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Study to control the devastating leather microbes using biogenically synthesized Ag nanoparticles

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ABSTRACT: During the manufacturing and distribution of leather, the biodeterioration is due to bacteria (pre-tanning) and fungi (post-tanning) in hides and leather. It leads to a substantial economic loss in the leather industry. Traditionally, biocides like TCMTB, BMT, CMC, and OPP are used to prevent microbial growth, which might deteriorate the quality of leather based materials. Recently, plant mediated metal nanoparticles are gained much attention in various biomedical applications such as antibacterial and antifungal activity. Silver nanoparticles (AgNPs) are potent antimicrobial agents against microbial pathogen, which can act as a capping agent on leather's surface, preventing the material from degradation. The green synthesis of AgNPs lies in its cost-effectiveness and the abundance of raw materials. Four potential plants were identified for the synthesis AgNPs among ten plants screened. Among all plants, four plants could synthesize silver nanoparticles effectively at lower concentrations (1mM). The green synthesized AgNPs have been further characterized by UV-Visible, FTIR, SEM-EDX analysis to confirm the presence of nanoparticles. Accordingly, our aim of the work is to avert the development of the microorganism growing on leather material by applying AgNPs, and their activity was screened against various microbial pathogen.

KEYWORDS: Biosynthesis; Silver nanoparticles; SEM-EDX; Antibacterial; Antifungal.

I. INTRODUCTION

Leather is a distinctive commodity that bridges the rustic farmer to the vouge world. Annually, 60% of leather production has a market value of $ 2,276.39 billion US dollars, according to a recent report from Economic times and the government of India in the year 2019-2020 [1]. India is the 2nd largest producer of foot-ware and leather garments and ranked the fourth-largest exporter, with an 8 % share in the global import of saddlery and harnesses. The quality of this expensive piece of an accessory depends solely on the methods followed during processing and distribution, wherein cleanliness becomes an important criterion. The raw fleshy material used during the initial phase of production attracts several microbes like *Bacillus anthracoides*, *Staphylococcus spp*., *Streptococcus faecalis* [2], *Bacillus subtilis* [3], *Bacillus pumilus* [4], *Escherichia coli*, *Pseudomonas aeruginosa* [3], *Bacillus megaterium* [5], with its nourishing environment. It leads to the putrification during the pre-tanning stages. Different measures are taken to prevent bacterial attacks, including the addition of highly concentrated salt solutions. Similarly, after post-tanning conditions, the leather is rich in lipid and protein. It attracts various fungal species like *Aspergillus niger* [6], *Aspergillus fumigatus*, *Aspergillus ochraceus* [5], *Penicillium rugulosum*, *Penicillium funiculosum* [7], and *Trichoderma* spp [3]. These fungal species colonize and deteriorate the quality of the finished leather products, thereby contributing to its loss.

To prevent deterioration of leather from microbial attack, certain chemical microbicides like 2-(thiocyanomethylthio) benzothiazole (TCMTB), ortho-phenylphenol (OPP), chlorometacresol (PCMC) DIMTS, etc. are available [8]. 2-10% of these chemicals are used during pre-and post-tanning processes [9], but they have become an environmental threat creating unsustainable waste and human toxicity [10]. These drawbacks have to be taken care of to produce products of high quality. Modern researchers have developed several technologies employed to solve these industrially developed problems. One among them is “nanotechnology” which has been exploited massively nowadays. The methods for the synthesis of nanomaterials are broadly classified into three ways. Among these, the physical and chemical processes have become hazardous and quite unstable [11].

In contrast, biological nanoparticle synthesis has proven advantageous and environmentally friendly, low cost and less toxic in biomedical applications. Plants, yeast, and bacteria have several metabolites that aid in reducing silver nitrate in nanoparticles that are inexpensive, easily accessible, and nontoxic. The synthesized nanomaterials can be metallic or non-metallic, with numerous applications. However, metal-based nanoparticles like gold, silver, copper, palladium, and platinum are preferred due to their unique characteristics like surface plasmon resonance and optical properties [12]. Besides, gold and silver are noble metals that have gained much attention in biomedical applications like antimicrobial, anti-cancer, tissue engineering, gene, and drug delivery.

