. Prof. Dr. Deval Prasad Bhattarai, Department of Chemistry, Amrit Campus, Tribhuvan University, Kathmandu, Nepal, hereby submitted has been approved for partial fulfillment of the requirement for completion of her Master of Science (M.Sc.) Degree in Chemistry. This dissertation has not been submitted to any other university or institution previously for the award of a degree.

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RECOMMENDATION LETTER

This is to certify that the dissertation entitled **"Characterization and antimicrobial study of silver nanoparticles prepared using *Artemisia vulgaris* leaf extract"** has been carried out by Ms. Sandhya Parajuli as a partial fulfillment for the requirement of M.Sc. Degree in Chemistry under my supervision and guidance. The work presented herein is genuine and performed originally by her and has not been submitted elsewhere for any other degree. She has performed this research work sincerely and satisfactorily. I, therefore, recommend this dissertation for approval and acceptance.

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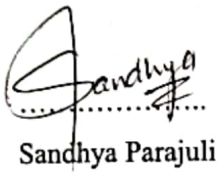
DECLARATION

I, Sandhya Parajuli, hereby declare that this dissertation entitled **"Characterization and antimicrobial study of silver nanoparticles prepared using *Artemisia vulgaris* leaf extract"** being submitted to the Department of Chemistry, Amrit Campus, Institute of Science and Technology (IoST), Tribhuvan University (T.U.), Nepal for the partial fulfillment of the requirement in Master of Science (M.Sc.) Degree in Chemistry is carried out by me under the supervision of Associate Professor Dr. Puspa Lal Homagai. This work is genuine and originally performed by me. It has not been submitted elsewhere for any other degree program. Any literature, data or works done by other researchers are duly cited and acknowledged.



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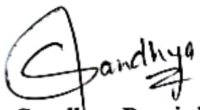

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ABSTRACT

Synthesis of nanoparticles by green method is most convenient approach. Herein, silver nanoparticles of different ratio (1:3 and 1:5) were synthesized using the leaves extract of *Artemisia vulgaris*. The Leaves extract was characterized by qualitative chemical tests and spectroscopic measurements. Bioactive compounds which acted as reducing, capping, and stabilizing agents were characterized by qualitative chemical tests and spectroscopic measurements. Formation of silver nanopartilces was monitored by UV-visible spectroscopic measurement followed by characterization using FTIR, XRD, SEM and EDX. FTIR reflected the possible functional groups associated for the formation of nanoparticles. XRD pattern revealed the crystalline nature of nanoparticles. From the interpretation of powder form diffraction peaks of XRD, the average size of as synthesized nanoparticle was found as 28 nm. EDX revealed that the nanoparticles are mostly made of silver, with a little carbon and oxygen, where content of Ag was 91.31 % by mass % in 1:5 AgNPs. Similarly, FE-SEM results showed that as-synthesized products were in nanosize dimension. Antimicrobial acitivity of synthesized nanoparticles against Gram positive bacteria (*Bacillus subtilis*), Gram negative bacteria (*Escherichia coli*), and fungus (*Candida albicans*) was studied. These particles showed good zone of inhibition indicating their good potential to control the bacterial and fungal growth.

Keywords: *Artemisia vulgaris*; leaf extract, green chemistry; AgNPs; antibacterial activity; antifungal activity;

शोधसार

हरित बिधिद्वारा न्यानो कणहरु बनाउने बिधि अत्यन्त उपयोगी उपागम हो। यो अनुसन्धान कार्यमा विभिन्न अनुपातका चादी न्यानो कणहरुलाई कुनै बाहिरी घटाउने वा क्यापिङ्ग एजेन्टको अतिरिक्त प्रयोग बिना तितेपातीको पातहरुको निकासीबाट हरित बिधिद्वारा सश्लेषण र जाँच गरिएको छ । संश्लेषित न्यानो कणहरुको प्रभावकारी ढङ्गले युवी- भिसिवल स्पेस्ट्रोस्कोपी, एफ.टि.आई.आर., एक्स.आर.डि., एस.ई.एम. र ई.डि.एक्स.द्वारा बिशेषता जाँच गरियो । चादी न्यानो कणहरुको युवी-भिसिवल अवशोषण प्रोफाइल ४३० न्यानो मिटरमा शिखर देखियो, जुन चादी न्यानोकणहरुको एक बढ्दो संख्याको गठन सङ मेल खान्छ । एक्स.आर.डि.ढाचाले न्यानो कणहरुको क्रीस्टलीय प्रकृति प्रकट गय ो। एक्स. आर. डि. अध्ययन अनुसार संश्लेषित न्यानो कणको औसत आकार २८ न्यानो मिटर आयामको रुपमा फेला पर्यो जुन एफ.ई.एस.ई.एम. अध्ययनले प्रदशन गरेको सङ राम्रो सम्झौतामा छ । ई.डि.एक्स. अध्ययनका अनुसार संश्लेषित न्यानो कणहरुमा प्रचुर मात्रामा चादी र केही मात्रामा कार्बन र अक्सिजन पनि देखिएको छ । हरित संश्लेषित न्यानो कणहरुले ग्राम सकारात्मक जीवाणु (*Bacillus subtilis*), ग्राम नकारात्मक जीवाणु (*Escherichia coli*) र त्यस संगै फङ्गल (*Candida albicans*) को बिरुद्दमा प्रभावी रोगाणुरोधी गुण प्रदशिर्त गर्यो । तितेपातीको निकासि बिभिन्न औषधि र बायोमिडिकल अनुप्रयोगहरुको लागि बाहुमुल्य छ, जस्ले निस्सन्देह चिकित्सा क्षेत्रमा यसको व्यावसायिक व्यवहार्यता स्थापित गर्नेछ ।

शब्द कुन्जिका: तितेपाती; पात निकासी; हरित संश्लेषण; चादि न्यानोकणहरु; जीवाणुरोधी गतिबिधि; एन्टिफङ्गल गतिबिधि

LIST OF ABBREVIATIONS

µL:	Microliter
Ag:	Silver
AgNPs:	Silver Nanoparticles
AVLE:	*Artemisia vulgaris* leaf extract
EDX:	Energy Dispersive X-ray Spectroscopy
FE-SEM:	Field Emission Scanning Electron Microscopy
FTIR:	Fourier Transform Infrared Spectroscopy
JCPDS:	Joint Committee On Powder Diffraction Standards
mm:	Millimeter
~~Nm~~: nm	Nanometer
NPs:	Nanoparticles
UV:	Ultra Violet
XRD:	X-ray Diffraction
ZOI:	Zone of Inhibition

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CHAPTER 1

INTRODUCTION

1.1.Background

In recent research scenario, the prefix 'nano' is found to be used as keywords in each and every stream. Actually the term 'nano' is derived from the Greek word 'nanos' or Latin word 'nanus' which means "dwarf" (Kolahalam et al., 2019). A nanometer is roughly about the length of 10 hydrogen atoms or 5 aligned silicon atoms (Goyal, 2017). Those materials which have at least one of their dimensions in nanoscopic range (1-100 nm) are called nanomaterial. Nanomaterials could be nanoparticles, nanorods, nanotubes, etc. Hence, all nanoparticles are nanomaterials but all nanomaterials are not nanoparticels (Neupane, 2018). The nanoparticles have a smaller size and large surface to volume ratio, thus these substances exhibited new, un-parallel, preferable, and remarkable noble physical and chemical properties, and have larger surface area (Aritonang et al., 2019). Due to the unique qualities and properties of NPs when compared to large particles such as form, size, and distribution, Nanoparticle synthesis and characterization have exploded its popularity in recent decades and these materials can be used in verities of fields including the environment, optics, foods, sensor, chemical industries, healthcare, drugs delivery, computer science and information technology, cancer therapy, etc. (Roy, 2017; Song & Kim, 2009).

Nanotechnology is the science which deals with the study of nano-sized materials by controlling their shape and size. It alters matter at the atomic and the molecular levels while reducing materials at the nanoscale range to increase functionality (Tahvilian et al., 2019). The nanotechnology getting attention in almost all engineering branches and should have good knowledge of physics, chemistry, materials science, solid state and biosciences in order to understand nanotechnology (Kolahalam et al., 2019).

Although nanotechnology has just recently attracted attention, it has long been known to have been in use. The nanomaterials and their formation procedure in nanoscale have been know from dates back to ancient time (Tolochko, 2009). However, the credit of nanoscience goes to a physicist, Richard Feynman for establishing and generalizing of this field (Feynman, 1960). Along with this, equal credit goes to Prof. Norio Taniguchi for his contribution in the field of nanoscience (Goyal, 2017).

Various discoveries regarding nanomaterial like carbon nanotubes, fullerenes, and quantum dots with unique mechanical, thermal, and electrical properties have been reported in that era of nanotechnology (Iijima, 1991; Kroto et al., 1985). Hereafter, Nanotechnology became one of the latest scientific progresses leading to developing a new form of material fabrication by controlling and manipulating material structure on a nanoscale (Ebrahiminezhad et al., 2016). In future, mankind activities such as living, working, and communicating fields will be controlled by nanotechnology. So this field is now become mature and multidisciplinary with varieties of applications in multidimensional fields such as electronics, medicines, energy, and material science.

