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Green Fabrication and Biological Evolution Ag-Ni Alloy Nanoparticles Using *Psidium Guajava* Leaf Extract

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ABSTRACT

Green plant materials in the form of extract support the bio reduction reaction as a reducing and capping agent. The present work reports the synthesis of Ag-Ni alloy nanoparticles using *Psidiumguajava* leaf extract as a precursor and AgNO₃ and NiSO₄ metal solutions as resource metals. Especially, silver and nickel alloy nanoparticles (Ag-Ni ANPs) are synthesized by bio reduction method using *Psidiumguajava* leaf extract. The extract can be easily used as a reducing, stabilizing, and capping agent for the effective green synthesis of Ag-Ni ANPs. The synthesized Ag-Ni ANPs were well characterized by analytical tools for its phase formation. FT-IR spectral analysis confirms the bio derived Ag-Ni ANPs compounds by absorption below 1000 cm⁻¹. The UV Visible spectra result showed that strong absorption peak at 430 nm, a characteristic band of green synthesized Ag-Ni ANPs. The XRD study confirmed the monoclinic structure with crystalline phase formation of Ag-Ni ANPs sample. SEM and EDX confirmed the regular spherical arrangement with some particle agglomeration surface morphology along with the presence of Ag-Ni ANPs. The antimicrobial activity of Ag-Ni ANPs was evaluated against gram positive and gram-negative bacteria strains and fungal pathogen. The obtained result indicated that good activity against bacteria as well as fungi.

Key words: *Psidium guajava*, Leaf extract, Ag-Ni ANPs, Antibacterial, and Antifungal

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1. Introduction

Owing to their distinctive features including optical, electrical, sensorial, catalytic, magnetic and antimicrobial characteristics *etc.* of hetero-metallic alloy nanoparticles (ANPs) have been attracted much to the researchers due to its nano-size level with large surface area [1-2]. Despite their size, bimetallic clusters are becoming more and more prevalent because of their molecular composition and

atomic distribution. In addition, the unique physical characteristics namely high surface area, quantum effect, mobility and chemical properties of bimetallic ANPs are greatly enhanced by their synergistic effects, which include the lattice strain, electron effect, ensemble effect and bifunctional effect [3]. Bimetallic ANPs involved in several applications across the area mainly oxygen reduction reactions (ORR), synthetic organic chemistry, antimicrobial activity, biomedical, optoelectronics, solar cells, magnetic recording, catalyst in energy storage cells *etc.*[4]. Additionally, a few bimetallic heavy alloys are employed in hydrogen dissociative membranes, shape memory materials for civil constructions, biomedical materials and super alloys for aero plane engine systems [5].

Numerous synthetic approaches including biosynthetic, physical and chemical methods have been used to synthesize the nanoparticles. In general the chemical methods employed are very expensive and are associated with using noxious and harmful chemicals that directs the various threats to the environment [6]. Therefore, compared to conventional chemical processes, the synthesis of nanoparticles involving green technique has been earned more advantages [7-8]. These techniques lead to reduce toxicity, boosts in the stability and are eco-friendly, sustainable, inexpensive. Consequently, make them excellent for massive production [9, 10]. Additionally, they provide an opportunity to synthesize the nanoparticles of many different sizes and shapes that can be tuned for certain applications. Numerous phytochemical constituents with redox properties, such as flavonoids, terpenoids, polysaccharides and phenolic, are found in plants. Hence, these are more favorable and extensively used for nanoparticles synthesis by green method approach [11-13]. Green synthesized nano carriers, which rely on their metals, offer distinct advantages in medical and biomedical applications, including drug delivery, immunotherapy, regenerative medicine, dentistry, wound healing, tissue treatment, diagnostics, anticancer, antimicrobial activity, therapeutics with improved efficacy and bio-sensing platforms [14-16]. Green nanotechnology can also be applied in environmental and agricultural sectors for purification of water, detection of pollutants and crop enhancement, insect control, nutrient delivery systems respectively [17-18]. Additionally, the synthesized nanoparticles through ecofriendly pathway can contribute towards manufacturing of sustainable electronic and energy storage devices with enhanced performance [19].

Ag-Ni ANPs are a recent addition to the nanoparticle family, receiving attention because of their peculiar synthesis involving immiscibility of silver and nickel, dissimilarity in their lattice systems, smaller surface energy and related computational studies [20-22]. Additional intriguing

features of these ANPs include the coupled robust surface plasmon resonance (SPR) of silver in the visible spectrum, which is caused by size-dependent intrinsic ferro /super paramagnetic and the coherent action of conduction band electrons contrary to the reduced electrochemical potential of nickel [23]. Literature reviles that, a large number of Ag-Ni nanoparticles are believed for possessing a phase segregated core shell form since considering the reduction potential of Ag^+ reduces to Ag^0 far more quickly than Ni^{2+} does to Ni^0 [24-26]. To address these issues, just a few investigators have employed new strategies to synthesis authentic Ag-Ni ANPs in recent years [27]. Ag-Ni ANPs were synthesized through gas condensation, γ -radiation, and vapor quenching and chemical methods. Ag and Ni nano particles were considered for various applications including medical, environmental and electronic fields [28, 29]. Therefore, to the best of our knowledge, very few reported on the green synthesis of metal-metal alloy nanoparticle using plant extract as a capping or reducing agent to assess antimicrobial activity. Present work is reporting green synthesis of Ag-Ni ANPs using Psidium Guajava plant leaf extract and is well characterized by various characterization techniques. Evaluation of the antimicrobial activity of the prepared alloy sample was carried out against gram-positive and gram-negative bacteria strains and fungal Pathogen by using disc diffusion technique.

