GREEN SYNTHESIS OF BIMETALLIC NANOPARTICLES USING NEEM LEAF EXTRACT AND THEIR ANTIMICROBIAL ACTIVITY

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ABSTRACT

Green synthesis is the most favorable choice to synthesize nanoparticles, due to its biocompatibility for various applications. In this paper we synthesized Ag, Cu, bimetallic Ag-Cu and mixture of Ag and Cu nanoparticles using neem leaf extract. The neem leaf extract is indeed rich in phytochemical compounds, and some these compounds like alkaloids, flavonoids serve as capping and reducing agent. Synthesized nanoparticles were characterized by XRD, UV analysis, SEM and EDX. Average size of synthesized bimetallic nanoparticles using Debye Scherer equation has been calculated to be 24.21nm.The bimetallic Ag-Cu nanoparticles showed a characteristic plasmon peak at 550nm which was broad and red shifted. The antimicrobial activity of green synthesized Ag, Cu, Ag-Cu bimetallic ,mixtures of Ag and Cu and chemically synthesized Ag-Cu nanoparticles were investigated against Escherichia coli.

Keywords: Green synthesis, Bimetallic Nanoparticles, Phytochemicals, Biocompatibility and Antimicrobial activity.

INTRODUCTION

Green synthesis of bimetallic metal nanoparticles may be a upward research avenue due to their potential applications. Green synthesis is defined as the ability of organisms and organic compounds to reduce metal ions and stabilize them into nanoparticles, minimizing environmental impact and ensuring the safety of both the synthesis process and the resulting materials. For synthesis of nanoparticles through green synthesis method various biological entities, such as plants and plant products, algae, fungi, yeast, bacteria, and even viruses, can serve as valuable resources. Using diverse biological entities numerous studies have documented the synthesis of a variety of metal nanoparticles, including Ag, Au, Cu, Pt, Cd, Pd, Ru, Rh, and others. The research interest in the field of biological synthesis of nanoparticles using different parts of plants such as root, steam, leaves flower, fruit, latex, seed coat, and seed extract is experiencing a notable and continuous increase each year [1]. The synthesis of a wide variety of metallic nanoparticles has been investigated extensively, with numerous studies employing various plant extracts as reducing agent [2,3,4]. The plant's composition of various biomolecules, such as amino acids, alkaloids, aldehydes, flavones, ketones, proteins, phenolics, polysaccharides, saponins, tannins, terpenoids, and vitamins, serves as a vital factor in the reduction of metals and are toxic to microbial cells. This eco-friendly approach is gaining attention in various applications, including nanomedicine, catalysis, and materials science, due to its reduced environmental impact compared to traditional methods. Importance of reducing agent is to provide the free electrons needed for deionization and the formation of nanoparticles [5]. In the present paper neem leaf extract is used as reducing agent in the synthesis of silver, copper and silver-copper nanoparticles. *Azadirachta indica*, which is a common plant known as Neem. It belongs to *Meliaceae* family and is known for its medicinal property . Each part of the tree has various medicinal value. The neem extract contains 140 biomolecules [6]. This poses a challenge in precisely identifying the specific reducing and stabilizing agents involved in nanoparticle synthesis. The stabilization of NPs is important for its functions and various applications [7]. Flavonoids are the major contributors of green synthesis of nanoparticles. The skeleton of flavonoids comprises of two phenyl rings (A and B), associated by an oxygenated heterocycle ring C, and is hydroxylated in several positions. These compounds have important roles in plants because they participate in the response to biotic and abiotic stresses [8]. Metal nanoparticles can exhibit various compositions, including mono-metallic (composed of a single metal element), bi-metallic (consisting of two different metal elements), tri-metallic (incorporating three distinct metal elements), or even poly-metallic (involving multiple metal elements) structures. Bimetallic nanoparticles are more advantageous than the monometallic nanoparticles from both scientific as well as technological point of view [9,10]. Different methods like co-reduction method, successive reduction method and electrochemical method to synthesize and characterize bimetallic nanoparticles [11]. Alloying of two different elements may result within the structural changes of the bimetallic nanoparticles. Several factors influence the structure of nanoalloys including the relative strengths of metal-metal bonds, the surface energy of bulk materials, relative atomic sizes, charge transfer, and specific electronic/magnetic effects [12]. Most noble metal bimetallic nanoparticles use reduction methods, while most transition metal bimetallic nanoparticles are synthesized using thermal methods [13]. Various bimetallic nanoparticles so far synthesized are Platinum based bimetallic nanoparticles**,** Copper based bimetallic nanoparticles**,** Nickel based bimetallic nanoparticles**,** Iron based bimetallic nanoparticles**,** Palladium based bimetallic nanoparticles**,** Gold based bimetallic nanoparticles**.**[14]**.** Cu-Ag nanoparticles were synthesized using Kigelia africana fruit by [15,16] through reflux at 120 °C. Merugu et al, synthesized 10nm size spherical shape bi-metallic Ag/Cu nanoparticles using the leaf extract of *Majorana hortensis* plant and studied antibacterial effect [17]. Zarina Ansari et al. designed a noble, facile, cost-effective, and environmentally benign method for the synthesis of silver, copper, and silver-copper bimetallic nanoparticles that exhibit Raman signals. The method involves the utilization of agro waste as the source of polyphenols [18]. A study documented the synthesis of Ag-Cu bimetallic nanoparticles employing the fungal strain Aspergillus terreus [19]. Aloe vera leaf was employed in the synthesis of Ag-Cu nanoparticles on cotton fabric, showcasing potential applications for wound dressing [20]. In a study documented by Al-Haddad, [21] Cu-Ag nanoparticles approximate size of 26 nm were synthesized using the aqueous extract of date palm tree.. So far, only a few works on the biogenic synthesis of alloy/core shell nanostructures have been reported [22]. This initiated our present work where we have synthesized monometallic Ag, Cu, mixture of Ag and Cu and Ag-Cu bimetallic nanoparticles using neem leaf extract at room temperature and antimicrobial activity against *E. coli* (Gram-negative bacteria), is being investigated.

