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GREEN SYNTHESIS OF SILVER, COPPER AND ZINC NANOPARTICLES USING TABERNAEMONTANA DIVARICATA AND ITS APPLICATION.

Biotechnology

Sruti S Nair PG & Research Department of Biotechnology, Women's Christian College, Chennai -6, Tamilnadu, India.

Anchana Devi. C* PG & Research Department of Biotechnology, Women's Christian College, Chennai -6, Tamilnadu, India.*Corresponding Author

Pushpa. N PG & Research Department of Microbiology, Cauvery College for Women, Tiruchirappalli – 18, Tamilnadu, India.

ABSTRACT

Nanoparticles are particles of any shape with size ranging from 10 to 100 nm. Synthesis of metal nanoparticle is a single step process, which is inexpensive and eco-friendly method. Metals such as silver, zinc and copper can be used for the production of nanoparticles. Tabernaemontana divericata have been reported to exhibit antimicrobial, antioxidant, antitumor and analgesic effect. It's used for wound healing and treatment of various diseases like skin diseases, aches, ascaris, scabies eye diseases and pain. Nanoparticles of silver, zinc and copper were biosynthesised from the stem extract of Tabarnaemontana divaricata. Characterization studies were carried out using UV spectrophotometer, FTIR & SEM analysis. The nanoparticle were tested for it potential antibacterial activities. Among the three, AgNPs nanoparticle exhibited more antimicrobial activity than the CuNPs, and ZnNPs. The biomolecules in T. divericata stem extract with the potential to reduce and cap the bio reduced AgNPs were assessed with FTIR spectral measurements.

KEYWORDS

Tabarnaemontana divericata, Nanoparticles, Biosynthesis, Antimicrobial Activity, UV- Spectrophotometer, FTIR, SEM.

INTRODUCTION

Tabernaemontana divericata is shrub which is commonly seen in India. It usually grows in to a height of six feet. Almost all parts of the plant can be used for the treatment of various diseases. It's widely used in Ayurveda, Siddha and Unani. *Tabernaemontana divericata* have antimicrobial, antioxidant, antitumor and analgesic effect. It's used for the healing of wound and treatment of various diseases like skin diseases, aches, ascaris, scabies eye diseases and pain (Vazhacharickal, P.J. and Nair, S.S., 2018) *Tabernaemontana divaricata* is commonly known as pinwheel flower or crape jasmine. The flower is also known as milk flower because the stem exudes milky latex, when it's broken. *Tabernaemontana divaricata* belongs to the family of Apocynaceae and the subfamily Rauvolfloideae. It's called Nandhiar vattai in Tamil, Nandhiar vattam in Malayalam, Nandi vardhanam in Telugu, Nandi battalu in Kannada (USDA, N., 2015). Plants are good sources for nanoparticle synthesis as they are free from toxins and act as natural capping and reducing agents.

Plant Taxonomy

Kingdom- Plantae
Division- Magnoliophyta
Class- Magnoliopsida
Order -Gentianales
Family- Apocynaceae
Genus -*Tabernaemontana*
Species -*divaricata*

Nanoparticles have a great potential use in biological studies including antimicrobial activity. Nanoparticles are particles of any shape with size ranging from 10 to 100 nm. Nanoparticles have wide range of application in the field of physics, chemistry, biology, material science and medicine (Sajesh Kumar, N.K. *et al*., 2014). Nanoparticles have optical, catalytic, electronic and antimicrobial effect. Nanoparticles can be synthesised using chemical as well as physical method. Nanoparticles can be created using gas condensation, ion implantation, pyrolysis, hydrothermal synthesis (Hajipour, M.J. *et al*., 2012). Metals have inhibitory effect on microbes. Synthesis of metal nanoparticle is a single step process, which is inexpensive and eco-friendly method. Metals such as silver, zinc and copper can be used for the production of nanoparticles. Silver nitrate, copper sulphate, copper nitrate, zinc sulphate, zinc oxide is mainly used of the synthesis of nanoparticles (Sajesh Kumar, N.K. *et al*., 2014). Traditionally Silver, copper and zinc metals have been used in ancient medicine for treatment of various wounds and diseases. These metals in small quantities act as effective broad spectrum antimicrobial agent with low toxicity (Farooqui, M.A. *et al*., 2010). The most important application of silver and silver nanoparticles is in medical industry such as tropical ointments to prevent infection against burn and open wounds (Ip, M. *et al*., 2006) Generally studies of various nanoparticles are reported to possess antifungal, anti-inflammatory, antiviral, anti-angiogenesis, and antiplatelet activity (Wiley, B.J. *et al*., 2006). Hence the present study intends to explore the potential antimicrobial activity of Silver, Zinc and Copper nanoparticles synthesized from Stem extracts of *Tabernaemontana divericata.*

