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Antibacterial analysis of *Ocimum sanctum* mediated silver and copper nanoparticles

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ABSTRACT

Background and Aim

Synthesis and development of newer antimicrobials are necessary to overcome the emerging and re-emerging infectious diseases. To evaluate the *in vitro* antibacterial activity of the *Ocimum sanctum* mediated silver and copper nanoparticles.

Methods and Results

The aqueous solution of the silver and copper, and crude extract of *O. sanctum* was evaluated. The color change, uv spectra, scanning electron imaging, atomic force microscopic and x-ray diffraction determinations, pH, nitrate and sulphate reductase assay were assessed for *O. sanctum* mediated silver (Ag-O.s) and copper (Cu-O.s) nanoparticles and finally *in vitro* anti-bacterial activity was performed. Copper is very effective than silver with maximum inhibition of 20mm against *Staphylococcus aureus, Klebsiella pneumoniae* and *Pseudomonas aeruginosa.* Further, the characterization of Ag-O.s and Cu-O.s nanoparticles revealed that the color change to dark brown and bluish tinge; pH upto 5 and 6 respectively. The SEI revealed of Ag-O.s and Cu-O.s as 15nm and 26nm; XRD as crystalline cubic lattice with 41.6nm and polycrystalline wurtzite with 69.2nm respectively. The AFM detected with 71 clusters with 12mm size of each and 48nm distance between two clusters, 177 particles were counted in one cluster whose size is 63nm with the peak height of 130nm for Cu-O.s. Bactericidal nature of Cu-O.s and Ag-O.s showed better results against *Pseudomonas aeruginosa, E. coli* etc.

Conclusion

The herbal mediated silver and copper materials were proved as nanoparticles and have effective bactericidal nature compared to the crude activity of herbs and metals.

Keywords: *Ocimum sanctum*, Copper, Silver, Nanoparticles, AFM, XRD, SEI, Bactericide

INTRODUCTION

Surgical Site Infection (SSI) are considered as the third most nosocomial infectious status as described by National nosocomial infection surveillance system [1, 2]. SSI is developed within 5-30 days after surgery which directly contributes the patients' morbidity and mortality. The triangular relationship between host, pathogens and environment may determine the prevention or initiation of wound infection [3, 4]. The identification, differentiation and diagnosis of SSI are difficult that leads to improper diagnosis, inappropriate antibiotic prescription, evolving of antibiotic resistance microbes and some mild to severe side effects [5].

Risk of wound infection is majorly created by microbial contamination, thus originates from the patient [6, 7] or by exogenous/ endogenous microbial group of pathogens, the most common microbes involved in SSI are Streptococcus sp [8], *Escherichia coli*, Methicillin Resistant *Staphylococcus aureus* (MRSA), *Bacillus* sp and *SSI are Streptococcus sp* [5, 9, 10]. The increase in the emergence of antibiotic resistant strains lead to treatment failure is a major challenge in the healthcare industry, in the recent decades had to search a new approaches and method for finding a new compound against drug resistant pathogens. Now, scientists accepted that the metal nanoparticles from herbal plants are also a novel area that proved biophysical chemistry [11, 12, 13].

Even though, the remarkable development in the use of techniques used for surgery, antibiotic selection procedure, aseptic operative rooms and surgical site infections remains a serious issues [14, 15]. Antibiotic resistance gram positive isolates showed maximum sensitivity to vancomycin, teicoplanin, linezolid and gram negative isolates were highly sensitive against meropenem and amikacin [16, 17]. Combinational drugs may have a high chance of exhibiting bactericidal and bacteriostatic nature against drug resistant pathogens due to its synergestic approaches [18].

Apart from antibiotics, chemically synthesized and derived drugs, green mediated metal nanoparticles are more or less equal effect against microbial pathogens with minimal health related risks [19, 20]. In the last decades, plants are the major source of antimicrobial agent in various countries, nearly 60-90% of peoples in the developing countries majorly using plant derived medicine, thus to treat human infectious diseases [21, 22, 23, 24, 25].

Generally metals are basic constituent to all living cells and get nourished when in combination with other nutrients, but once it reaching the minimal level (in micron) which was highly toxic [26], because of this nature, metal have a capacity to destroy microbes when its reach very minute size. Basically, silver and copper plated materials were used on those days there were not aware about its microbicidal nature. Technology development which helps to synthesize metal nanoparticles from herbal plants to perform specific function when compare to the bulk form of metal alone [27].