MATERIAL AND METHODS:

Preparation of Neem Leaf Extract:

Neem leaves were collected from botanical garden, Kondapur, located at Hyderabad. To eliminate any potential contaminants and dirt adhering to leaves, a thorough cleaning process was carried out by rinsing leaves twice with tap water and once with distilled water. A quantity of 25g leaves were sliced into small pieces, which were added to 100 mL of deionized water. This mixture was stirred for 10 min at constant temperature 40^{o}C using a magnetic stirrer to release intracellular materials from the neem leaves into the solution. The extract was then cooled to room temperature and filtered using whatman filter paper No.1.The extract was stored in a refrigerator in order to be used for further experiments.

The precursors $AgNO_3$ and $CuSO_4.5H_2O$ were used to obtain Ag, Cu and Ag-Cu nanoparticles. Three 0.1 M solutions were prepared separately from these precursors in 100 mL deionized water each.

Synthesis of Ag Nanoparticles:

For synthesis of Ag nanoparticles. A total of 30ml of 0.1M of $AgNO_3$ was added to 20mL of Neem leaf extract in a 250ml of conical flask at room temperature with constant stirring on a magnetic stirrer for overnight. A colour change from transparent to orange was observed, this is an initial indicator of the formation of silver nanoparticles. The obtained mixture was incubated in a dark chamber to moderate the risk of auto-oxidation of the silver nitrate. After aging the solution for 24 hours, the precipitate formed was carefully filtered and washed with distilled water and then subjected to centrifugation at 6000 rpm for 15 minutes. The nanoparticles pellets obtained from the centrifugation were collected and subsequently dried in an oven at 80 °C

Synthesis of Cu Nanoparticles:
For synthesis of Cu nanoparticles, A total of 30ml of 0.1M of $CuSO_{4.}5H_2O$ was added to 20mL of Neem leaf extract in a 250ml conical flask at normal temperature with constant stirring on a magnetic stirrer for overnight. The biosynthesized Cu nanoparticles were verified by a colour change from light green to reddish brown. Brown precipitates settled at the bottom. After aging the solution for 24 hours, the nanoparticles were gathered and subjected to washing with distilled water to eliminate impurities. Subsequently, the washed nanoparticles were left to dry at room temperature, resulting in the formation of a powdered state for the copper nanoparticles.

