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ANTIBIOFILM ACTIVITY OF GREEN SYNTHESIZED SILVER NANOPARTICLES OF *MENTHA PIPERITA* L. (PEPPERMINT) AGAINST *ESCHERICHIA COLI*

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Keywords
Mentha piperita; silver nanoparticles; green synthesis; Escherichia coli; biofilm; antibiofilm activity; nanotechnology.

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Abstract

Background: *Biofilm formation by Escherichia coli contributes to persistent bacterial infections and reduced antimicrobial effectiveness. Green-synthesized silver nanoparticles (AgNPs) have emerged as promising antimicrobial agents because of their potential antibacterial and antibiofilm properties. This study aimed to investigate the antibiofilm activity of green-synthesized silver nanoparticles using Mentha piperita L. (peppermint) against biofilm-forming E. coli.*

Methods: *Clinically isolated E. coli strains were obtained from the Microbiology and Biotechnology Research Laboratory, Abasyn University, Peshawar, Pakistan. Aqueous extracts of peppermint were prepared and used for the green synthesis of silver nanoparticles. The synthesized AgNPs were characterized using ultraviolet-visible (UV-Vis) spectroscopy, Fourier-transform infrared spectroscopy (FTIR), and scanning electron microscopy (SEM). Biofilm formation was assessed using the tube adherence method, while the antibiofilm activity of AgNPs was evaluated quantitatively using the microtiter plate assay. Percentage biofilm inhibition was calculated by comparing the optical density of treated samples with that of untreated controls.*

Results: *UV-Vis spectroscopy demonstrated characteristic absorption peaks within the range of 350–460 nm, indicating the formation of silver nanoparticles. FTIR analysis identified several functional groups, including*

an O–H stretching band at 3298.2 cm⁻¹, suggesting the involvement of plant-derived phytochemicals in nanoparticle stabilization. SEM analysis revealed predominantly spherical and rounded nanoparticles with an approximate average diameter of 40 nm. The synthesized AgNPs demonstrated an antibiofilm inhibition of 65.33% against E. coli, indicating substantial inhibition of biofilm formation under the experimental conditions.
Conclusion: *Green-synthesized silver nanoparticles using Mentha piperita demonstrated promising in vitro antibiofilm activity against E. coli. The findings highlight the potential of peppermint-mediated AgNPs as an alternative plant-based approach for controlling bacterial biofilms. Further studies involving different bacterial isolates, concentration-dependent assessments, toxicity evaluation, and in vivo investigations are recommended to establish their therapeutic potential.*

Introduction

The increasing prevalence of antimicrobial resistance (AMR) has become a major global health concern, creating an urgent need to develop alternative antimicrobial agents. One of the major factors contributing to persistent bacterial infections and treatment failure is the ability of bacteria to form biofilms. Biofilms are structured communities of microorganisms attached to living or non-living surfaces and surrounded by a self-produced extracellular polymeric substance (EPS). This protective matrix consists mainly of polysaccharides, proteins, and extracellular DNA, which facilitate bacterial attachment, structural stability, and protection against environmental stress. Biofilm-associated bacteria demonstrate greater tolerance to antimicrobial agents and host immune responses than planktonic bacteria, contributing to persistent and recurrent infections (Ballén et al., 2022).

Escherichia coli is a Gram-negative, facultatively anaerobic bacterium commonly found in the intestinal tract of humans and animals. Although many strains are harmless, pathogenic and opportunistic strains are responsible for several infections, particularly urinary tract infections (UTIs), gastrointestinal diseases, and bloodstream infections. The ability of *E. coli* to form biofilms is an important virulence characteristic that contributes to bacterial persistence and treatment failure. Biofilm development involves several stages, including initial surface attachment, irreversible adhesion, microcolony formation, maturation, and bacterial dispersal. Structures such as type 1 fimbriae, curli fibers, flagella, and extracellular polysaccharides contribute to these processes. Once established, biofilms provide physical and physiological protection against antibiotics and host immune mechanisms, making their eradication particularly challenging (Ballén et al., 2022). Therefore, identifying alternative compounds capable of inhibiting biofilm formation and disrupting established biofilms is essential.