1.2. Classification of Nanomaterials

1.2.1. Classification on the basis of dimensions

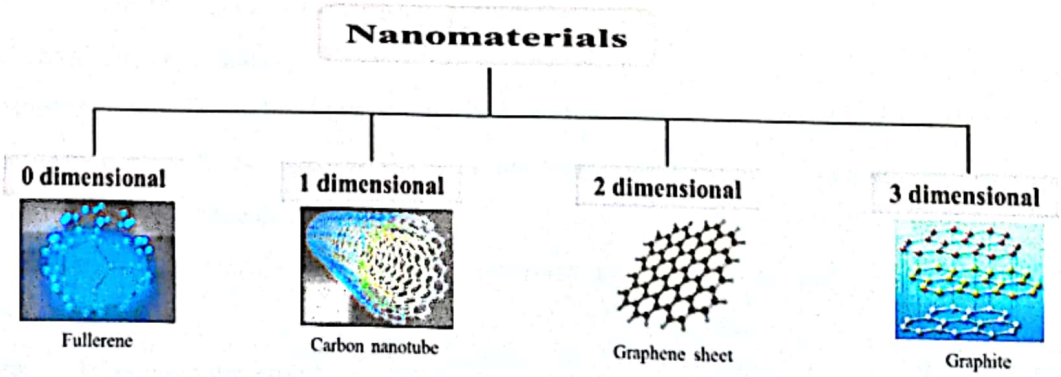
On the basis of dimensions, nanomaterials can be classified into four distinct classes (Vollath, 2008).

I. **Zero dimension nanomaterials (0-D):-** All the three dimensions of the nanomaterials are in the nanoscale range. Nanoparticles will come into this classification.

II. **One dimension nanomaterials (1-D):-** In this, any one dimension will be in nanoscale range and remaining two dimensions are out of the nanoscale range. Nanorods or Nanotubes or Nanowires are related to this class.

III. **Two dimension nanomaterials (2-D):-** Any two dimensions are in nanoscale range and remaining one dimensions is out of it. These include Nanofilms, Nanolayers, and Nanocoatings.

IV. **Three dimension nanomaterials (3-D):-** In any dimensions, these nanomaterials are not in nanoscale range. These includes nanocomposites, core shells, multi nanolayers, bundles of nanowires, bundles of nanotubes.



Scheme 1: Classification of nanomaterials on the basis of dimensions.

1.2.2. Classification on the basis of composition

On the basis of composition, their size, and properties, nanomaterials can be classified in following categories.

I. **Carbon-based nanomaterials**: The main constituent for this type of nanomaterials is the carbon. Carbon nanotubes, fullerenes, and graphene are related to this type of nanomaterials. They have unique electrical, thermal, and mechanical properties, making them suitable for various application (Iijima, 1991; Vollath, 2008).

II. **Metal-based nanomaterials:** Metal ions with divalent and trivalent cations serve as the precursors for metal nanomaterials. The metal ions are converted to metal nanoparticles using reducing agents. This includes nanoparticles of metals such as Au, Ag, Pt, and metal oxide such as ZnO, TiO_2. They can be used in multidisciplinary areas such as catalysis, sensing, and biomedical engineering (Babu & Antony, 2019; Mirkin et al., 1996).

III. **Semiconductor nanomaterials:** They have both metallic and non-metallic properties. They exhibit wide band gaps by modifying it shows different properties. These are widely used in photocatalysis, solar cells, and electronic devices (Kolahalam et al., 2019).

IV. **Polymeric nanomaterials:** These include dendrimers, polymersomes, and polymeric nanoparticles. They are used in areas such as drug delivery, imaging, and tissue engineering (Tomalia et al., 2007).

V. **Biological nanomaterials:** These include viruses, liposomes, and exosomes. They are used in areas such as drug delivery, gene therapy, and vaccines (Johnstone et al., 1987).

1.3. Synthesis approach

Nanoparticle synthesis has been extensively explored utilizing chemical and physical methods, but the development of dependable nanoparticles production technology is a crucial feature of nanotechnology.

There are two synthesis approaches categorized as 'top- down' and 'bottom- up' approaches.

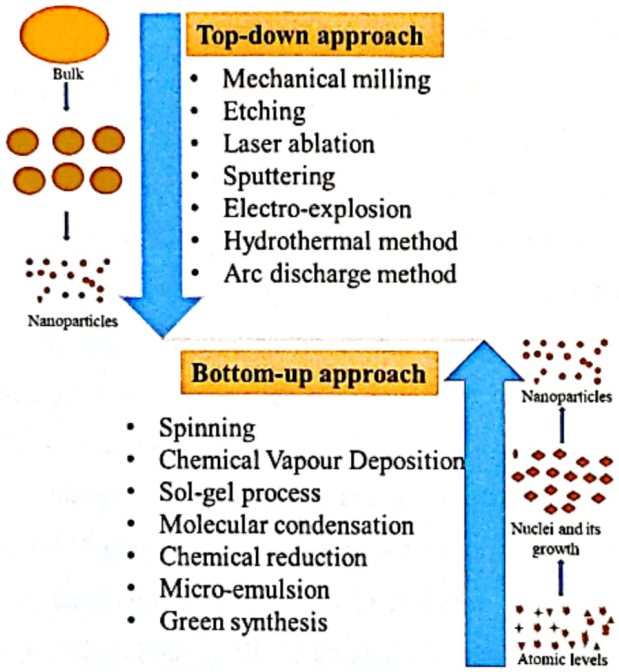
I. **Top-down approach**

The larger materials are broken down into tiny pieces using mechanical and chemical methods in the "top-down" method. The biggest drawback of this method is how challenging it is to achieve the correct particle size and shape

(Thakore et al., 2014). Mechanical alloying, high pressure torsion, nanolithography, and other synthesis methods are examples of different sorts of approaches (Kanchi & Ahmed, 2018).

II. Bottom-Up approach

The larger materials are produced by the chemical fusion of the smaller ions when nanoparticles are condensed in either the liquid or gaseous phase in the "bottom-up" method (Kolahalam et al., 2019). The appropriate nanosized materials are produced using this method from atoms, molecules, clusters, or smaller particles. By including a capping agent, it creates homogeneous, stable nanoparticles that can be altered in terms of size, shape, and content (Kanchi & Ahmed, 2018).



Scheme 2: Synthesis approach of nanomaterials

There are various methods such as co-precipitation, hydrothermal, sol-gel, chemical vapor deposition, wet impregnation, thermal decomposition, complex directed hybridization, direct heating, electrospinning, microwave synthesis, biosynthesis, etc. for the synthesis of nanomaterials (Das & Srivastava, 2018). However, they are limited due to high cost, toxicity, and accessibility. In this context, green method of nanomaterial synthesis can be the best alternative approach.

1.4. Green synthesis of Nanoparticles

A green chemistry method is the bottom up approach to synthesize nanomaterials by utilizing plant extract, microorganisms, biological templates, etc. as capping or stabilizing agents (Daphne et al., 2018). The main theme of green chemistry is to find new ways to create the best chemical products and methodology by obeying the principle of green chemistry by utilizing low cost, non- hazardous, renewable, and easily available materials as raw materials (Anastas & Warner, 1998).

Nanoparticle biosynthesis is an efficient, one-step method of bio-reduction. The process by which plant metabolites reduce metal ions to metal atoms is known as bio-reduction. A large scale manufacturing with minimal contamination has resulted from the synthesis of nanomaterials employing a variety of sources, including bacteria, fungi, algae, biological template, and plant extract, due to the growing popularity of green technology (Ahmad et al., 2019). Among these, synthesis of nanomaterials using bacteria, fungi, algae, and biological templates are limited because of their super specificity towards nanoparticles. So, in the broad-spectrum synthesis of nanomaterials plant extracts are highly recommended. Plants are regarded as highly desirable system for nanoparticles range of bioactive secondary metabolites with profound reducing potential (Pandey et al., 2013). The biosynthesis of nanoparticles has received a lot of interest since it is straightforward, inexpensive, and non-toxic, producing more stable metal nanoparticles and demonstrating the superiority of plant extract as a material (Zheng et al., 2015). And a notable advantage is that no separate reducing, capping, stabilizing agents are to be added in the whole synthesis process i.e. plant extract itself functions as all (Khan et al., 2018; Marslin et al., 2018).

1.5. Antimicrobial activity

A substance or agent has antimicrobial activity when it has the capacity to prevent the growth or eradicate microorganisms including bacteria, viruses, fungus, and parasites. The prevention and treatment of infectious illnesses, as well as a number of industries like healthcare, agriculture, and food preservation, all rely heavily on it (Varaldo et al., 2020). Depending on their intended function, they can be categorized into different groups, such as anti-biotic, anti-viral, anti-fungal, anti-parasitic, etc. (Abushaheen et al., 2020; Spellberg et al., 2008). The microbes that harm the host should be poisoned by these anti-microbial agents, but not the host itself. Herbal plants have been used long to treat ailments in human history. The growth of the

pharmaceutical industry has eliminated numerous human-devastating diseases. As a result, concerns about illness and human health have always exists each new technological creation. Many medications that operate as anti-microbial agents have been used for many years, however due to excessive usage of certain drugs, certain bacteria and fungi have developed resistance to them. Due to the development of drug-resistant microbes and the demand for other methods to treat infectious illnesses, the hunt for effective antimicrobial drugs has assumed growing importance (Varaldo et al., 2020).

