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Characterization of *Azadirachta indica* Mediated Copper and Silver Nanoparticles – A Comparative Approach.

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ABSTRACT

Background: In the modern scientific approach, green nanotechnology plays a vital role in the formulation of newer and novel biomolecules which have wide biomedical applications. The applications and values of the synthesized nanoparticles have its own action based on the mode of synthesis and the effect may differ according to the substrate. The physical and chemical methods of nanoparticle synthesis are cumbersome in duration of synthesis, physico-chemical modification and contaminations, expensive and environmentally toxic. But the biological mediated nanoparticles synthesis has great advantages in its non-toxic nature and ability in large scale production. Objective: The objective of the present study is to synthesize and characterize *Azadirachta indica* leaf powder mediated silver and copper nanoparticles. **Methods:** The methodology was standardized according to the synergistic effect of metal solution and test herbal plant that catabolized to form nanoparticles, thereby in vitro antibacterial activity is possible by performing well cutting method. **Results:** The physico-chemical characterization of both the nanoparticles revealed that the synthesized molecules are nano in size mainly by its color formation and size dependent phenomena. Both silver and copper nanoparticles were highly effective against *Escherichia coli, Proteus* sp, *Staphylococcus aureus* and *Pseudomonas aeruginosa*; moderate level of inhibition was observed against *Enterococcus* sp and *Klebsiella pneumoniae* and low level of inhibition was observed against *Micrococcus* sp by copper nanoparticles whereas no inhibition was recorded by silver nanoparticles. **Conclusion:** The green synthesized nanoparticles are having acceptable level of bactericidal activity compared with the crude *A. indica* extract and metal solution controls.

Keywords: Green nanoparticles, Copper and Silver, *Azadirachta indica*, antibacterial activity

INTRODUCTION

Literally, antibacterial agents are the broad source of substances which kill and/or disrupt the growth and replication the pathogenic bacteria. Most of the commercially available antibiotics are defined based on the source as natural, chemically modified and chemically mediated/ derived synthetic compounds.[1,2] Due to misuse of antibiotics, the target action on five modes including disturbing cell wall synthesis, dysfunction of cytoplasmic membrane, inhibition of protein synthesis, modification in the functions of metabolic enzymes and suppress the DNA replication get failed. This leads to drug failure or emergence of antibiotic resistance against these antimicrobial targets and developed physical, chemical, enzymatic and biological modifications in the bacterial cells.[3,4] These non-functional molecules and non-responded cellular targets enhance the exploration of resources through alternative sources for the development of newer antibacterial agents with molecular understanding for the successful elimination of pathogens from the tissue or environment.

Historically, India is one of the countries, traditionally utilized the natural resources for systemic and superficial ailments. After the invasion of modern medicine, the targeted antimicrobial agents of synthetic nature provide smart and earliest treatment, and fast recovery; but lot of adverse reactions like mild (rashes) to severe (multiorgan dysfunction, multiorgan failure, cancer etc.) that threat the life. Due to this unpleasant environment, there is an urgent need of exploring alternative drugs to overcome the infectious diseases. Lot of studies defined the exploration of medicinal herbs (green molecules) as new approach for targeting various diseases; but large scale

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production and drug commercialization are not possible due to ethical disturbances of not permitting clinical trials, non-motivation of young scientists, political interferences, poor drug manufacturing policies and non-stepping of the pharmaceutical companies.[1-3]

For a long period of time, the usage of metals in our traditional medicine (bashpam, sooranam, kashayam etc) and house hold practices in form of metal vessels to store and prepare various food and related materials that exhibit wide range of antimicrobial properties. Due to urbanization and modernization, some external pressure diverted our previous generation for the usage of other metals like stainless steel, aluminium etc. that are highly carcinogenic. The negative advertisements pushed the current generation to use the modular kitchen materials from condensed environment to nonstick metal vessels that are very high risk of forming various organ specific cancers.[2,3]

