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Medicinal Importance of *Emblica officinalis* leaf extract nanoparticles and their biological evaluation

ABSTRACT

Green Synthesis of Nanoparticles is a novel field of nanotechnology that outperforms both biological and chemical approaches in terms of biocompatibility, cost-efficiency, scalability and environmental friendliness. Bacteria, fungi, plant and algae have lately been used to produce metals and metal oxide nanoparticles as an alternate method. In the present study, the green synthesis of Silver and copper nanoparticles was carried out using leaf extract of Emblica officinalis as a reducing agent and their antibacterial activity against human pathogens. Biosynthesis of nanoparticles were carried out using methanolic leaves extract of Emblica officinalis. Nanoparticles were characterized by UV-Visible Spectroscopy, X-ray Diffraction patterns(XRD), Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). These biogenic particles were tested for antimicrobial activity by disc diffusion method against Escherichia coli & Staphylococcus aureus. Biosynthesized nanoparticles showed potent biological activity and effective radicals scavenging activity. Methanolic leaf extract of Emblica officinalis acts as an excellent capping agent for the formation of silver & copper nanoparticles and demonstrates immense biological activities. Hence, these particles can be used as antibacterial, antioxidant as well as cytotoxic agent in treating many medical complications. It can be concluded that the silver and copper nanoparticles constitute an effective antimicrobial agent against common pathogenic bacteria. This could be a significant achievement in contending with many dynamic pathogens.

Keywords: *Nanoparticles, Green synthesis, XRD, Emblica officinalis, Biological agents*

1. INTRODUCTION

Green particles is the ideal approach to minimize the effectively of nanomaterial manufacturing and application while also reducing the risk of problems associated with other methods [1]. Biogenic nanoparticles have potential applications and have gained considerable interest in different fields such as biomedical filed, agriculture information technology, optical, environment, energy, and sensors [2]. These metals particles include Silver Copper Zinc and Iron [3]. Ultimately, our aim is to provide a strategy for “green” synthesis and associated components that will help researchers working in this area while also serving as a useful reference for readers interested in the subject in general [4]. Green synthesis methods utilize biological activity such as bacteria, algae, viruses, fungi and plants that are used for the synthesis of nanoparticles are non-pathogenic in nature because they should not interfere with the application of synthesized nanoparticles [5]. Bacterial infections most illnesses caused by bacteria are associated with the consumption of infected fresh products. The U.S. Center for Disease Control and Prevention (CDC) estimates that forty-eight million people get sick from a food borne illness each year [6], meaning, that microbial pathogens are responsible for one of the leading causes of life-threatening illnesses around the world. Among the most common food borne disease-causing bacteria, in the United States, are *E. coli* and *S. aureus* [7]. The consumption of food contaminated with these pathogenic bacteria could cause diarrhea, abdominal cramps and nausea. However, it could also lead to chronic illnesses such as cancer, brain and neural disorders, kidney and liver failure [8]. Hence, the attention has been focused on the production of novel nanoparticles- based on materials with enhanced antimicrobial properties[9]. It is well-known that Silver and copper is a potent antimicrobial agent whose properties have been exploited to inhibit bacterial growth and destroy the cellular structure of microorganisms [10]. In

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this paper, we present synthesis of silver and copper nanoparticles produced using a biological method. This synthesis route provides an enhanced alternative to conventional synthesis method as it leads to the formation of smaller and monodisperse metallic nanoparticles in a shorter time, thus decreasing the use and quantity of toxic reagents [11]. UV-Vis analyses were carried out to confirm the presence of Silver and Copper in the nanoparticles form. The NPs were characterized in terms of crystallinity through XRD, and morphology and nanoparticle size distribution by TEM and SEM [12].The antibacterial activity of the nanoparticles was tested against *E. coli* and *S. aureus*, all of which are well known to be responsible of the most common food borne illnesses [13]. To the best of our knowledge, the use of E. officinalis plant extract for greener synthesis of Copper & Silver nanoparticles has not been reported. Hence the present study was carried out to synthesize and characterize the copper & silver nanoparticles using E. officinalis plantextract. The aim of the present study is the green synthesis, characterization, and applications of plant-derived Nanoparticles using E. officinalis leaf extract as a reducing/stabilizing agent and to investigate their antibacterial activity against human pathogenic bacteria.