AgNPs have pronounced antibacterial properties due to their larger surface area, thus providing better interaction with microbial pathogens. Hence, researchers have performed various studies regarding antimicrobial activities of silver nanoparticles (Alomari, 2020; Rasheed et al., 2017; Shanmuganathan et al., 2018, 2018; Soon et al., 2020).

1.6. Plant description

A medicinal plant *Artemisia vulgaris* L. (commonly known as Titepati in Nepal) belongs to the family asteraceae is a rhizomatous perennial weed that infests the landscape, agronomic setting, waste area and road side (Weston et al., 2005). The genus *Artemisia* is native to Europe, Asia, South Africa, and the pacific islands and being naturalized in regions from temperate region of southern North America to the Himalayan mountains (3700 m), A.V. has not been reported only in Antarctica continent (Kadereit & Jeffrey, 2007).

1.6.1. Taxonomic classification

Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Order: Asterales
Family: Asteraceae
Genus: *Artemisia*
Species: *vulgaris*
Common Name: Mugworth
Nepali Name: Titepati

Figure 1.1: *Artemisia vulgaris* plant

1.6.2. Morphological Characteristics

Artemisia vulgaris is the herb with 70-150 cm long, highly branched shrub with many headed and creeping rhizomes but no rosette or runners. Although, some herbs are highly branched and short, others are un-branched and tall (2 m). The flowers are red, brown and yellowish in color and almost glabrous. *A. vulgaris* is characterized by having pinatissect or bipinatissectas leaves with lanceolate or oblong segments, soft, white silver dorsally (Abiri et al., 2018). *Artemisia vulgaris* has been utilised since antiquity for ethno pharmacological purposes such as antibacterial, antipyretic, anti-fertility, antitumor, and anti-malarial activities (Shanmuganathan et al., 2018).

1.7. Statement of problem

An unchecked use of antibiotics has led to an increase in both gram positive and gram negative bacterial strains. Lack of selectivity in the treatment, along with its slowness and high cost, made it hard for the average person to afford medical expenses. As a result, the silver nanoparticles' antibacterial capability may be useful in creating antibacterial medications to fight harmful pathogenic strains.

So in terms of eco-friendly convenience and cost effectiveness, it is more dependable to use natural plant sources such as *Artemisia vulgaris* leaf extract. Biological synthetic process offers a wide range of environmentally acceptable methodology, low cost and minimum time required, also helps to find the gaps.

1.8. Objectives of the study

1.8.1. General objectives

The general objective of this study is to carry out the green synthesis of silver nanoparticles using *Artemisia vulgaris* leaf extract and study of its antimicrobial activities.

1.8.2. Specific objectives

The specific objectives of the study can be figured out as follows;

- Preparation of methanol extracts and conduction of its phytochemicals screening.
- Green synthesis of AgNPs by using AVLE.
- Characterization of AgNPs.
- Examine the antimicrobial activities.

CHAPTER 2

LITERATURE REVIEW

Out of various NPs, many NPs such as ZnO, FeO, SiO_2, CeO_2, and TiO_2 are extensively used because of their favorable photocatalytic capabilities. Elemental metals of nano-metric dimensions, such as Ag, Au, Fe, Cu, Pt, Pd, Ni, and Co are widely employed for antibacterial, optical, catalytic, electrical, and sensing application, as well as doping agents. Conductors and semiconductors are composed of Au, Cu, Si, and Co nanowires (Ghosh et al., 2011; Goswami et al., 2017; Mittal et al., 2013; Quinn et al., 2005).

Due to the unique qualities and properties of NPs when compared to large particles such as form, size, and distribution (Roy, 2017), nanotechnology can be used in a variety of application including the environment, food, optics, healthcare, chemical industries, biosensor, medication delivery, cancer therapy, etc. Nano-biotechnology provides a critical technique for the development of a fair, nontoxic, and eco-friendly metal NPs production process that has the ability to reduce metals through metabolic discipline (Elangovan et al., 2015; Roy, 2017).

Silver NPs, among the various NPs that have been developed to date, have piqued the interest of researchers due to their mysterious properties, which include high electrical and thermal conductivity, surface enhanced Raman scattering, chemical stability, high catalytic activity and antimicrobial activity. Silver ion has the strength to Damage bacterial cell, i.e. When the silver ion enters the bacterial cell, the DNA molecule transforms into a intensify form and misses out its reproduction power leading to bacterial cell death (Priyadarshini et al., 2019). Because silver plays a unique role in antimicrobial, catalytic, and biological systems the synthesis of AgNPs has grown in importance as an antimicrobial agent against the ever increasing threat posed by antibiotic resistant microbes (Nadagouda et al., 2009).

Ostad et al., (2010), used a nanoparticle-based strategy and found that giving breast cancer cells and tamoxifen-resistant cancer cells a low dose of tamoxifen followed by AgNPs had a potential synergistic anticancer impact against both cancer cell lines. Similarly, the versatile silver embedded magnetic NPs were gracefully synthesized and successfully used for targeting breast-cancer cells and floating leukemia cells (Jun et al., 2010). When compared to antibiotics like Gentamycin, the produced NPs have

a number of antibacterial advantages. When clinical bacterial infections were exposed to AgNPs , it was discovered that the NPs disrupted cell replication by influencing membrane permeability and finally triggering cell death by degrading cell growth (Kasithevar et al., 2017).

In an earlier study, antibacterial activity of AgNPs made from *Vitennegundo* extract has been demonstrated against *E.coli* and *St. aureus* (Zargar et al., 2011). For *B. cereus*, *B. subtilis* and *S. aureus*, the zone of inhibition created by AgNPs developed with *Acorouscalamus* rhizome extract was measured to be 15, 17, and 16 mm respectively (Nakkala et al., 2014). Similarly, it was found that the AgNPs derived from papaya fruit exhibited notable antibacterial activity against *E. coli* and *P. aeruginosa* (Jain et al., 2009). Well diffusion technique was used to study the antibacterial activity of green synthesized AgNPs from *Solanum tuberosum* extract against gram positive and gram negative bacteria. This study showed the most antibacterial activity was observed against *E. coli* (28 mm) and *St. aureus* (18 mm) followed by *M. luteus* (15 mm) and *P.aeruginosa* (13 mm). The diameter of the zone of inhibition towards antibacterial activity, area less than 9 mm was considered as inactive, 9-12 mm partially active while 13-18 mm as active and 18 mm as very active (Tokuşoglu & Üunal, 2003).

Likewise, green synthesis of AgNPs using extract of medicinal plant *C. gigantea* has shown potential antimicrobial, antipyretic, cytotoxic, antioxidant, anticancer, larvacidial activity and anti-proliferative activities. The effectiveness of the nanomaterials created using C. gigantea can be employed in an economical and environmentally friendly way to ward off mosquitoes. The way by which silver nanoparticles kill larvae is that they readily pass through the cell membrane of the organisms, ultimately leading to cell death (Ajith et al., 2019).

In the another study done by Mathew et al., (2020), reported that the antibacterial activity evaluated for synthesized AgNPs showed that biosynthesized AgNPs from the flower extract have resistance against Gram 7 positive bacteria like *B. subtilis*. This type of nanoparticle biosynthesis is an environmentally acceptable, nontoxic approach. Salayová et al., (2021), reported green synthesis for the production of silver nanoparticles using five different aqueous plant extracts namely *Berberis vulgaris, Brassica nigra, Capsella bursa-pastoris, Lavandula angustifolia* and *Origanum vulgare* was investigated in this study. Extracts from these plants were successfully

characterized by UV-visible spectrometer, XRD, Zeta-potential, FT-IR, TEM, etc. The XRD analysis or investigation confirmed the presence of Ag^0 in the nanoparticles and interactions between the bioactive compounds of the plants and provided AgNPs were evident in the FTIR spectra. TEM indicated that the nanoparticles exhibited a bimodal size distribution, with the smaller particles being spherical and the larger having a truncated octahedron shape. In addition, the antimicrobial activity of the AgNPs was tested against five bacterial strains. All the synthesized NPs exhibited enhanced antimicrobial activity at a precursor concentration of 5 mM compared to the control substance, gentamicin sulphate, with the best results observed for AgNPs prepared with *B. nigra*and *L. angustifolia* extracts.

According to the study done by Alsammarraie et al., (2018), green synthesis of AgNPs using extract of Turmeric powder where plant biomaterials were used as a reducing as well as capping agent. After 24 hrs of reaction, yellow color of extract changed to dark brown-reddish due to reduction of silver ions to AgNPs. It was characterized using UV-Vis spectroscopy, FT-IR, TEM and EDS. UV-spectra showed maximum absorbance at 432 nm whereas TEM reveals that the shape of AgNPs was spherical in size with average particle size of 18±0.5 nm. EDS reveals strong signals of silver element. In this study, green synthesized AgNPs shows high and efficient antimicrobial activities against two food-borne pathogens (*Escherichia coli* and *Listeria monocytogens)*. TEM and SEM image reveals that there were significant shrinkage and damage of bacterial cell wall, and leakage or loss of bacterial intracellular contents. A significant reduction ($p<-0.05$) of bacterial counts just after 4 hrs of exposure was observed. These results indicate that green synthesized AgNPs can be utilized as an antimicrobial means to inhibit the growth of pathogenic bacteria for applications in agricultural and food industries.