2. Experimental

2a). Materials and methods

Chemicals like metal salts, polyvinyl alcohol and reagents used in the experimentation were of AR (analytical grade) and double distilled water was used as a solvent for the preparation various solutions. Environmental friendly bio reduction method was used for the synthesis of Ag-Ni ANPs using Psidium Guajava plant leaf extract.

2 b).Preparation of leaf extract of Psidiumguajava

Fresh Psidium guajava leaves about 600 gm were collected from Botanical Garden of Vijayanagara Sri Krishnadevaraya University, Ballari, Karnataka, India. Plant leaves, fruit and its budding fruit with leaves were given in Figure 1(a-c) respectively. These leaves were washed thoroughly with distilled water to remove impurities then dried. The dried leaves samples were cutted in to small pieces and were powdered using a mixer grinder. 10 gm of prepared Psidium guajava leaves powder were dissolved in 100 mL of distilled water and then heated to 60 °C for °C min and was cooled to room temperature, then filtered using Whatman No. 1 filter paper and store it at 5-6 °C for further analysis . The procedures were summarized pictorially in Figure 2(a-c) respectively.



Figure 1: a) Leaves of *Guava*, b) Fruit of guava plant c) Flower of guava plant;

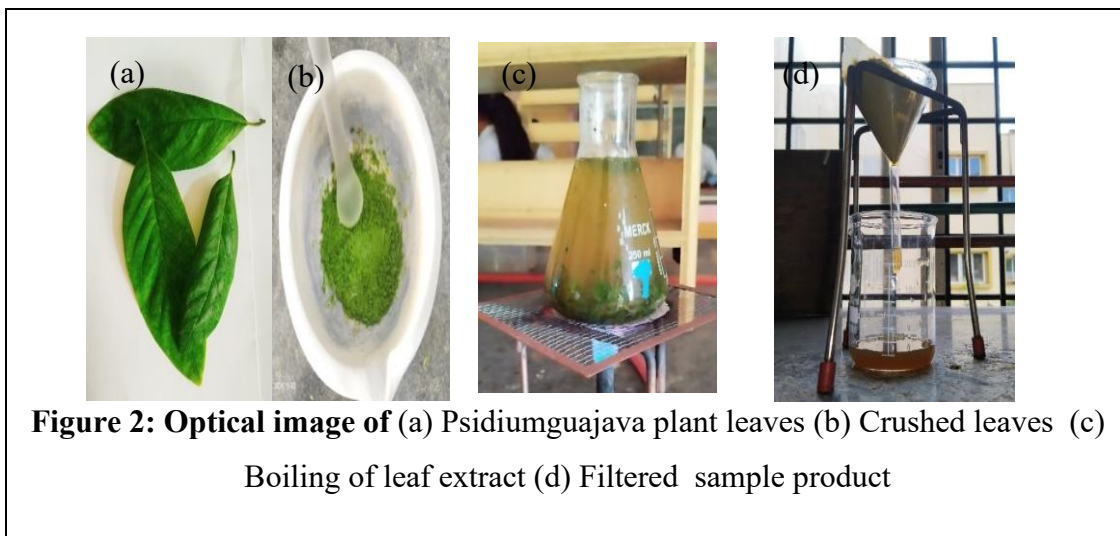


Figure 2: Optical image of (a) *Psidiumguajava* plant leaves (b) Crushed leaves (c) Boiling of leaf extract (d) Filtered sample product

2 c). Synthesis of Ag-Ni ANPs

According to scheme 2, Ag-Ni ANPs were synthesized by employing the plant-mediated co-reduction approach. A known volume of *Psidiumguajava* leaf extract was mixed with an appropriate concentration of 0.1 N of silver nitrate and nickel sulphate solutions as shown in Figure 3(a-b). This reaction mixture was heated up to 70 °C and kept aside for 24 hours at room temperature to complete the bio reduction reaction. The formation of Ag-Ni ANPs was indicated by change in color as dark brown colour. The formed nanoparticles were centrifuged at 4000 rpm about 5 minutes. The collected centrifugation was recrystallized to obtain Ag-Ni ANPs as shown in Figure 3(c). Scheme for complete synthesis of Ag-Ni ANP is shown in scheme-1.

