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Green synthesis of silver and silver-zinc doped nanoparticles using *Anamirta cocculus* leaf extract: And their antifungal activities

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Abstract
Metallic nanoparticles are widely employed in every phase of science, engineering and medical field. Also several studies have proven their antimicrobial significance. Synthesis of metallic nanoparticles is still charming arena to scientists for exploring novel dimensions for their respective worth which is generally attributed to their corresponding small sizes. Conventionally nanoparticles are synthesized using chemicals as reducing agents by chemical method which pose to various environmental risks due to their general harmfulness. It engenders the serious concern to develop environmentally friendly processes. Hence, biological approaches are shown quite interest to fill the void; for green syntheses of nanoparticles using biological molecules derived from plant sources in the form of extracts. In the present study, rapid, simple approach has been applied for synthesis of silver and silver doped zinc nanoparticles using aqueous *Anamirta cocculus* leaf extract. The plant based biological molecules of *Anamirta cocculus* undergo highly controlled assembly for making them suitable for the metal nanoparticle syntheses. Various techniques were carried out to characterize synthesized nanoparticles such as FTIR, UV, SEM, and EDX. Investigation on antifungal activity showed that the nanoparticles exhibit good potential antifungal activity against *Apergillus flavus* and *Apergillus terreus.* Results confirmed this protocol as simple, nontoxic, rapid and eco-friendly an alternative conventional chemical methods.

Keywords: green synthesis, anamirta cocculus, Ag, Ag-Zn doped nanoparticle and antifungal activity

Introduction

Nanotechnology is a dynamic field of modern research dealing with strategy, manipulation of particle's structure and synthesis of nano-size particles. Applications of nano-particles are increasing rapidly on various fronts because of their completely novel or enhanced properties based on morphology, size, and their distribution. Nanoparticles applications are rapidly gaining renovation in a various fields such as, food technology, cosmetics, health care, environment, drug delivery, health, mechanics, chemical industries, catalysis, optics, space research, electronics, light emitters, energy technology, electron transistors, photo-electrochemical applications, and nonlinear optical devices. These remarkable growths on nanotechnologies have opened applied frontiers and novel fundamentals include the production of nano-materials and investigation on utilization of their mysterious physicochemical and optoelectronic properties. The metallic nanoparticles contain significant antimicrobial properties due to their large surface area to volume ratio. It gives immense interest for researchers due to the growing microbial resistance against metal ions, antibiotics and the development of resistant strains. Among the different metal nanoparticles, silver (Ag) nanoparticles are most significant product in nanotechnology since it has gained vast beneficial unique properties such as good conductivity, chemical stability, most important antibacterial, antifungal, anti-viral, and catalytic activity. It has gained potential applications in biomedical field such as antiseptic sprays and fabrics, being added to wound dressings, and topical creams. It shows a broad biocide influence against microorganisms through the disruption of their unicellular membrane. Because of their wide range of applications, synthesis of Ag nanoparticles is of much interest to the research community. Commonly, chemical and physical methods of nanoparticles synthesis are quite expensive and pose to significant hazardous to the environment which involve use of toxic and perilous chemicals. Thus, the development of biologically-inspired environmentally friendly experimental processes for the syntheses of nanoparticles is gaining importance into nanotechnology. When compare to chemical and physical methods, green syntheses of nanoparticles has several advancements such as cost effective, environment friendly, and easily scaled up, no need to use high pressure, temperature, energy and toxic chemicals [1].

A lot of reports have been published to till date on biological syntheses of Ag nanoparticles using fungi, bacteria, and plants; because of their reducing properties typically responsible for the reduction of metal compounds in their respective nanoparticles. Since it is mandatory to maintain high aseptic conditions, biological methods of Ag nanoparticle synthesis by microbe mediated system is not of industrially feasible. Hence; the use of plant extracts for this purpose is potentially advantageous over microorganisms because of the ease of improvement, elaborate process of maintaining cell cultures, and less biohazard. Furthermore, use of plant extracts also reduces the cost of isolation microbes and their culture media. Keeping the view, the present study was carried out to synthesize and characterize the nanoparticles using plant leaf extract. The synthesis of Ag nanoparticles from various plants species like leaf extracts of *Euphorbia hirta* and *Nerium indicum* [2] papaya fruit extract [3], *Cinnamomum camphora* [4], *Emblica of icianalis* [5] *Carica papaya* [6], *Parthenium hysterophorus* [5] and several work has been reported. However, the potential plants as biological materials for the synthesis of nanoparticles are yet to be fully explored. In this study, *Anamirta cocculus*, the climbing plant, which is common in south Asia, was used as plant source for production of Ag-Zn doped nanoparticles. Further synthesized nanoparticles were applied to determine its antifungal activity against *A.flavus* and *A.terreus* in laboratory condition and for dermatology aspect in future.