Yousef et al [13] reported that zinc oxide and manganese oxide nanoparticles were effective against common microbes like *E. coli* and *B.subtilis*. The effectiveness of zinc nanoparticles over the other microbes was also studied [14]. Chemically synthesized copper [15] and titanium oxide [16] NPs were utilized to improve the finishing of animal skin products and arrest the growth of microorganisms [17]. Silver nanoparticles (AgNPs) have gained significant interest among noble metals because of their superior characteristics like good conductivity, non-hazardous chemical stability, and antimicrobial properties [18]. However, this material has already been synthesized chemically to prevent the growth of microorganisms on leather products [19]. However, the use of chemicals on leather materials was found to contribute to human toxicity and environmental issues.

Advantages of AgNPs synthesis using such an approach (on large scales) can be made non-hazardous and, if used in lower concentrations, maintain good stability, in a cost-effective procedure, with the slightest modification of the materials and so on [20]. Several mechanisms are reported for the antimicrobial power of silver nano-particles. The rapid breakdown of AgNPs releases ionic silver, thereby inactivating the important microbial enzyme (like thiol), eventually leading to the death of the organism [21, 22]. Green synthesis of AgNPs was reported using sources like bacteria [23], fungi [24], algae [25], and plants [26]. However, plants are commonly used as they are chemical-free and can act as natural capping agents, thereby enhancing the applicability of nano-particles [27]. Plants are lavish among various proteins and secondary metabolites, including flavonoids, saponins, quinones, and terpenoids, which predominantly help to reduce silver nitrate to silver ions. Therefore, plant extracts are used for successful synthesis in the field of phyto-nano technology. In the present study, the following ten plants: *Tridax procumbens* [28], *Amaranthus virdis*, *Embelia ribes* [29], *Aloe vera* [30], *Lawsonia inermis* [24], *Ocimum tenuiflorum* [31], *Murraya koenigii* [32], *Acalypha indica* [33], *Azadirachta indica* [34], *Ocimum sanctum* [35] were selected based on their reports on antimicrobial activity. Leaf extracts of these plants act as natural reducing agents and help in the synthesis of AgNPs [27]. According to the literature review, these plants showed potent activity against viruses, bacteria, and fungi which can be attributed to the various phytochemicals like alkaloids, saponins, and many more [35, 28]. Due to its strong antimicrobial property and rich secondary metabolites, the efficiency of the synthesized AgNPs against microbes increased. Among these ten plants, *Acalypha indica*, *Amaranthus viridis*, *Embelia ribes*, and *Ocimum tenuiflorum* were further studied based on their initial characterization. These four plants are abundantly found in various parts of India, making them cost-effective compared to the other biological materials.

II. MATERIALS AND METHOD

Materials

Analytical grade silver nitrate ($AgNO_3$) (99.5% purity) was obtained from Sisco Research Laboratories Pvt. Ltd (SRL), Mumbai, India. Standard cultures for antimicrobial assays were stepped up from Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh, India.

Plant sample collection

Fresh leaves of the above-mentioned potential ten plants were collected from Stella Maris College, Chennai, Tamil Nadu, India, and identified by the Department of Botany, Stella Maris College, Chennai. The collected plant leaves were thoroughly surface cleaned with gushing tap water to pull out all debris attached, followed by washing with deionized water. The leaves were then air-dried at room temperature.

Preparation of aqueous extract

The aqueous extracts of the ten plant leaves (*Acalypha indica*, *Aloe vera*, *Amaranthus virdis*, *Azadirachta indica*, *Embelia ribes*, *Murraya koenigii*, *Lawsonia inermis*, *Ocimum sanctum*, *Ocimum tenuiflorum*, *Tridax procumbens*) was prepared. About 10 g of the finely chopped leaves were crushed thoroughly with mortar and pestle and transferred into a beaker containing 100 ml of Milli Q water. The concentrate was boiled at 60°C for 10 min. The plant extract was cooled, sieved through Whatman filter paper 1 , and stored at 4ºC for future use [33].