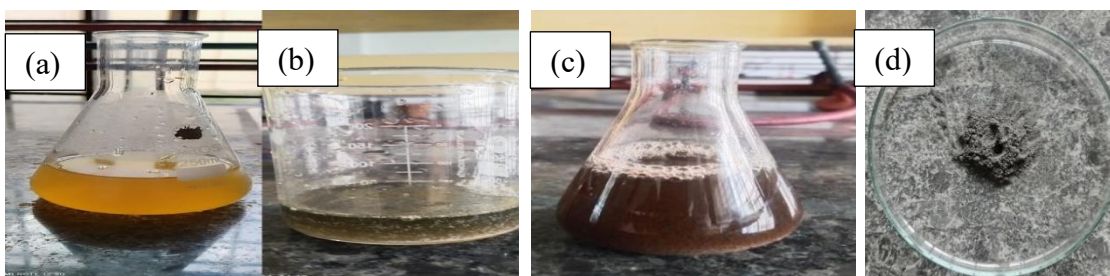
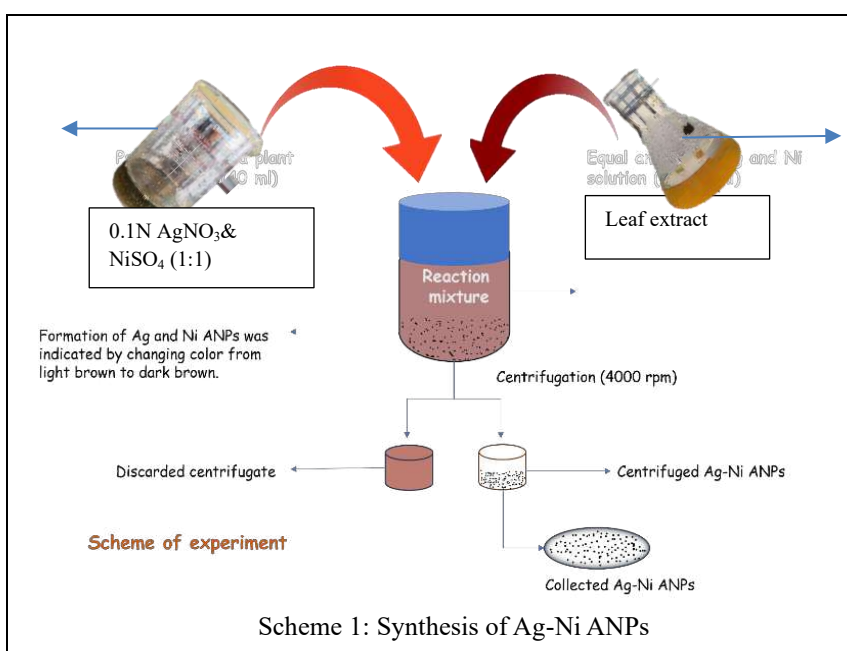


Figure 3: Optical image of (a) Prepared leaf extract (b) Mixture of AgNO_3 and NiSO_4 metal solution (c) Reaction mixture (d) Ag-Ni ANPs



2 d). Antibacterial Activity:

Antibacterial activity of different compounds was tested by agar well diffusion assay with some modifications. Petridishes were prepared by pouring 20 mL of sterilized NA media under aseptic condition and allowed to solidify. After solidification of the media, 100 μL of standardized test microbial inoculums of *Escherichia coli*, *Bacillus subtilis* and *Klebsiella* were spread uniformly using sterile cotton swabs. 6 mm diameter agar is drawn from plate to form a well using sterile cork borer. Antibiotic gentamycin was used as positive control and DMSO as negative control. After keeping at 4°C for 4 hours for the diffusion of antibacterial metabolites, thereafter plates were

incubated at 37 °C for 24h. The diameter of the inhibition zone around the well is measured in millimeter (mm) and the average of three repeated agar discs were taken to assess the strength of antibacterial activity.

2 e). Antifungal Activity:

Antifungal activity of different compounds was tested by agar well diffusion assay with some modifications. Petri dishes were prepared by pouring 20 ml of sterilized PDA media under aseptic condition and allowed to solidify. After solidification of the media, 100 μ L of standardized test *Aspergillusniger* and *Fusarium* was spread uniformly using sterile L-shaped loop. 6 mm diameter agar is drawn from plate to form a well using sterile cork borer. Antifungal Nystatin was used as positive control and DMSO as negative control. After keeping at 4°C for 4 hours for the diffusion of antibacterial metabolites, thereafter plates were incubated at 28°C for 72h. The diameter of the inhibition zone around the well is measured in millimeter (mm) and the average of three repeated agar discs were taken to assess the strength of antibacterial activity.