Materials and Methods

Plant Extract Preparation and Biosynthesis of Nanoparticles

The Fresh and mature *Anamirta cocculus* leaves were collected from Siddha medicinal plants garden, Mettur in the month of February. Leaves were washed in tap water to remove the dirt and dust on the surface of the leaves. 45g of finely chopped *Anamirta cocculus* leaves were added and rinsed to 225ml of distilled water. Again it was rinsed with 225ml of double-distilled water. The chopped leaves were taken in a ratio of 1:5 (45gm: 225ml) of double-distilled water and maintained at 80 ºC in oil bath along with magnetic stirrer for 90 min. After boiling, the leaf extract was cooled at room temperature and filtered conferring 120ml of transparent yellow color leaf broth. Then, the solution it was divided into two beakers (Named as A and B) of 60ml each. 4gm of $AgNO_3$ solution was added in beaker A for the green sythesis of Ag nanoparticles. 4gm $AgNO_3$ and 0.75gm of zinc acetate were added in the beaker B for the green synthesis of Ag-Zn doped nanoparticles and allowed at ambient condition to react. At different time intervals, the change of color in reaction mixture was observed from transparent yellow to dark brown that indicated the formation of Ag-Nanoparticles. The nanoparticles solutions were collected and UV-vis spectral analysis was carried to investigate the reduction mechanism of Ag ion in to nanoparticles in solution. The nanoparticles solutions were centrifuged and the excess liquid phases were removed by evaporation in an air-dryer yielding black colored nano powder [7].

Characterization of Nanoparticles

Bio-reduced nanoparticle samples were used to observe the optical absorbance between 190 and 800 nm with a UV-vis spectrometer (HITACHI, Model U-2800 spectrophotometer) at room temperature operated at a resolution of 1 nm. FT-IR spectra study was carried out to identify the presence of potential biomolecule and functional groups using Perkin Elmer spectrometer having a in the wavelength range 500-4000 cm^{-1}. It also determines the possible physiochemical interactions among the components in a plant extract formulation. The results of it were compared for the shift in functional peaks of the critical value. The surface morphology of synthesized nanoparticles was investigated by scanning electronic microscope (FEGSEM Zeiss, Ultraplus model). Energy-dispersive X-ray (EDX) analysis at 20kV was undertaken to determine elemental composition of the particles [8].

Assessment of Antifungal activity

The antifungal activity of the synthesized silver-zinc doped NPs was tested against the fungi, *Aspergillus flavus* and *Aspergillus terreus* by well diffusion method. The fungal strains were grown in potato dextrose broth (PDB) for 72 h and used for the study. Well grown fungi was swabbed (50µl approximately) on the potato dextrose agar (PDA) media with sterile cotton buds. Various concentrations of Ag-Zn NPs (50, 75 and 100µl) were added to the center of the well having 7mm of diameter. The fluconazole antibiotic was used as a positive control. All the petriplates were incubated at 25 ºC for 72 h in incubator and the zone of inhibition (mm) surrounding the was measured [9].

Chemical constituency identification of Plant extract

Test for alkaloids

The extract of *Anamirta cocculus* was heated on a boiling water-bath with 5ml Hydrochloric acid. After cooling, the mixture was filtered and treated with a few drops of Meyer's reagent and Hager's reagent (saturated picric acid solution). Formation of yellow colored precipitate indicates the presence of alkaloids.

Test for Tannins

The aqueous extract of *Anamirta cocculus* was added with 10 ml of bromine water. Decoloration of bromine water showed the presence of tannins.

Test for Terpenoids

The aqueous extract of *Anamirta cocculus* of about 5ml is added with 2ml of chloroform and then boiled with 3ml of Sulphuric acid concentrated. A grey color formed which showed the entity of terpenoids.

Test for saponins

5 ml of aqueous extract of *Anamirta cocculus* is shaken vigorously with 5 ml of distilled water in a test tube. The formation of stable foam was accepted as an indication of the presence of saponins [10].

Test for Phenol (Ferric Chloride Test)

5ml aqueous extract of *Anamirta cocculus* is treated with 5% ferric chloride. The occurence of deep blue color indicates the presence of phenol.

Test for Amino acid (Ninhydrin Test) 5ml aqueous extract of *Anamirta cocculus* is treated with Ninhydrin solution. The occurence purple color indicates the presence of amino acid.