Synthesis of Ag-Cu Nanoparticles: Under optimal experimental conditions, Ag-Cu nanoparticles were produced through a simple one-pot biosynthetic method. In this procedure, 20ml of each metal salts of AgNO3 and $CuSO_4.5H_2O$ were continuously stirred together at room temperature in a 250 mL beaker. To this mixture of metal salts, 20 mL of neem leaf extract was introduced slowly to minimize the risk of particle agglomeration during the synthesis process and the mixture was stirred for 2h. The confirmation of Ag-Cu bimetallic nanoparticle synthesis was evidenced by a visible color change in the experimental solutions, shifting from light orange to grey. Following this, the resulting grey reaction mixture underwent centrifugation at 8000 rpm for 15 minutes to separate the reaction precipitate. Subsequently, the acquired Ag-Cu bimetallic nanoparticles underwent thorough washing with distilled water multiple times. The Ag-Cu nanoparticles was then dried at 80°C for 12 hours. This multi-step process ensures the separation, purification, and drying of the Ag-Cu bimetallic nanoparticles, yielding a finalized product ready for further characterization and application.

Mixing of Ag and Cu Nanoparticles
For the preparation of mixtures of Ag and Cu nanoparticles, nanoparticles were prepared separately similar to Ag-NP protocol and then mixed together [23].

Antimicrobial Activity of Nanoparticles
The antimicrobial efficacy of Ag NPs, Cu NPs, and Ag-Cu NPs was investigated against pathogenic strain *E.Coli* utilizing an agar diffusion assay. Initially, bacterial cultures were cultivated on Nutrient Agar (NA) slants, formulated with specific components including 5.0g of peptones, 15.0g of agar, and appropriate quantities of meat extract, sodium chloride, and yeast extract per liter of distilled water. Following solidification of the culture medium, wells were carefully fashioned using an 8mm cork borer. Subsequently, 100µl of freshly prepared Ag NPs, Cu NPs, and Ag-Cu bimetallic NPs were introduced into the wells created in the culture medium plates. The plates were then refrigerated at 4°C for 15 minutes to allow for nanoparticle diffusion. Thereafter, they were incubated for duration of 24 hours at 37°C in an incubator, fostering bacterial growth and interaction with the nanoparticles. This methodological approach provides a robust framework for assessing the antimicrobial potential of, boiled neem leaf extract, Ag NPs, Cu NPs, green and chemically synthesized Ag-Cu bimetallic NPs and mixture of Ag and Cu against *E. coli* offering valuable insights for potential applications in combating bacterial pathogens.

Characterization
The XRD pattern, crystal size and structure of synthesized Ag, Cu and Ag-Cu bimetallic nanoparticles were characterized by using X-ray diffractometer and Debye Scherer Equation. For optical properties UV-Vis analysis was used. The surface morphology and microstructure of the prepared particles are monitored with SEM. EDX analysis was used to evaluate elemental composition of the synthesized nanoparticles.

RESULTS AND DISCUSSION

Probable Mechanism of Formation of Nanoparticles
The green approach utilizes the inherent properties of plant biomolecules to facilitate the formation of nanoparticles, the precise mechanisms underlying phytosynthesis remain a subject of ongoing research and are not yet fully understood**.** The term phytosynthesis refers to the process by which plants or plant extracts facilitate the synthesis of nanoparticles**.** In general, the synthesis of metallic nanoparticles from plant extracts involves three distinct phases; the reduction phase, growth phase and termination phase. During the reduction phase upon

introducing metal salts to the plant extract, metal ions form intricate bonds with proteins and water-soluble compounds, primarily through -OH and -COOH functional groups and metal ions(M^+) undergo reduction to form zero valent metal atoms (M^o) by transfer of electron. During the growth stage, the zero-valent metal atoms (M^o) undergo a process of aggregation, coming together to form nanometallic particles with diverse shapes which include linear structures, rod-shaped formations, triangular configurations, hexagonal arrangements, or cubic morphologies. The aggregation is mainly caused by high surface energy and thermodynamic instability of the nanoparticle surfaces. The biomolecules in plant extract like protein, phenol and flavonoids play a major role in preventing aggregation of nanoparticles and also helps to lower the surface tension in a suspension and make the particles easy to disperse within it. In the termination phase, biomolecules with antioxidant properties attaches around the metal nanoparticles, contributing to the maintenance of metal nanoparticles stability.