2. MATERIAL AND METHODS

Collection of Plant Material

Healthy, disease-free leaves of *E.officinalis* were collected during the month of August from Jaipur, Rajasthan India. The collected leaves were washed thoroughly in tap water and then in detergent water and were finally rinsed with distilled water until no foreignmaterial remained. The freshly cleaned leaves were left todry in sun light for approximately 10 days after dried leaves used for further experiment.

Preparation of Plant Extract

Take 500 gm of dried leaves were washed twice in tap water and rinsed thrice in distilled water. Then they were surface sterilized by 70% isopropyl alcohol for 1 min, cut into small pieces, dried in the micro-oven, and ground into powder using an electronic blender. About 100 g of leaf powder material was uniformly packed into a thimble and run in soxhlet extractor. It was extracted with methanol for the period of about 5–6 cycles. After that extracts were filtered with the help of Whatman No. 1 filter paper. The filtrates were then evaporated under reduced pressure and dried using a rotary evaporator at 55ºC. Then the extract was kept in refrigerator at 4ºC for future experiments [14].

Synthesis of Silver and Copper oxide Nanoparticles

For reduction of silver ions, 10 ml of collected filtrate was treated with 90 ml of silver nitrate aqueous solution (21 gm of AgNO3 powder in 125 ml of Milli Q water) and incubated at room temperature for 10 mins. Similarly for reduction of copper ions, 15 ml of collected filtrate was treated with 85 ml of copper sulphate aqueous solution (21 gm of $CuSO_4$ powder in 125 ml of Milli Q water) and incubated at room temperature for 10 mins. After 8 hours of incubation, the solution was centrifuged with 12,000 rpm for 20 min, and their pellets were redispersed in sterile distilled water. The centrifugation and redispersion were repeated three times to ensure the complete separation of nanoparticles. After drying, purified nanoparticles were resuspended in de-ionized water and stored in a freezer for further study [15].

Characterization of Nanoparticles

Purified nanoparticles were characterized for their morphology using a UV-Visible Spectroscopy, ZEISS EVO series Scanning Electron Microscopy model EVO-50 (SEM), IIT Delhi, Philips CM200 model of Transmission Electron Microscopy and X-Ray Diffraction (XRD) Rigaku Miniflex 600 model [16].

Antibacterial Activity

Copper and silver oxide nanoparticles synthesized using *E. officinalis*leaves extract were tested for antimicrobial activity by agar well diffusion method against different pathogenic microorganisms *E. coli* and *S. aureus*. Each strain was swabbed uniformly onto the individual plates using sterile cotton swabs. The sterile disc approximately 6mm in diameter of Whatman filter paper discs were prepared by applying 50µg/ml and 100µg/ml of synthesized nanoparticles for bacterial cultures and also applying for Standard antibiotics streptomycin sulphate. The disc was dried in hot air oven until it gets fully dry. The pure cultures of bacteria were subculture on Muller Hinton Agar. The agar suspension is poured into sterile petri-plates and allowed to solidify. Than the pathogenic bacterial strains *E. coli* and *S. aureus* fresh overnight cultures were spreaded evenly over the entire surface of the plate by swabbing in three directions.The sterile discs so prepared were kept in the centre of all petri plates after they were fully dried and incubated at 37°C for bacterial cultures. The standard antibiotic discs Streptomycin sulphate purchases from CDH (laboratory reagent) New Delhi were used. The activity was clearly visible after 24-48

hrs. for bacterial cultures. The zone of inhibition was noted for all the petri plates [17].