MATERIALS AND METHODOLOGY

Sample Collection and Preparation of Plant extracts

Tabernaemontana divaricate stem samples free from diseases were collected from Chennai, Tamilnadu, India (Fig 1). The plant material was taxonomically identified and authenticated by Department of Botany, Women's Christian College, Chennai, India. It was then washed thoroughly 2-3 times with tap water and once with sterile water. 10 g of fresh stem were finely chopped and added to 100 mL of distilled water (1:10 ratio) and left undisturbed at room temperature for 48-72 hrs. Similarly ethanol extract was prepared in a capped bottle to prevent evaporation. The extracts were filtered with Whatman paper number 1. Filtrate was collected and stored for further analysis.

Green Synthesis of Nanoparticles:

Synthesis of Silver Nanoparticles (Singhal, G. *et al*., 2011)

Synthesis of silver nanoparticles was brought about by 0.1 M of aqueous solution of silver nitrate ($AgNO_3$). 5 mL of stem extract of *Tabernaemontana divaricate* was added to 45 mL of 0.1 M $AgNO_3$ solution for bio reduction process at room temperature overnight.

Synthesis of Copper Nanoparticles (Murthy, H.C. *et al*., 2020)

0.2 M aqueous $Cu(NO_3)_2{\cdot}3H_2O$ solution was prepared and stored in brown bottles. 10 ml of plant stem extract was slowly dropwise added under constant stirring with 40 ml of 0.2 M Cu $(NO_3)_2{\cdot}3H_2O$ solution (1: 4 ratio). The mixture was incubated at room temperature for 24 hrs. The color change from blue to light brownish indicates the formation of Cu Nps.

Synthesis of Zinc Nanoparticles (Singh, N. and Haque, F.Z., 2016)

10 mL of stem extract sample was added to 100 mL of 0.1 M Zinc Sulfate ($ZnSO_4$) aqueous solution. Prepared NaOH was slowly added to the solution under continuous stirring until the pH reached to 8. It was then kept on a magnetic stirrer at 60°C for 6 h. The change of colour of the solution to yellowish-brown color indicates the formation of the zinc oxide nanoparticles.

Characterization of Nanoparticles

UV-Visible Absorbance Spectroscopy

UV-Visible spectroscopy analysis of synthesized nanoparticles was carried out using Shimadzu UV-Visible absorption spectrophotometer. (0.5 mL) volume of each sample (Silver, Copper and zinc) nanoparticles was taken and analyzed at room temperature.

UV-Visible Spectra was used for monitoring the reaction between metal ions and the extract.

For silver nanoparticles wavelength in nanometers from 340 to 800 nm was used. The reduction of silver ions and formation of silver nanoparticles was observed and the reading were noted (Zhang, W. et al., 2006). The formation of ZnO-NPs was monitored using a UV-Vis spectrophotometer at 300–600 nm (Karimzadeh, M.R. *et al*., 2020) and formation of CuNPs was observed at 200-400 nm (Sarkar, J. *et al*., 2020).

Fourier-transform infrared (FTIR) Analysis:

Fourier transform infrared (FT-IR) spectrometric analysis was performed to delineate the functional groups in the extract that reduce ions to NPs. For FTIR measurements, the synthesized nanoparticle solutions were centrifuged at 10000 rpm for 30 minutes. The pellets of silver, zinc and copper nanoparticles were washed thrice with 5 mL of deionized water to get rid of the free proteins or enzymes that are not capping the nanoparticles. The pellets were dried by using vacuum drier. It was analyzed by FTIR using potassium bromide (KBr) pellet method. Scan range was between 4000 and 400cm−1 for copper silver and zinc nanoparticles where, possible biomolecules bonding interactions can be studied. (Rajendran, S.P. and Sengodan, K., 2017)

Scanning electron microscopy (SEM):

SEM is the type of electron microscope produces image of a nanoparticles using focused beam of electrons. The electrons interact with atoms in a sample, provides information on morphological and topographical characterization of the nanoparticles had been well established by the electron microscopic studies (Nair, P.M.G. *et al*., 2014).