2 f). Analytical Techniques for characterisation

The powder X-ray diffraction patterns were recorded on a JEOL JDX-8P diffractometer using $\text{CuK}\alpha$ radiation (1.54 Å) at 30 kV. The Fourier transform infrared (FT-IR) spectrum of the samples were recorded on a Perkin-Elmer FT-IR spectrometer (Model No. 1000) in the range 4000-400 cm^{-1} at a resolution of 4 cm^{-1} . JEOL JSM-6380 LA scanning electron microscope with energy dispersive micro analysis of X-Ray (EDAX) is used to study particle morphology with metal confirmation of the sample. Optical absorption and Raman traces were carried out by Shimadzu UVPC-1601 spectrophotometer and Deitanu Rock Hound Nuspec 2.0 respectively. Thermal characterization was conducted using a Mettler Toledo Star instrument.

3. Results and Discussions

3 a). Visual observation of colour change

Physical confirmation of the synthesized sample Ag-Ni ANPs (the reduction process Ag^+ and Ni^{2+} to Ag^0 and Ni^0 nanoparticles) was done by visual observation of the color change in the solution. Figure 3(a) and 3(b) shows that, variation of colour change of the mixture compared to its metal salt solutions indicates the reduction reaction. Further, dark brownish colour indicates the chemical change by complete green reduction.

3 b). FT-IR study

Infrared study is undertaken to know the bonding nature of the prepared Ag-Ni ANPs sample and its spectral record is shown in Figure 4. The presence of the -OH group in the moisture content of Ag-Ni ANPs sample is indicated by the broad peak at 3331 cm^{-1} in the FT-IR spectra. The spectrum displayed the stretching and bending frequencies at 1616 cm^{-1} , 1382 cm^{-1} and 1099 cm^{-1} , which correspond to aromatic -C=C-, -CH₃ and -C-O- functional groups, respectively, indicating the presence of natural pigments such as polyphenols, flavonoids, quercetin and tannins. Peaks appeared at 828 cm^{-1} and 612 cm^{-1} , are due to the presence of metal-metal (Ag-Ni) bond and the formation of metal oxide (Ni-O or Ag-O) respectively. It is confirmed from the analysis of FT-IR spectrum that, the carbonyl groups, amino acid and proteins have the stronger ability to bind the metal. It is also highlighting that, the possible formation of the metal nanoparticles by the proteins to prevent agglomeration and thereby stabilizing the medium. Further, it is learnt that possibly the biological molecules could perform the dual functions of forming and stabilizing of the Ag-Ni ANPs in the aqueous medium. Similarly, the groups of carbonyl proved the absorption of flavanones or terpenoids on the surface of metal nanoparticles. Moreover, in the absence of other strong ligating agents in sufficient concentration is possibly by the interaction of carbonyl or π -electrons.

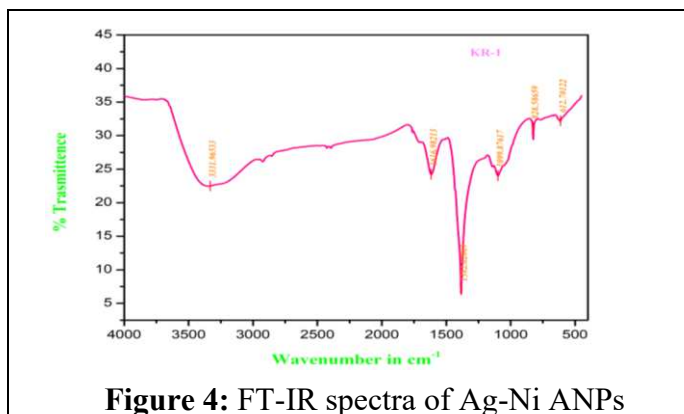


Figure 4: FT-IR spectra of Ag-Ni ANPs

3 c). UV-Visible study

It is the efficient technique used for the sample analysis by identifying the possible absorption. For the successful synthesis process, this stands a testimony for the process evidence. The optical absorption spectrum of the prepared alloy sample is scanned to know its absorption characteristics. The UV-Visible spectra of synthesized Ag-Ni ANPs showed the reduction of Ag⁺ and Ni⁺² ions to alloy nanoparticles and it is effectively examined by absorption of the surface resonance peak (SRP) at 331

nm which confirms the absorption in the visible region. The wavelength range of SRP is a typical signature of Ag-Ni ANPs, which confirmed the formation of Ag-Ni ANPs during course of the reaction. Generally, a peak appears at 331 nm is due to $n-\pi$ transition as shown in figure 5. It also proved the alloy nature of the bimetallic nanoparticles but not of Ag and Ni metals physically embedded monometallic nanoparticles. The forming of colloidal gel solution that contains both Ag and Ni is indicated by the shifting towards red side. Reduction of metal ions and formation of stable alloy nanoparticles occurred rapidly in short span of time. This substantiates the successful synthesis of Ag-Ni ANPs and highlights their potential applications in biomedical and bioscience applications.