Formation of Ag Nanoparticles

When silver nitrate ($AgNO_3$) reacts with water (H_2O), it undergoes dissociation. In this reaction, silver nitrate dissociates into silver ions (Ag^+), nitrate ions (NO^{3-}), and water (H_2O).

The reaction can be represented as follows:

$$AgNO_3 + H_2O \rightarrow Ag^+ + NO^{3-} + H_2O\text{+Neem Leaf extract } (e^-) \longrightarrow \text{Ag NPs}$$

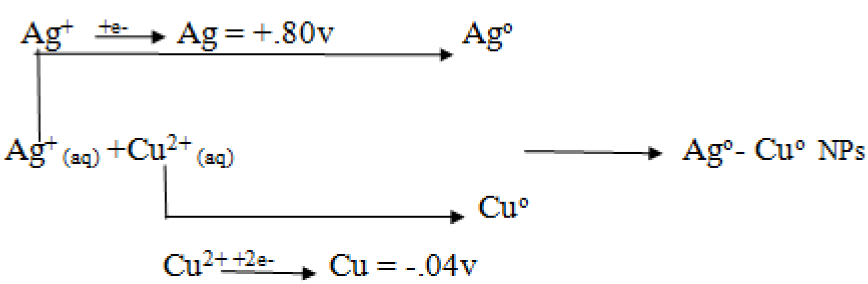
Formation of Cu Nanoparticles

Copper sulfate pentahydrate ($CuSO_4$•$5H_2O$) dissociates in water to form copper ions (Cu^{2+}) and sulfate ions (SO_4^{2-}). The neem leaf extract is added to the solution. This extract likely contains various phytochemicals as reducing agents, which can reduce the copper ions to form copper nanoparticles.

$$CuSO_4.5H_2O + H_2O \longrightarrow Cu^{2+} + SO_4^{2-} + 5H_2O\text{+Neem Leaf extract } (e^-) \longrightarrow \text{Cu NPs}$$

Formation of Ag-Cu Nanoparticles

The hydroxyl groups present in neem are responsible for the reduction of silver ions to Ag nanoparticles, Cu ions to Cu nanoparticles and Ag-Cu bimetallic nanoparticles [24]. Due to the higher reduction potential of Ag^+ metal ions compared to Cu^{2+} metal ions, the reduction rate of $Ag^+ \longrightarrow Ag^0$ is faster than $Cu^{2+} \longrightarrow Cu^0$. The phytochemicals, not only facilitate the conversion of metal ions into metal nanoparticles but also prevents agglomeration of Ag-Cu. Due to large variation in lattice constants of silver and copper, Ag-Cu nanoparticles are more prone to agglomerate.



X-ray Diffraction (XRD) Analysis:

The XRD pattern of synthesized Ag, Cu and Ag-Cu nanoparticles is shown in Fig 1(a),1(b) & 1(c). Peaks observed at 2θ values for Ag nanoparticles is 38.1°, 46.1°,64.39°&77.00° corresponds to (111), (200), (220) & (311) planes of cubic structure silver (JCPDS card No. 04-0783). The intense peak at 38.1° corresponds to (111) plane and indicates Ag nanoparticles are crystalline in nature. Similarly for Cu nanoparticles are 43.30°, 50.48°& 74.10° corresponds to (111), (200) & (220) planes of cubic structure copper (JCPDS card No. 04-0836) no other peaks for copper oxides are present. Ag-Cu bimetallic nanoparticles is 38.51°,44.65° ,64.79° & 78.0°corresponds to (111), (200), (220) & (311). All of the above Ag and Cu diffraction peaks were observed in bimetallic Ag-Cu nanoparticles, indicating that the bimetallic nanoparticle contains both Ag and Cu phases, the results are similar to results reported by S.I. Thakore et al. [25]. From Fig 1(c) no other characteristic peaks were observed, indicating the high purity of the Ag-Cu bimetallic material. However, no diffraction peaks indicating copper nanoparticles in various phases are detected, if detected it may damage its properties particularly in the area of water treatment. Additionally, the absence of copper oxide peaks signifies that the Cu nanoparticles are shielded from oxidation, owing to the presence of capping agents on their surface. The lines in a powder diffraction pattern are of finite breadth but if the particles are very small, the lines are broadened than usual. The broadening decreases with the increase in particle size. Other peaks at 2θ values in XRD pattern of Ag can be due to the presences of phytochemical compounds in the leaf extract.