Antimicrobial assay of synthesised nanoparticles

Antibacterial analysis was done both Gram positive and negative bacteria (*Staphylococcus* spp, *Streptococcus* spp, *Bacillus* spp, *Escherichia coli*, *Pseudomonas aeruginosa*) by well-diffusion method. Overnight cultures were prepared in nutrient broth and inoculated with the organisms and incubated at 37°C for 24 h. 0.1 ml of bacterial culture was used for spread plate on Muller Hinton agar medium, Wells were punched using sterile well puncher. 200 µl of silver, copper and zinc nanoparticles were loaded on to the wells to each culture plate and incubated at 37°C for 24- 48 h. Antibacterial activity was measured based on the zone of inhibition obtained around the well. Silver nitrate, copper sulphate and zinc sulphate solutions were used as standards to compare and check the efficiency of green synthesized nanoparticles. Ampicillin (10 µg/ml) was used as standard.

Antifungal analysis was carried out using *Candida spp. Pencillium spp and Aspergillu niger* on SDA media using agar well diffusion method. The zone of inhibition was observed after 72 hours of incubation at 37°C.

RESULTS AND DISCUSSION

Sample Collection and Preparation of Plant extracts

The sample was extracted using distilled water and ethanol. The stem of *Tabernaemontana divericata* and distilled water was taken in 1:10 ratio, grinded, filtered and stored in closed glass vessel. (Figure 1) Stem was collected freshly from *Tabernaemontana divericata* to obtain the maximum amount of extract. Mechanical extraction using motor and pistil made the extraction easier. Aqueous and ethanol was used as solvents, various studies have reported the efficiency of aqueous and solvents containing alcohol are good for the extraction of plant materials. (Do, Q.D. *et al*., 2014)

Figure 1: *Tabernaemontana divaricate* plant, Stem and extract preparation.

Synthesis of Nanoparticles

Silver nanoparticles

Silver nanoparticle was synthesised from the stem extract of *Tabarnaemontana divaricata*. And 1mM of silver nitrate was used for the synthesis of silver nanoparticle. Colour change was observed from mild yellow to brown indicated the formation of silver nanoparticle. (Figure 2)

Copper nanoparticles

The synthesis of copper nanoparticle takes place as a result of the reduction of copper ions in the solution. The biomolecule present in the plant sample which is added along with the copper sulphate solution helps in the reduction of copper ions and capping the biosynthesised nanoparticle. The colour change was observed from light blue colour was pale yellow after incubation period. (Figure 3)

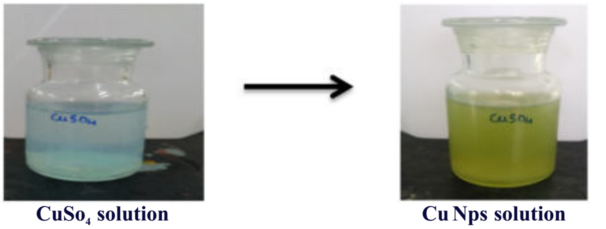
Zinc nanoparticles

The synthesis of zinc nanoparticle takes place as a result of the reduction of zinc ions in the solution. The colour change was observed from colourless to pale green after incubation period. (Figure 4)

The change in colours in synthesis, is due to the surface Plasmon excitation of metal ions, which is the primary evidence for the formation of nanoparticle. (Singh, P. *et al*., 2018; Thirumurugan, A. *et al*., 2010)