Nanotechnology has emerged as a promising approach for addressing antimicrobial challenges through the development of nanoparticles with distinctive physicochemical and biological properties. Among different metallic nanoparticles, silver nanoparticles (AgNPs) have received considerable attention because of their broad-spectrum antibacterial and potential antibiofilm activities. Their antimicrobial effects involve multiple mechanisms, including interaction with bacterial cell membranes, increased membrane permeability, release of silver ions, generation of reactive oxygen species (ROS), and interference with essential cellular components such as proteins and nucleic acids. These mechanisms can compromise bacterial structural integrity, disrupt metabolic activities, and ultimately cause cell death. The multiple antimicrobial mechanisms of AgNPs make them attractive candidates for investigating alternative strategies against resistant and biofilm-forming bacteria (Mikhailova, 2025).

Conventional nanoparticle synthesis frequently involves chemical reducing agents, stabilizers, and organic solvents that may generate hazardous by-products and environmental concerns. In contrast, green synthesis provides an environmentally friendly and comparatively economical approach using biological materials, particularly plant extracts, as reducing and stabilizing agents. Plant-derived phytochemicals, including flavonoids, phenolic compounds, terpenoids, and alkaloids, can facilitate the reduction of silver ions (Ag^+) into metallic silver nanoparticles while contributing to their stabilization. These naturally occurring compounds may also influence nanoparticle characteristics and biological activities. Consequently, plant-mediated synthesis has gained considerable research interest as a sustainable alternative to conventional nanoparticle production (Mikhailova, 2025).

Mentha piperita L., commonly known as peppermint, is an aromatic medicinal plant belonging to the family Lamiaceae. It is widely recognized for its pharmaceutical and antimicrobial properties. Its leaves contain several biologically active phytochemicals, including menthol, menthone, flavonoids, phenolic compounds, and other terpenoid constituents. These compounds contribute to its antioxidant, anti-inflammatory, and antimicrobial activities. The presence of diverse phytochemicals makes M. piperita a suitable biological material for green synthesis of metallic nanoparticles. Its leaf extract can potentially participate in silver-ion reduction and nanoparticle stabilization, producing biologically functional AgNPs. Previous research has demonstrated the successful synthesis of silver nanoparticles using peppermint leaf extract and reported antibacterial activity against bacterial pathogens, including *E. coli* (Hussain et al., 2023). Similarly, investigations involving other mint species have demonstrated promising antimicrobial properties of plant-mediated silver nanoparticles (Khan et al., 2023).

Despite these promising findings, antibacterial activity against planktonic bacteria does not necessarily establish the ability of nanoparticles to prevent biofilm formation or eliminate established biofilms. Therefore, specific investigations are required to evaluate their effects on bacterial attachment, biofilm development, and mature biofilm disruption. Assessing different nanoparticle concentrations can further establish their concentration-dependent antibiofilm effectiveness.

Therefore, the present study aims to investigate the antibiofilm activity of green-synthesized silver nanoparticles using Mentha piperita L. leaf extract against Escherichia coli. The study evaluates the antibacterial and antibiofilm potential of the synthesized nanoparticles at different concentrations. The findings may contribute to understanding the potential application of peppermint-mediated silver nanoparticles as plant-based nanomaterials for controlling bacterial biofilms and may provide a foundation for further investigations into their mechanisms of action and biomedical applications.

Aims

The purpose of the current study was to determine the antibiofilm activity of AgNps synthesized from *Mentha piperita* against *E. coli* isolates by mi micro titer plate assay while the

Objectives

1. Develop *in vitro* biofilm of *E. coli* isolates in micro titer plate.
2. Utilize the *Mentha piperita* (root, stem and leaves) extracts for the synthesis of AgNps.
3. Characterize AgNPs by TEM, FTIR and UV –Visible Spectra analysis.
4. Determine the biofilm inhibition potential of AgNPs through tube inhibition assay.

MATERIAL AND METHODS

Sample collection

In the current study, clinically isolates *Escherichia coli* were collected from Microbiology and Biotechnology Research Laboratory, Abasyn University Peshawar. Then stored for further process on suitable temperature. Afterward, the isolates were re-identified by Gram staining procedure and biochemical tests and were

processed for biofilm formation in the laboratory (Risal *et al.*, 2018).

Plant Collection and processing

In this study 60gram peppermint plants were collected from district North Waziristan, Khyber Pakhtunkhwa and then were identified taxonomically at the Department of Botany University of Peshawar. The sample was cleaned by removing the dust particles and unwanted materials. Then plant was spread on clean surface and shade dried at room temperature under dust free condition and then grinded to fine pieces. The similar procedure was done by (Donlan *et* al.,2001).

Study Area

All the material in the study were provided by the Department of Microbiology and Biotechnology, Abasyn University Peshawar. The research work is performed at Abasyn university Peshawar and PCSIR laboratories.