3. RESULTS AND DISCUSSION

In the present study, *E. Officinalis* leaf methanolic extract is used to produce silver and copper oxide nanoparticles. The color change from green to dark brown and the absorbance peak at about 439 nm indicated the formation of nano-particles. Reduction is followed by an immediate change in colour from light brown to black and change in pH of the solution. On mixing, E. officinalisleaf methanolic extract with the aqueous $AgNO_3$, solution and FeCl3, it changed the color of the solution immediately and reducing the pH, which indicates the formation of silver and copper nanoparticles. It was observed that the pH changed from high acidic to low acidic. For reduction of copper ions, 15 ml of collected filtrate was treated with 85 ml of copper sulphate aqueous solution (21 gm of $CuSO_4$ powder in 125 ml of Milli Q water) and incubated at room temperature for 10 min, resulting in the formation of sea green to dark navy brown color indicating the synthesis of silver nanoparticles. From the leaf extract of *Eugenia jambolana,* silver nanoparticles synthesis was carried out and their phytochemical screening was evaluated [18]. Earlier, reports are available regarding the formation of AgNPs and their biological applications from *Syzygium cumini* [19] *Eugenia caryophyllata* [20]. From the leaf extract of *Eugenia uniflora,* silver nanoparticle formation was carried out and their antibacterial and antidiabetic potential were evaluated [21]. It is essential that these NPs be precisely and thoroughly characterized in order to ensure reproducibility in their production, biological activity, and safety. For this purpose, a wide range of physicochemical methods are used to very precisely characterized the synthesized NPs including ultraviolet-visible spectroscopy, Fourier transform infrared spectroscopy (FTIR), attenuated total reflection (ATR), Raman spectroscopy, photoluminescence analysis (PL), dynamic light scattering (DLS), UV-visible diffuse reflectance spectroscopy (UV-DRS), transmission electron microscopy (TEM), scanning electron microscopy (SEM), atomic force microscopy (AFM), field emission scanning electron microscopy (FE-SEM), X-ray diffractometer (XRD), X-ray photoelectron microscopy (XPS), energy dispersion analysis of X-ray (EDAX), thermal gravimetric differential thermal analysis (TG-DTA), or nuclear magnetic resonance (NMR) [22-24].

UV-Vis Spectroscopy Analysis

Formation and stability of silver and copper oxide nanoparticles in sterile distilled water is conformed using UV-Vis spectroscopy in a range of wavelength from 300nm to 800nm as shown in Figure 1. The synthesis of silver and copper oxide nanoparticles of the surface Plasmon resonance of silver occurs at 331nm and 451nm where as copper wavelength showed 310nm and 561nm, steadily increasing with the time of reaction without much change in the peak wavelength. In this study, after mixing of extract and silver nitrate solutions a color change of extract was observed over the progression of time which may be due to the reduction of the silver ions leading to the excitation of Surface Plasmon Resonance (SPR) of the AgNPs [25]. To confirm this, UV spectra analysis was carried out and a peak was observed at 451nm & 561nm of silver & copper nanoparticles which showed a stable range for nanoparticles formation. In *Syzygium cumini* the UV spectra of synthesized nanoparticles was observed at~450 nm with particle size 3.5 nm from the XRD analysis [26] and also in Eugenia unifora UV spectra of synthesized nanoparticles was observed at 44 nm having its particle size was ranging from 25 to 50 nm. [27]

X-ray diffraction pattern (XRD) Analysis

X-ray diffraction pattern (XRD) was recorded for the synthesized Silver Nanoparticles and Copper Nanoparticles. Three distinct diffraction peaks at 35.04°, 43.23°, and 64.37° were indexed with the planes (111), (200), and (220) for the face-centered cubic silver as per (20). The X-ray diffraction pattern of as prepared copper nanoparticles is shown in Figure 2. Three peaks are observed at 2θ values of 39.46°, 45.65° and 65.73° and indexed as (111), (200) and (220) respectively, which confirmed the monophasic nature of pure copper with face centered cubic symmetry. The peaks are broad with fair intensity indicating the nanocrystalline nature of copper powder. The well resolved and intense XRD pattern clearly showed that the Ag NPs formed by the reduction of Ag+ ions using *Emblicaofficinalis* leaf extract are crystalline in nature. Similar results were reported for Silver Nanoparticles in the literature [14-17, 28, 32-35]. The low intense peak at 77.34 belongs to (311) plane [28]. The result indicated that the formation of a typical monoclinic structure for all weight fraction of copper oxide & iron oxide nanoparticles. Moreover, sharp peaks confirmed CuO Nps is highly crystalline nature which is good agreement with (JCPDS card no. 801268 [29-30].