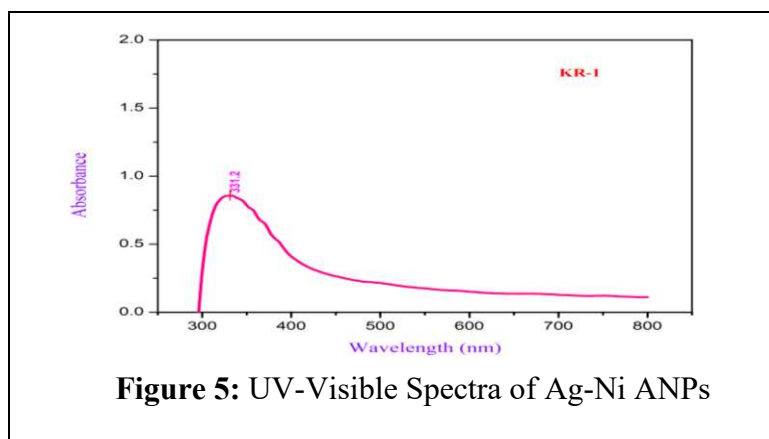


Figure 5: UV-Visible Spectra of Ag-Ni ANPs

3 d). EDX study

In order to confirm the elemental composition of green synthesized Ag-Ni ANPs, the EDX instrumentation has been employed and obtained EDX patterns is shown in figure 6. Inset table in the pattern showing elemental data obtained by EDX scan. The peak position and their intensity reveal the type of element present and % of the element in the sample respectively. The sample produces a strong signal at 3 keV which reveals the presence of silver nanoparticles. Due to the presence of the strong Ag signal weakens the signals of Ni metal. The EDX pattern illustrates the chemical constituents present in Ag-Ni ANPs including Ag and Ni elemental segments confirms the formation of Ag-Ni ANPs.

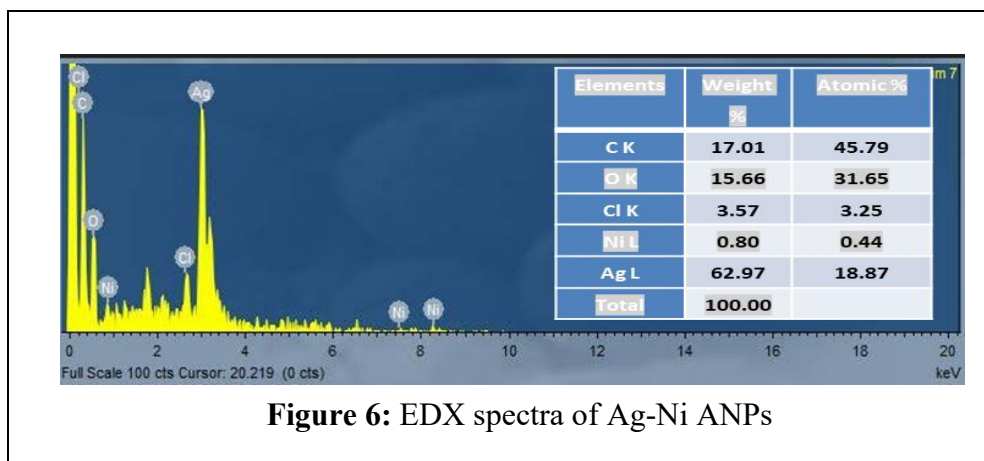


Figure 6: EDX spectra of Ag-Ni ANPs

3 e).Raman study

Raman spectroscopy was used to characterize the prepared ANPs in order to determine its molecular structure, chemical and bonding environment. Figure 7 shows the Raman spectrum of green derived Ag-Ni ANPs. As illustrated in Figure 7, one distinct peak at 1545 cm^{-1} is observed which can be attributed to the formed metal oxide involving Ag-O or Ni-O vibration for subsurface species. Although subsurface species may facilitate molecular oxygen adsorption, it could also be a probable consequence of surface restrict by Ag-Ni ANPs.

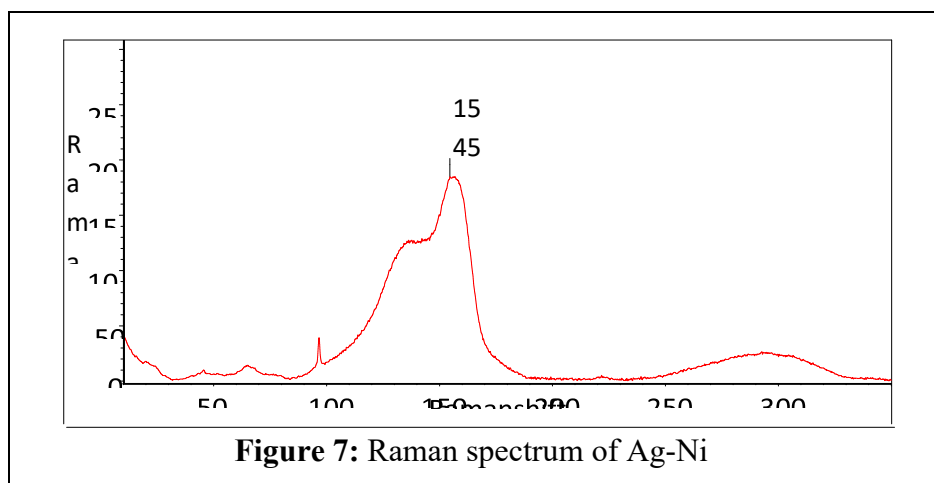


Figure 7: Raman spectrum of Ag-Ni

3 f). Thermo gravimetric analysis (TGA)