Synthesis of silver nanoparticles

To synthesize AgNPs, 90 mL of 1mM $AgNO_3$ solution was prepared in a closed Erlenmeyer flask. To the solution, 10 mL of plant extract was added, stirred well, and incubated for 6 hours in a dark environment. A transition of colorless solution to reddish-brown colour was observed, which indicated the formation of reduced silver nanoparticles AgNPs [33].

Characterization of synthesized silver nanoparticles

An aliquot of each sample solution was taken in a 2 mL cuvette, and the absorbance was monitored from 200–700 nm using UV–Vis spectrophotometer (Shimadzu UV 2450, Japan). Further, different analytical tests were performed, including Fourier transform infrared spectroscopy (FTIR) (Frontier Perkin Elmer FTIR, USA) and Scanning electron microscopy and Energy dispersive X-ray spectroscopy (SEM-EDX) (Phenom Pro, USA).

Anti-bacterial activity

The agar well diffusion method was performed to determine the anti-microbial activity of the synthesized AgNPs. These organisms namely Gram +ev (*Bacillus cereus* (MTCC 4079), *Staphylococcus aureus* (MTCC 737) *Streptococcus pyrogens* (MTCC 442), Gram -ev (*Escherichia coli* (MTCC 1687), *Pseudomonas aeruginosa* (MTCC 1688), and *Serratia* sp (MTCC 97). Fresh overnight (24hrs) bacterial culture ($1x10^5$ cells/mL) was spread evenly on the surface of nutrient agar with the help of cotton swabs. Then, with a sterile cork borer, holes of 6 mm were punctured. Varying volumes (25-75µl) of AgNP were loaded aseptically into the holes, and the plate was incubated for 24 hrs. Ampicillin was used as the positive control [36].

Anti-fungal activity

The antifungal assay was accessed out by well diffusion method using four different fungal species *Aspergillus fumigatus* (MTCC 343), *Aureobasidium pullulans* (MTCC 153), *Penicillium pinophilum* (MTCC 2009), and *Trichoderma virens* (MTCC 2023). The fungal organisms were maintained at 37ºC on Potato Dextrose Agar (PDA) agar. After 48 hours of incubation, one loop of each fungal culture was mixed with distilled water and uniformly swabbed on individual PDA agar plates. Three different concentrations of synthesized AgNPs (25µl, 50µl, and 75µl) were added to the punctured wells and were incubated for 48 hrs. Fluconazole was used as the standard [37].

III. RESULT AND DISCUSSION

The formation of AgNPs was anticipated because of the reaction between the phytoconstituents present in the extract and the silver nitrate [38]. This results in the initial observation of change in colour from yellow to orange and then brown as the reaction proceeds observed only in four plants (Figure 1 (a)) [39] due to the excitation of surface plasmon vibrations. The visual observation of the synthesized AgNPs was assessed by UV-Vis experiments (Figure 1 (b)) which represent the SRP peaks at 420, 427, 436, and 419 nm for Ai-AgNPs, Av-AgNPs, Er-AgNPs, and Ot-AgNPs, respectively. The reliable results were interpreted by Varghese Alex et al [40]

The triple role of reducing, capping, and stabilizing function of the selected plant extract and its functional groups were accessed by FTIR analysis, which reveals several bands between 3388 cm^{-1}, 2421 cm^{-1}, 1674 cm^{-1}, 1390 cm^{-1},1066 cm^{-1}, and 827 cm^{-1} respectively for all the AgNPs (Fig 2). This demonstrates the presence of O-H, N-H stretching, Alkyne, Amide C=O stretching, C-H Alkenes stretching, amine and carbonyl group stretching are observed [41]. And these results are ageerable with the AgNP synthesized from leaf extract of *Clitoria ternatea* and *Solanum nigrum* [42] due to the

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presence of phytoconstituents responsible for the nanoparticle formation.

