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CHARACTERIZATION AND EVALUATION FOR ANTIBACTERIAL ACTIVITY OF GREEN SYNTHESIZED NANOPARTICLES OF *LEONOTIS NEPETIFOLIA*

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Keywords:

Green synthesis, *Leonotis nepetifolia*, Antibacterial activity, Silver nanoparticles

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ABSTRACT: The current research is to develop an easy and eco-friendly method for synthesizing Flower silver nanoparticles [FAgNPs] and Stem silver nanoparticles [SagNPs] using an aqueous extract of flower and stem *Leonotis nepetifolia*,(L.) R. Br. (family: Lamiaceae), and to evaluate their antibacterial properties. The obtained sample of synthesized FAgNPs and SAgNPs were characterized by UV–vis spectrophotometer, Zeta potential, X-ray Diffraction [XRD], Field Electron- scanning electron microscope [FE-SEM], and Fourier transform infrared spectroscopy [FT-IR] analysis. The formation of FAgNPs and SAgNPs was confirmed by the observation of band between 300 nm to 700 nm UV–vis spectrum. The crystalline structure of FAgNPs and SAgNPs with a face- centered cubic (FCC) was confirmed by XRD. The responsible phytochemicals for the reducing and capping agents of FAgNPs and SAgNPs were observed with FT-IR spectra analysis. The FE-SEM monograph revealed the spherical shape and size of FAgNPs and SAgNPs. Hence synthesized AgNPs were found to be in nano size and stable. Furthermore, the biosynthesized FAgNPs and SAgNPs indicated adequate antibacterial activity against clinical pathogen, *Staphylococcus aureus* [Gram-positive] and *Klebsiella pneumonia* [Gram-negative] bacteria. Minimum Inhibitory Concentration (MIC) was found to be 25 µg/ml.

INTRODUCTION: Exploiting the potential of herbal plants is one of the green synthesis routes, and it is significant because the current therapeutic approaches have toxicity problems and microbial multidrug resistance issues. Plants have been a vital source of novel pharmacologically active compounds, with many blockbuster drugs being derived directly or indirectly from plants. In the 21st century, 11% of the 252 drugs considered basic and essential by the WHO were exclusive of flowering plant origin [1].

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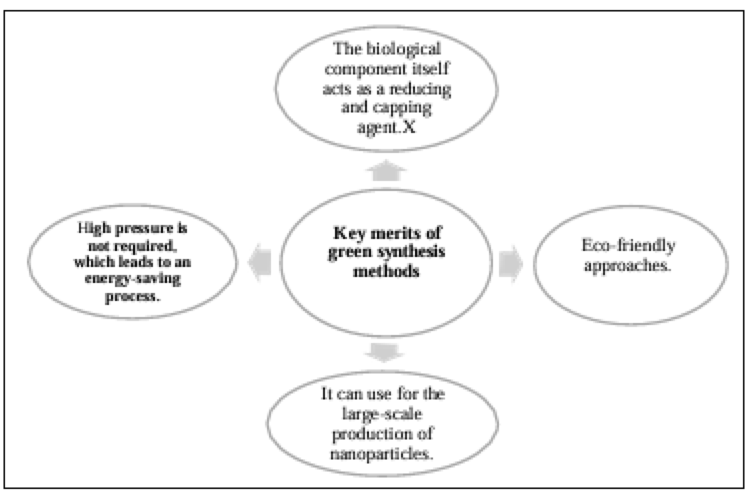
The World Health Organization (WHO) estimated in 1985 that approximately 65% of the world population the primarily relied on plant-derived traditional medicines for their primary health care, while plant products also play an important, though more indirect role in the health care systems of the remaining population, who mainly reside in developed countries [2].