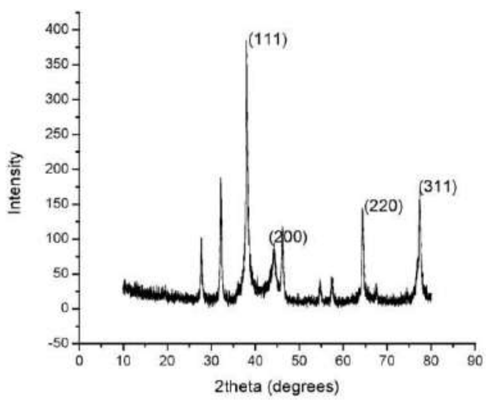


Fig (1a) XRD of Ag nanoparticles

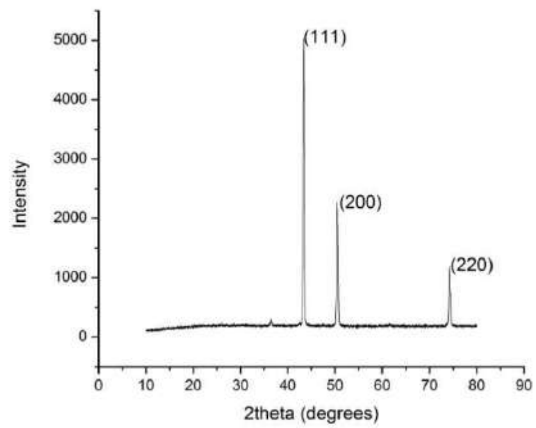


Fig (1b) XRD pattern of Cu nanoparticles

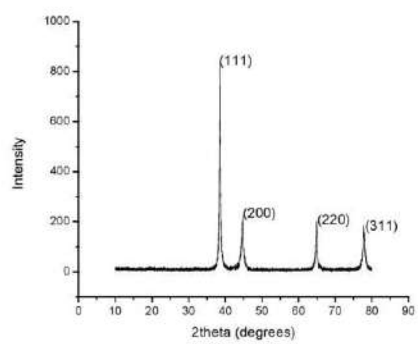


Fig (1c) XRD pattern of Ag-Cu nanoparticles

Particle Size Calculation using Debye Scherer Equation:

Debye Scherrer equation was used to determine the crystallite size from XRD diffraction pattern $D=.9\lambda/\beta\cos\theta$ (1)

where K is the Scherrer constant (shape factor, its value is 0.9), λ is the X-ray wavelength ($\lambda = 0.154$ nm), β is the line broadening at half the maximum intensity (FWHM) in radians, θ is the Bragg angle, (the position of the diffraction peak maximum) and D is the averaged dimension of crystallites in nanometers.

Using above equation Ag nanoparticle size has been calculated to be 26. 7nm.Similarly Cu nanoparticles and Ag-Cu nanoparticles size has been calculated to be 39.49nm and 24.21 nm respectively. The size of Ag-Cu nanoparticles is smaller than the Ag nanoparticles and Cu nanoparticles. The presence of silver ions among the nanoparticles reduces the collision frequency of the particles. Consequently, this control over collision frequency leads to the regulation of particle growth.