Recently, green synthesis of AgNPs using extract of medicinal plant *A.vulgaris* has shown potential antimicrobial, antioxidant and anti-proliferative activities (Alomari, 2020; Rasheed et al., 2017; Soon et al., 2020). Thujone, caryophyllene oxide, α-thujone, 1, 8-cineole, trans-cryophyllene and linalool were investigated as major active in the essential oil of *A.vulgaris* (Yildirim et al., 2016). The essential oil from *A. vulgaris* is reported to be 90% effective in repelling *Aedesaegypti*, a mosquito strain that transmits dengue and yellow fever. Furthermore, gold NPs synthesized from *A. vulgaris* shows better activity than essential oil regarding damage of cells of

Aedes aegypti. Hence, this method is promoting better action against dengue fever vectors (Sundararajan & Kumari, 2017). It was found that the iron NPs synthesized in *A. vulgaris* extract were potentially useful for environmental remediation (Kouhbanani et al., 2018).

A. vulgaris leaves extract (AVLE) was used to synthesize AgNPs due to its reducing potential, without adding other capping agents. Green synthesized NPs with AVLE remarkable antibacterial effects against pathogenic bacteria such as *E. coli*, *St. aureus*, *P. aeruginosa*, *Klebsiella pneumonia* and *Haemophillus influenza*. In vitro antioxidant assays revealed that AgNPs have a cytotoxic effect on human epithelical cells and MCF-7 cells when tested using the MTT methods (Reddy et al., 2014).The studied on the antibacterial activities of AgNPs prepared by AVLE against five human pathogens namely *E. coli*, *St. aureus*, *P. aeruginosa*, *K. pneumonia* and *H. influenza* by disc diffusion method revealed that the AgNPs displayed significant inhibition activities against all the pathogens. The highest value of the inhibition zone was recorded against *St. aureus*(18±0.27 mm) and the lowest value was recorded against *V. cholera* (12±0.18 mm) by AgNPs (Rasheed et al., 2017).

According to the study done byAlomari, (2020), the AgNPs have been successfully synthesized by using *Artemisia vulgaris* leaf extract through green approach. Formation of AgNPs was characterized by using UV-visible, FT-IR, XRD, TEM, AFM and SEM analysis. The UV-visible spectroscopy revealed the characteristic peak of AgNPs at 430 nm and TEM revealed the average size of AgNPs from 2.90-200 nm. Thus formed AgNPs were successfully examined against the gram positive (*Staphylococcus aureus, Bacillus cereus)* and gram negative (*Escherichia coli, Pseudomonas aeruginosa)* bacteria and it was found that AgNPs shows excellent antibacterial activity against gram positive bacteria.

Study done by Soon et al., (2020), the AgNPs have been synthesized by *Artemesia vulgaris* and examined against wound bacteria. The characteristic peak of AgNPs was found at 427nm as revealed by UV-visible spectrometer. TEM showed the AgNPs displayed average size of 20-50 nm. This study revealed that the AgNPs using *Artemesia vulgaris* extract were more effective in inhibiting the growth of gram negative bacteria than gram positive ones.

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

3.1.1. Sample collection and study area

Artemisia vulgaris leaves were gathered from Sainamaina municipality ward No: 11, Rupandehi, Nepal in September, 2022. Laboratory of Amrit Campus is the research area for this study.. The leaf of *Artemisia vulgaris* has been used this study.

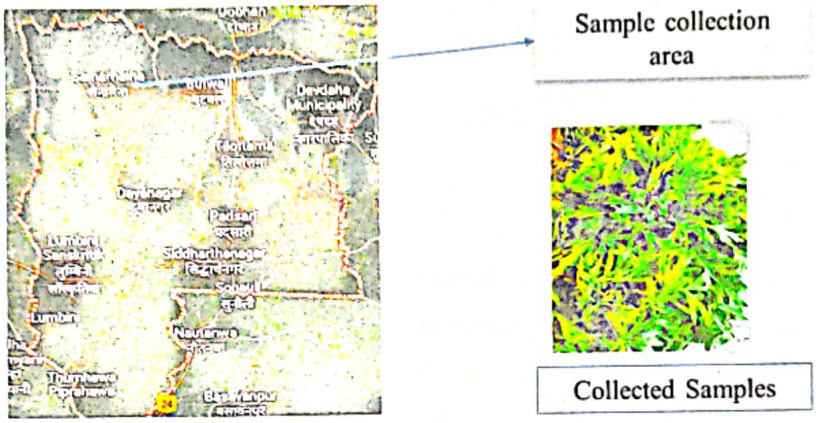


Figure 3.1: Sample collection area and collected samples.

3.1.2. Equipment/Instruments

a. Digital weighing balance
b. Magnetic stirrer
c. Volumetric glassware
d. UV Visible spectrometer (Labotonics, LT2802)
e. FTIR (PerkinElmer 10.6.2)
f. XRD (Rigaku diffractometer)
g. SEM and EDX (Hitachi, Tokyo, Japan)

3.1.3. Chemicals

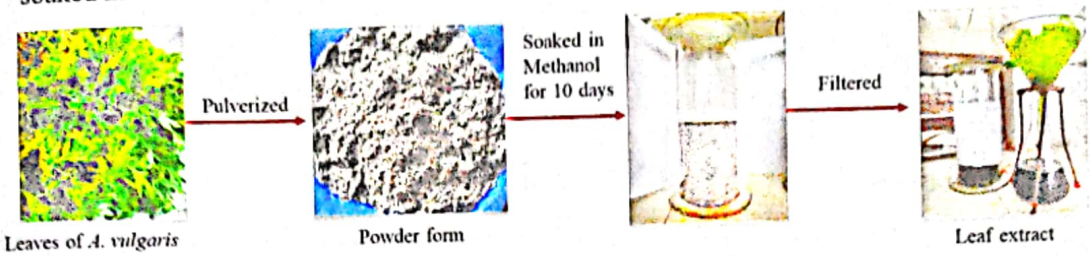
All the chemicals used for this study were of analytical grade and were used as received without further purification.

a. Methanol (99.8 %, EMPARTA®)
b. Silver Nitrate (99.8%, Qualigens)

3.2 Preparation of Extract and Chemical Analysis

3.2.1 Preparation of extract

Artemisia vulgaris leaves were washed with distilled water, dried in shade for 10-12 days, and then crushed to a fine powder using the herbal disintegrator. The leaf extract was prepared by cold percolation method where 100 g of leaf powder was soaked in 750 mL methanol for 10 days then filtered to obtain extract.



Scheme 3: Preparation of methanolic extract of*Artemisia vulgaris* leaves

3.2.2 Phytochemical analysis

Phytochemical analysis helps to determine the bioactive compounds present in the extract. Presence of the bioactive compounds in the prepared extract was confirmed by observing the color reaction using specific reagent for specific bioactive compounds. Standard protocol was followed to investigate the phytochemical constituents (Appendix table A_1).

3.3 Reagent Preparation

3.3.1 Preparation of $AgNO_3$ solution

8.2 g of silver nitrate was dissolved in 500 mL distilled water to prepare 0.01N Silver Nitrate solution.

3.3.2 Synthesis of AgNPs;

Silver nanoparticles were synthesized by adding 10mL and 6mL plant extract separately into the Beaker with 30mL 0.01 N $AgNO_3$ solution while stirring on magnetic stirrer at room temperature. After 45 minutes, color was changed to brown and thus formed precipitated product was filtered and washed with distilled water and ethanol and then it was dried. Finally pure AgNPs has been collected.

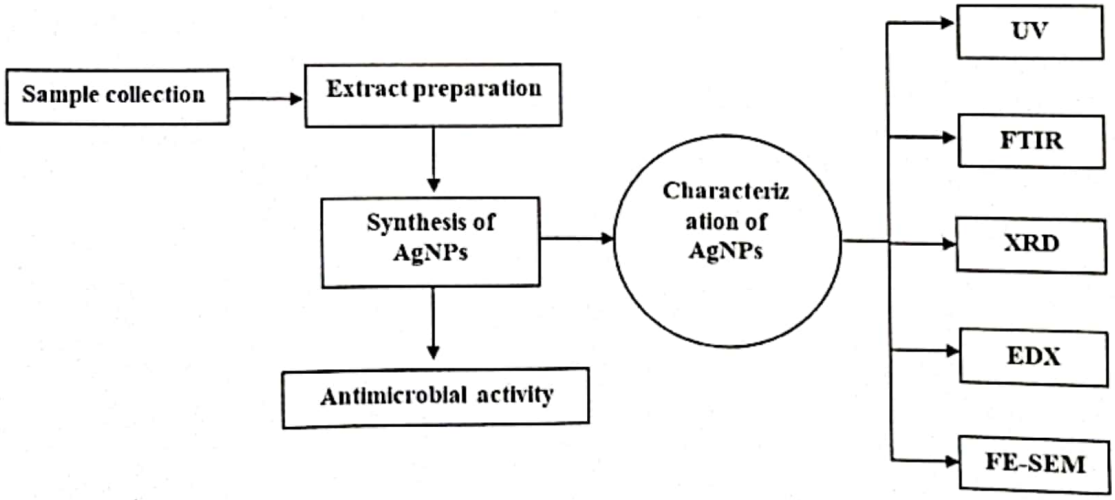
3.4 Physicochemical Characterization

Structural and morphological characterization were carried out using different instruments, such as UV spectroscopy, FT-IR and XRD. The phytochemical test was based on the chemical test method where visual changes were noted.