Chemicals

Different chemicals were used during this research project including, PBS, LB broth solution, agar, NaCl, AgNO3, hydrogen peroxide, spirits, MHA media, MRS agar.



Figure 1.. **The conversion of Mentha leaf in (Mint) extract**



Figure 2. Picture of prepared plant powder

Sample preparation for silver nano-particles

The extracts of aqueous were made ready from peppermint plant. Take different concentration of fresh healthy and mature peppermint were washed altogether in Distilled water H_2O. After that the plant was crushed into small pieces and put into 100ml Distilled H_2O and heated up to 60c for fifteen minutes. The whole materials were then passed through filter (Whitman No.1) paper with pore size of 0.6 micrometer. In the last the filtrate again filtered with filter paper having 0.6 micrometer. Now, the prepared aqueous solution was used for the development of silver nanoparticles (Praba *et al.*, 2015). The extracts were used for the reduction of Ag+ ion, so the solution was incubated in the dark condition at room temperature for 24hr, to avoid photo activation of sliver nanoparticles. The color changed from colorless to yellowish brown confirmed that Ag+ is reduced to Ag+ Nanoparticles and was verified by the use of UV-Visible spectroscopy also implemented by (Banerjee *et al.*, 2011).

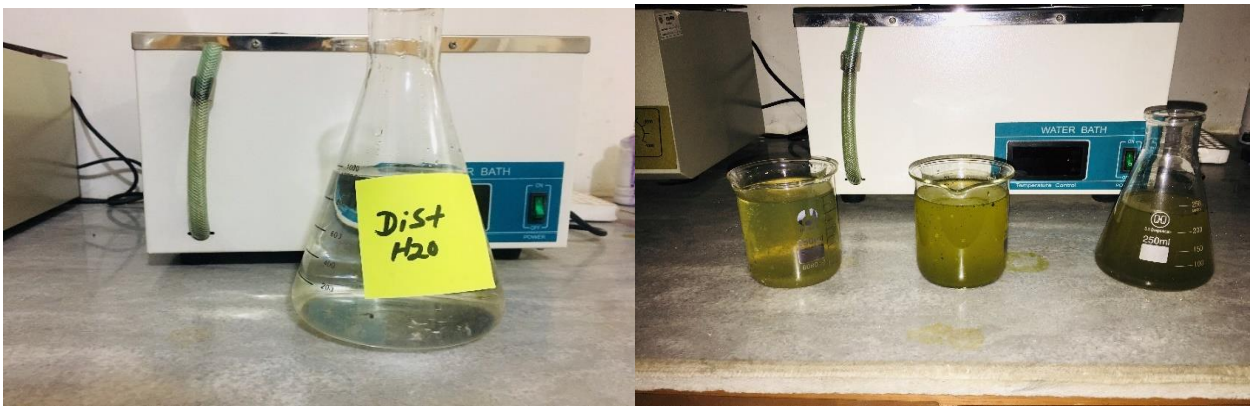


Figure 3# Sample preparation for silver Nano-particles AgNPs

FTIR analyses of SNP, s

FTIR technique was used for identify SNP, s based on functional groups and their absorption during the procedure which characterized the SNP, s synthetized from the peppermint plant. FTIR analyses was performed in PCSIR lab Peshawar (FTIR Model IR Prestige-21(shimadzu japan).



Figure 2. FTIR Model IR Prestige-21(shimadzu japan)

U-V analyses of silver nanoparticles

Synthesized SNP, s was characterized by UV-visible spectrophotometer in PCSIR lab Peshawar. This characterization shows the size, diameter, shape, and morphology of the nanoparticles. SNP, s synthesized from aqueous extract of plant leaves are estimated done by UV spectrophotometer in the range of wavelength from 200-700nm. The spectrophotometric analyses was carried out by the

UV-Visible double beam spectrophotometer – 1700 (shimadzu japan).



Figure 3.UV-Visible double beam spectrophotometer – 1700 (shimadzu japan)

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SEM Analyses of silver nanoparticles

This method is used for the highest magnification of a picture while using electron instead of light. The Scanning Electron microscope (SEM) was used to amplify picture of SNP, s. The SEM pictograph was obtained through (Hitachi S-4500 SEM) a drop of simple was putted on the carbon coated grid, bloating paper was used to remove the addition sample to develops a thin film. The sample loaded grid was then dried under mercury light for 5 munities. The dried sample loaded grid was then analyzed through SEM to obtained pictograph of the sample (Das *et al.,* 2014).