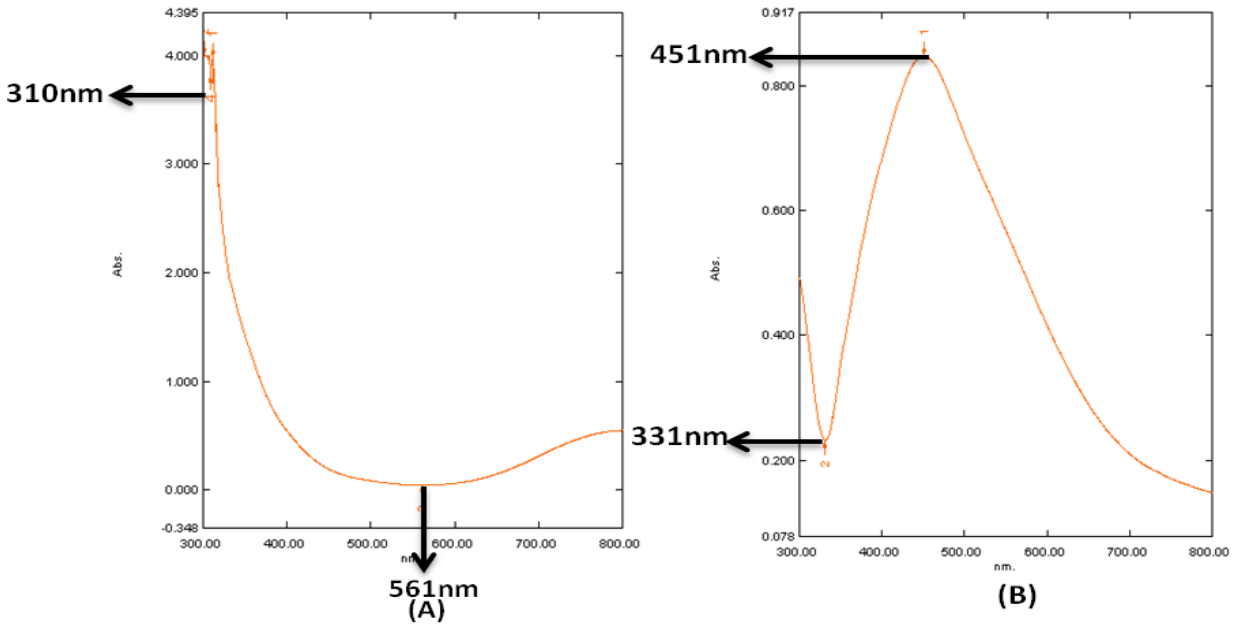
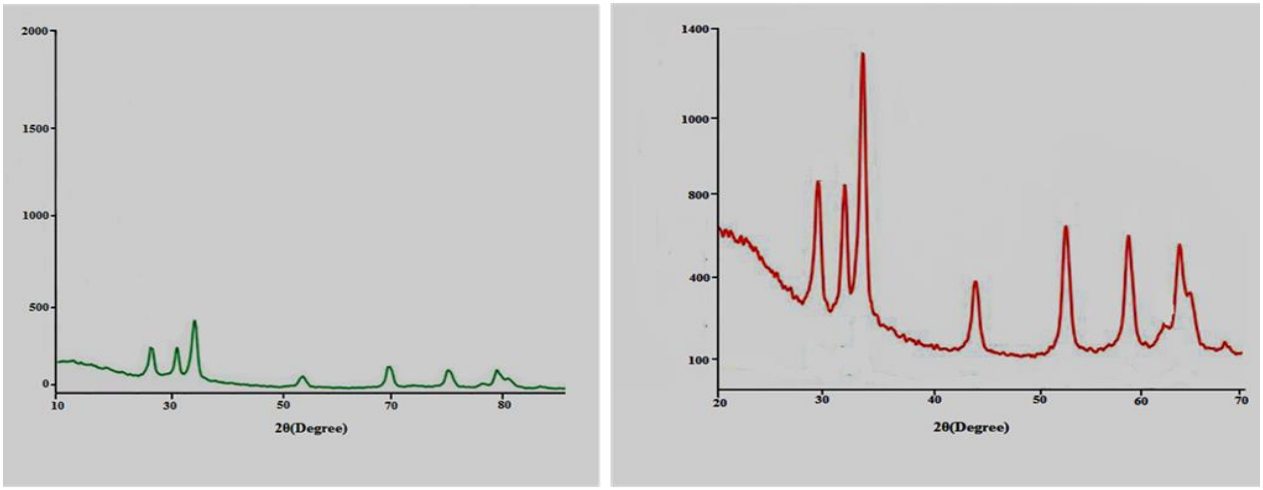


Figure 1. 1 UV – Vis absorption spectra of copper nanoparticles (A) silver nanoparticles sinthesized by biological method



(A) *(B)*

Figure 2. XRD patern of sinthesized copper nanoparticles (A) and silver nanoparticles (B)

Scanning Electron Microscopy Analysis

Scanning Electron Microscopy analysis was done using ZEISS EVO series SEM model EVO-50 machine. Thin films of synthesized and stabilized copper and silver nanoparticles powder was placed on carbon tape coated very small amount of the sample on the grid extra solution was removed using blotting paper and then the film on the SEM grid were allowed to dry by putting it under a mercury lamp for 15min. and sample was analyzed for morphology and size of the copper and silver nanoparticles. The SEM image showed relatively crystalline spherical shape nanoparticles formed with diameter range 50-1000 nm. SEM analysis shows uniformly distributed copper and silver nanoparticles on the surface of the cells, because those dispersing in the solution may also deposit on to the surface of the cells whereas they showed only change of particles surface due to increased permeability.