Nano-sized drug delivery systems of herbal drugs have potential for enhancing activity and overcoming the problems associated with plant medicines, especially in treating chronic diseases such as bacterial infection, asthma, diabetes, cancer, and others. Nanoparticles have been synthesized using different methods such as chemical, physical and biological methods. Green synthesis of nanoparticles is a branch of nanotechnology. Green syntheses are required to avoid the production of unwanted or harmful by-products through the build- up of reliable, sustainable, and eco-friendly synthesis procedures. The use of ideal solvent systems and natural resources (such as organic systems) is essential to achieve this goal. Green synthesis of metallic nanoparticles has accommodated various biological materials (e.g., bacteria, fungi, algae, and plant extracts). Among the available green synthesis methods for metal/metal oxide nanoparticles, utilizing plant extracts is a relatively simple and easy process to produce nanoparticles at a large scale, compared to bacteria or fungi mediated synthesis [3]. The metallic nanoparticles are nano-sized metals with dimensions (length, width, thickness) within the size range of 1–100 nm. Metallic nanoparticles, which are essential products of the nanobiotechnology field, have gained significant attention in biomedical technology. It has derived from gold, silver, palladium, cobalt, and zinc [4, 5]. Silver is a general cost-effective, regularly utilized metal in our daily lives and also an anti-microbial agent. Ag has been used to synthesize NPs with a broad range of usages such as preparing food dispensation, contemporary ointments, and therapeutic implants [6]. The anti-microbial activity of silver nanoparticles (Ag-NPs) appears significantly high. Silver is more toxic element to microorganisms than many other metals in the following sequence: Ag > Hg > Cu > Cd > Cr >Pb> Co > Au > Zn > Fe >Mn> Mo > Sn (Zhao and Stevens, 1998). Ag-NPs exert more efficiency than silver ions and other silver salts in mediating their anti-microbial activity [8]. *Leonotis nepetifolia*, also known as Christmas candlestick or Lion's ear, is a plant genus Leonotis belonging to Lamiaceae. The plant has widely distributed in Pantropics and tropical Africa, and southern India. It develops to a height of 3 metres and has whorls of striking lipped flowers, most usually orange. The aqueous extract of *Leonotis nepetifolia* contains many polar bioactive compounds such as alkaloids, flavonoids, tannins, terpenoids, and glycosides. These compounds are potent anti-microbial agents [9]. Many literature papers reported the synthesis of silver nanoparticles using *Azadirachta Indica*, *Oryza sativa*, *Berberis aristata*, carob leaf, *Saraca indica*, *Gymnema slyvestre*, *Ipomoea pes-caprae stem*, *Caralluma fimbriyata* stem, and *Zephyranthes rosea* flower. Some literature reveals that the methanolic extract of leaf, flower, and stem shows potent antibacterial activity, and that various phytoconstituents like alkaloids, terpenoids, tannins, steroids, etc., motivate the synthesis of nanoparticles of this extract and their evaluation as antibacterial. Literature also reveals attempts to synthesize silver nanoparticles of *Leonotis nepetifolia* whole plant extract and its antibacterial activity. Still, no silver nanoparticles were synthesized specifically from the flowers and stem extracts where potential antibacterial activity was reported. This paper investigates the green synthesis and characterization of Flower silver nanoparticles (FAgNPs) and Stem silver nanoparticles (SAgNPs) along with their evaluation as antibacterial.



MATERIAL AND METHOD:

Collection and Identification of Plant Material: *Leonotis nepetifolia* Flowers and Stems were collected from the Umred Road, Nagpur. The plant was taxonomically authenticated by the

Department of Botany, Rashtrasant Tukdoji Maharaj Nagpur University.

Preparation of Plant Extract: Freshly collected flowers and stems were shade dried at room temperature for 6-7 days until they became rigid, and then grinded into powder using a Mechanical grinder. A total of 10g of flower powder was boiled with 100ml of double-distilled water in 250ml of the beaker for 30min at 60ºC. The crude extract was allowed to cool it attains room temperature and then filtered through Whatman Filter Paper 1. The purified extract was preserved in the refrigerator at 5ºC for further work. A similar method was used to prepare the stem crude extract.

Synthesis of Silver Nanoparticles: Prepared 1mM of aqueous silver nitrate (AgNO3) solution and 2.5 ml of fresh plant was added in a beaker containing 47.5 ml of 1mM AgNO3 solution. The mixture was stirred with a magnetic stirrer for 30 min at 60ºC.

Initially, turned the colorless solution gradually turned Reddish-brown designated the bio reduction of Ag+ to Agº and the formation of flower silver nanoparticles (FAgNPs). The reactant solution was stored in the dark for 12-16 hr, and then refrigerated for further use. A similar method was used for the synthesis of stem silver nanoparticles (SAgNPs).

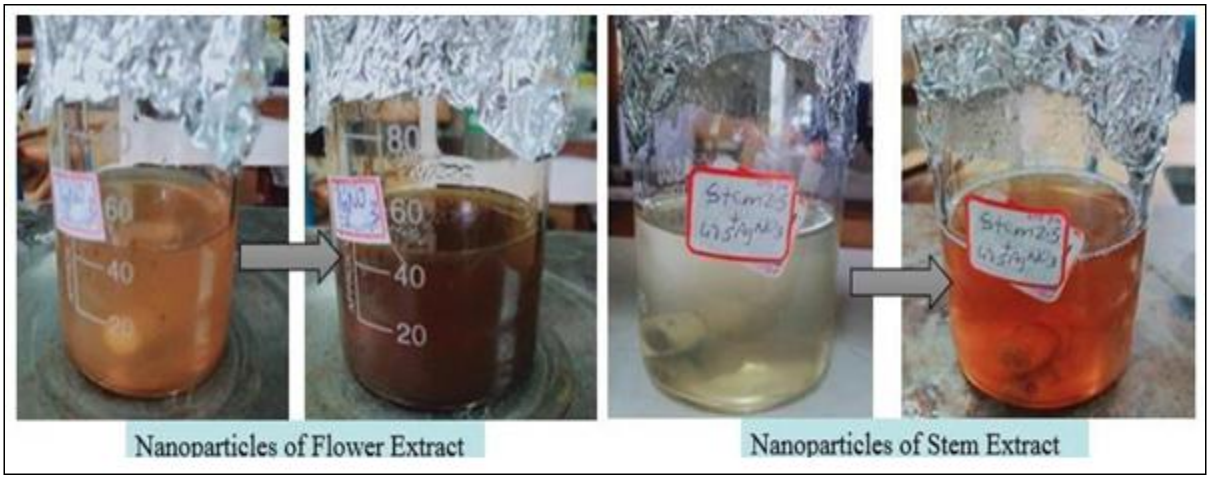


FIG. 1: SYNTHESIS OF SILVER NANOPARTICLE FROM *L. NEPETIFOLIA* STEM AND FLOWER EXTRACT

Characterization of Silver Nanoparticles:

UV-visible Spectrophotometry, Particle Size, and Zeta Potential Analysis: The silver ion reduction was monitored by measuring the UV-visible spectrum of the reaction medium after diluting solution, ranging from 300-700nm, using a UV-visible Spectrophotometers. A UV visible JascoV-650 spectrophotometer recorded the UV-visible spectra. Laser Particle Size Analyzer LS POP (9)-BGD 249 Binged Instrument determined the particle size distribution profile of biosynthesized FAgNPs and SAgNPs. The zeta sizer is an indicator of surface charge potential, an essential parameter for understanding the stability of the nanoparticles in aqueous suspensions. The potential of FAgNPs and SAgNPs was measured using a Malvern Zetasizer Nano ZS (UK).

FTIR Investigation: FTIR measurements were conducted on the plant extract and synthesized silver nanoparticles to study the Ag nanoparticle and their associated molecules. The FAgNPs and SAgNPs were centrifuged at 8000 rpm for 20 min. The AgNPs were dried and analyzed using a Bruker alpha II FTIR Instrument with a range of 400-4000 cm^{-1}.

The spectrum obtained was compared with a reference chart to identify functional groups present in the sample.

X-ray Diffraction Studies: XRD was used to evaluate the crystalline structure of FAgNPs and SAgNPs and their lattice and made on the powder samples at room temperature.

The prepared samples were obtained by placing them on a glass slide and dried under a hot air oven at 50 °C. Analysis was performed using Bruker X-ray diffractometer instrument at 27 °C, with a Cu X-ray source at 1.5406Ao wavelength in thin- film mode.

FE-SEM Analysis: Field Electron-Scanning electron microscopy (FE-SEM) analysis of FAgNPs and SAgNPs was determined using Zeiss SUPRA 55-VP FE-SEM Electron Microscope. FESEM with a Schottky thermal field emission source. It provides an ultimate imaging resolution in the range of 2nm-200um and includes a blanker and a control system for electron beam lithography.

Antibacterial Activity: The biosynthesized FAgNPs and SAgNPs were analyzed for their antibacterial activity against *Staphylococcus aureus* and *Klebsiella pneumonia* by Kirby-Bauer Disk Diffusion Susceptibility test [disc diffusion method] according to CLSI [2007]. The bacterial cultures were collected from the Department of Biotechnology, LIT premises, Nagpur, Maharashtra.

Minimal Inhibitory Concentration (MIC): The MIC is the lowest concentration of anti-microbial agents that completely inhibits 99% of the microorganism growth. MIC values were measured to confirm the effectiveness of the synthesized silver nanoparticles.

RESULT AND DISCUSSION: The aqueous extract changed from colorless to dark brown and reddish-brown after adding 1mM AgNO3 solution and mixing with a magnetic stirrer for 30 minutes. **Fig. 1.** This color change due to the high content of phenols and flavonoids in the aqueous flower extract of *Leonotis nepetifolia*, indicates surface plasmonresonance synthesized AgNPs. Bio reduction of Ag+ions was pragmatic in the 1mM AgNO3 solutions from *L. nepetifolia* chemical compounds.

Uv-Vis Spectrophotometer:

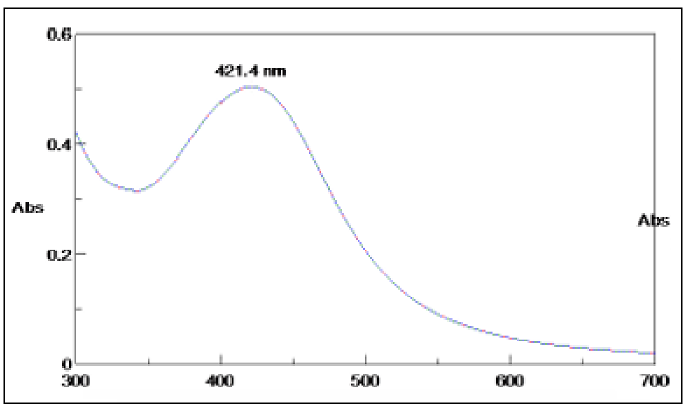


FIG. 2A: UV-VISIBLE SPECTRA OF FAgNPs

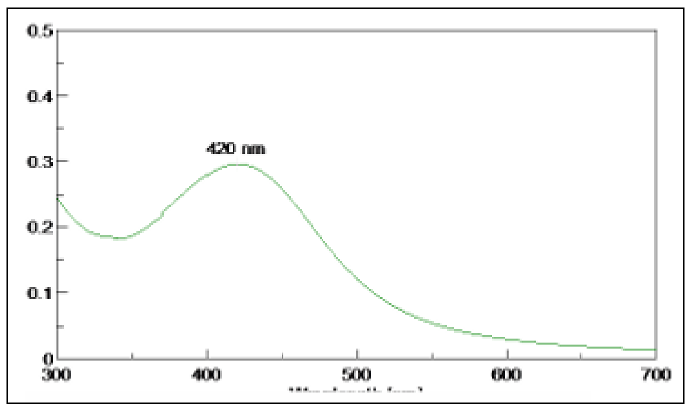


FIG. 2B: UV-VISIBLE SPECTRA OF

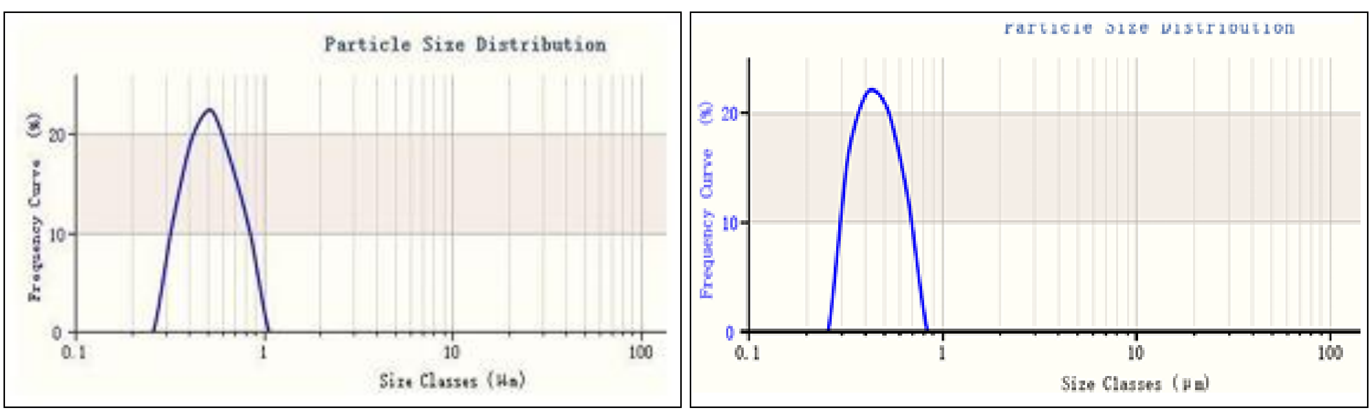


FIG. 3: PARTICLE SIZE DISTRIBUTION OF FAgNPs AND SAgNPs

Fig. 2 shows the UV–vis absorption spectra of synthesized FAgNPs and SAgNPs of *L. nepetifolia*. The maximum absorbance peak for synthesized FAgNPs occurred at 421.4 nm [Abs=0.505375] range, while for SAgNPs occurs at 420nm [Abs.-0.296101], it specified the development of AgNPs respectively. Our similar outcome coincides with Awwad *et al.* (2013), who studied the synthesis of AgNPs using Carob leaf extract, confirmed through a spectrum of the absorption peak at 420nm. **Fig. 3**

Particle Size was analyzed of FAgNPs **Fig. 3A**, and SAgNPs **Fig. 3B** were 0.45µm and 0.46µm respectively.

Zeta Potential (Sizer): The zeta potential results showed the stability of the particle, and the particle carried a net charge of FAgNPs and SAgNPs were measured -12.7mV and -12.3 mV, respectively in **Fig. 4.** It stated that produced nanoparticles had negatively charged on their surface, which indicates the stabilization of AgNPs. Hence, FAgNPs and SAgNPs showed a negative zeta potential and were stable at room temperature.

The expression of negatively charged values specified that the capping molecules that exist on the surface of silver nanoparticles involve a negatively charged molecule and are also responsible for the moderate stability of nanoparticles (Mittal *et al.*, 2014) [10].