Figure 4. Hitachi S-4500 SEM university of Peshawar Model: JSM5910

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Biofilm Formation Testing with tube adherence method

Formation of biofilm from *E. coli* will only be our main concern in this study. In this method cultivated organism was transferred into a tube containing Trypticase Soy broth (TSB) and incubate for 24 hr. at 37°C. After centrifugation, the supernatant was discarded accordingly and the pallet in the tube was washed by phosphate buffer saline. Then in the process, the tube inner lining was exposed with 0.1% (v/w) crystal violet for the purpose of staining and then again washed with saline water and finally was dried. Visible lining was indicate biofilm at the bottom of tube wall (Gao *et al.*, 2019).

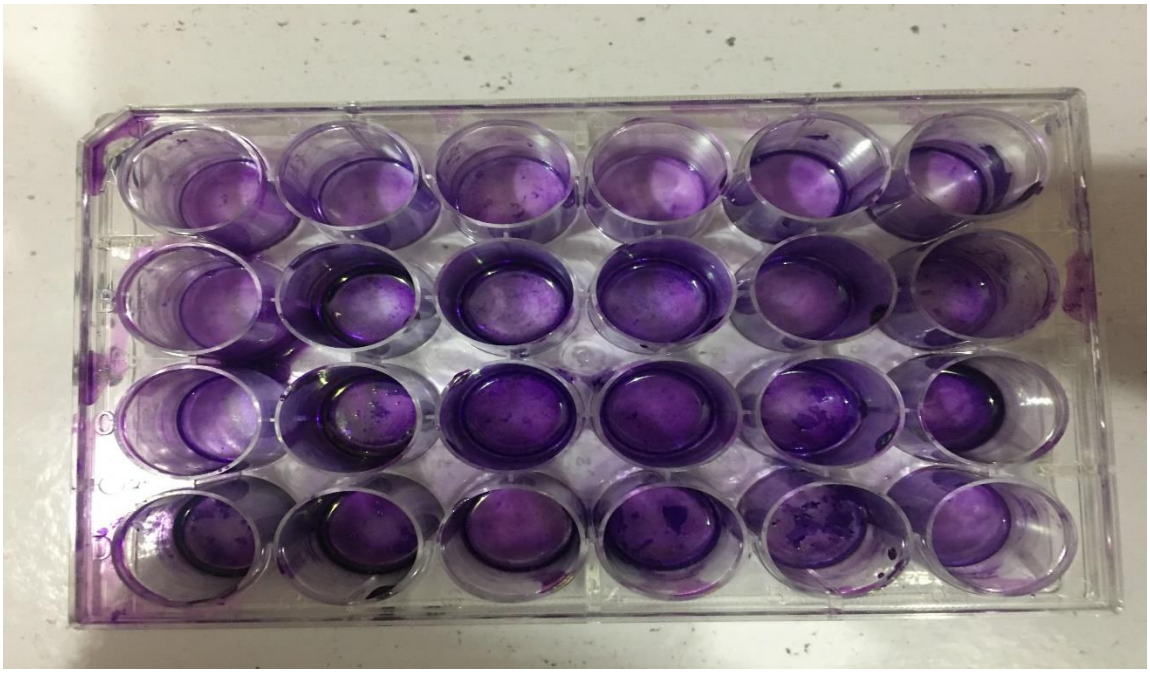


Figure 5. Biofilm Formation Testing with tube adherence method

Anti-biofilm activity Measurement by micro-titer plate method

The optical density of control and experimental well were noticed. After obtaining these values following formula was used:

$$Percentage\ inhibition = \frac{OD\ Negative\ control - OD\ Experimental\ x\ 100}{OD\ Negative\ control}$$

Antibiofilm activity of AgNps by micro-titer plate method

The anti-biofilm activity of AgNPs we used micro titer method which is a quantitative bases measurement. Micro titer plate was labeled for each concentration of AgNPs in triplicate (1-100 mg/ml) and first three wells were labelled for control (without AgNPs). Tubes of optical density value of 0.001 were used for each *E. coli* isolates in triplicate. AgNPs in 100ul concentration was added to each well of the plate. Then nutrient media was added to all wells to complete total 1ml. For Blank (untreated or control) 50ul of sterilized distilled water was added instead of AgNPs. Add Ciprofloxacin at amount of 0.0025 mg/ml (MIC value) was used as the positive control. Plates was then incubated at 37°C in shaking incubator at 120 RPM, for 24 hr. Bacterial suspension from

wells were transferred into a Cuvette and OD (optical density) was determined by spectrophotometer at 600 nm wave length for the checking of AgNPs effect on bacterial growth. The microliter plates were further treated with Crystal violet dye (0.1% W/V) and the excessive stain was removed by using tap water and then air dried. The stained pallet was re-dissolved by adding 1ml acetic acid (33% v/v) in each well. Then liquid from each well was transferred to Cuvette and OD (optical density) was determined at 595 nm (Martinez-Gutierrez *et al.*, 2013). The mean absorbance of the samples was determined, and percentage inhibition of biofilm was determined using the equation below (Sandasi *et al.*, 2008)