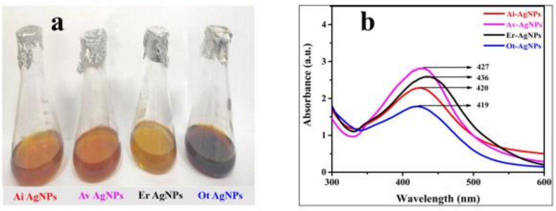

Fig 1: (a) Formation of AgNPs (b)UV-Visible spectrum analysis of synthesized AgNPs.

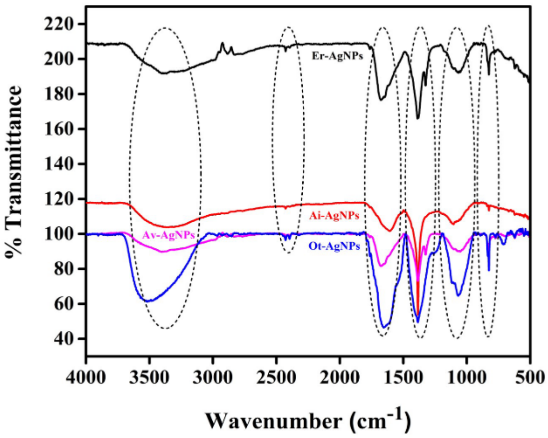

Figure 2: FTIR spectra of AgNPs synthesized from plant extracts

The synthesized nanoparticles Ai-AgNPs, Av-AgNPs, Er-AgNPs, and Ot-AgNPs seem spherical, aggregated with polydispersed (Figure 3). And this result is agreeable with AgNPs extracted from *Coccinia grandis* leaf extract [43]. And also, the resultant data were clearly distinguishable owing to their size difference, which was got interpreted by the corresponding wavelength by a shift towards the nano regime due to the quantum size effect [44]. Ot-AgNPs have the least particle size (4-20 nm), Ai-AgNPs as the second least particle size (22-35nm), Av-AgNPs have the second-largest on size analysis (32-49 nm), Er-AgNPs result in the largest size among the four nanoparticles (42-49 nm). Thus, the order of wavelength towards the nano regime matches the order emphasized from the size analysis.

AgNPs	% Ag	% O	% K

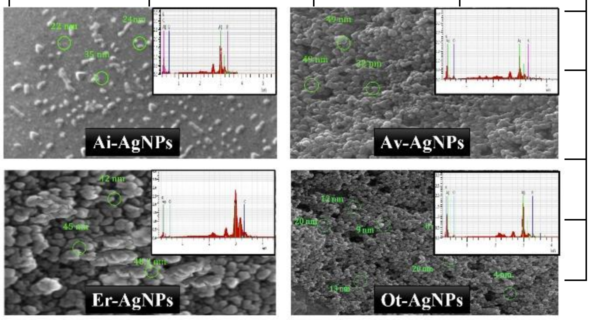

Table 1. Elemental analysis of EDX

Figure 3: FE-SEM and EDX spectrum of AgNPs synthesized from plant extracts

Elemental composition analysis of the AgNPS was concluded to have an optical absorption peak at 3 keV because of the SPR [45]. In this study, the elemental constitution of silver was 64 % (Ai-AgNPs), 67 % (Av- AgNPs), 93% (Er-AgNPs) and 72% (Ot-AgNPs) respectively (Table 1). Though the EDX profile shows a prominent signal for silver, It also has an appreciable percentage of oxygen, which may originate from the biomolecules that present on the surface of AgNPs, indicating the responsiveness of oxygen as a reducing agent [45]. And the negligible percentage of potassium signal is due to the phytoconstituents, which act as a capping agent [46] present in the plant extract and even because of the impurities while synthesizing. This confirms the complete reduction of silver compounds to AgNPs as shown in the spectrum [47].