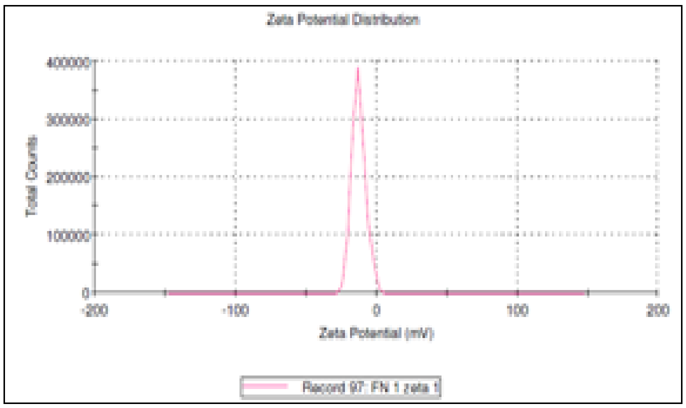


FIG. 4A: ZETA POTENTIAL OF FLOWER $A_{G}NP_{S}$

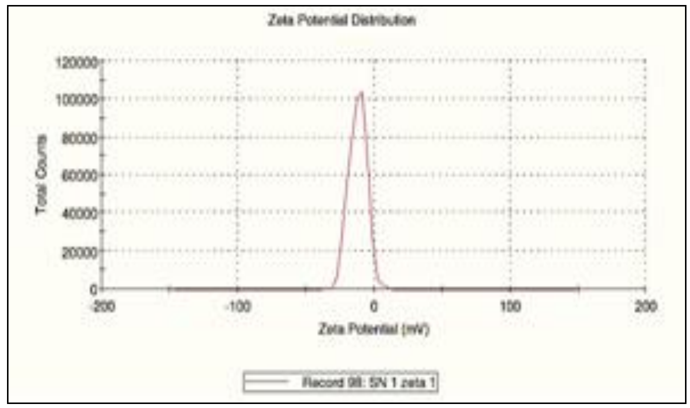


FIG. 4B: ZETA POTENTIAL OF STEM $A_{G}NP_{S}$

Fourier Transform Infrared Spectrum [FTIR]: Fig. 5A shows the synthesized AgNPs and the flower extract analyzed by FTIR. The secondary metabolites present in the fresh extract were responsible for the reduction and stabilization of AgNPs and observed FTIR spectrum.

The FTIR spectrum of FAgNPs showed peaks at 669, 765, 860, 1380, 1423, 1548, 1636, 3253 cm^{-1}. It also implies the presence of several functional groups in the aqueous flower extract and FAgNPs mentioned in **Table 1.**

TABLE 1: FTIR PEAKS 770-735 OF FLOWER EXTRACT AND 765 FAgNPs

Sr. no.	Functional Group	Normal Range	Assigned
1	O-H Stretching	3400-3200	3266
2	O-H Stretching	3550-3200	3253
3	C=C Stretching	1650-1600	1636
4	N-O Stretching	1550-1450	1548
5	C-H Stretching	1485-1340	1423,1380
6	C-H Bending	817-668	860-669
7	C-C Bending	770-735	765

TABLE 2: FTIR OF STEM EXTRACT AND SAgNPs

Sr. no.	Functional Group	Normal Range	Assigned
1	O-H Stretching	3550-3200	3253
2	C=O Stretching	1680-1630	1636
3	C=C Stretching	1550-1450	1546
4	C-H Stretching	1485-1340	1423
5	C-C Stretching	1375-1275	1380,1235,1039
6	C-N Stretching	1200-1350	1281
7	C-O Stretching	1150-1040	1195,1039
8	N-O Stretching	860-800	861
9	C-H Bending	900-700	817-668

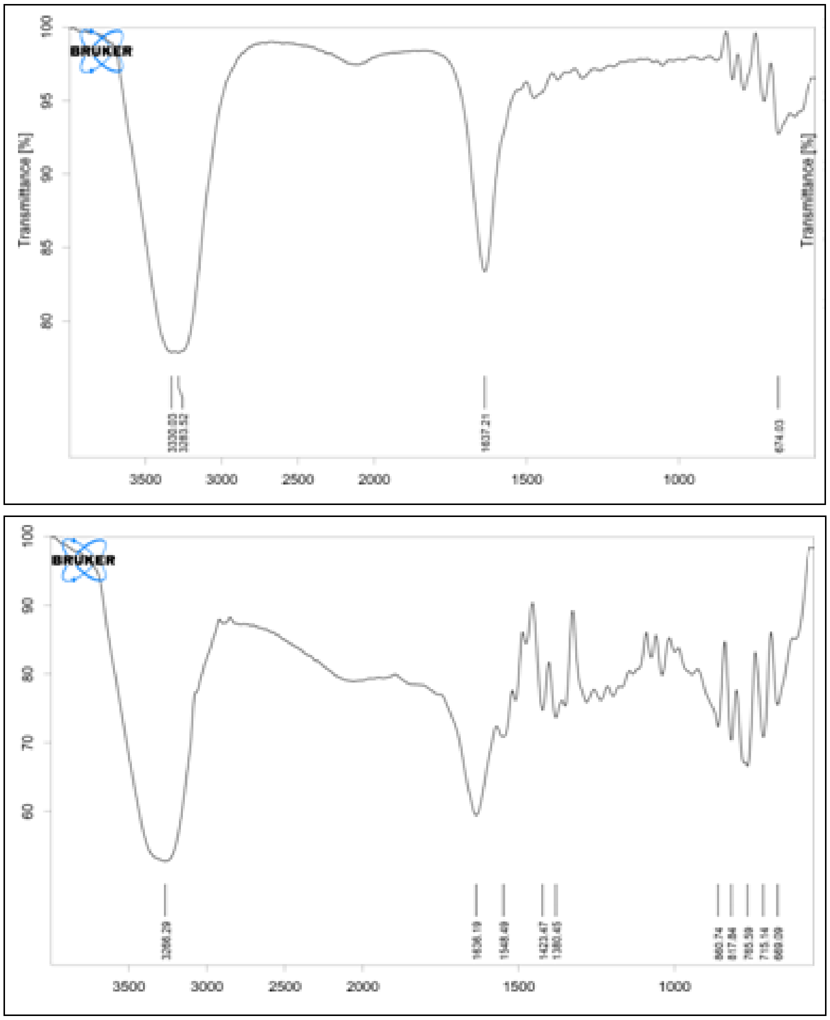
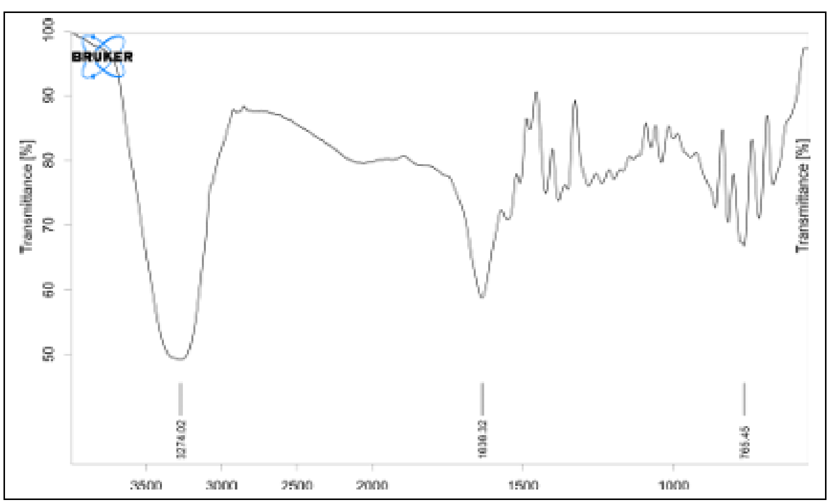