UV-visible spectroscopy was used to monitor as well as determine the formation of AgNPs. AgNPs dispersed in ethanol was taken in quartz cuvette and the absorbance due to surface Plasmon resonance of particle was recorded in the range of 350-460 nm at scan interval of 5 nm using UV- visible double beam spectrometer (Labotonics, model LT2802). It was carried out in the Department of Chemistry, Amrit Campus, Kathmandu.

Fourier Transform Infrared (FTIR) Spectroscopy was used to identify the functional group associated with the sample as a stabilizing and reducing agent. The FTIR spectra were recorded with an FTIR spectrometer (PerkinElmer 10.6.2) at the cut off range 400-4000 cm^{-1} with scan interval 4 cm^{-1}. It was also carried out in the Department of Chemistry, Amrit Campus, Kathmandu.

The crystallinity and crystal phase of the obtained materials were probed by an X-ray diffraction (XRD) instrument (Cu Kα (λ = 1.5406 Å) radiation on a Rigaku diffractometer, JNCASR, Bengaluru, India). The crystal structure of the synthesized AgNPs was examined by XRD. The surface morphology of as-synthesized material was studied using field emission scanning electron microscopy (FE-SEM, Hitachi, Tokyo, Japan) equipped with energy dispersive x-ray spectroscopy (EDX). The overall experiment was carried out based on the following framework.



Scheme 4: Systematic organization of the study work

3.4.1. Antimicrobial activity

Antibacterial and antifungal test was determined using Agar well diffusion methods for plant extract, 1:3 AgNPs, and 1:5 AgNPs. Inhibitions of bacterial and fungal growth were tested by zone of inhibition.

First, Agar surface was inoculated by spreading a volume of microbial inoculums over the entire agar surface; it was incubated in bacteriological incubator for 24 hrs at 37 °C. Next day, the agar plate was then separated with sign pen for different nanoparticles and filter paper was kept in the disc plate on four sides. 5 µL of standard kanamycin was loaded into respective sections with the help of micro pipette. Kanamycin was used as standard for gram positive (*Bacillus subtilis*), gram negative (*Escherichia coli*), and fungus (*Candida albicans*), respectively. Small pinch of nanoparticles was kept in respective sections of plate. Then kept in incubator for 15 minutes and each plate was then observed for the zone of inhibition (ZOI) produced by antibacterial and antifungal activity. ZOI was measured by the use of scale.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Phytochemical analysis

Phytochemical test of the methanol extract of *Artemisia vulgaris* leaves was carried out using standard procedure (Appendix table A_1) and the obtained results are tabulated in table

Table 4.1. Showing the results obtained by phytochemical test

Alkaloids	Absent
Flavonoids	Present
Saponins	Present
Terpenoids	Present
Quionens	Present
Polyphenols	Present
Proteins	Present
Glycosides	Present

Alkaloids were not detected in the leaf extract, according to the research, but bioactive substances including flavonoids, saponins, terpenoids, quinones, polyphenols, proteins, and glycosides present. For the creation of nanomaterials, these bioactive phyto-constituents were thought to function as stabilizing and reducing agents. The study by Thangjam et al., (2020) revealed that the leaves extract of Artemisia vulgaris included phytochemicals such flavonoids, triterpenoids, glycosides, polyphenols, Saponins, and proteins. The results and our findings are in strong agreement.

4.2. UV-Vis spectroscopy

Finding of UV-vis spectroscopic characterization are shown in figure 4.1. The UV-Vis spectra of produced AgNPs showed a distinct peak at 430 nm, indicating the finely dispersed AgNPs. In the similar experiment, AgNPs synthesis in aqueous solution was monitored by recording the absorption spectra at a wavelength range of 300-600 nm (Leela & Vivekanandan, 2008). The peak at 430 nm was discovered to be a characteristic of the AVLE variant metabolites and proteins, which play an important role in the reduction of silver ions into synthesized NPs. Our findings are in agreement with the result obtained by (N. Ahmad et al., 2011; Sagar& Ashok, 2012; Sinha et al., 2015).

Rasheed et al., (2017), reported that the peak was detected at 420 nm for the AgNPs synthesized of aqueous extract of *A. vulgaris* L. In another study, the synthesized AgNPs from AVLE have been reported at 427 nm (Soon et al., 2020). According to the study done by Alomari, (2020), prominent peak of AgNPs was observed at 431 nm. In the same way, the AgNPs developed from *Acorouscalamus* rhizome extract displayed broader peak around 400 nm (Nakkala et al., 2014). Similarly, several researchers have been reported that the peak of AgNPsappearsto be around this region (Thatoi et al., 2016; Zahir & Rahuman, 2012).

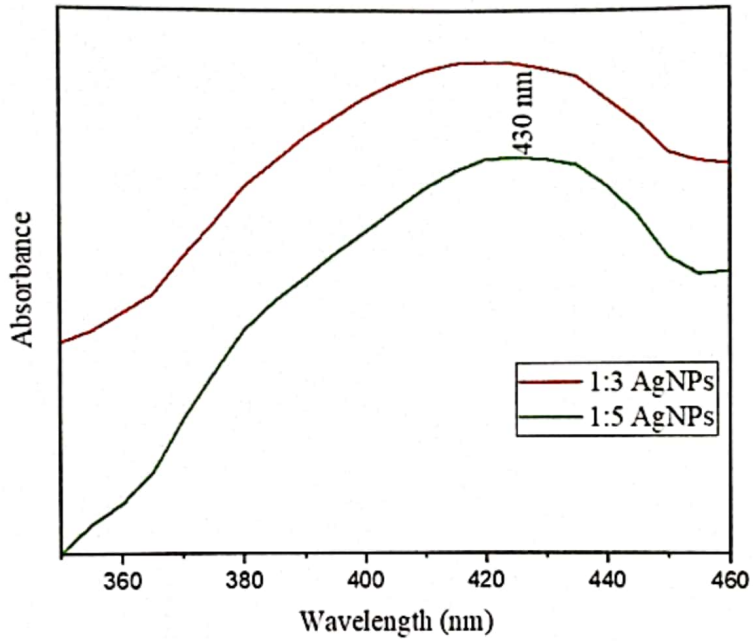


Figure 4.1: UV-Vis spectra of 1:3 AgNPs and 1:5 AgNPs .

4.3. FT-IR

The functional groups present in as-synthesized products were investigated using FTIR Spectroscopy. The FTIR spectra of plant extract and synthesized nanostructure were shown in figure 4.2. In the spectra of leaves extract presence of protein stabilizing agents was demonstrated by strong peaks such as the board spectrum at 3323 cm^{-1} was due to the O-H bond stretching. Similarly, the C–H stretching of alkane has resulted in a band at 2945 cm^{-1} and 2833 cm^{-1}. C=O stretching of carbonyl compounds are resulted in a band at 1656 cm^{-1}. The C–H bending of alkane gives rise

to band at 1449 cm^{-1} and 1413 cm^{-1}. An absorption peak at 1114 cm^{-1} was ascribed to C-N stretching, and band at 1022 cm^{-1} was attributed to ester and tertiary alcohol. These peaks assigned were carried out accordance to the Spectrometric identification of organic compounds (Silverstein & Bassler, 1962). The functionalities present in methanolic extract of leaves of *Artemisia vulgaris* were found to be well indexed with some previously published report (Karki et al., 2023). Furthermore, the stretching peaks at 400-500 cm^{-1}may corresponds to the formation of Ag confirms the production of AgNPs.

In another study done by Rasheed et al., (2017) have reported that the strong absorption peaks at 3419, 3151, 1619, 1400, 1069 cm^{-1}. The intense peaks at 3419, 3151 cm^{-1} in AV-AgNPs spectrum attributed the O-H stretching of the phenolic group. Similarly, the peaks at1 stretching of 1619, 1400, 1069 cm^{-1} designated the carbonyl stretching of –C=O, aromatic stretching of C-N and –C-O or –C-O-C, respectively which agreed well with previous reports (Nakkala et al., 2014; Poljansek & Krajnc, 2005).