The antibacterial study was considered a noteworthy application to use the green synthesized AgNPs, because of its proposed mechanical attributions to combat the bacterial species, both indirectly and directly. Though, the exact mechanism for antimicrobial assessment was yet to be clarified. But as per the postulation made by the researchers, the antimicrobial activity may either be due to the direct action of AgNPs or because of the silver ions (Ag+) present in the AgNPs.

In bacteria, the indirect mechanism arouses because of the release of Ag+ from AgNPs, which act as a Lewis acid and proficiently binds with the major bio-molecular components like sulphur, phosphate, imidazole, and carbonyl groups (Lewis base) that remains in and on the bacteria by a strong affinity and electrostatic force [48]. On the atmost layer of the cell, Ag+ binds with the outer-membranal molecules

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leading to bacterial envelope disruption and cellular membrane permeation [49]. Upon injection of Ag+ into the cell, it proficiently binds with the thiol group present on the respiratory enzyme and deactivates the respiratory enzyme. Thus it affects oxidative phosphorylation, interrupting ATP production [48]. And even simultaneously pretends to increase the cellular oxidative stress, which leads to increased ROS and free radical production in the cells. Apart from this, the Ag+ binds with the reduced glutathione (GSH) to form glutathione disulfide (GSSG) (an oxidized form), which collapses the normal scavenging mechanism of GSH on maintaining the free radical and ROS, leads to hyper-oxidation of cellular components [48, 50]. Even the Ag+ provokes a worthy impact on the central dogma of the bacterial cells, due to the binding of Ag+ with imidazoles and other components like sulfur, phosphate, and carbonyl groups present on the genetic matter and pretends the base pairs to intercalate, and leads in disruption of hydrogen bonds. It hinders cellular division, reproduction, and replication [51]. And also, the Ag+ results in protein deactivation by forming a stable bond between AS-Ag from the ASH (Thiol) group present in protein or enzyme [52], which ultimately distorts ions transport system across the cellular membrane, ATP generation, the 3D structure of the protein, cellular morpho, and even results in the constraint protein synthesis due to the ribosomal denaturation [49].

The direct mechanism postulates that AgNPs act on the cell membrane by interaction with sulfur-containing protein on the cell wall and also due to the attraction of positively charged AgNPs with the negatively charged cellular membrane by an electrostatic attraction that leads to the infiltration and subsequently pretends the cell to death [53]. Apart from their action on the membrane, AgNPs can even penetrate the cell. Then the penetrated AgNPs affect the bacterial signal transduction, which causes apoptosis and impairment in cell multiplication. Even the AgNPs up-regulates the free radical and ROS due to the downregulation of antioxidant and respiratory enzyme [54]. Also, AgNPs interact with protein and DNA to pretend bacterial dysfunction and death [55].

Both the direct and indirect role occurs in a parallel system, making AgNPs an excellent tool to combat leather microbes. This study worked to assess the efficiency of AgNPs from a leaf extract towards both gram + ev and gram -ev bacteria with the highest concentration of approximately 10^6 CFU/mL. Among four nanoparticles, Av-AgNP was found to have the least effective zone of inhibition (Fig 4) at 16mm because of its large particle size and the phytoconstituents like phytoanticipins and hydrolytic enzyme present in the plant extract, which was justified by Gonzalez-Lamothe et al [56]. Whereas Ot- AgNP provides a maximum zone of inhibition around 21mm (Fig 4) due to its small particle size, because small particle size has a large surface area that results in effective surface contact with microbes, as described by Tang and Zheng 2018 [48] and even the active constituent present in *Ocimum tenuiflorum* like eugenol (1-hydroxy-2-methoxy-4-allylbenzene), methyl eugenol, linalool, and 1,8-cineole might provide an effective role in antibacterial activity as proven by Ijaz 2020 [57]. Though apart from nanoparticle size and the phyto-constituents, even the bacterial species might have a role in an effective antibacterial activity due to the cell wall arrangement.