FIG. 5A: FTIR OF FLOWER EXTRACT AND FAGNPS

Similarly, **Fig. 5B** showed the synthesized SAgNPs, and the stem extract was analyzed. The exhibited peaks were observed in range 3253, 1636, 1546, 1423, 1380, 1281, 1195, 1039, 861, 765, 668. The various peaks, along with their position and mode of vibration, are tabulated in **Table 3.** The FTIR spectrum revealed the presence of carbonyl and nitro functional groups from phenolic group, which helps in the formation, reduction, and stabilization of AgNPs.



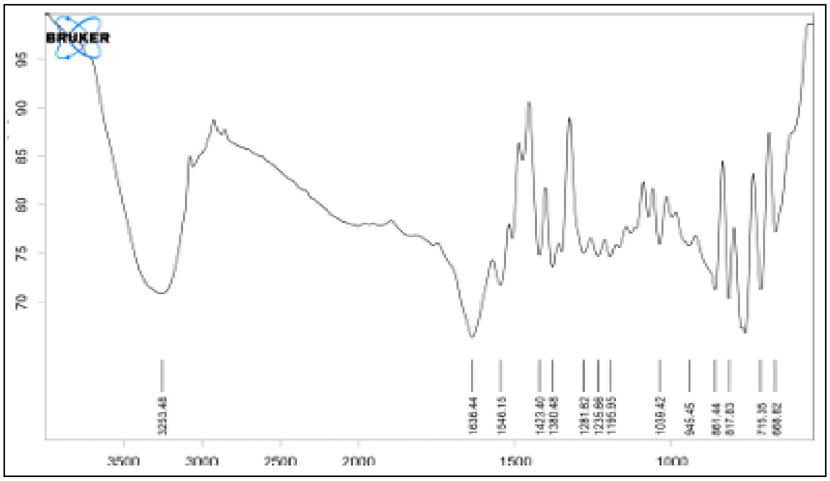


FIG. 5B: FTIR OF STEM EXTRACT AND STEM AGNPS

X-Ray Diffraction Analysis: The *Leonotis nepetifolia* FAgNPs and SAgNPs have crystalline nature confirmed by XRD analysis **Fig. 6.** The XRD showed evidence of diffraction peaks at [111], [200], [220], and [311] appeared. These peaks indicate the crystalline face-centered cubic (FCC) nature of the FAgNPs and SAgNPs, respectively, which concur with the catalog of the Joint Committee on Powder Diffraction Standards [JCPDS]. The intense extra peaks at 2h points of *Leonotis nepetifolia* AgNPs result from the association of AgNO3 for the synthesis of AgNPs (Rajakumar *et al.*, 2017; Taha *et al.*, 2019). The expanding of Bragg's peaks shows the arrangement of nanoparticles, and Debye-Scherer's condition as certain the mean size of the AgNPs was 24 nm. The examples of XRD propose that crystallization of the bioorganic stage happens on the outside of AgNPs. Our outcomes confirm the results of Rajakumar *et al.* (2017) and Taha *et al.* (2019) [11, 12].