The increase in the re-emergence of usage of metals as solid materials in households, temples and restaurants are become modernized that give a positive sign of re-entering into our traditional system of living. To observe such socio-functional changes, the usage of metal nanoparticles became an alternative and attractive bio-material to act against pathogens that are multi-resistant. The size, charges, drug delivery nature and specific targets are the major characteristic features of the metal based nanoparticles that exhibiting microbicidal nature.[5] The specific mode of action of nanoparticles is still unknown due to its visceral antimicrobial targets on pathogens and broad spectrum nature.[4,6] Nanoparticles are synthesized by various methods including physical, chemical and biological or biological mediated methods; while biological synthesis using microbes or medicinal herbs is cost effective, simple and reliable method.[7-9]

Herbal mediated metal nanoparticle synthesis provide more biocompatible than the chemical and physical synthesis; may have a chance to disturb the size and avail the residues of toxic chemicals respectively. Herbal mediated nanoparticles are produced using numerous plants of Pelargonium graveolens, Medicago sativa, Azadirachta indica, Lemongrass, Aloevera, Cinnamomum, Camphor etc.[10,11] The metal based nanoparticles are less toxic to human beings and highly effective against virus, bacteria and several eukaryotic microorganisms at very low concentrations.[12,13]

The test herbal plant Azadirachta indica (Neem) is an aromatic herb contains alkaloids, glycosides, tannins, saponins and minerals like Ca, Ma, Cu, Zn, P, K, Na and Mg which is the major source of bio-reduction and also act as a stabilizer for the synthesis of metal nanoparticles. Over thousand years, neem leaves were used for healing because of its phytonutrients and essential oils thus have an antibacterial and germicidal activity.[14] Thus the present study aims to compare the physico-chemical characterization and antibacterial activity of the *Azadirachta indica* mediated silver and copper nanoparticles.

MATERIALS AND METHODS

Collection and preliminary processing of plant

The leaves of *Azadirachta indica* were collected and cleaned. Then it was allowed to shade dried to remove the moisture thoroughly. The dried leaves are grind to make a powder. Then it was stored in the air tight container for future use.

Bacterial gallery for bactericidal analysis

The pathogenic bacterial species isolated from wounds and characterized by standard bacteriological procedures were included in this study including *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Proteus* sp, *Klebsiella pneumoniae, Enterococcus* sp *and Micrococcus* sp. The overnight incubated bacterial broth cultures were used for further analysis.

Synthesis of copper and silver nanoparticles

For the synthesis of Copper (Cu) and Silver (Ag) nanoparticles, 100ml of 4% copper sulphate and 10^{-3}mM silver nitrate solution was added to 5g of A. indica leaves powder. The mixture was agitated thoroughly without forming bubbles and was kept for incubation at dark condition for the synthesis of herbal mediated metal nanoparticles.

Characterization of synthesized nanoparticles

The initial physical analysis (color change) was noted manually from 0 time to 72 hours by analyzing every doubling hours. The color change from light (pale green) to dark (brown to black) indicated nanoparticle synthesis. Then the pH was assessed again from 0 time to 72 hours; thus the increase in the pH by time increases denoted nanoparticle synthesis.[15]

The spectrophotometric assessment for analyzing the increase in optical density (OD) value was carried out. Additionally nitrate reductase assay and sulphate reductase assay were performed in order to cross check the bioreduction from nitrate to nitrite and sulphate to sulphite respectively.[16-18]

All the mixture flasks were allowed to filter separately through Whatman No.1 filter paper aseptically and the filtrate was centrifuged at 6000rpm for 20 minutes to separate the pellet from the solution. The size dependent phenomena of the synthesized nanoparticles were done by scanning electron imaging (SEI). The pellets were further diluted accordingly and used for the analysis of in vitro antibacterial activity.

Antibacterial assay

Muller Hinton Agar – Well cutting method was used to determine the antibacterial wound isolated pathogens against the synthesized herbal mediated metal nanoparticles. Each bacterial pathogen was seeded separately on to the surface of the agar plates and 2mm diameter wells were punched and add various concentrations (1 - 5%) of *A. indica* mediated silver and copper nanoparticles separately. Then all the plates were allowed to incubate at 37oC for 24 hours and assessed for determining zone of inhibition (the marker of antibacterial assay).

RESULTS

Color change

The color change from pale to dark color indicated the formation of nanoparticles. At the initial the addition of *A. indica* leaves powder with the copper and silver solution are observed as pale color [Figure 1a]; later after incubation time, the mixture gets darker in color [Figure 1b], thus indicated the reduction of sulphate and nitrate in solution.