Recently, *Ocimum sanctum* leaves were used to synthesize silver and gold nanoparticles [28, 29] because of the presence of eugenol compound in tulsi helps to the action of antimicrobial activity. Synthesis of *O. sanctum* mediated silver nanoparticles reveal excellent antimicrobial activity was a cost effective method [30]. Here, we simultaneously synthesis and characterize *O. sanctum* mediated silver and copper nanoparticles to analyze the antimicrobial activity of isolated from wounds.

MATERIALS AND METHODS

Collection and preparation of herb

Ocimum sanctum leaves were collected and preliminarily processed. Then, test herb was allowed to shade dried and grinded to make a powder. Then the powdered herb was stored in the air tight container until further use.

In vitro antibacterial activity of metal solutions

The various concentrations of silver (10^{-2} to 10^{-5} mM) and copper solutions (1 to 4%) were prepared freshly and *in vitro* antibacterial activity was performed using Mueller Hinton agar- well cutting method. The bactericidal nature (zone of growth inhibition) was measured in mm.

In vitro antibacterial activity of *O. Sanctum* crude extract

The various concentrations (1 to 5%) of acetone extract of *O. sanctum* were prepared freshly and *in vitro* antibacterial activity was performed using Mueller Hinton agar – well cutting method. The

bactericidal nature (zone of growth inhibition) was measured in mm.

Synthesis of silver and copper nanoparticles

The initial step of nanoparticle synthesis is by mixing 100ml of 4% copper sulphate and 10^{-3} mM silver nitrate solutions with 5g of herbal powder. The solution mixture was mixed well and allowed to incubate at room temperature. During the incubation time, at some point the colour of the solution mixture were turned from pale to dark. Then the mixture was allowed to filter through Whatman No.1 filter paper and the filtrate was centrifuged at 6000 rpm for 20 minutes to separate the debris from the solution. Then the biosynthesized nanoparticles from *O. sanctum* were allowed to characterize.

Characterization of synthesized nanoparticles

The synthesized nanoparticles were characterized by physical, chemical and biological methods. In the physical method of characterization color change, uv spectra, SEI, AFM and XRD were done; chemical characterization pH, nitrate and sulphate reductase assay and antimicrobial activity was done.

Color change

At the initial the addition of test herbal leaves powder with the copper and silver solution are not gives colour. After the incubation time, the mixture gets darker in colour, thus indicate the reduction of sulphate and nitrate in the solution.

Calorimetric analysis

Optical Density value of copper and silver solution alone was recorded, before the addition of test herbal powder. After the addition of leaves to the silver nitrate and copper sulphate solution, the initial and final OD value was recorded.

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 *O. sanctum* solution. Initial and final pH of the each solution was recorded.

Nitrate and sulphate reductase assay

The spectrophotometric assessment for analyzing the Increase in 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 [31, 32] and sulphate to sulphite [33] respectively.

Shape and size determination

Further, the synthesized nanoparticles were physically characterized for the exploration of shape and size. For size specific phenomena, scanning electron imaging was performed thereby nano or micron sized particles measured. By atomic force microscopy, the study of agglomeration of the nanoparticles including clustering count and its size, distance between the clusters, number of nanoparticles in one cluster, size of single particle and peak evaluation heights were determined. The X-ray diffraction was performed to evaluate the shape and size of the particles thereby it was confirmed as nanoparticles.

Antibacterial assay

Agar well cutting method was used to determine the antibacterial wound isolated pathogens were included in this study. Each bacterial pathogen was added separately on to the Muller Hinton Agar (MHA) plates. Making wells on the agar plates and add various concentrations (1- 5%) of silver and copper nanoparticles. Allow all the plates were incubating at 32^{o} C for 24 hours. The zone of inhibition was observed and measured in millimeter.

RESULTS AND DISCUSSION

The biomolecular analysis of herbal reduced metals whose particle sizes are exhibited as nanoscale has its own toxic effects to pathogenic microorganisms. “Lowering the molecular size attack the higher scale organism” according to this principle, the herbal based materials whose material size below the micron have enough reducing, capping ability and antimicrobial activity [34, 35]. The biggest question is about the environmental toxicity.