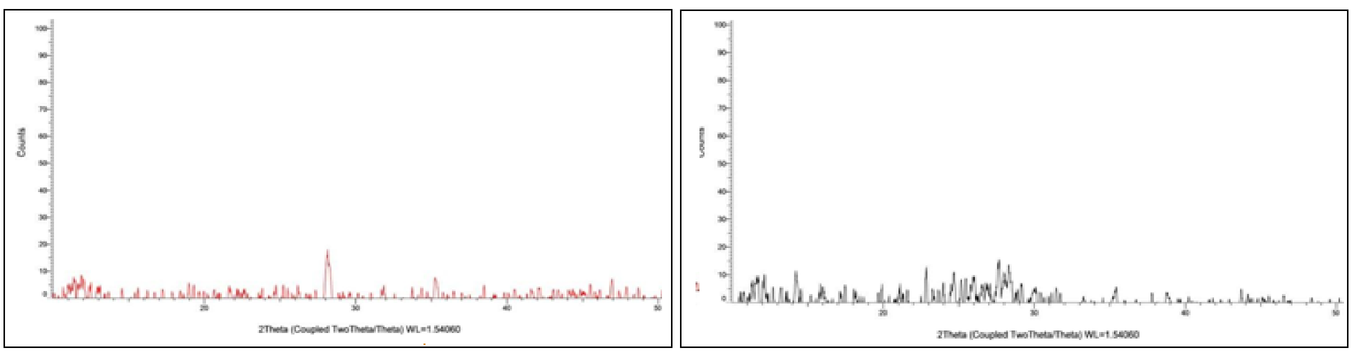


FIG. 6: XRD OF FAGNPS AND SAGNPS

Field Emission -Scanning Electron Microscopy Analysis [FESEM]: The size, shape, and morphology of silver nanoparticles were analyzed using a Field Emission Scanning Electron Microscopy. The obtained FESEM images showed uniformly distributed silver nanoparticles on the surface of the cells in **Fig. 7a. & 7b.**

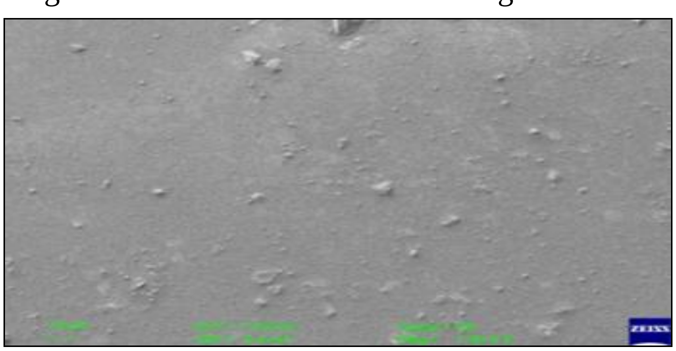


FIG. 7A: FE-SEM OF FLOWER AGNPS

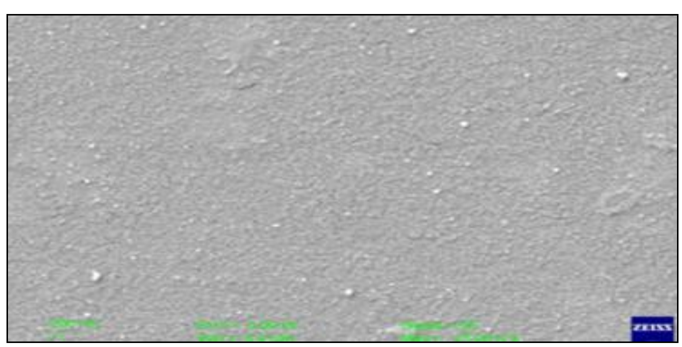


FIG. 7B: FE-SEM OF STEM AGNPS

The synthesized FAgNPs have an irregular shape and morphology distribution in figure.7, from fig. A. The images showed the particles have non-uniformly distributed with varying particle sizes. In figure b, the particles were spherical, ranging from 10 to 200nm of SAgNPs. Thus, the green synthesized FAgNPs and SAgNPs with the smooth-surfaced rectangular plate- like morphology, and the FESEM analysis revealed the agglomerated particles.

Antibacterial Activity: Biosynthesized *L. nepetifolia* flower extract and FAgNPs was against pathogenic bacteria like *Staphylococcus aureus* (Gram-positive), *Klebsiella pneumonia* (Gram-negative) to ensure its antibacterial activity. Notably, the zone of inhibition for *L. nepetifolia* extracts formed, which indicates the extract also has the potential inhibition against bacterial pathogens. But the AgNPs have good antibacterial activity, as shown in **Fig. 8.** The microbial halo that has formed around the well indicates the synthesized AgNPs had antibacterial activity. The flower and stem AgNPs react with the bacteria cell wall and inhibit the respiratory enzymes present in the bacterial cell walls. The synthesized AgNPs have better inhibition in gram positive bacteria than gram-negative bacteria.