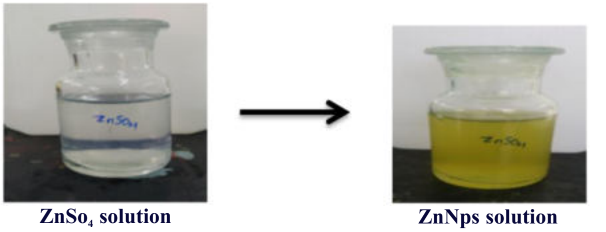
$AgNo_3$ solution **Ag Nps solution**

Figure 2: Synthesis of Silver nanoparticles



$CuSo_4$ solution **Cu Nps solution**

Figure 3: Synthesis of Copper oxide nanoparticles



$ZnSo_4$ solution **ZnNps solution**

Figure 4: Synthesis of Zinc oxide nanoparticles

Characterization of Nanoparticles :

UV spectrophotometer

The reduction of metal ions was subjected to spectral analysis by using the UV–Visible spectrophotometer. Sharp rise in absorbance peaks was due to the effect of surface plasmon resonance in the reaction mixture. Reduction of silver ions into AgNPs was observed as a result of the change in color, The sharp absorption of AgNPs was observed at 480 nm(Ahmad, N. and Sharma, S., 2012) . For copper nanoparticles, an absorbance peak was around 270 nm, which was specific for copper oxide nanoparticles (Saif, S. *et al*., 2016). The absorption peak for the synthesized ZnO NPs is 360 nm, correlates with the range of absorption of ZnO NPs, which is 360–380 nm. (Nagarajan, S. and Kuppusamy, K.A., 2013)
(Figure 5)(Table 1)

Following UV spectrophotometer studies, the nanoparticles were tested for antibacterial and antifungal activity and the potentially active nanoparticle was selected and further analysed using FT IR and SEM to identify the functional group and study the morphology and size of nanoparticle.

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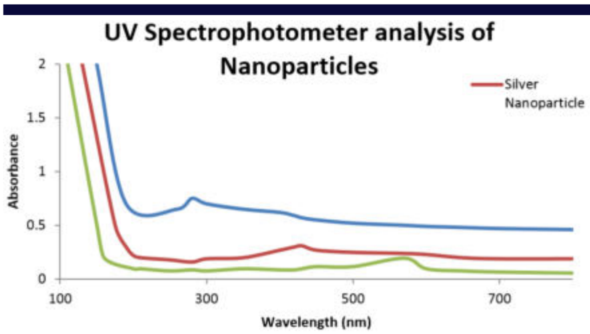

Figure 5: UV Spectrophotometer analysis of nanoparticles

Table 1: Peak absorbance value of Nanoparticles

S.no	Nanoparticle	Maximum Peak absorbance in nm
1.	Silver	480
2.	Copper	270
3.	Zinc	360

FTIR- Fourier Transform Infrared Spectroscopy

The results of FTIR analysis of the AgNPs biosynthesised from *Tabarnaemontana divericata* stem extract shows different stretches of bonds shown at different peaks. The peaks were observed at 1639.70, 2110.54, 2363.02, 3322.66. The peak at 1639.70 indicate the presence of C=C – the stretching of alkenes, which is a weak bond and C=O – the presence of primary amides, which is a strong bond.The peak at 2110.54 indicates the presence C≡C in the biosynthesised AgNPs, which is a weak bond. The peak at 2363.02 shows the medium bond of C-H stretching. The peak at 3322.66 indicates the nanoparticles have amines (N-H) medium, and OH groups of alcohol, phenol and organic acids. (Figure 6; Table 2)

FTIR spectral measurements were performed to find out the potential Functional group in *Tabarnaemontana divericata* stem extract, which is responsible for the reducing and capping the bio reduced AgNPs showed the structure and respective bands of the biosynthesised AgNPs and the stretch of the bond. The stretch and bonds obtained were also similar to the results reported by Devaraj, P. *et al*., 2014.