Statistical Analysis

Statistical analysis was done by Different tests like t-test and Fisher exact test was used to determine the significance of differences. Results were declared significant when a *p*-value of less than 0.05 is obtained.

RESULTS

The present study was conducted to discuss about the synthesis, characterization and anti-biofilm activity of peppermint plant against Escherichia - coli producing biofilm.

4.1. Characterization of silver Nano-particles

UV-Spectrophotometer analyses of silver Nano-particles

This technique provided information about the optical band and having a great importance. Figure 7 portrays several peaks silver nanoparticles between 350-460 nm. The noticed different peaks because of different volumes of plant extracts and silver nitrate solution in the process of synthases of silver nanoparticles. The upper black line indicates the maximum absorption peaks against smaller size had prepared in 10:1 ratio, whereas others show minimum absorbance with different ratio. This black line also indicated the Ag-NPs are more stable and less partials size are compared to the other ratio of silver nitrate solution.

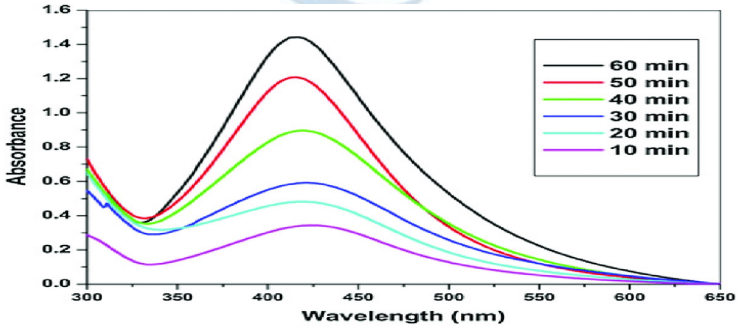


Figure 6. UV-visible absorption spectrum of (Ag-NPs) synthesized by mint plant

FTIR analysis of pepper mint Nanoparticles

This analysis was done for the purity and intact form of Ag nanoparticles. The two known regions in this particle, functional group region and finger print region is given in figure. After FTIR analysis, the peak shows the absorption and flattened curve shows no absorption and hundred percent transmission of the beam. In the functional group, organic compound portrayed absorption, whereas, the metals depicted absorption in the finger print region of the silver Nano-particles due to vibration of the respective atomic molecules. Moreover, 3298.2^{cm-1} represent O-H because of deformation and stretching in water molecule absorbed on the surface of the prepared silver Nano-particles. The wave count for the functional group was noted as; 1643.35,1633.71,1614.42,1588.13,1519.91,1504 .48,1371.39,1357.89,1255.88,1207.44,1151.50,1 028.06,991.97,804.32,696.30 and 612.36.