TGA of Ag-Ni ANPs provides valuable insights into their thermal stability, composition and decomposition behavior. TGA monitors the weight change of a sample as it is heated over a controlled

temperature range, giving crucial information about various thermal processes, such as oxidation, reduction, or decomposition as well as the presence of impurities or adsorbed species. The obtained TGA trace is as shown in Figure 8 indicates the three step weight loss of the sample. The first weight loss in between 25°C to 330°C region is due to elimination of moisture content in the sample. The second step weight loss from 330°C to 480°C is loss of one or two metal nanoparticles and carbon dioxide gas from the ANPs. The third weight loss from 480°C on wards the gradual and steady weight loss over the broad temperature range represents high order of thermal stability of Ag-Ni ANPs sample and is found to be thermally stable up to 820.0 °C.

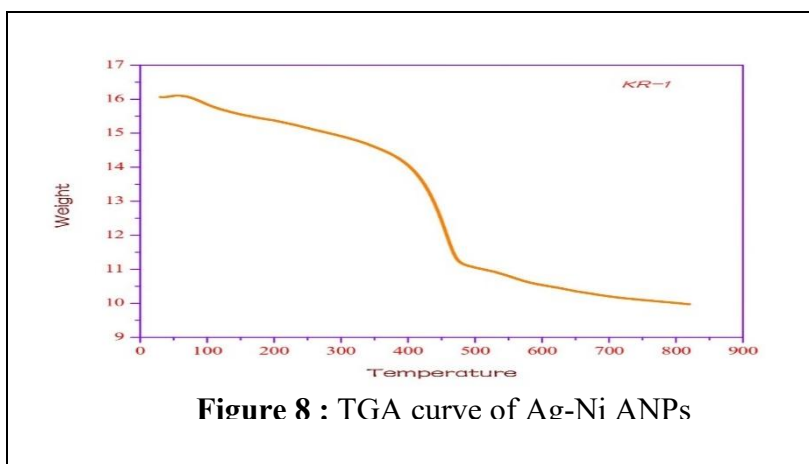


Figure 8 : TGA curve of Ag-Ni ANPs

3 g). Dynamic light scattering study

The DLS study of green-derived Ag-Ni ANPs focuses on characterizing the size distribution and stability of nanoparticles and is shown in Figure 9. The average particle size and polydispersity index (PDI) of green derived Ag-Ni ANPs were evaluated by using Nano-Zeta-Sizer. The average size of the green derived ANPs which are formed by the Psidium Guajava plant leaf extract is 53 nm and the PDI value of 0.434 indicates the moderate polydisperse displaying very broad size distribution of green derived Ag-Ni ANPs.

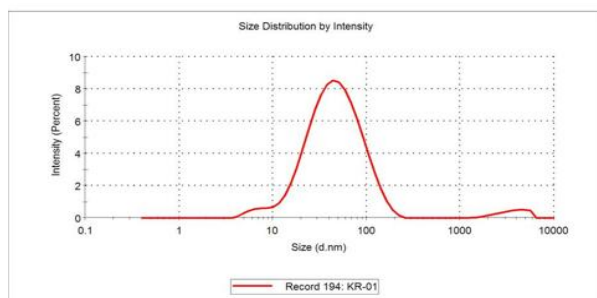


Figure 9:DLS Spectra of Ag-Ni ANPs

3 h). X-Ray Diffraction Study (XRD)

Indexed X-ray diffraction pattern of the bio fabricated Ag-Ni ANPs is shown in Figure 10. Highly intense Bragg's reflections of both Ag and Ni were observed in the pattern and are indexed with reference to JCPDS file of Ag (JCPDS file No. 65-2871) and Ni (JCPDS file No. 00-004-0850). Presence of both Ag and Ni reflections in the single pattern confirms the formation of Ag-Ni ANPs. The observed XRD results clearly indicating that, alloy nanocomposite synthesized by the green route is crystalline in nature with cubic structure.