This study has its own manner of evaluating the bactericidal effect of un-processed metal solutions and crude herbal compound. As a result, *in vitro* aqueous solution of silver were analyzed for bactericidal nature and showed maximum inhibition towards *S. aureus*, *K. pneumoniae* and *P. aeruginosa*. The copper solution revealed the

intrinsic bactericidal activity to *S. aureus, K. pneumoniae, P. aeruginosa* and *Serratia* sp. The detailed antibacterial activity of the test metal solutions against clinical pathogens was depicted in table 1. In this study, the concentration of 4% and 10^{-3} concentration of copper and silver solutions showed maximum inhibition, thus this concentration was taken for consideration to evaluate the herbal mediated metal nanoparticles.

Table 1: *In vitro* antibacterial activity of metal solution

Bacterial isolates	Copper sulphate				Silver nitrate			
	1%	2%	3%	4%	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Salmonella typhi	5	10	13	16	8	8	5	-
Flavobacter sp.	10	12	14	16	16	16	12	7
Enterococcus sp.	-	5	8	11	11	11	5	-
Salmonella paratyphi B	-	-	7	10	5	5	-	-
Staphylococcus aureus	10	14	16	20	20	20	14	9
Klebsiella oxytoca	6	10	12	15	9	9	-	-
Providencia rettigeri	10	12	15	18	10	10	5	3
Shigella sonnei	10	12	13	13	-	-	-	-
Klebsiella pneumoniae	10	13	17	20	20	20	13	7
Pseudomonas aeruginosa	17	20	20	20	20	20	20	12
Serratia sp.	13	15	20	20	8	8	-	-
Escherichia coli	10	12	14	16	16	16	12	5
Proteus mirabilis	15	16	18	18	18	18	16	11

The antibacterial effect of crude aqueous extract of *O. sanctum* was methodologically done and the results revealed that the maximum inhibition was observed against *S. aureus, P. aeruginosa, K. pneumoniae* and *E. coli* at the concentration of 250µg (Figure 1).

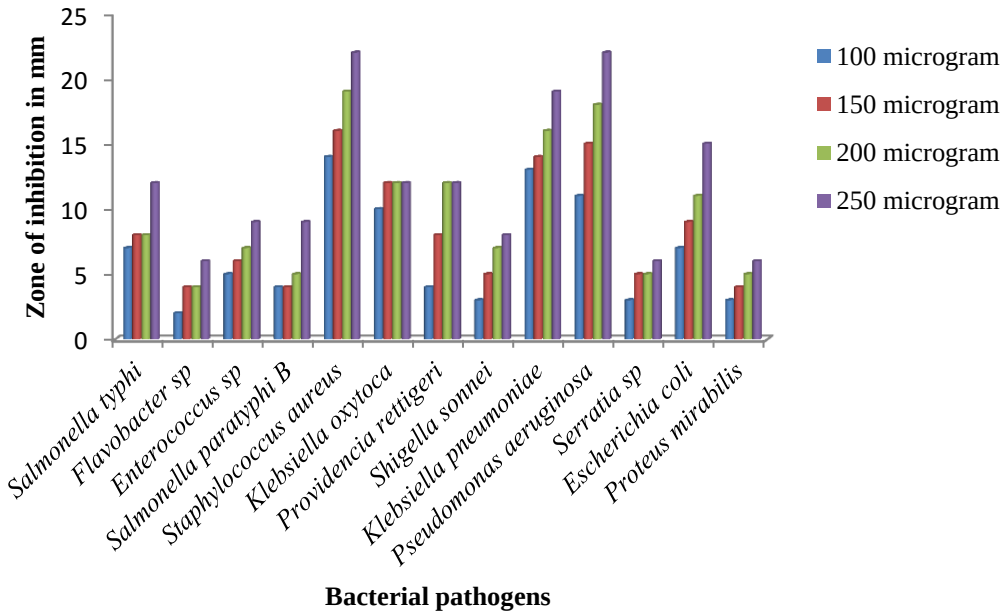


Figure 1: *In vitro* antibacterial activity of *O. sanctum*

The silver and copper nanoparticle synthesis through herbs (O. sanctum) was analyzed thereby it was preliminarily characterized by color change from pale to dark color. The silver complex is

colourless initially later changed to dark brown to black threads (Figure 2a and b); whereas copper was initially observed as pale blue and later changed to dark (Figure 2a and b). All these changes were recorded within 24 hours.

The synthesis of metal nanoparticles using herbs can be easily observed and preliminarily evaluated by the color change of the mixture, thus changed from light colour to yellowish to brown colour. This colour change arises from the excitation of vibrations of the surface plasmon resonance due to oscillation of free electrons accumulation occurrence [36, 37, 38, 39].