Test for Protein (Xanthoproteic Test)

The Aqueous extract of anamirta cocculus about 5ml is treated with a few drops of conc. Nitric acid. Formation of yellow colour indicates the presence of proteins.

Test for Carbohydrates

Molisch's Test

Aqueous extract of *Anamirta cocculus* is treated with two drops of alcoholic ά- naphthol solution in a test tube. Carefully, incline tubes and pour drop wise conc. Sulphuric acid using a dropper, along the sides of test tube. Formation of violet colour at the junction or interface of two liquids indicates the presences of carbohydrates.

Benedict's test

Aqueous solution of *Anamirta cocculus* is treated with Benedict's reagent (Sodium carbonate + sodium citrate and copper sulphate solution), then the mixture is heated on a boiling water bath for 5 minutes and cooled. Orange red precipitate indicates the presence of carbohydrates.

Results and Discussion

The presences of photochemical constituency in *Anamirta cocculus* leaf extract were clearly shown in tables 1. That test to indicate the presence of various bioactive secondary products. Fig 1 was clearly shown the colure differentiation that was identified the result. The similar result was reported in *Cledrodendrum inerme, Dennotia tripetala, Pongamia glabra, O. Sanctum.* This test to indicate the presence of various bioactive secondary product which would be responsible for their five common plant attributes. Phytochemicals such as alkaloids, terpenoid, steroids, saponins were processed the standard methods, phytochemical analysis of plant was need to discover and extended to novel therapeutically agents with improved efficiency. In this process deals with the secondary based on phytochemical test of five plants this test to indicate the presence of various bioactive secondary product which would be responsible for their five common plant attributes. Phytochemicals such as alkaloids, terpenoid, steroids, saponins were processed the standard methods, phytochemical analysis of plant was need to discover and extended to novel therapeutically agents with improved efficiency. In this process deals with the secondary based on phytochemical test of five plants this test to indicate the presence of various bioactive secondary product which would be responsible for their five common plant attributes. Phytochemicals such as alkaloids, terpenoid, steroids, saponins were processed the standard methods, phytochemical analysis of plant was need to discover and extended to novel therapeutically agents with improved efficiency. In this process deals with the secondary based on phytochemical test of five plants Clerodendrum inerme, Pongamia glabra, Sanctum, Pennetia tripetala, Abutilon indicum, were contain some specific phyto-constituents. This test to indicate the presence of various bioactive secondary product which would be responsible for their five common plant attributes. Phytochemicals such as alkaloids, terpenoid, steroids, saponins were processed the standard methods, phytochemical analysis of plant was need to discover and extended to novel therapeutically agents with improved efficiency.

Table 1: Phytochemical properties of *Anamirta cocculus* plant extract

S. No.	Phytochemicals present	Result
1.	Alkaloids	+
2.	Terpenoids	+
3.	Tannins	-
4.	Phenols	+
5.	Proteins	+
6.	Amino acids	+
7.	Carbhohydrate	+
8.	Glucoside	+
9.	Saponification	+

Fig 1: Phyotochemical results of *Anamirta cocculus* extract

Ultraviolet Visible Spectroscopy

It is well known that silver NPs exhibit strong absorption band in the visible region. In this experiment, addition of plant extract of *Anamirta cocculus* with aqueous solution of silver nitrate led to the change in the color of the solution yellowish to reddish brown.

The optical properties of silver Nps and silver-zinc dopped Nps were investigated by UV-Vis absorption spectroscopy. Plasmon resonance peak detected at 344.3, 382.6, and 764.0 nm with absorbance values, 0.182, 0.180, 0.197, respectively, for the silver NPs. The same is depicted in Fig. 2(a). Plasmon resonance peak detected at 362.9, 1,005.7, and 1,079.1 nm with absorbance values, 0.157, 0.132, 0.132, respectively, for the silver-zinc NPs. The same is depicted in Fig. 2(b).