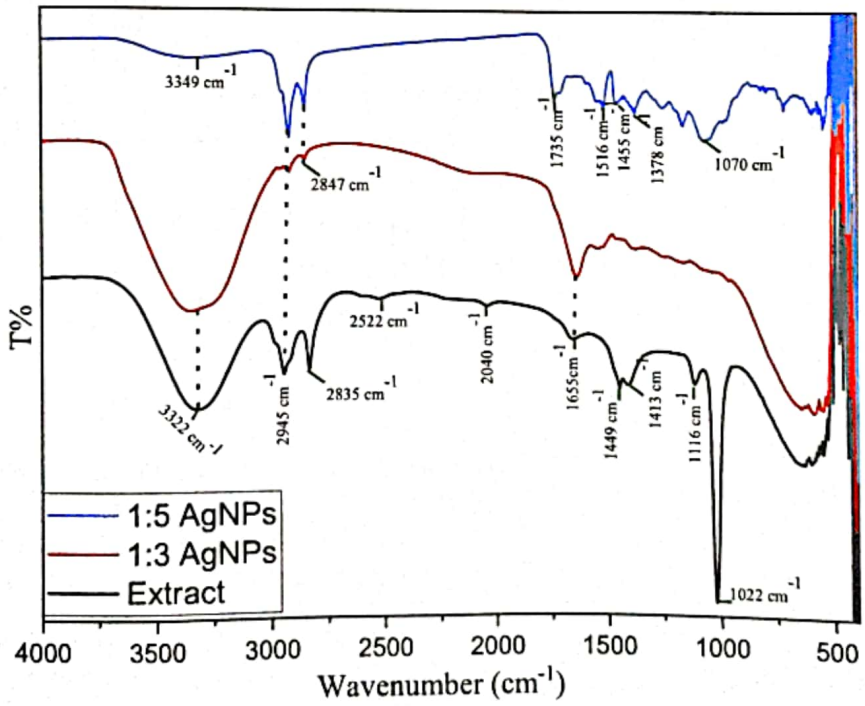


Figure 4.2: FT-IR spectra of *A. vulgaris* leaf extract only and green synthesized AgNPs.

4.4. XRD

The crystallite nature and size of the as-synthesized nanoparticles were carried out by using X-ray diffraction spectroscopy. The XRD patterns of the green synthesized AgNPs using *A. vulgaris* leaves extract is shown in figure 4.3. The XRD patterns of AgNPs were appeared at 2θ values of 27.78°, 32.35°, 38.09°, 44.53°, 54.78°, 57.53°, 64.45°, 67.37°, and 76.74°. The four distinct diffraction peaks at 2θ values of 38.09°, 44.53°, 64.45°, and 76.74° were well indexed to the (111), (200), (220), and (311) reflection plane of cubic structure of silver, respectively, with JCPDS card no: 90-13050, space group: Fm-3m (Suh et al., 1988).

Furthermore, along with these representatives peaks of silver, some additional peaks were also observed at 2θ values of 27.78°, 32.35°, 46.36°, 54.78°, 57.53°, and 67.37° were well matched to the peaks from the JCPDS card no: 76-1393 for silver oxide.

Presence of some of these peaks was due to the oxidation of silver during the long term storage before the characterization. Similar studies of XRD patterns for Silver nanoparticles have been reported in elsewhere (Rautela & Rani, 2019; Vanaja & Annadurai, 2013).

The average crystallite size of the green synthesized silver nanoparticles was calculated by using Debye-Scherrer formula,

$$D=\frac{K\lambda}{\beta\cos\theta}$$

Where,

D= Crystallite size of materials

λ= Wavelength of Cu Kα radiation (0.15406 nm)

θ= Bragg's angle

β = Corrected half width of the diffraction peak (in radian)

K= Shape factor which usually equals to 0.94

The average crystallite size of green synthesized AgNPs was found at around 28 nm.

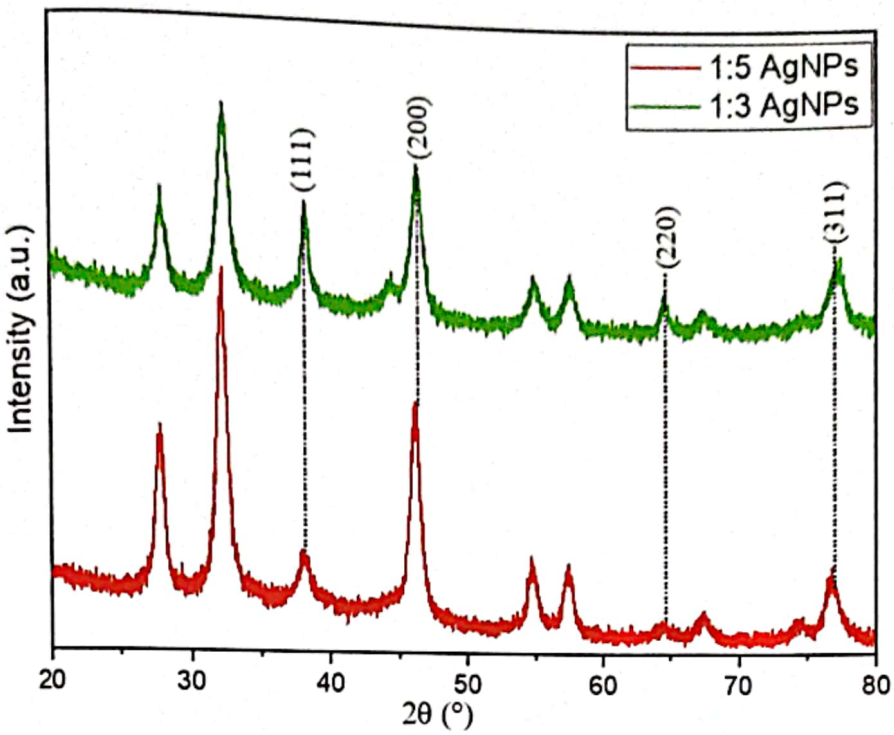


Figure 4.3: XRD patterns of green synthesized AgNPs.

4.5. FE-SEM and EDX analysis

Using field emission scanning electron microscopy (FE-SEM) coupled with energy dispersive X-rays (EDX) and elemental mapping, the surface morphology of as-produced silver nanoparticles was investigated. The morphology of silver's surface with various magnifications was shown in figure 4.4 and figure 4.5. The picture demonstrates the homogeneously generated, narrow size distribution of the silver nanoparticles. The formation of silver nanoparticles occurs at the nanoscale range. Plant metabolites have an important role in the synthesis and stabilization of AgNPs, as seen by the biomolecule coating of the biosynthesized AgNPs in the FE-SEM pictures. These findings concur with those reported in (Dakshayani et al., 2019; Mortazavi-Derazkola et al., 2020; Soon et al., 2020).

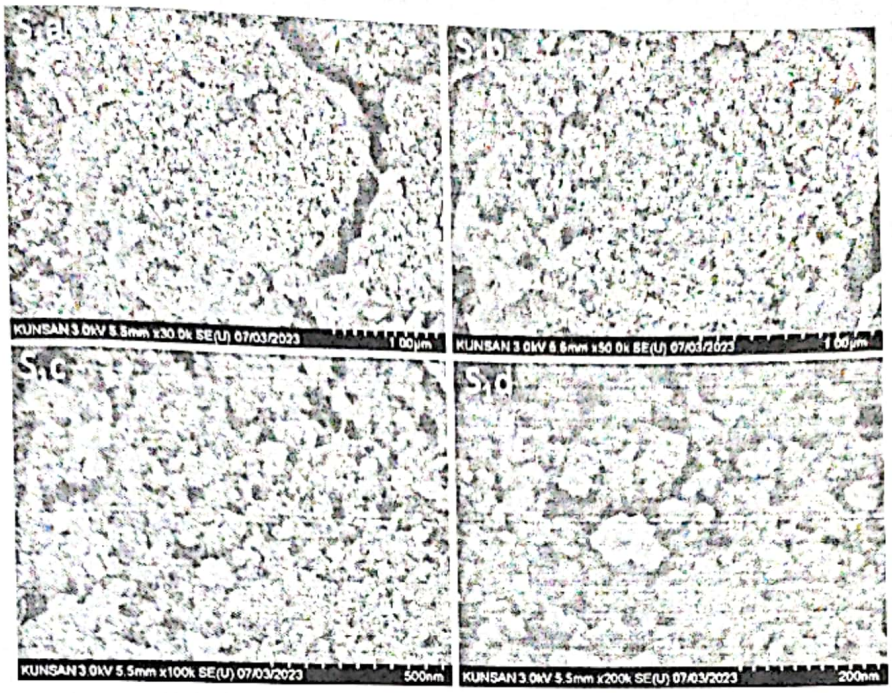


Figure 4.4: FE-SEM images of 1:3 AgNPs at different magnification. S_1a (×30k magnification in 1μm), S_1b (×50k magnification in 1μm), S_1c (×100k magnification in 500 nm), and S_1d (×200k magnification in 200 nm).