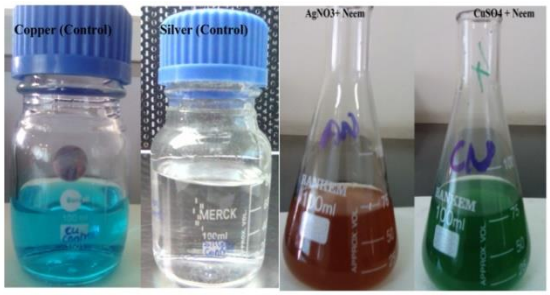


Figure 1a: Silver and copper control Figure 1b: Silver and copper solution with test herb

Calorimetric assay

The OD value using simple calorimetric assay of copper and silver solution alone was recorded, before the addition of *A. indica* powder. After the addition of leaves to the silver nitrate and copper sulphate solution, the initial and final OD value was recorded [Table 1].

Table 1: OD value of test herb with copper sulphate and silver nitrate solution (AI- Ag: *Azadirachta indica* with silver nitrate solution & AI-Cu: A. indica with copper sulphate solution)

Time Duration	Optical Density (OD) value	
	AI-Ag solution	AI-Cu solution
Distilled water	0.00	0.00
Metal solution alone	0.02	1.69
0time	0.61	1.64
1 hour	0.60	1.62
2 hours	0.57	1.12
4 hours	0.52	1.04
8 hours	0.48	0.99
16 hours	0.45	0.92
32 hours	0.40	0.89
64 hours	0.38	0.87

UV-Vis Spectrophotometer

The UV-Vis spectra recorded from the test herbal mediated metal nanoparticles at different times of reaction is presented in [Figure 2a and 2b]. The strong surface plasmon resonance centered at 420 nm clearly increases in intensity with time. The solution was extremely stable, with no evidence of flocculation of the particles even several weeks after reaction.

The spectra in low wavelength region recorded from the reaction medium exhibited absorption and it was attributed to aromatic amino acids of proteins. This observation indicated the release of proteins into solution by test herb and suggested a possible mechanism for the reduction of the metal ions of both silver and copper present in the solution.

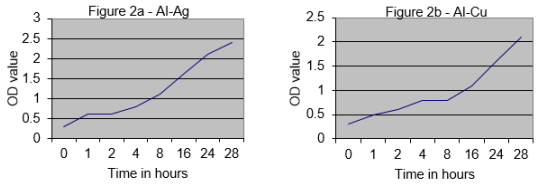


Figure 2a and 2b: UV-Vis Spectra recorded as a function of time of reaction of aqueous Solution of 10^{-3} mMAgNO3 and CuSo4 with the AI biomass (07SD) at 420nm

pH value

The pH of the solution is important while the synthesis of nanoparticles, because of the reduction of copper sulphate and silver nitrate with the *A. indica* solution, the value get reduced. Initial and final pH of the each solution was recorded [Figure 3].

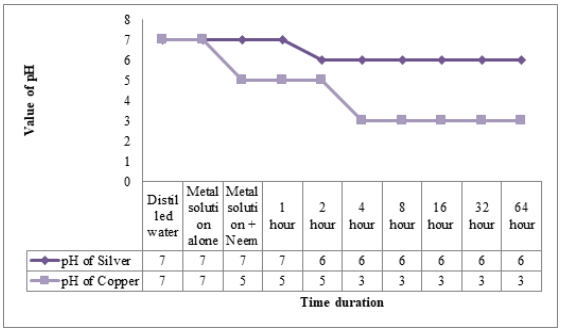


Figure 3: Reduction of pH value of copper and silver herbal mixture

Nitrate reductase assay

The nitrate reductase assay provides the confirmation of the presence of nitrate reductase, thereby we confirm the reduction of nitrate to nitrite. The maximum OD with 0.10 and 0.12 was observed among Ag and Cu herbal solution respectively [Table 2]. The major peaks of the calorimetric intensity at 460 nm corresponding the intensity maximum of 2,3-diaminonapthotriazole. This may be further preceded to analyze the

antimicrobial activity of the compounds obtained which having the characteristic features of azole groups. The [Table 2] showed the nitrate reductase through the reaction of nitrite with 2,3 – diaminophthalene.