Transmission Electron Microscopy Analysis

Transmission Electron Microscopy analysis of copper and silver nanoparticles sample was dispersed in double distilled water a drop of thin dispersion is placed on a “staining mat”, carbon tape

coated grid is inserted in to the drop with the coated side upwards. After about 15 minutes, the grid is removed and air dried. Then screened in Philips CM-200 model of transmission electron microscopy at an accelerating voltage of 80kv. The abstained nanoparticles are in the range of size 50-200nm and few particles are agglomerated; it is evident that there is variation in particles size and the average size estimated. It was found that the average size and distribution by taking micrograph from drop coated films of the copper and silver nanoparticles shows that most of them are crystalline spherical with the average size range from 200nm which correlated with the morphology of the nanoparticles which is highly variable, with crystalline spherical and occasionally triangular nanoparticles observed in micrograph. Moreover, the nanoparticles were not in direct contact even within the aggregates, indicating stabilization of the particles by a capping agent.

Analysis of Antimicrobial Activity

The antibacterialassay was performed against two bacterial pathogens using green-synthesized silver and copper nanoparticles. Silver and Copper oxide nanoparticles at concentration of 150µg/ml showed excellent antibacterial activity against S. aureus & E. coli. as compared to standard antibiotics Figure 3. In previous studies, it was reported that nanoparticles the green antibacterial synthesized silver activity against Staphylococcus aureus, Escherichia coli, Proteus vulgaris showed the maximum inhibition of bacterial zone.Silver & Copper Nanoparticles exhibit a high antibacterial effect due to their well developed surface which provides the maximum contact with the environment. Furthermore, toxicity is presumed to be size and shape dependent because small size of nanoparticles may pass through cell membranes. Insides a bacterium, nanoparticles can interact with DNA, thus losing its ability to replicate which may lead to the cell death. Green synthesized nanoparticles also have the more effective antimicrobial zone inhibition of the pathogens. To enhance the antimicrobial activity of the leaf extract we have tried to prepare AgNPs from the extract to access the effect of the nanoparticles, we have used it to inhibit the growth of biofilms by S. aureus. As it has been seen that nanomaterial is better at combating microbes than normal crude extracts, our present investigation will help evaluate the antimicrobial effect of Emblica officinalis AgNPs. Adhesion or attachment of microorganisms to a substrate is the first step towards colonization and this strategy has been used for microbial biofilm production [31]. In this study, a new approach was undertaken by synthesizing nanoparticles from biomaterial and using them against biofilm-producing microorganisms to test their effects on them. Silver and Copper oxide nanoparticles synthesized at various concentrations showed higher antimicrobial activity as compared to standard antibiotics (Table 1 & 2).