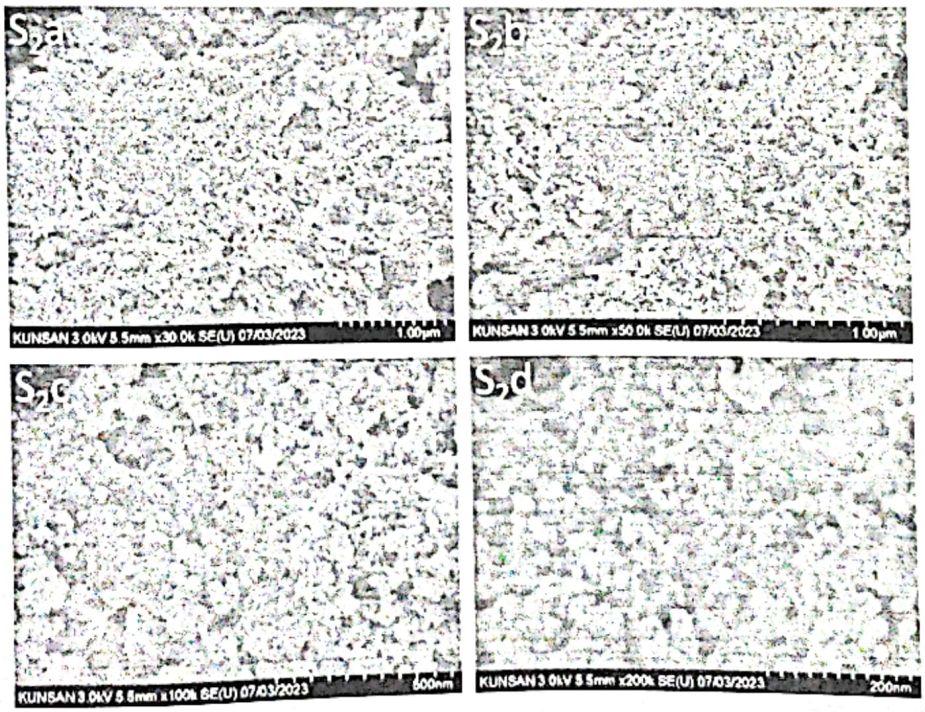


Figure 4.5: FE-SEM images of 1:5 AgNPs at different magnification. S_2a (×30k magnification in 1μm), S_2b (×50k magnification in 1μm), S_2c (×100k magnification in 500 nm), and S_2d (×200k magnification in 200 nm).

Additionally, energy dispersive X-ray with elemental mapping was carried out to guarantee the synthesized product, as seen in Figure 4.6. The outcome reveals that the nanoparticles are mostly made of silver, with a little carbon and oxygen. The methanolic extract of *Artemisia vulgaris* leaf was used to create the nanoparticle, and it also acted as a capping and stabilizing agent. Such organic molecules that are attached to a surface may include carbon and oxygen. Additionally, some of the aerial oxidation that silver may have experienced during proper storage, which contributed to the element's existence. Silver, however, makes up the bulk of the nanoparticle's composition.

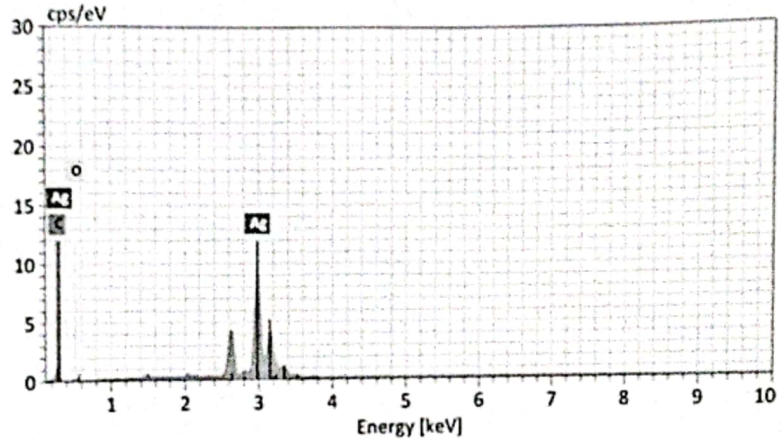


Figure 4.6. EDX spectra of 1:5 AgNPs.

Table No.4.5: Elemental composition of 1:5 AgNPs from EDX.

Element	At. No.	Mass [%]	Mass Norm. [%]	Atom [%]	abs. error [%] (3 sigma)
C	6	3.98	4.05	24.22	2.18
Ag	47	91.31	92.85	61.84	8.72
O	8	3.05	3.10	13.94	2.53
		98.35	**100.00**	**100.00**	

EDX spectral analysis demonstrated higher counts at 3 keV due to silver, thus confirming the development of silver nanoparticles. Observation of some other peaks such as peaks of carbon and oxygen in vicinity of silver major peaks corresponded to that C and O elements are characteristic of plant extract. Strongest signal of silver appeared in EDX spectra indicate the presence of large amount of silver nanoparticles

and weakest signals from carbon and oxygen were also recorded. The study was found to be consistent with literatures (Rasheed et al., 2017; Ahamed et al., 2011).

Figure 4.7 represented the elemental mapping of as synthesized product and revealed the presence of C, O, Ag and EDX layer mapping of different element in as synthesized product.

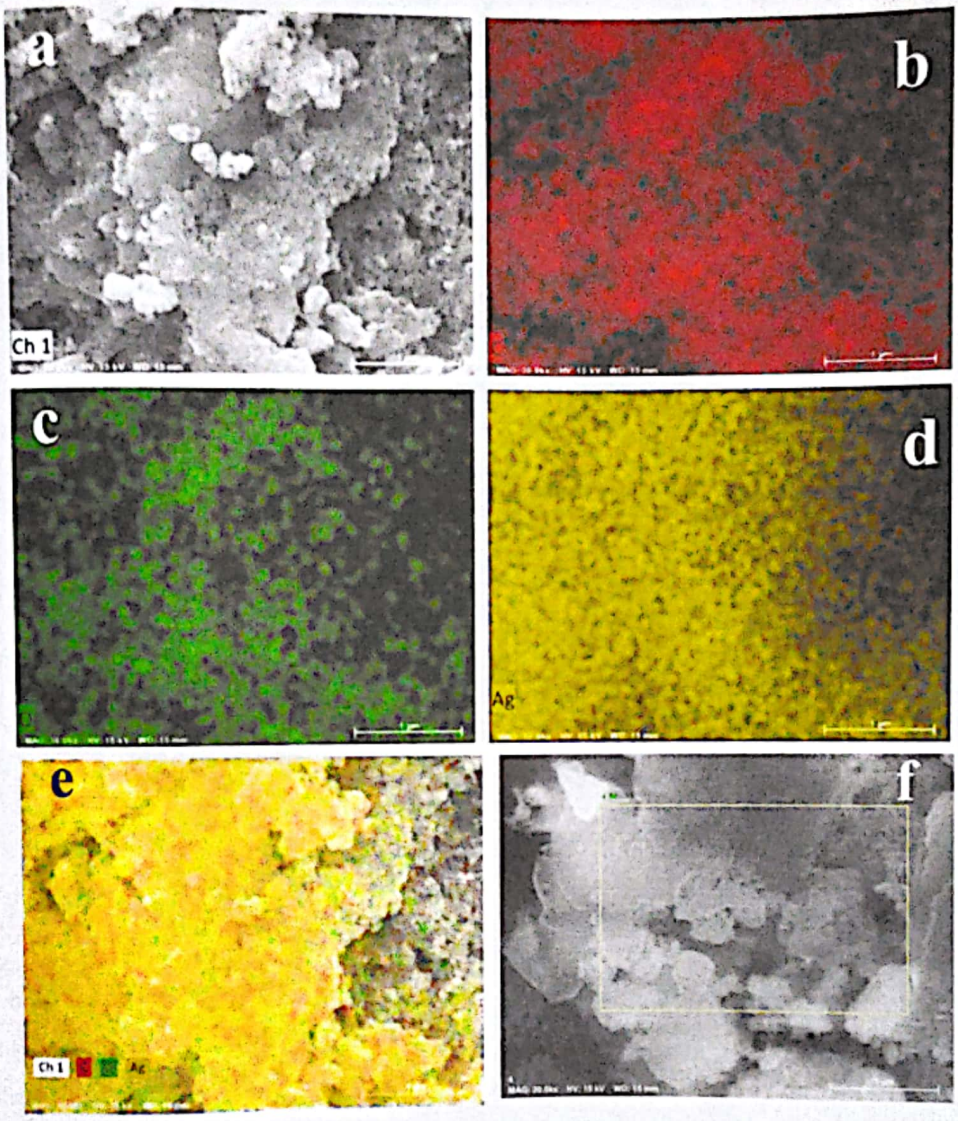


Figure 4.7. Elemental mapping of (d) Ag, (b) C, (c) O, (e) EDS layered image of 1:5 AgNPs, and (a) and (f) 3-D FE-SEM image of 1:5 AgNPs in μm scale.