Figure 2a: Silver and copper control; Figure 2b: Silver and copper with test herb

The calorimetric value of the synthesized nanoparticles were evaluated by uv visible double beam spectrophotometric method, thereby the peak spectra showed decreased OD value verses time duration (Figure 3). The reduction in the OD value of the mixture indicated that the particles size were condensed and exacerbated as less concentrated molecules. By this we cannot confirm the molecule as nanoparticles, thus further extension of the work done to understand the enzymatic break down and physical characterizations (shape and size).

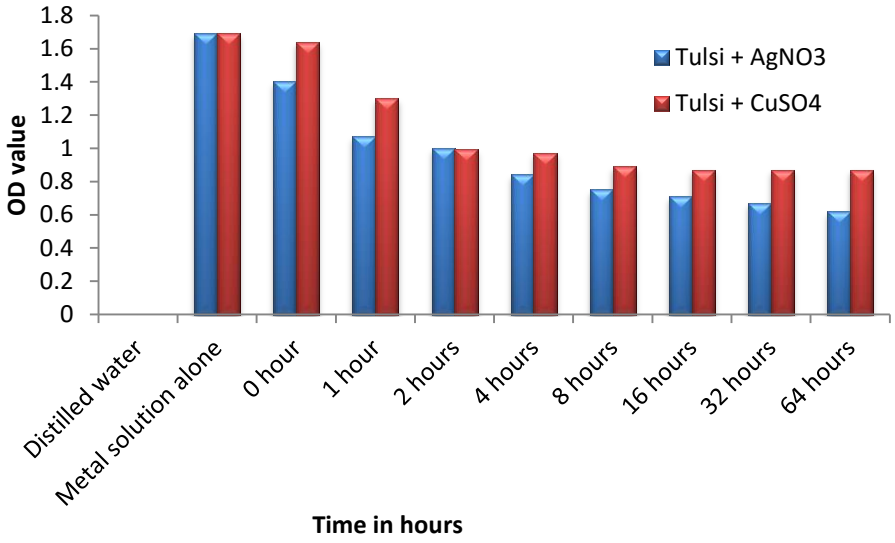
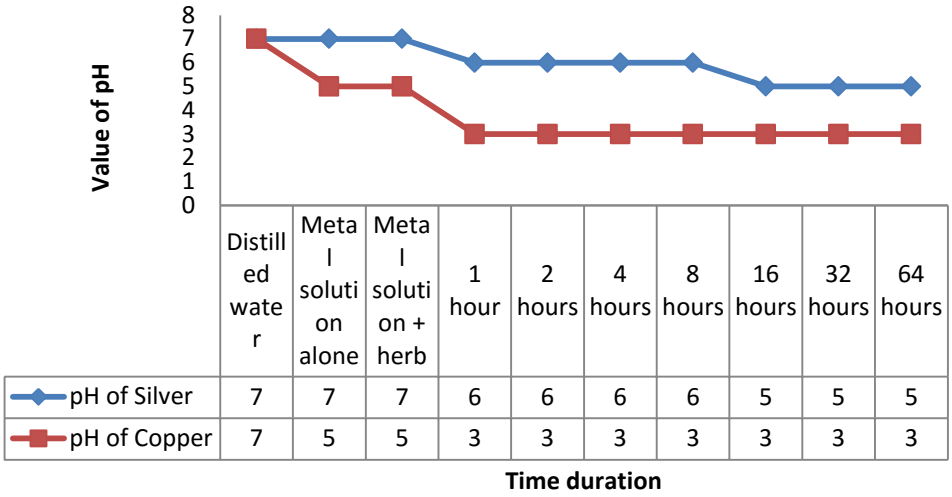


Figure 3: Calorimetric value of metal herbal mixture

The pH of the mixture was also supported by reducing from 7 to 5 and 3 among silver and copper – herbal mixture respectively. On comparing with silver, the copper – herbal mixture showed better results drastically after 60 minutes of interactions (Figure 4). The evaluation of the pH of solutes is the major factor for the synthesis thus influence on the size, shape and morphology of the nanoparticles.

Reaction of pH stimulate the electrical charge of the biomolecules which might be change the reducing and capping ability of the metal solution to synthesize biomolecules [40, 41, 42]. This study supported the same where pH was changed due to the reduction of metal ions with the compounds of herb.