The reason could be that the smaller size of particles which lead to increased membrane permeability and cell destruction.. The zone of inhibition increases with increasing concentration of silver and copper nanoparticles as the nanoparticles bind with cytoplasmic membrane and killed the bacterial cell. As compare to copper nanoparticles, electrostatic attraction of silver nanoparticles causes damage of bacterial cell membrane to the formation of pits on the surface, and these structural changes take place due to cell expiration [32-35]. Previous studies have demonstrated the antimicrobial effect of iron oxide nanoparticles. Naseem and Farrukh evaluated the antimicrobial efficiency of IONPs by five different plants [36]. They observed that the iron oxide nanoparticles synthesized via Lawsonia inermis and Gardenia jasminoides have good antimicrobial effect against Salmonella enterica, Proteus mirabilis, Escherichia coli and Staphylococcus aureus.Our results are in agreement with Chifiriuc et al (2012) who reported Fe3O4 nanoparticles covered with Rosmarinus officinalis essential oil had strong inhibitory activity on biofilm-forming C.tropicalis and C. albicans [37]. The present results coincides with Beher etal who reported ZnO and CuO NPs had best antibacterial behavior against both Gram-positive and Gram-negative bacteria compared with Fe2O3 NP. The antimicrobial activity of the IONPs closely correlates to the oxidative stress generated by reactive oxygen species (ROS). ROS including hydrogen peroxide, superoxide radicals (O2–), hydroxyl radicals (–OH) and singlet oxygen could cause damage to DNA and proteins in pathogenic organisms such as bacteria and fungi [38].Silver oxide and Copper oxide nanoparticles interfere with the bacterial cell membrane and bind with mesosome cell organelle and after that reduce the mesosomal function and increase the ROS generation. Nanoparticles interact with thiol groups in protein which induced the inactivation of the bacterial protein synthesis as well as DNA replication [39-40]. Similarly, oxygen associates with silver and copper reacts with the sulfhydryl (−S-H) groups on cell wall to remove the hydrogen atoms causing the sulfur atoms to form an R-S-S-R bond, blocked the respiration, and causing the lethal effect of bacterial cells [41]. Nanoparticles naturally interact with the membrane of bacteria and disrupt the membrane integrity; silver ions and copper ions bind to sulfur, oxygen, and nitrogen of essential biological molecules and inhibit the bacterial growth.

Table 1. Screening of Silver nanoparticles against bacterial strains tested

Bacterial cultures	Silver nanoparticles			Streptomycin sulphate		
	50 µg/ml	100 µg/ml	150 µg/ml	50µg/ml	100 µg/ml	150 µg/ml
Staphylococcus aureus	20 mm	32mm	40mm	18mm	27mm	37 mm
Escherichia coli	22 mm	37 mm	45mm	15mm	30mm	35 mm

Table 2. Screening of Copper nanoparticles against bacterial strains tested

Bacterial cultures	Copper nanoparticles			Streptomycin sulphate		
	50µg/ml	100µg/ml	150µg/ml	50µg/ml	100µg/ml	150µg/ml
Staphylococcus aureus	22 mm	34 mm	46mm	18mm	27mm	37 mm
Escherichia coli	25 mm	39 mm	49mm	15mm	30mm	35 mm

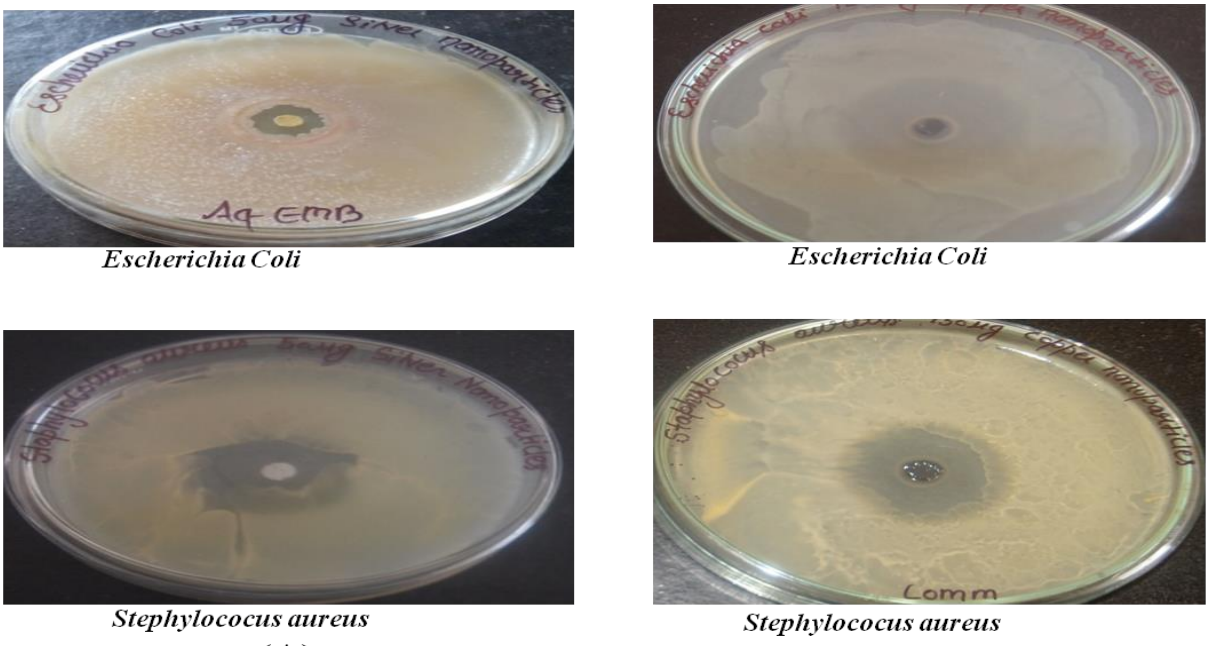


Figure 3. Antimicrobial activity studies on two selected bacteril culture against (A) silver nanoparticles (B) copper nanoparticles