Table 3 Here, we compared the zone of inhibition of plant extracts, 1Mm silver nitrate, synthesized FAgNPs, and SAgNPs. Whereas FAgNPs was more potential than the SAgNPs due to flavonoids constituents in flowers.

TABLE 3: ZONE OF INHIBITION

Sr. no.	Compound	Concentration	Zone of Inhibition[mm]	
			S. aureus	*K. pnemoniae*
1.	Standard (Amoxicillin)	200ug/ml	19	21
2.	Flower Extract	200ug/ml	18	17
3.	Silver nitrate	100ug/ml	14	13
4.	Flower, Silver, nanoparticles	50ug/ml	20	22

Sr. no.	Compound	Concentration Zone	E of Inhibition [mm]	
			S. aureus	*K. pnemoniae*
1	Standard (Amoxicillin)	200ug/ml	20	15
2	Stem Extract	200ug/ml	14	11
3	Silver nitrate	100ug/ml	18	22
4	Stem Silver nanoparticles	50ug/ml	16	17

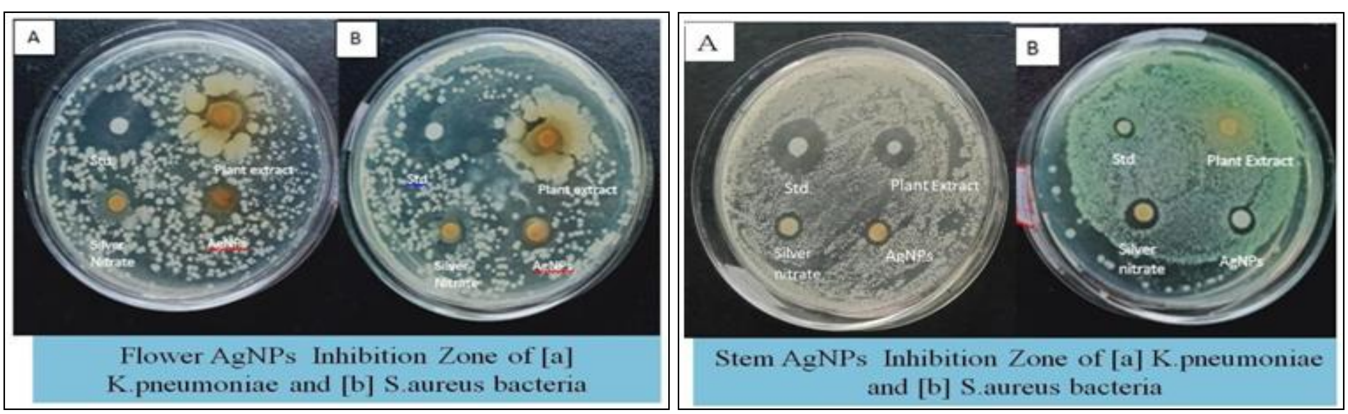


FIG. 8: ANTIBACTERIAL ACTIVITY OF FLOWER AgNPs AND STEM AgNPs

The Minimum Inhibitory Concentration [MIC] determined the lowest concentration of silver nanoparticles that inhibited the visible growth of *Staphylococcus aureus* (Gram-positive), *Klebsiella pneumonia* (Gram negative). The MIC [ug/l] for silver nanoparticles is 50ug, for silver nitrate 1mg, and a standard antibiotic is 0.1mg. The zone of inhibition ranged from 10-25mm. Our present results are under the previous findings, demonstrating that biosynthesized AgNPs have shown intense antibacterial activity [M. Gomathi *et al.* 2020 and Maheshwaran *et al.* 2020] [13, 14].

CONCLUSION: Synthesis of AgNPs from the biological agent is eco-friendly, rapid, low-cost, and proficient in synthesis at room temperature.

The current research has developed an easy and eco-friendly method for synthesizing Flower silver nanoparticles and Stem silver nanoparticles using an aqueous extract of *Leonotis nepetifolia*. The obtained sample of synthesized FAgNPs and SAgNPs was characterized by UV–vis spectroscopy, Zeta potential, XRD, FE-SEM, and FTIR analysis. U-vis absorption spectra confirmed the biosynthesized FAgNPs and SAgNPs supporting surface plasmon resonance reading. XRD spectra confirmed the structure of FCC and crystalline nature in FAgNPs and SAgNPs, respectively. The reducing and capping agents of FAgNPs and SAgNPs are due to phytochemicals and by FT-IR spectra analysis. The FE-SEM monograph revealed the spherical shape and size of FAgNPs and SAgNPs. The synthesized AgNPs demonstrated significant antibacterial activity of synthesized *L. nepetifolia* FAgNPs and SAgNPs especially against *S. aureus*, suggesting their potential in biomedical applications. Thus, this safe and eco- friendly synthesis process to develop AgNPs, demonstrating effective biological properties in the future. Further studies are necessary to get more applications of these biosynthesized FAgNPs and SAgNPs.

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CONFLICT OF INTEREST: No conflict of interest.

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