	Distilled water	Metal solution alone	Metal solution + herb	1 hour	2 hours	4 hours	8 hours	16 hours	32 hours	64 hours
pH of Silver	7	7	7	6	6	6	6	5	5	5
pH of Copper	7	5	5	3	3	3	3	3	3	3

Figure 4: Reduction of pH value of solution mixture (copper and silver with test herb)

The nitrate and sulphate are bio-reducted by the test herb – *O. sanctum*, thereby evidences of formation of nitrite and sulphide observed. The elevated OD with 0.8 and 0.11 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 that are having the bactericidal properties. This reaction showed the nitrate reductase through the reaction of nitrite with 2,3 – diaminophthalene. It was observed that there is a reasonable reduction affinity for magnesium ions which are essential for assay of sulphate reductase activity.

The residual sulphate reductase activity was also done and comparing the assay reaction time, with enzymatic action revealed some observable reduction (Table 2). The bio-reduction was also characterized by the enzymatic analysis of nitrate and sulphate reduction revealed the theorem of enhanced antimicrobial properties. No evidences were so far created to proof this data [43]. Currently, very few methods can be adopted for the determination of metal reductase activity and the methodology has been optimized in order to confirm the enzymatic reduction and increase the efficacy of nanoparticles. There was high correlated phenomena thereby the study and the data will be correlated in future for standardizing the principles of nano-molecular synthesis [43, 44].

Table 2: Calorimetric analysis of nitrate and sulphate reduction

Time duration	Optical density at 460nm	
	Os-Ag solution	Os-Cu solution
0	0.18	0.27
30 mins	0.3	0.3
1 hour	0.4	0.5
2 hours	0.4	0.5
4 hours	0.5	0.6
8 hours	0.6	0.7
16 hours	0.7	0.9
24 hours	0.8	0.11

The scanning electron imaging revealed the better resolute of images thereby the particle size were analyzed as 15 and 26.3nm while magnified at 500X of Cu-O.s and Ag-O.s respectively (Figure 5). The characteristic particle agglomeration initiated from cluster size to size of the single particle was executed. The maximum of 86 clusters with 10mm sized and 72nm distance between the clusters were determined while evacuating *O. sanctum* mediated silver nanoparticles. One hundred and thirty seven (137) number of particle in one cluster was counted and the size of the single particle was approximately estimated as 36nm whose particle peak is 125nm height (Table 3).

Two different shapes were observed by X-ray diffraction where Cu-O.s and Ag-O.s exhibited cystalline cubic lattice (Figure 6 A) and polycrystalline Wurtzite structures (Figure 6 B) respectively. The inter-relationship analysis of effectiveness of light and electron microscopy considers substantial reach to synthesize and characterize newer biomolecules which are sub-cellular in nature and utilize the Applications and principles of physical and life sciences principles in order to obtain nano images with molecular specificity and spatial resolution. This phenomena has its own limitations to countenance the technical challenges for standardizing the sensitivity and specificity to achieve the reliable and efficient co-registration of optical and electron images. In this study, we characterize the images between 15 and 26nm by SEI, 36 and 63nm by AFM and 42 and 69nm by XRD for Ag-O. s and Cu- O.s respectively very few contaminations found but predominantly stable, spectrally-distinct cluster formation with controlled surface chemistry [45].

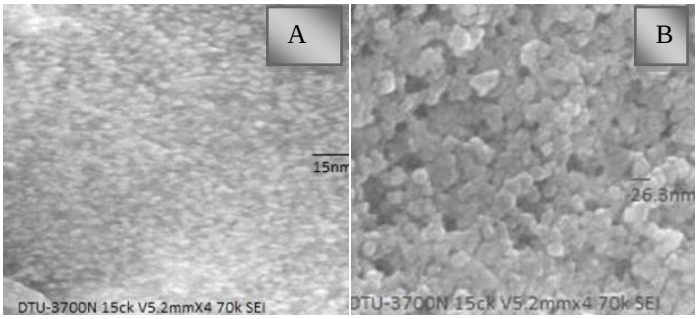


Figure 5: SEI of Cu-O.s (a) and Ag-O.s (b) at 500X

Table 3: Atomic force microscopic descriptions of Cu-O.s and Ag-O.s

Characters	Unit descriptions	Cu-O.s	Ag-O.s
Size of the cluster	Millimeter	12	10
Number of clusters	Numbers	71	86
Distance between two clusters	Nanometer (nm)	48	72
Number of particles in one cluster	Number	177	137
Size of the particles	nm	63	36
Height of particles' peak	nm	103	125