4.6. Antimicrobial activities

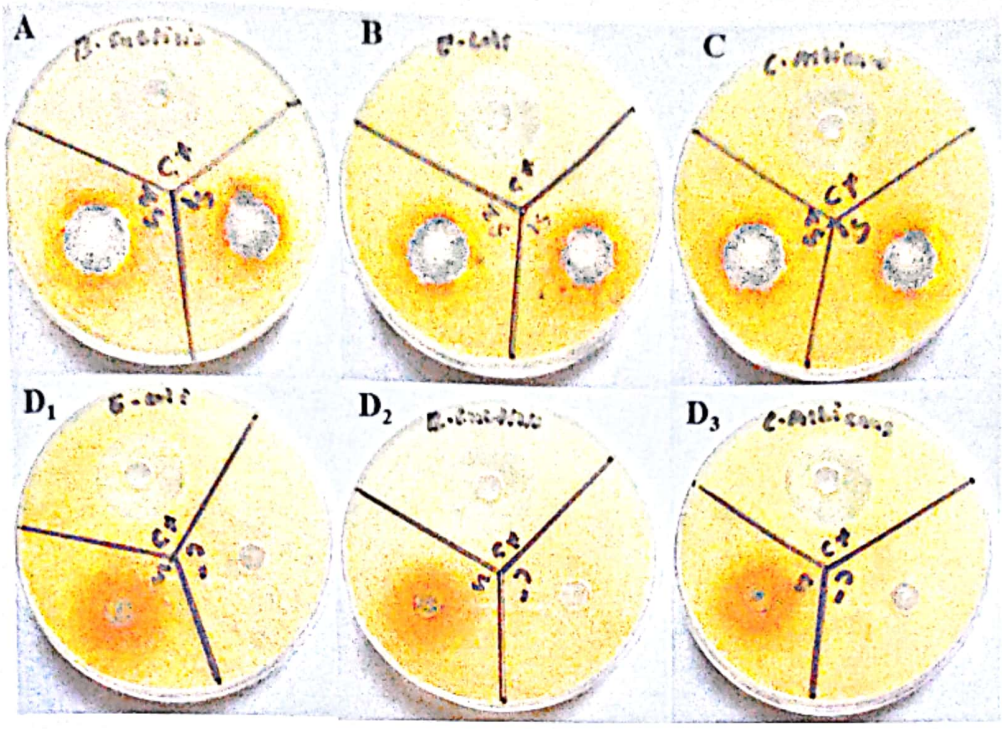
In this study, the antimicrobial activity of green synthesized AgNPs using AVLE was studied against Gram positive bacteria (*Bacillus subtilis*), Gram negative bacteria (*Escherichia coli*), and fungi (*Candida albicans*) by agar well diffusion method. Thus obtained results were showed in Table 4.7. *Kanamycin* was used as a positive control to compare with the obtained results of as-synthesized AgNPs to observe their antimicrobial efficiencies.

Table 4.7: Antimicrobial activity of as-synthesized AgNPs using *Artemisia vulgaris* leaves extract

Sample	Zone of inhibition (mm)		
	Bacterial Species		Fungal species
	G^+	G^-	
	Bacillus subtilis	*Escherichia coli*	*Candida albicans*
Extract (100 µL)	5	5	4
1:3 AgNPs (5 mg)	4	5	4
1:5 AgNPs (5 mg)	5	6	6
Kanamycin (5 µg/mL)	9	9	9

Green synthesized AgNPs showed the potential antimicrobial activity against Gram positive bacteria (*Bacillus subtilis*), Gram negative bacteria (*Escherichia coli*), and fungi (*Candida albicans*).AgNPs of 1:5 ratio showed best efficiency as compared to 1:3 AgNPs and leaves extract alone. The zone of inhibitions of 1:5 AgNPs for *Bacillus subtilis, Escherichia coli, and Candida albican* were found as 5 mm, 6 mm and 6 mm, respectively and are comparable to the positive standard control *Kanamycin* (9 mm zone of inhibition). However, the antibacterial activity of 1:3 AgNPs against *Bacillus subtilis* was found to be less with 4 mm inhibition zone, which are less as compare to both extract as well as 1:5 AgNPs. In addition, the antimicrobial activity of leaves extract alone and 1:3 AgNPs were found to be same except for gram positive bacteria *Bacillus subtilis*. The zone of inhibition shown by

leaves extract, 1:3 AgNPs, and 1:5 AgNPs against gram positive, gram negative bacteria, and fungi are manifested in figure 4.8.



Where, S= plant extract, ^1S= 1:3 AgNPs, S_2= 1:5 AgNPs, C^+=positive control, and C^-= Negative control

Figure 4.8: Antimicrobial activity of green synthesized AgNPs against (A) *B. subtilis*, (B) *E. coli*, (C) *C.albicans*, and of AVLE against D1, D2, D3 (*E. coli, B. subtilis,* and *C. albicans*, respectively)

According to the studies done by Rasheed et al., (2017), the antibacterial activities of NPs were investigated using the disc diffusion method against five human pathogens, namely *Es.cherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumonia* and *Haemophilus influenza.* The highest value was recorded against *St. aureus* (18±0.27 nm) and the lowest value was recorded against *V. cholera* (12±0.18 nm) by AgNPs. Similarly, in the study done by Alomari, (2020), green synthesized AgNPs showed significant activity against gram positive bacteria (*Bacillus subtilis, Staphylococcus aureus*), and gram negative bacteria (*Escherichia coli, Pseudomonas aeruginosa*), whereas the fungi (*Aspergillus flavus* and *Candida albicans*) showed hight resistance towards AgNPs with 0.0 inhibition zone. Meanwhile, our finding is slightly differing from his results. As our study showed

good antifungal activity against *Candida albicans* and have good agreement with the study done by Kumararaja et al., (2019), who found that AgNPs prepared from *A. vulgaris* had good antifungal activity. This could be due to the environment's impact on the active compound's nature and concentration in the plant.

4.8. Antimicrobial mechanism

Researchers have proposed different mechanisms accounting for the antibacterial effect of silver NPs. AgNPs have pronounced antibacterial properties due to their larger surface area, thus providing better interaction with microbial pathogens. NPs adhere to the cell membrane followed by bacterial colonization. Since bacterial membranes are made up of several sulfur-containing proteins, AgNPs specifically interact with these proteins and other cellular components containing phosphorus and sulfur in the cell. Since the bacterial plasma membrane is home to the components of the electron transport chain (ETC), the energy harvesting system, and the active transport of ions and molecules, any change in membrane organization leads to inhibiting the growth of bacteria.

In addition, NPs releases silver ions into bacterial cells inactivating respiratory enzymes and thereby leading to the production of reactive oxygen species (ROS), ultimately producing an enhanced bacteriostatic effect (Logeswari et al., 2015; MubarakAli et al., 2011). However, the exact mechanism showing the action of AgNPs is not yet clearly identified. It's also worth noting that the antibacterial effectiveness of nano silver varies depending on particle size (Panáček et al., 2006), production process, and other factors. AgNPs with a smaller size and a spherical form have a stronger antibacterial effect (Benakashani et al., 2016).

Morones et al., (2005), reported that the smaller size of AgNPs with greater surface area confers a stronger bactericidal effect compared with larger particles. In addition, investigations confirming that the antibacterial activities of biosynthetic AgNPs against Gram-negative and Gram-positive bacteria showed that Gram-negative bacteria were more reactive than Gram-positive bacteria, due to differences in membrane structure and composition (Kim et al., 2007). Therefore, the higher susceptibility of Gram-negative bacteria may be due to this difference.

CHAPTER 5

CONCLUSION

Green approach for nanoparticles synthesis is convenient, peaceful, inexpensive, fast, and provides excellent and relevant results without the interference of dangerous chemicals. In this study, Ag nanoparticles were successfully synthesized using leaf extract of *A. vulgaris* L. As synthesized AgNPs exhibited surface plasmon resonance at 430 nm, XRD analysis showed the crystalline peaks of silver nanoparticles and grain size was calculated based on the Debye scherrer formula shows the 28 nm diameter which is in good agreement with those exhibited in FE-SEM study. To the biomedical point of view, the newly synthesized nanoparticles found to be act as clear antimicrobial agents. It was confirmed that biosynthetic silver nanoparticles show excellent antibacterial performance against Gram-positive bacteria (*Bacillus subtilis*) and Gram-negative bacteria (*Escherichia coli*), and also showed antifungal activity against *Candida albicans*. The extract from *A. vulgaris* has potential value for various biomedical and pharmaceutical applications, and will certainly establish its commercial viability in medicine.

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Appendix

Table A_1: Phytochemical test procedure

S.N.	Experiment	Observation	Inference
1	Test for alkaloids a. Mayer's test 3 drops of Mayer's reagent+ 2 mL of extract, shake well	Yellowish ppt.	Presence of alkaloids
	b. Dragendorff's test 3 drops Dragendorff's reagent+ 2 mL extract, shake well	Yellowish ppt.	Presence of alkaloids
2	Test for flavonoids 5 mL dil. Ammonia solution+ extract+ conc. Sulphuric acidfrom side of the tube	Yellow color	Presence of flavonoids
3	Test for terpenoids 2 mL $CHCL_3$ + 5 mL extract + 3 mL conc. H_2SO_4 slowly	Reddish brown color	Presence of Terpenoids
4	Test for saponins 5 mL extract + 20 mL distilled water, shake vigorously	Appearance of frothing	Presence of saponins
5	Test for quinones 2 mL extract + Conc. HCl	Yellow colored ppt.	Presence of quinones
6	Test for polyphenols 3 drops of 5 % $FeCl_3$ + 2 mL extract, shake	Black color	Presence of polyphenols
7	Test for glycosides 3 drops of Molish's reagent + 2 mL extract, shake well + few drops Conc. H_2SO_4 slowly from the side tube and allow to stand for few minutes	Violet ring at junction of two layers	Presence of glycosides
8	Test for proteins Biuret Test: 2 mL 5 % NaOH + 2 mL extract + $CuSO_4$ solution	Pink color	Presence of proteins

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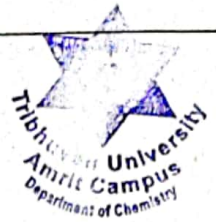

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