UV-Vis Spectroscopy Analysis:

UV- Vis is found best technique for analyzing the optical properties and helps to estimate the band gap and wavelength of the material. The aggregation and dispersion phenomena among Ag, Cu and Ag-Cu nanoparticles were detected with UV–Vis absorption spectra the broad plasmon peaks. The narrow plasmon peaks obtained confirmed to the well dispersion of nanoparticles. In UV-V is spectra recorded for the reaction solution of reduced silver nitrate and copper (II) sulfate pent hydrate by leaf extract of Neem is shown Fig 2(a) & 2(b). The variation in the size, shape, and properties of the accumulated nanoparticles can be attributed to the differences in the composition and concentration of biomolecules present in plant extracts. The single SPR band for Ag-Cu composition in Fig. 2(c) indicates the formation of nanoparticles with an alloy [24]. The presence of a single absorption peak at an intermediate ratio indicates that the silver and copper components have combined to form a homogeneous alloy with distinctive optical properties. This finding supports the formation of a well-integrated bimetallic structure rather than the presence of separate, discrete silver and copper entities. Optical spectroscopy is indeed a valuable technique for confirming the formation of nanosized copper and silver particles, as well as their alloy.

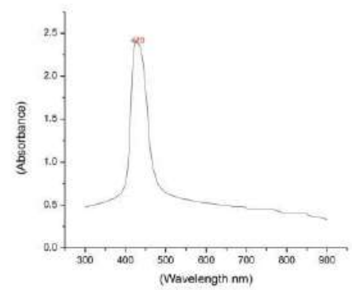


Fig 2(a) Ag NPs

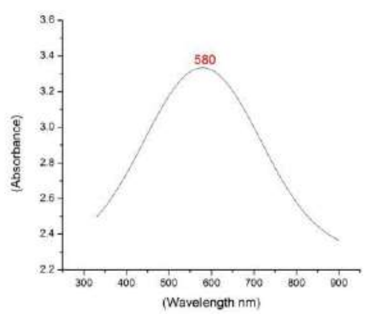


Fig 2(b) Cu NPs

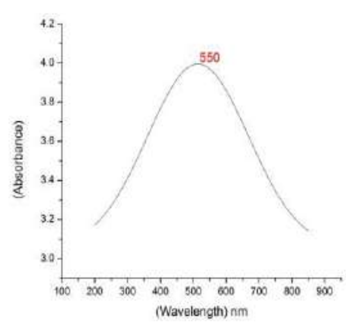


Fig 2(c) Ag-Cu NPs

The maximum absorbance peak was seen at 420nm, 580nm & 550nm for Ag, Cu and Ag-Cu nanoparticles respectively. Plasmon resonance peak depends on the particle sizes. The surface plasmon resonance band gives helpful data about the size of the synthesized nanoparticles. This is due to the frequency and width of surface plasmon resonance depends on the size of the nanoparticles, dielectric constant of the material and the surrounding medium [26].

SEM:

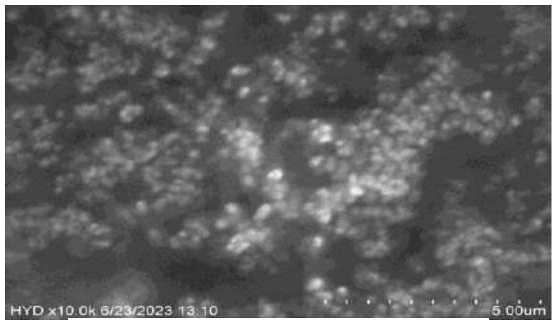


Fig. 3 SEM of Ag-Cu nanoparticles

The SEM images confirm the structural and morphological characteristics of the bimetallic nanoparticles. The SEM analysis depicted in Fig.3 revealed the presence of semi-spherical agglomerated clusters in the bimetallic silver-copper nanoparticles, consistent with previous findings by Zaleska-Medynska et al. [27]. Specifically, the silver nanoparticles exhibited a spherical shape, while the copper particles exhibited a cubic hexagonal structure.