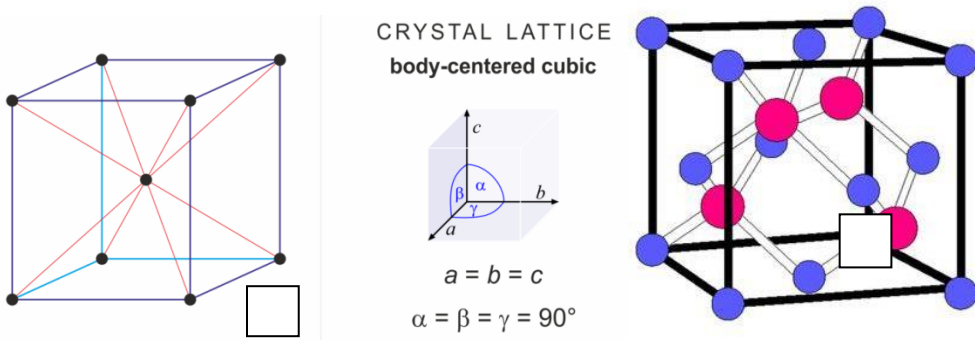


Figure 6: A model crystalline cubic lattice (A) and wurtzite (B) [46]
[Source: glossary.periodni.com]

The results of *in vitro* antibacterial activity of *O. sanctum* mediated silver and copper nanoparticles were measured as zone of inhibition and data was compared. The maximum inhibition was observed against *Proteus* species (40mm) followed by *Micrococcus* species (35mm) while using Cu-O.s. The silver nanoparticles also have more or less effective bactericidal nature against *Pseudomonas aeruginosa* (40mm) followed by *Klebsiella pneumoniae* and *E. coli* with 39mm each. The detailed antibacterial potential of O. sanctum mediated metal nanoparticles were depicted in table 4. The synthesis of effective antimicrobial agents is the challenging task for overcoming the state of drug failure and antimicrobial resistance.

The byproduct synthesis and environmental compatible bio-molecular production are the choice of current and future herbonanoceutical industries [47]. Through the scientific method of biocompatible formation of newer therapeutics [48], this study may provide some sort of information that explore the antibacterial potential of silver nanoparticles induced by *O. sanctum*, and study on the molecular mechanism of copper nanoparticles potential against gram positive and gram negative bacterial pathogens are to evaluated [49]. Effective inhibition towards *Proteus* species, *Micrococcus* species, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *E. coli* were studied and exhibited (Table 4).

Table 4: Antibacterial activity of inhibition against bacterial pathogens

Bacterial pathogens	**Level of zone of inhibition by O. s mediated silver nanoparticles (mm)**	**Level of zone of inhibition by O. s mediated copper nanoparticles (mm)**
Escherichia coli	39	32
Staphylococcus aureus	37	28
Pseudomonas aeruginosa	40	30
Proteus sp.	37	40
Klebsiella pneumoniae	39	27
Enterococcus sp.	25	10
Micrococcus sp.	Nil	35

The synthesis, manipulation, characterization and biological activity are sermonized and advocated for the proof of evidence of constructing green nanomaterials using eco-friendly metals solutions by biological entities for potential applications [50]. The optimization of the laboratory techniques and appropriate factors for reactions including light intensity, pH of the solution, interaction time and temperature are enhancing the nanoparticle synthesis [33].

The observation of various shapes also currently attracts the scientists to identify the exact mechanism of reducing the aqueous metal ion and herbal extract mixture [50]. In various studies the triangular Ag [51] and nanoprisms [52] synthesized by chemical and photochemical methods are impregnated but in this study crystalline cubic lattice and polycrystalline wurtzite elevated with 41.6 and 69.2nm sized molecules. By this sub-cellular microscopic analysis, we can characterize the particle size distribution, agglomeration and aggregation state, number of clusters and particles, and nanomaterials' surface characters [53].

CONCLUSION

Thus by this study, it was anticipated that the synthesis and characterization of O. sanctum mediated silver and copper nanoparticles initially and later confirmed the nano scale images through SEI, XRD and AFM. Further, it will be extended to analyze the exact nanochemistry of the synthesized bio-molecules for the pharmaceutical expansion.

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