4. CONCLUSIONS

The growing demand for green chemistry and nanotechnology has pushed for the development of green synthetic methods for the production of nanomaterials using plants, microbes and other natural resources. The silver and copper nanoparticles prepared from *E.officinalis* leaf extract were observed under UV–Vis Spectroscopy monitored at 451 nm & 561 nm and their crystallinity nature was confirmed from their XRD study. The antimicrobial activity depends upon the concentration of silver and copper nanoparticles to produce the most significant effects against the pathogenic bacterial growth. AgNPs are found to be very effective against biofilm production by bacteria. Toxicological studies are also required to eradicate any kind of intoxication in a mouse model or human being. Once the NP is found nontoxic or safe in vivo studies, the nanoparticles can be utilized for the treatment of various diseases such as diabetes, arthritis, hypertension, etc.

This green-synthesized method is rapid, facile, convenient, less time consuming, environmentally safe, and can be applied in a variety of existing applications. This plant leaf extract compounds can be extended to the synthesis of other metal and metal oxide nanoparticles. This study springs a new approach for synthesizing nanoparticles from the leaves of *E. officinalis* which is found out to be inhibiting biofilm production and bacterial colonies can be a significant achievement in contending many dynamic pathogens. So, the present work can be considered an attempt to exploit the active

principle present in the leaf of *E. officinalis* to cure various ailments.

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Conflict of interest

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IZVOD

LEKOVITI ZNAČAJ NANOČESTICA EKSTRAKTA LISTA *EMBLICA OFFICINALIS* I NJIHOVA BIOLOŠKA PROCENA

Zelena sinteza nanočestica je nova oblast nanotehnologije koja nadmašuje i biološke i hemijske pristupe u smislu biokompatibilnosti, ekonomičnosti, skalabilnosti i ekološke prihvatljivosti. Bakterije, gljive, biljke i alge se u poslednje vreme koriste za proizvodnju metala i nanočestica metalnih oksida kao alternativne metode. U ovoj studiji, zelena sinteza nanočestica srebra i bakra je sprovedena korišćenjem ekstrakta lista Emblica officinalis kao redukcionog agensa i njihove antibakterijske aktivnosti protiv ljudskih patogena. Preliminarna fitohemijska analiza metanolnog ekstrakta E.officinalis pokazala je prisustvo tanina, saponina, flavanoida i fenola, od kojih su flavonoidi i saponini najinhibitivniji prema svim patogenima. Emblica officinalis definitivno poseduje snažno antimikrobno dejstvo protiv bakterijskih patogena i može se koristiti u lečenju različitih bolesti izazvanih ovim organizmima. Biosinteza nanočestica je sprovedena korišćenjem metanolnog ekstrakta listova Emblica officinalis. Nanočestice su okarakterisane UV-vidljivom spektroskopijom, uzorcima difrakcije rendgenskih zraka (XRD), skenirajućom elektronskom mikroskopijom (SEM) i transmisijskom elektronskom mikroskopijom (TEM). Ove biogene čestice su testirane na antimikrobnu aktivnost metodom disk difuzije protiv Escherichia coli i Staphilococus aureus. Biosintetizovane nanočestice pokazale su snažnu biološku aktivnost i efikasnu aktivnost uklanjanja radikala. Metanolni ekstrakt lista Emblica officinalis deluje kao odlično sredstvo za zatvaranje za formiranje nanočestica srebra i bakra i pokazuje ogromne biološke aktivnosti. Stoga se ove čestice mogu koristiti kao antibakterijski, antioksidativni, kao i citotoksični agens u lečenju mnogih medicinskih komplikacija. Može se zaključiti da nanočestice srebra i bakra predstavljaju efikasan antimikrobni agens protiv uobičajenih patogenih bakterija.

Ključne reči: *nanočestice, Green sinteza, KSRD, Emblica officinalis, Biološki agensi.*

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