EDX:

Energy Dispersive X-ray Spectroscopy is an analytical technique used to determine the elemental composition of a sample. It works by measuring the characteristic X-rays emitted by elements when they are exposed to a high-energy electron beam, such as those in an electron microscope. EDX provides information about the elemental composition. The sample should be prepared in a way that allows the electron beam to interact with the material. This typically involves creating a thin section or a polished surface. The sample is bombarded with a high-energy electron beam. This interaction causes the sample to emit characteristic X-rays. The spectrum is then analyzed to identify the peaks corresponding to the characteristic X-rays of different elements. The intensity of each peak is proportional to the amount of the corresponding element present in the sample.

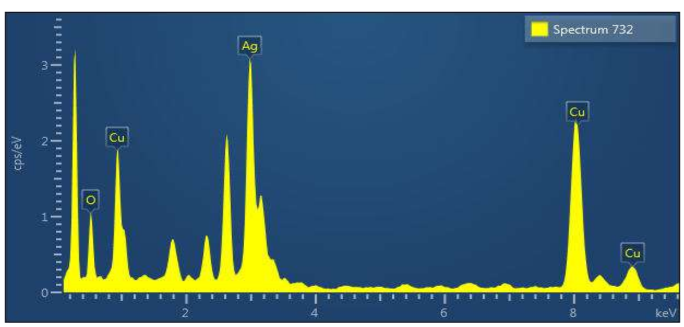


Fig 4 EDX of Ag-Cu nanoparticles

From Fig. 4 the application of EDX analysis confirmed the presence of both silver (Ag) and copper (Cu). Additionally, the EDX examination provided quantitative data, revealing that the Ag-Cu nanoparticles consist of approximately 43.94% by weight of silver and 43.95% by weight of copper. The presence of 12.11% oxygen in the EDX spectrum is likely attributed to the rich diversity of phytochemicals found in neem leaf extract, some of which naturally incorporate oxygen atoms. This emphasizes the crucial contribution of these phytochemicals in the synthesis process.

Antimicrobial Activity

The antibacterial activity of nanoparticles involves several mechanisms that collectively contribute to inhibiting or killing bacterial cells. Here are some of the key mechanisms.

Direct Physical Contact

Nanoparticles can physically interact with bacterial cell membranes. The high surface area of nanoparticles facilitates strong interactions, disrupting the membrane integrity and causing leakage of intracellular components.

Generation of Reactive Oxygen Species (ROS):

Certain nanoparticles, like silver, copper and zinc oxide, can generate ROS (e.g., superoxide radicals, hydrogen peroxide, hydroxyl radicals) upon exposure to biological environments. ROS can damage bacterial DNA, proteins, and lipids, leading to cell death.

Metal Ion Release:

.In the present investigation, the antibacterial activities of prepared green synthesized nanoparticles and chemically synthesized nanoparticles were studied on *E. coli* (Gram Negative bacteria). The zone of incubation around Ag NPs, Cu NPs Ag-Cu NPs, mixture of Ag and Cu nanoparticles, chemically synthesized Ag-Cu nanoparticles and neem leaf extract bacterial culture is shown in Fig.6a and Fig.6b. Some metallic nanoparticles release metal ions (e.g., Ag^{+}, Cu^{2+}) into the surrounding environment. These ions can disrupt cellular functions and lead to bacterial cell death. The numerical value of the inhibition zone is given in Table 1. From the table (1) The boiled water extract from neem leaves exhibited a moderate antibacterial effect against *E.coli*. Antibacterial activity of mixture of Ag and Cu nanoparticles is intermediate between pure silver and copper nanoparticles. Green synthesized Ag-Cu alloy nanoparticles exhibited highest antibacterial effect than chemically synthesized Ag-Cu nanoparticles which indicated that phytochemicals are also contributing to the antibacterial effect. The results follow previous studies in which the antibacterial action of silver and copper nanoparticles by Raffi et al [28] and Minjie et al [29].