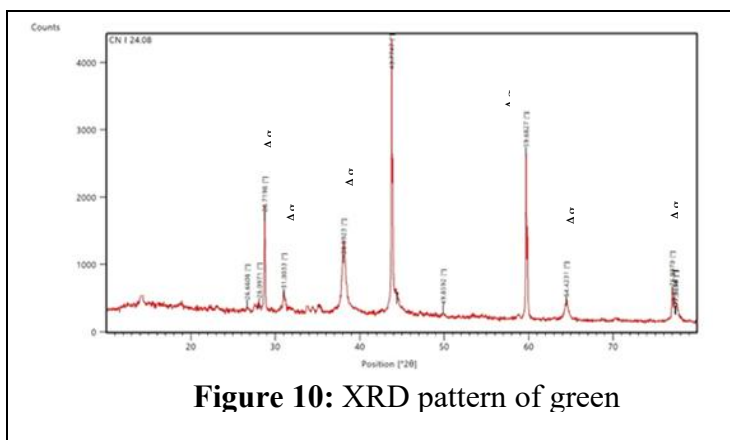
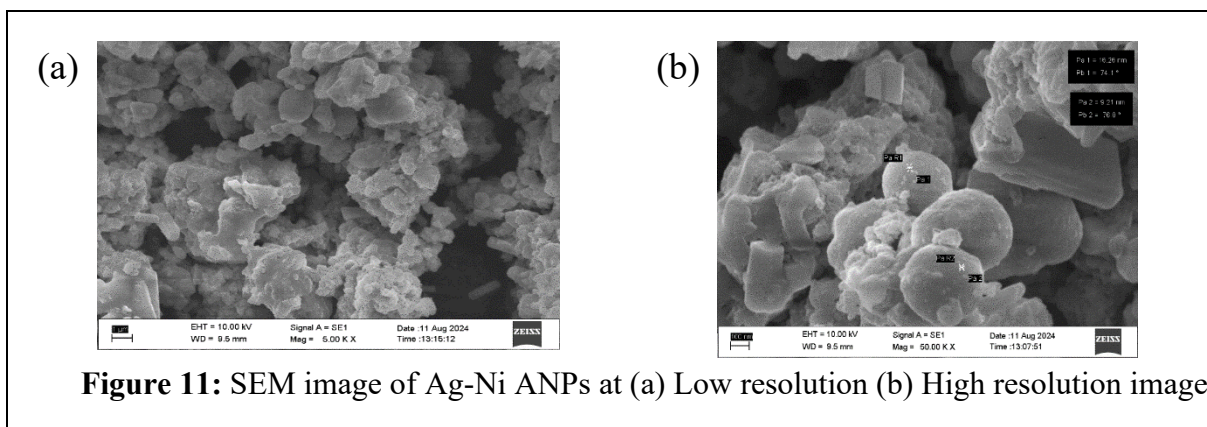


Figure 10: XRD pattern of green

3 i). SEM study

Surface morphology of the prepared byproduct alloy nanocomposite was well studied by SEM analysis. Figure 11(a-b) shows SEM images of synthesized Ag-Ni ANPs at low and high resolution respectively. Irregular shaped particles and finds occasionally regular with spherical arrangement with some particle agglomeration. The aggregation of the alloy nano composite with may be caused due to polarity and electrostatic attraction of alloy nanoparticle. It is also showing the dispersion of Ag and

Ni nanoparticles with each other forming high crystalline nature. Self-assembly of metal nanoparticles contribute to increased surface area leads many potential applications. The images were analyzed to identify potential interactions between the Ag and Ni metals and their close distribution. Careful examination of the SEM images revealed any synergistic effects arising from the simultaneous presence of Ag and Ni metals. This result offered strong support for the theory that Psidium Guajava plant leaf extract might act as reducing and capping agent during synthesis. However, it is clearly observed in high resolution image.

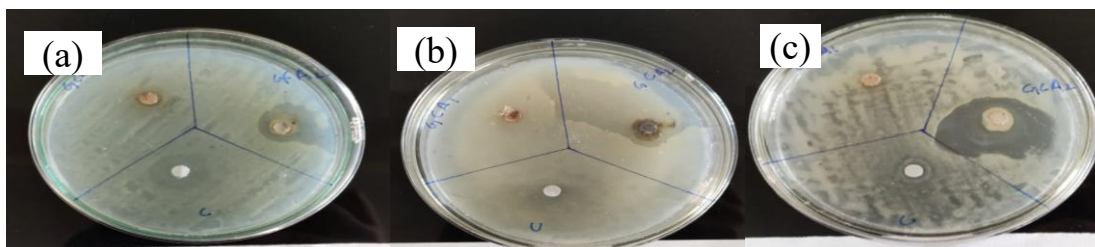


3 j). Antibacterial Assay

For assessing the practical application, antibacterial activity of Ag-Ni ANPs was performed. Fig12 shows the ZOI of antibacterial activity result of the sample. The formed Ag-Ni ANPs were screened for *in-vitro* studies to their antibacterial activity against *Escherichia coli*, *Bacillus subtilis* and *Klebsiella* using disc diffusion method. The plates were incubated immediately at 37°C for 24 hrs activity was determined by measuring the diameter of zones showing complete inhibition (mm) as shown in below table 2. The biosynthesized Ag-Ni ANPs showed better anti-bacterial activity (based on the zone of inhibition). The possible mechanism would be the production of electron-hole pairs when Ag-Ni ANPs with defects are activated by UV or visible light. Thus formed holes can split H₂O molecules into OH⁻ and H⁺ and electron addition converts dissolved oxygen molecules to superoxide radical anions (O₂⁻). Free radicals O₂⁻ and OH⁻ formed in the reactions can depolymerize some polysaccharides, breakdown of DNA strand and inactivate enzymes, leading to cell death. The other possible mechanism would be the binding of Ag-Ni ANPs to cell membrane proteins via electrostatic interactions, or nanoparticle accumulation in the cytoplasm or periplasmic region, disrupting cellular function and membrane disruption and disorganization leading to the death of the cells [30].