Table 2: Calorimetric assay of nitrate reductase assay by AI-Ag and AI-Cu solution

Time duration	Optical density at 460nm	
	AI-Ag solution	AI-Cu solution
0	0.2	0.3
30 mins	0.2	0.4
1 hour	0.4	0.5
2 hours	0.5	0.6
4 hours	0.6	0.7
8 hours	0.7	0.8
16 hours	0.8	0.10
24 hours	0.10	0.12

Sulphate reductase activity

It was observed that there is a reasonable reduction affinity for magnesium ions which are essential for assay of sulphate reductase activity. There is no significant difference was seen between the metals mediated herbal nanoparticle but observable reduction in the percentages was recorded when incubated at 30°C for upto 90 minutes before assay for residual sulphate reductase activity. By comparing the assay reaction time, there is no further reduction in percentage observed after 60 minutes in both the solutions [Figure 4]. No loss of activity was observed while the test was performed in the room temperature. Further optimization studies may be useful to understand the enhancement of activity in various temperatures and pH ranges of the solution in vitro. No significant improvements were seen upon inclusion of these agents during cell disruption or the assay of enzyme specifically to sulphate reduction activity.

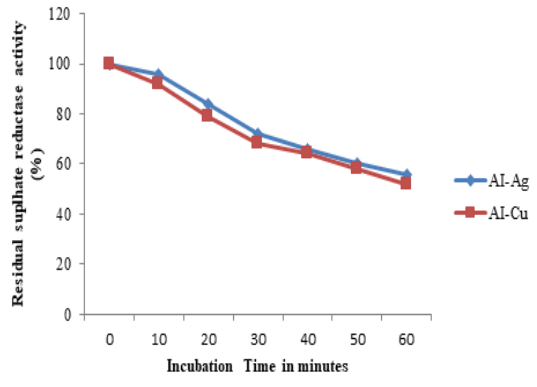
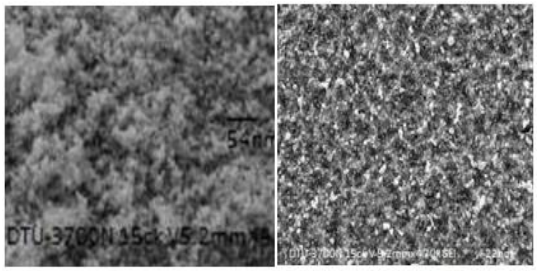


Figure 4: Residual sulphate reduction activity

Scanning Electron imaging

By imaging the nanoparticles by scanning electron principles, we obtain the figures of certainty to analyze the size of the nanoparticles to prove the size dependent phenomena of the synthesized nanoparticles. The herbal mediated silver solution showed the size of 54nm at 500 X magnification whereas copper solution supported with 30nm at 500X [Figure 5a and 5b]. This showed that the synthesized solution is in nanosize and are named and considered as nanoparticles. Thus from this section onwards, the solution is described as AI-AG Nps and AI-Cu Nps.



[5a: 54nm sized AI-Ag Nps; 5b: 30nm sized AI-Cu Nps]

Figure 5a: Scanning Electron imaging of AI-Ag Nps at 500X and Figure 5b: AI-Cu Nps at 500X

Antibacterial assay

The results of disc diffusion assay to determine the antibacterial activity of neem mediated silver and copper nanoparticles against wound pathogens were well analyzed thereby neem mediated silver nanoparticles showed maximum inhibition to *Pseudomonas aeruginosa* (39mm) followed by *Micrococcus* sp (38mm) and *Enterococcus* sp (31mm). Others also showed observation inhibition (Table 3). In this study, it was recorded that there is a better bactericidal action of neem mediated copper nanoparticles compared to silver. The highest inhibitory zone of 40mm (*Proteus* sp and *Micrococcus* sp) and lowest zone of 10mm was observed in *Enterococcus* sp. While comparing the bactericidal nature among the substrates, the inhibition rate of silver herbal nanoparticles is better among *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Enterococcus* sp, whereas copper herbal nanoparticles inhibited *Escherichia coli, Proteus* sp and *Micrococcus* sp better [Table 3].