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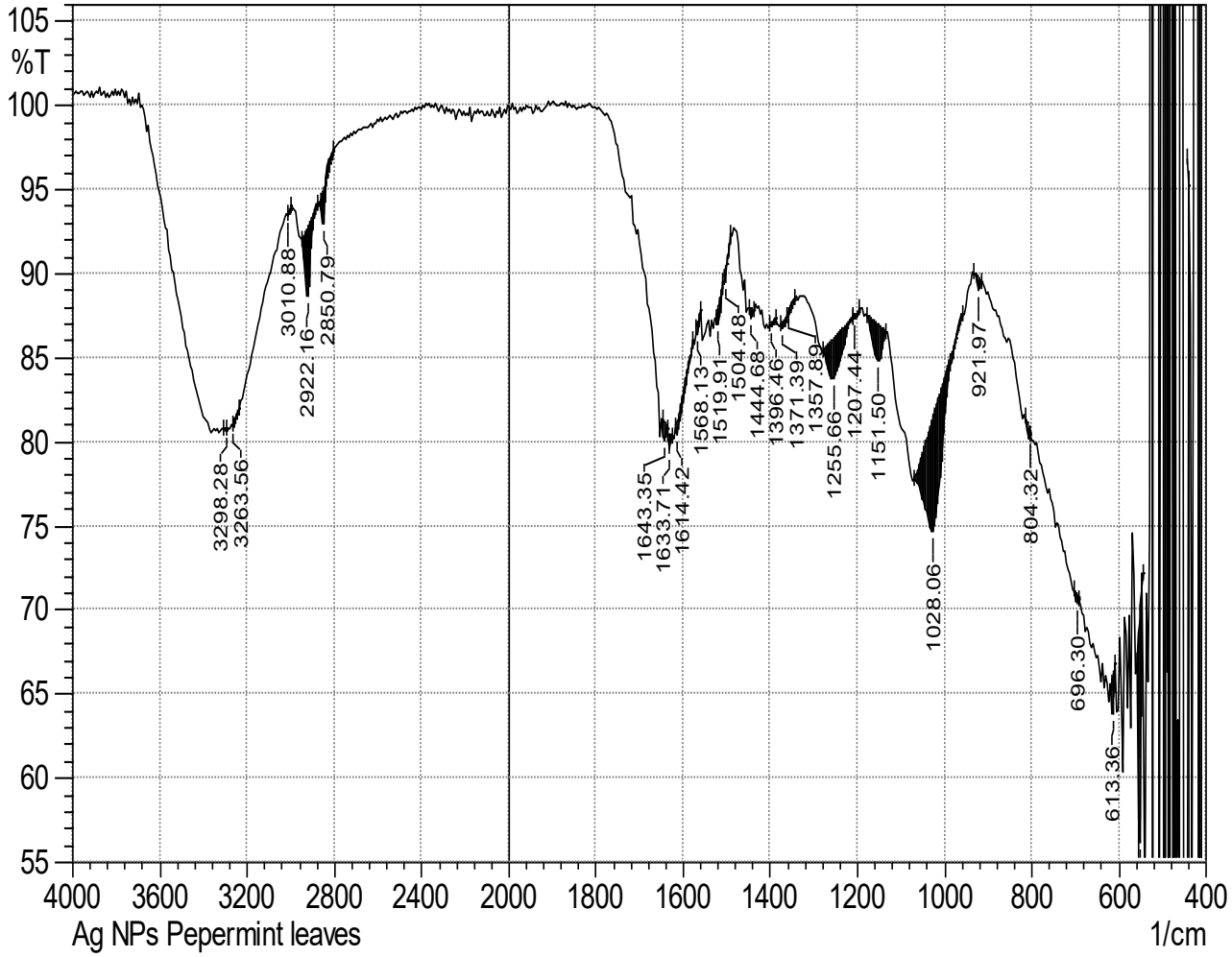


Figure 7:Characteristics of nanoparticles based on FTIR analysis

FE-SEM Analysis of Silver nanoparticles

There are white dots can be easily observed as shown in figure 6.1, the intact white patches provide information about the plant producing silver nanoparticles. Meanwhile, dull white lumps are also present possibly due to stacking of Ag-NPs. Ag-NPs, was prepared earlier, were found in the shape of spherical and round structures. Furthermore, the average size of these nanoparticles was noticed as 40nm in diameter. Meanwhile, the clear picture of small white dots like appearance illustrating the plant mediated silver nanoparticles. While comparing to the previous studies, present results are totally not in the line of previously reported results by Farhat *at* al where the sizes was observed between the range of 4nm -6.4nm (Khan *et* al., 2016