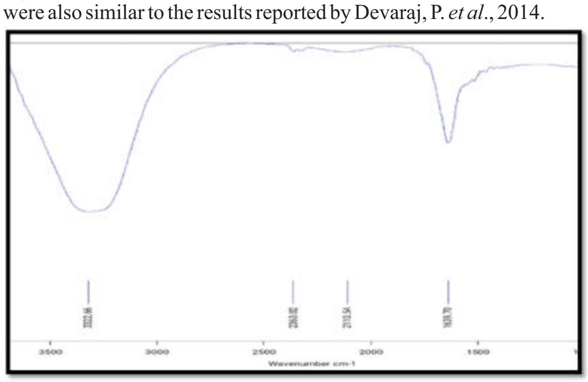

Figure 6: FTIR result of synthesised AgNPs

Table 2: Functional groups present in the silver nanoparticle –

Sl.No	Wave number (cm)	Assignments	Bond strength
1	1639.70	C=C – the stretching of alkenes	weak bond
2	2110.54	C≡C stretching of alkynes	weak bond
3	2363.02	C-H stretching	Medium bond
4	3322.66	amines (N-H) , and OH groups of alcohol, phenol and organic acids	Medium bond

Scanning Electron Microscopy (SEM) Analysis:

The SEM images obtained displayed the particle size of 2.5µm (Figure 7). The SEM image showed the presence of high-density and compact agglomerates of silver particles. The nature of AgNPs was found to be highly granulated. The images also showed the presence of various shapes such as spherical, ellipsoidal, and few irregular fused agglomerates. Similar kind of result was also obtained by Forough, M. and FAHADI, K., 2011

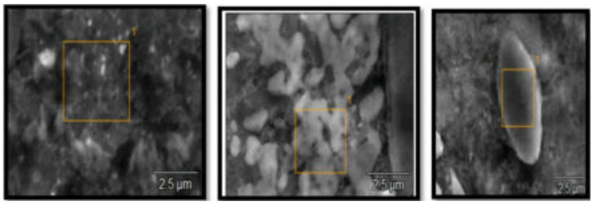

Figure 7 : SEM image of synthesized Silver nanoparticles

Antimicrobial Studies

The results of the antimicrobial studies of the synthesized NPs (Silver, Copper and Zinc), were evaluated using agar disc diffusion method against five bacterial strains are shown in Table 3 and three fungal strains in Table 4.

All the three nanoparticles showed antibacterial and antifungal activities. AgNps nanoparticle exhibited more antibacterial activity than the CuNps, and ZnNps. AgNps has more antibacterial activity in *Streptococcus sp* and *Pseudomonas sp*, with a 25 mm diameter of zone of inhibition. The least zone of inhibition for AgNps was observed in Bacillus *sp*. ZnNps exhibited low antimicrobial activity when compared to other two nanoparticles. *Aspergillus sp* had the highest zone of inhibition. Silver nanoparticle showed highest antimicrobial activity, followed by copper. The study results correlated with the finding of the study by Shahzadi, S. *et al*., 2018.

Table 3: Zone of inhibition of nanoparticles stem extracts of *Tabarnaemontana divaricata* against bacterial strains

S.no	Bacterial strains	Zone of inhibition of nanoparticles in mm			Standard Ampicillin (10µg/ml)
		Silver nanoparticles	Zinc nanoparticles	Copper nanoparticles	
1.	*E.coli*	18	11	15	15
2.	*Pseudomonas spp*	25	16	13	22
3.	*Staphylococcus spp*	20	15	16	22
4.	*Streptococcus Spp*	25	13	16	25
5.	*Klebsiella Spp*	23	11	12	26
6.	*Bacillus Spp*	18	11	15	23

Table 4: Zone of inhibition of nanoparticles stem extracts of *Tabarnaemontana divaricata* against fungal strains

S.no	Fungal strains	Zone of inhibition of nanoparticles (in mm)			Standard Fluconazole (10µg/ml)
		Silver nanoparticles	Copper nanoparticles	Zinc nanoparticles	
1.	*Candida spp*	17	14	13	22
2.	*Penicillium spp*	17	13	14	19
3.	*Aspergillus niger*	18	13	13	16

CONCLUSION

Stem extract of *Tabernaemontana divericata* were prepared using distilled water and ethanol. Freely water soluble terpenoids, steroids, alkaloids and proteins of T. divaricata play a vital role in the biosynthesis and stability of nanoparticles that are spherical in shape in an aqueous medium. Silver, nanoparticle biosynthesised from the stem extract, exhibited high antimicrobial activity, which was further used for the study. The current study envisions on the aspect of biosynthesis of different nanoparticles from the secondary metabolites of T. *divaricata* plant which are emerging sources of for antimicrobial therapy in modern medical applications.

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