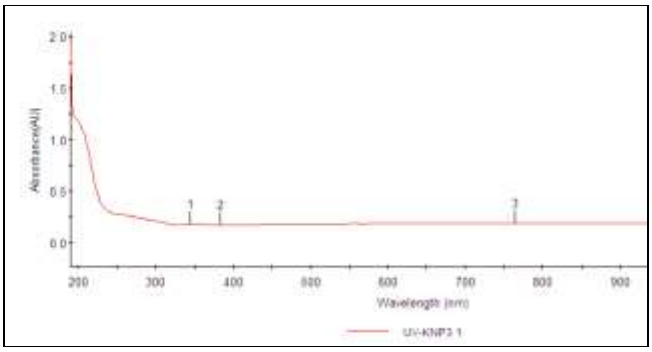

Fig 2(a): UV analysis of Silver nanoparticles

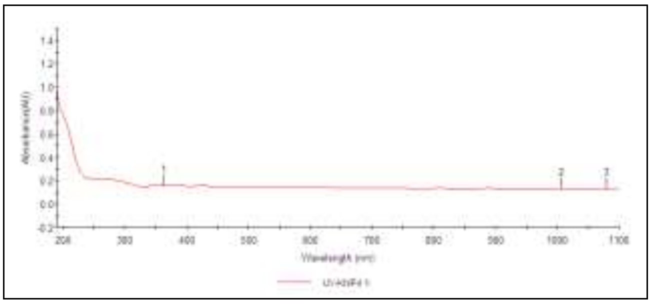

Fig 2(b): UV analysis of zinc -Silver nanoparticles

Scanning Electronic Microscope

Figure 3 shows the SEM images (Fig.3 (a) & (b)) of the same synthesized NPs. The particles were observed to be highly agglomerated, possibly an artifact of the centrifugation and subsequent drying required to prepare silver and silver-zinc doped nanoparticles samples for SEM analysis. The particles were found in the different range between 85-350 nm. Using a software, "IMAGEJ", It was found that the average particle size about 167.4 nm. Silver Nps and silver-zinc dopped Nps were subjected for the EDX analysis and the spectra from the same were given in Fig. 4(a) & (b). Silver nanocrystals typically display an absorption peak around 3keV and in the silver-zinc doped nanocrystals have a peak at 1keV due to their surface plasmon resonance. EDX analysis of NPs demonstrated a strongly well-defined silver signal at 3keV and zinc signal at 1keV along with weak carbon, oxygen and nitrogen peaks, with the latter weaker signals probably representing surface biomolecule capping structures originating from the leaf extracts.

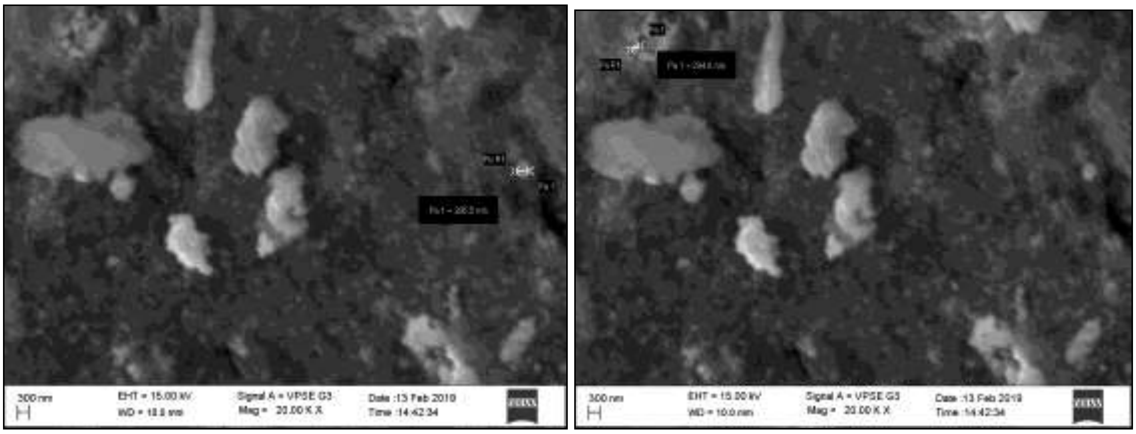

Fig 3(a) & (b): (a) SEM analysis of Ag Nanoparticles, (b) SEM analysis of Zn-Ag Nanoparticles