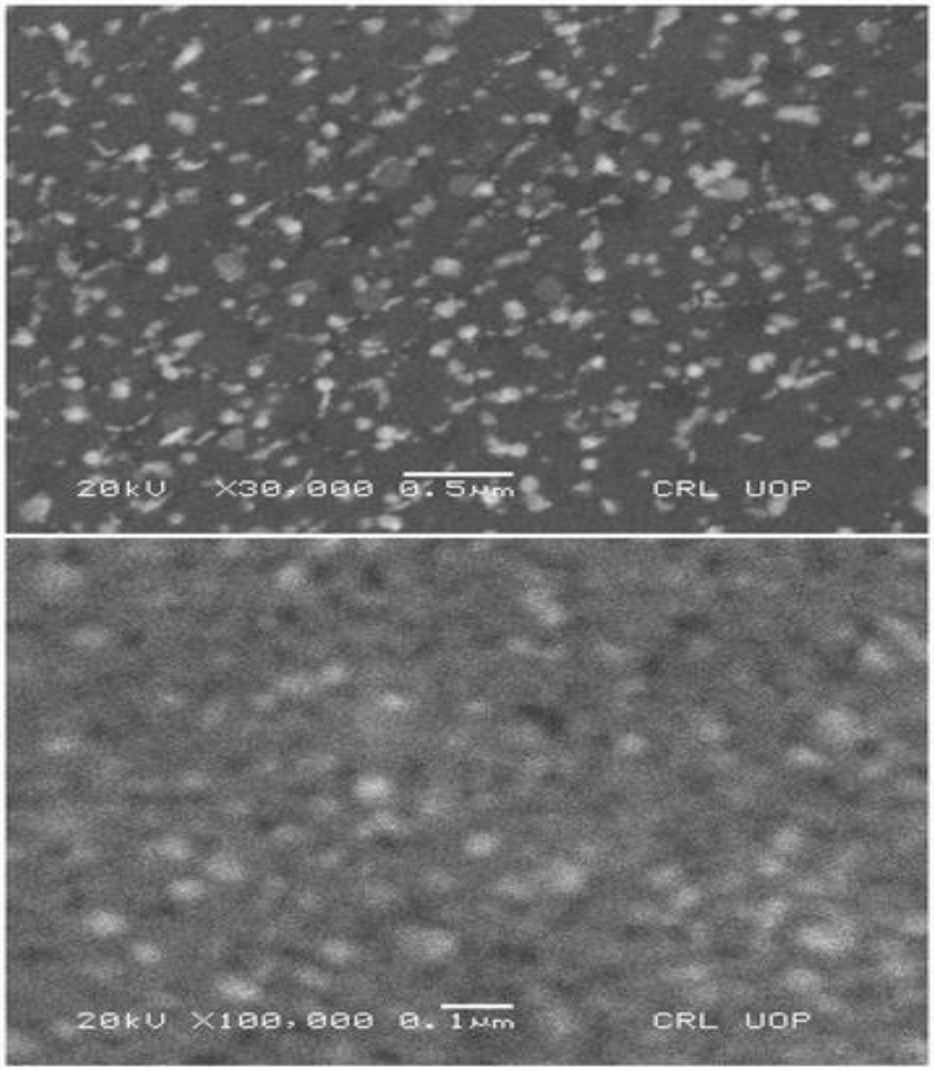


Figure 8: SEM images of Ag NPs that prepared using mint extract

Anti-biofilm activity of AgNP,s

Now, after the calculation we found percentage inhibition value of AgNps against biofilm of *E. coli* was 65.33%. After applying these nano-particles against the E-coli producing biofilm. The absorbance was noticed at 590nm. The mean absorbance of the sample was observed and percentage inhibition formula was used. In the equation, optical density of negative control and experimental sample were observed as 150 and 52, respectively.

4. DISCUSSION

In the area of Nano-technology, the attention has been drastically emerged about green synthesis of metals nanoparticle's due to easily accessible and minimal involvement of cost with less side effects. Silver nanoparticle's synthesis from plant extracts possessing great advantages compare to other metals nanoparticle's (gold, platinum, cobalt) as it has chemical's stability, preparation with low cost and minimal harmfulness. Moreover, the strong antibacterial, antifungals, and anti-inflammatory features as it has along with strong antioxidant properties make them unique. Meanwhile, it also plays a vital role in the cosmetic's composition, food products, dressing of wound, ointments, cream and antiseptics sprays (Noorsaiyyidah *et* al., 2013).

The plant peppermint (leaves, roots and shoots) was used as a main source for silver nano particles. The characterization was noted via scanning electron microscope (SEM) UV-spectrophotometer, and FTIR Fourier transom infra-red spectroscopy. Later, after the characterization the cultured bacteria was targeted by these particles to check the antimicrobial activity.

The technique UV-visible spectroscopy provided information about the optical band and having a great importance. The listed plant extract after filtration gives aqueous solution and then silver nitrate having silver ion exposed to the extract, the decreased rate of silver ions into silver nanoparticles was experiential change seen in his color and change in this color is due to the (SPRP) Surface Plasmon Resonance phenomenon, due to the presence of free electron in the surface of metal nanoparticles along with light wave creates a combined vibration and gives the SPR absorption band.