Table 2: Assay of antibacterial activity

Zone of inhibition (ZOI)	Extract	Ag-Ni ANPs	Control (Amoxicillin)
E.coli	12	18 mm	10 mm
Bacillus subtilis	14	18 mm	10 mm
Klebshiella	10	20 mm	5 mm

**Figure 12:** Antibacterial activity result for (a) E-coli, (b) Klebshiella and (c) Bacillus Subtillis

3 k). Antifungal Activities

The antifungal activities of prepared leaf extract and Ag-Ni ANPs samples were performed against *Candida albican*, *Candida lunata* and *Aspergillusniger* fungal strains and were performed with standard antibiotic fluconazole. Observed efficiency data is given in Table 3. The guava leaf extracts showed minimal antifungal activity compared to Ag-Ni ANPs samples. Previous research has identified two possible mechanisms for the interaction of nanoparticles with bacteria and fungi [31]. The synthesized Ag-Ni ANPs showed better anti-fungal activity than leaf extracts, the possible reason for increased action would be due to presence of Silver ions. These bio-fabricated Ag-Ni ANPs disrupts the cell morphology targeting to a great degree of representing into the fungal cells, making it friable, permeable and leaky eventually leads to death. The phyto-constituents of *Psidiumguajava* leaf have the ability to constrain the pathogens. Henceforth biologically fabricated Ag-Ni ANPs, the one used in the current study, can be considered as potent antifungal agents.

Table 3: Assay of antifungal activity

Zone of inhibition (ZOI)	Extract	Ag-Ni ANPs	Control (Flucanazole)
<i>Candida albican,</i>	10	12	8
<i>Candida lunata</i>	11	13	10
<i>Aspergillusniger</i>	13	16	11

Conclusions

In this study, Psidium Guajava plant leaf extract was used to synthesize Ag-Ni ANPs using green route as a low cost and eco-friendly green approach. The phytochemicals present in the Psidium Guajava plant extract plays a supreme impact in the nanoparticle formation. During the synthesis process Psidium Guajava plant leaf extract serves as a capping and reducing agent. The characterization by UV-visible spectroscopy, FTIR, scanning electron microscopy, Raman and XRD techniques endorse the successful designing of Ag-Ni ANPs. Both extracts and nanoparticles proved a remarkable antibacterial and antifungal activity, although the nanoparticles surpassed the activity as compared to the extract alone. The biogenic Ag-Ni ANPs are chemically stable and economically effective. Thus, from the results, it is concluded that the synthesized Ag-Ni ANPs has potent antimicrobial activity due to its enhanced surface area and smaller particle size of nanoparticles. Hence, Ag-Ni ANPs may provide an even worthier treatment after being converted into a proper dosage form for further applications.

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Author Contributions Statement

Arunkumar Lagashetty: Formal analysis, Investigation, Methodology. Kavya1, Krishna Rathod: Conceptualization, Visualization, Writing - Review & Editing, Supervision. Akshatha S, K: Investigation, Methodology, Visualization, Formal analysis. ChandraNaik: Formal analysis, Investigation, Methodology. Chetan Kumar K: Formal analysis, Investigation. Roja B.M:

Conceptualization, Visualization, Review & Editing. V.Veena: Conceptualization, Visualization, Writing - Review & Editing.

Data availability

The authors declare that the data supporting the findings of this study are available within the paper.

Funding declaration

The authors declare that they were not funded by any organization or fund.

Clinical trial.

This study is not a clinical trial. Therefore, clinical trial registration was not required, and no trial registration number is available.

Ethics declaration

Not applicable.

Consent to publish declaration

Not applicable.

Consent to participate declaration

Not applicable’.

Declarations

Competing interests

The authors declare no competing interests.

Ethics and consent to participate

The study adheres to the Biological Diversity Act, 2002, and guidelines from the Ministry of Environment, Forest and Climate Change (MoEFCC). No wild collection was involved; plants were sourced from a university-managed botanical garden. *Psidium guajava* plant is a widely cultivated, non-endemic, non-threatened species in India, Moreover, this plant is a tropical / subtropical fruit tree

grown commercially in Karnataka. This study was conducted using *Psidium guajava* L. plant material and in-vitro laboratory assays. No human participants, human biological samples, or identifiable personal data were involved in the study.

Human and animal rights statement

This study does not involve human participants or medical interventions; it focuses on plant-microbe interactions in an agricultural context.

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