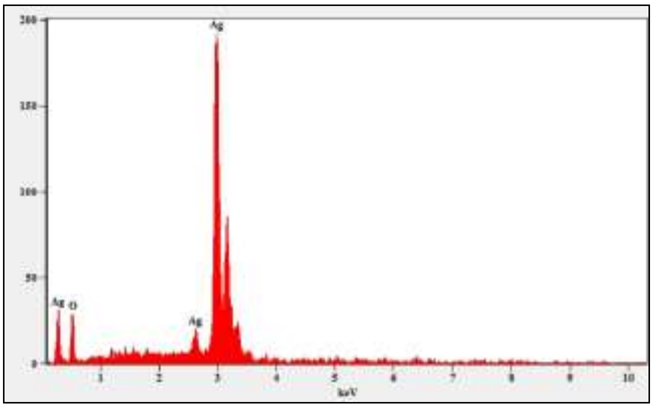

Fig 4(a): FTIR analysis of Ag Nanoparticles

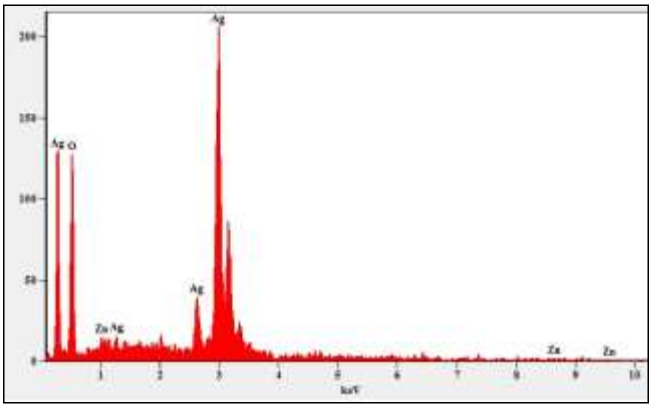

Fig 4(b): FTIR analysis of Zn-Ag Nanoparticles

Antifungal Activity

The antifungal activity of different concentrations (50, 75 & 100µl) of Ag-Zn doped NPs against two fungi, *A. flavus* and *A. terreus* by agar well diffusion method. Chart1 shows the zone of inhibition at different concentrations of NPs. The Figure 5 shows the clearance zone against the fungi growth. The result shows that with the increase in the concentration of nanoparticles, the zone of inhibition also increased. It clears that the zone formations of antifungal activity directly proportional to the concentration of synthesized Ag-Zn doped nanoparticles highly form the zone (21.33 mm) against *A. terreus* at the concentration of 100µl. Minimum inhibition (19.33 mm) was observed against *Aspergillus flavus*.

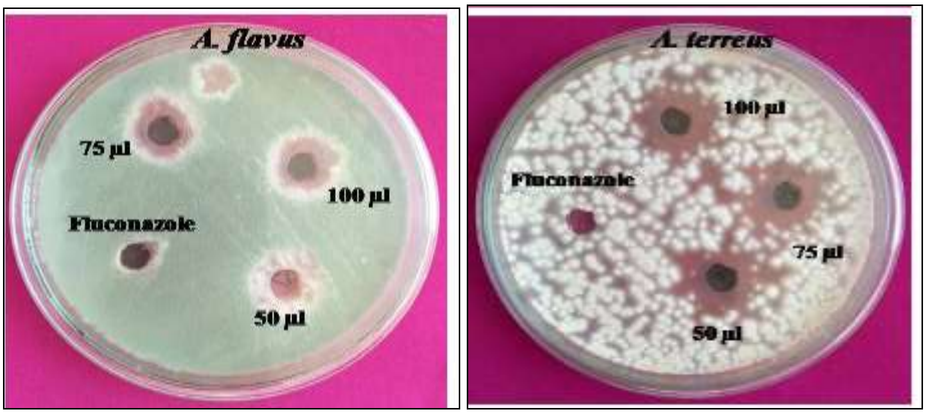

Fig 5: Zone of inhibition of Ag & Ag-Zn Doped nanoparticles synthesized against fungi

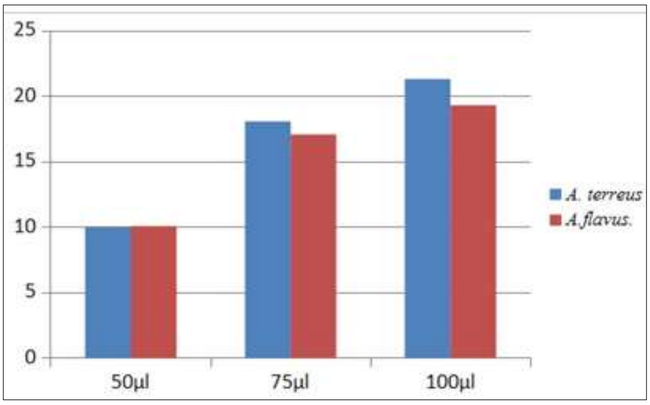

Chart 1: Anti fungal activity of different in concentration

Conclusion
A simple method used for synthesis of silver and silver doped zinc nanoparticles using *Anamirta cocculus* extract at room temperature. The synthesized nanoparticles excluded from external stabilizers or reducing agents. The synthesised silver doped zinc nanoparticles showed efficient antifungal activities against both *A. flavus and A. terreus* by agar well diffusion method. Benefits of using plant extract for synthesis is that it is energy efficient, cost effective, protecting human health and leading environment to lesser waste and safer products. The results showed that substantial amounts of silver and silver doped zinc nanoparticles were produced and that this was verified using UV-visible Spectroscopy. In this research work were suggested to applied in dermatological aspect in future.

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