Meanwhile, several peaks of silver nanoparticles between 350-460 nm was noted in this study. The noticed different peaks because of different volumes of plant extracts and silver nitrate solution in the process of synthases of silver nanoparticles. According to the previous study the maximum absorbance is dependent to silver nitrate solution and plant extract concentration (Mahesh *et al.*, 2007)

Subsequently, the upper black line indicates the maximum absorption peaks against smaller size had prepared in 10:1 ratio, whereas others show minimum absorbance with different ratio. This black line also indicated the Ag-NPs are more stable and less partials size are compared to the other ratio of silver nitrate solution. Meanwhile, a study was conducted by Farhat et al, examined that maximum absorbance is dependent to silver nitrate solution also by (Khan *et al.*, 2019). Moreover, it has been reported that the changes in the peaks is due to the morphological changes of the prepared nanoparticles (Shukla *et al.*, 2010).

Furthermore, FTIR technique was used for the purity and intact form of Ag nanoparticles. The two known regions in this particle, functional group region and finger print region is given in figure. After FTIR analysis, the peak shows the absorption and flattened curve shows no absorption and hundred percent transmission of the beam. In the functional group, organic compound portrayed absorption, whereas, the metals depicted absorption in the finger print region of the silver Nano-particles due to vibration of the respective atomic molecules. Moreover, 3298.2^{cm-1} represent O-H because of deformation and stretching in water molecule absorbed on the surface of the prepared silver Nano-particles. The wave count for the functional groupwasnotedas;1643.35,1633.71,1614.42,1588.13,1519.91,1504.48,1371.39,1357.89,1255.88,1207.44,1151.50,1028.06,991.97,804.32,696.30 and 612.36. The band recorded at 600 cm^{-1} is the band of metal oxide and this result is in the line of previously reported study by Raghunandan *et* al., (2011) and Ankamwar *et* al., (2005).

Lastly, for characterization SEM was used in which white dots can be easily observed in the studied nano-particles. In the results the intact white patches were noticed, provide information about the plant producing silver nanoparticles. Meanwhile, dull white lumps are also present possibly due to stacking of Ag-NPs. Ag-NPs, was prepared earlier, were found in the shape of spherical and round structures. Furthermore, the average size of these nanoparticles was noticed as 40nm in diameter. Meanwhile, the clear picture of small white dots like appearance illustrating the plant mediated silver nanoparticles. While comparing to the previous studies, present results are totally not in the line of previously reported results by Farhat *at* al where the sizes was observed between the range of 4nm -6.4nm (Khan *et* al., 2016)

Interestingly, while comparing this percentage inhibition value which is 65.33 % to previous studies is an effective in invitro treatment by AgNPs against biofilm of E.coli. These previous studies reported the biofilm formation by gram – negative and gram positive bacteria. Inhibition of biofilm is showed as percentage (%) biofilm inhibition. The numbers > 0 % to 50% show low activity, while the value of >50% show high activity

against the bacteria (Famuyide *et* al., 2019). According to the aforementioned study the quantitative measurement of antibiofilm was observed high as 100% in a S. Legati plant extract against E-coli, whereas 83% by E. erythrophylla (Famuyide *et al.,* (2019). Into our understanding, the only study by (Famuyide, I. M *et* al., 2019) was reported regarding quantitative analysis of anti-biofilm activity against E-coli, in which the author used dilution method. Due to this limitation of a smaller number of studies have been reported, we went for doing this process in our study where we found the results quite in the line of the mentioned study. Moreover, presently the MDR nature of the many bacteria has been drastically increased to many drugs which increased morbidity and mortality rate to great extent. On other hand the expensive antibiotics may also has a great influence in the development of these diseases. Currently mountain body of studies are being conducted to overcome the MDR feature of certain bacteria and enhance accessibility for those who cannot afford expensive treatment. Now, one of the main contributions in this is by phyto medicine analysis, loads of studies are being carried out so far in different parts of world. Currently, aiming the same thing we extracted nanoparticles to contribute in the development of innovative antibiotics from plant manta piperita. After completing this analysis as we discussed earlier, we found these nanoparticles quantitatively effective against the *E. col* (Famuyide *et* al., 2019).

Conclusion

We have concluded from the study that the use of medicinal plant for synthesis of AgNO3which is cost effective, rapid, and environment friendly and safe with potential use against microbe like E-coli. Furthermore, the synthesis of silver nanoparticles is very economical, safe, harmless, easy to synthesize. Peppermint has the capacity to produce SNP, s at room temperature for antimicrobial activity. FTIR analyses confirm that capping of AgNo3 by phytochemical by the functional groups presents in the peppermint.

Recommendations

The recommendations include, after this research it may work to be synthesized Nanoparticles from the same plant and should also be checked against insects, parasites and viruses in the future studies.

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