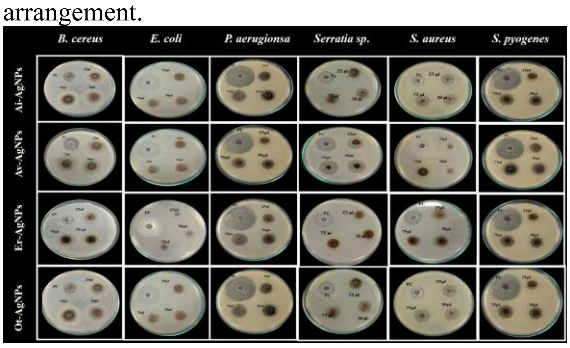

Figure 4: Antibacterial activity in well diffusion method

The silver nanoparticle was proved to be an effective antifungal agent against vast fungal species [58, 55]. The prominent property of AgNPs causes disintegration of cell wall integrity and impairment in DNA replication [59]. Besides, they are inactivating ribosomal subunit proteins, other cellular proteins, and enzymes essential for ATP production [60] and pretending to restrain the budding process. In the present study, these plant-mediated nanoparticles' antifungal activity was evaluated against four fungi growing on leather. Compared with the positive control, the inhibition of fungal growth increased for all the tested fungi. In addition, the assessment indicates that the higher inhibition rate was found to be around 21mm on average, at 75 µL for Ot-AgNP (Fig 5) compared to other AgNPs. That is due to the

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secondary metabolites like Phenylpropene (L. Linalool), Terpenoid (Camphor), Caryophyllene oxide, Bicyclic monoterpenes compounds that are present in the *Ocimum tenuiflorum* plant, which tends to exert the antimicrobial activity [61]. Whereas Ai-AgNP also showed a better result in exercising antifungal effect due to its tannin cardiac glycosides, alkaloids, flavonoids, terpenoids, and saponins [62].

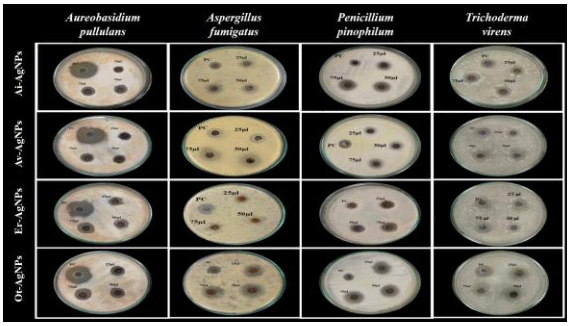

Figure 5: Antifungal activity in well diffusion method

But in comparison with the other two nanoparticles, Av-AgNP and Er-AgNP were accessed to have a large particle size than that Ot-AgNP and Ai-AgNP. Which pretends lower the inhibition rate due to the hindrance of binding with the proteins and complexity to penetrate the cell; according to the manifestation made by Ahmad et al [41], inhibition increases with a decrease in the particle size; vice versa. In summing up, AgNPs could be considered excellent broad-spectrum antifungal agents as per this result and the reports made by Bocate et al [63], Mare et al [64], Burduniuc et al [65].

IV. CONCLUSION

The study describes the hazard-free, biocompatible, and cheapest way of promoting AgNPs from four different plant extracts. The phyto-constituents present those plant extracts act as a reducing, capping, and stabilizing agent to provoke the green synthesis. The synthesized silver nanoparticle was accessed to check its originality by visual and spectro-photometrical analysis. Thus, emphasizing their UV visible peak around 430 nm with alkynes, alcohols, phenols, amines, and carboxyl groups proved to have a nano-sized particle. Even the synthesized silver nanoparticle was confirmed to have synergistic antimicrobial activity against the leather related bacteria and fungi, thus establishing their application in combating the spoilage of various leather materials. Moreover, this process could be easily stepped up for industrial applications to elevate the yield of the nano-structures and their commercial viability in the field of the leather industry.

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