Table 3: Comparative bactericidal nature of neem mediated silver and copper nanoparticles

S. No.	Bacterial pathogens	Zone of growth inhibition in mm	
		AI-Ag Nps	AI-Cu Nps
1	*Escherichia coli*	22	32
2	*Staphylococcus aureus*	30	28
3	*Pseudomonas aeruginosa*	39	30
4	*Proteus* sp	22	40
5	*Klebsiella pneumoniae*	30	27
6	*Enterococcus* sp	31	10
7	Micrococcus sp	38	40

DISCUSSION

In this study, it was recorded that the color change from pale color to intermittent and subsequent dark in color in the stipulated period of time indicated the formation of silver and copper nanoparticles. This color change highlighted that there is drastic changes in the shape and size of the particles from high molecular weight to lower and lowest.[19,20] Further, more studies suggested the importance of observing color changes from pale to dark for the indication of nanoparticle synthesis.[5,13,21,22]

The calorimetric assay of the synthesized particles showed mild to drastic reduction in their OD value indicated that the particle size and shape get reduced; thus the metals in solution catabolized by medicinal herb – neem consequently for the indication of monodispersion of nanoparticles.[23] Further this assay should be confirmed by uv double beam spectrophotometry.

From this investigation, we observed the UV spectra of the silver and copper nanoparticles in the range of 420nm which showed well defined Plasmon bands. The increase in maximum absorbance is due to the particle density which strongly depends on the amount of metal reduction at the surface of the medium. It is known that the color of metal particles is caused by the sum of the effects of absorption and scattering of visible light. In this study, multiple bands were recorded and closely justify the Mie's theory of confirming the single band for nanocrystals and multibands for nanoparticles.[24,25]

The analysis of pH explicitly depicts there is a gradual shift towards lowering the pH of the metal solution. It is noted that the sizes of the particles decrease when pH changed from highly acidic to neutral (3 to 7), indicated the size of the particles get reduced. If further the pH increased, then agglomeration of the particles was happened leads to non-determination of particle size exactly.[20] But in this study, the pH is maintained for first three hours, further reduced pH was observed; but no disturbances found in the nanosize formation that is confirmed by scanning electron imaging.

The sensitive determination of the best substrate for the analysis of nitrate reduction has the maximum activity. Numerous methods have been adopted to determine the nitrate reduction in the solution, but very few methods can be implemented and followed for the determination of reductase activity that have been optimized in order to confirm the enzymatic action and increase the nanoparticle synthesis. The principle behind these procedures was correlated highly in this study by determining the reduction of nitrate and sulphate.[26,27]

The size dependent phenomena are the central dogma for the determination of nanotechnological studies thereby the size of the particles or any other form should be measured by scanning or transmission electron microscopy that should not exceeding 100nm and are considered as nanomaterials. This study also supported the same by measuring 54 and 30nm sized particles in Ai-Ag and Ai-Cu complex. Thus we strongly found and recorded that the synthesized neem mediated silver and copper particles are nano in size and may have potent antimicrobial activity to kill microparticles. Various studies depicted that the scanning electron imaging are the incredible source of determining particle size in solute.[28-31]

It is instrumental that the step down particle size will kill and deteriorate the higher particle size due to the adhesive invasion. This is proved in this study by which the synthesized and characterized nanoparticles inhibit the growth in the culture plates of the test bacterial pathogens in vitro. Previous demonstrations also supported that the nanoparticles with synergistic combination including herbal mediated metal nanoparticles. This study showed the better inhibitory action of neem leaves mediated copper nanoparticles against wound infection causing bacterial pathogens. The silver nanoparticles also have certain bactericidal activity. Few studies described the importance of the sharing and synergizing the molecules among the substrates like herb and metal that are having more anti-infective characters.[32] Further exploration of such biomolecules in specific to chemistry, morphology, biochemical and biological interventions may provide the possible clue for the synthesis of nano sized particles and its antimicrobial efficiency.

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CONCLUSION

This study was initiated to identify the natural biomolecule from the synergistic action of herb and metal solution in condensed conditions. Further, it was characterized for the formation of biomaterials and to confirm the synthesis of nanomolecules. This study is an eye opener for research to include nitrate and sulphate reduction assay as a novel characterization to substantiate enzymatic catabolism of the bio to nanomaterials.

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