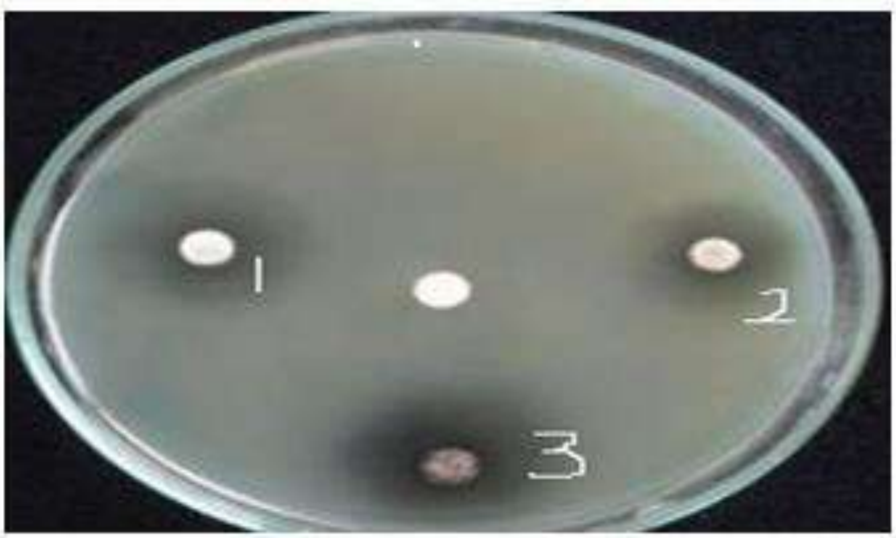


Fig 6(a) Antibacterial activity of Ag nanoparticles (1), Copper nanoparticles (2), Ag-Cu nanoparticles (3), against Escherichia coli

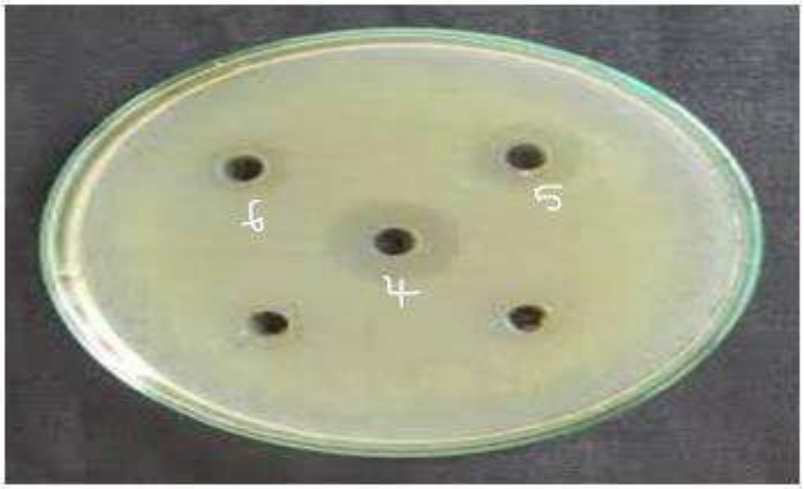


FIG.6 (B). Antibacterial Activity of Chemically Synthesized Ag-Cu Nanoparticles (4), Mix of Ag-Cu Nanoparticels(5), Neem-Leaf Extract (6) Against Escherichia Coli

Microorganism	Nanoparticle	Reducing Agent	Inhibition Zone in Mm
E Coli (Gram Negative Bacteria)	1. Silver	Neem Leaf Extract	13
	2. Copper	Neem Leaf Extract	11
	3. Silver-Copper	Neem Leaf Extract	23
	4. Silver-Copper	$Nabh_4$	20
	5. Mixture of Ag and Cu	Neem Leaf Extract	12
	6. Neem Leaf Extract	-	8

Table1: Antibacterial activity of Nanoparticles

CONCLUSION

This study emphasizes the eco-friendly synthesis of bimetallic silver-copper nanoparticles utilizing biomolecules extracted from Neem leaves. Comprehensive structural and morphological characterizations, conducted through UV, SEM, EDX, and XRD. XRD pattern confirmed the successful synthesis of silver-copper alloyed nanoparticles with minimal oxide content. The prepared bimetallic nanoparticles exhibited excellent antibacterial properties, displaying comparable zones of inhibition for gram-negative bacteria. The most significant antimicrobial impact was observed when Ag - Cu were alloyed. We anticipate that the bimetallic nanoparticles will present more exciting prospects for use in water treatment.

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