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BIOLOGICAL SYNTHESIS, CHARACTERIZATION AND ANTIFUNGAL EFFECT OF SILVER NANOPARTICLES AND THEIR FORMULATION

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ABSTRACT

Nanotechnology is a rapidly developing medical technology with several applications in the pharmaceutical sciences. The goal of the current study is to create a formulation that contains nanoparticles of silver and investigates their capability against specific fungal infections. Emblica Phyllanthus leaf was used for the preparation of nanoparticles and characterization of nanoparticles was done. It was seen that size of AgNPs was 27nm they were spherical in shape and no contamination was found in the extract. Before the preparation of formulation, the toxicity of AgNps was checked on the mice model, and the safest concentration of nanoparticles was selected. For the synthesis of the final concentration oil and water emulsion was prepared and AgNPs of 5% concentration were mixed. The disc diffusion method was used to check in vitro antifungal activity of AgNps and the nanoparticles were found to be effective against Candida, Aspergillus and Trichophyton. For invivo study, approval was taken from the University's ethical committee and Ayub Hospital and written consent was taken from the patients. Patients suffering from fungal infections such as tinea Mannum, tinea versicular, tinea capitis, and tinea faciei. were selected and the formulation was topically applied on the skin of the volunteers. Among the infections tine versicolar was the most common and prevalent in men ages (21-40). As a result, formation showed excellent antifungal effect 100% recovery was seen in patients and no harmful effect was seen.

Keywords: *Nanoparticles, Fungal Diseases, Antifungal Activity, Green Nanotechnology.*

INTRODUCTION

Nanoparticles are the simplest form of structures and their size ranges from 1-100nm.Due to large surface area and high energy nanoparticles exhibit unique properties. Different nanoparticles of metals like silver, platinum and gold had been synthesized. Metal nanoparticles shows strong antimicrobial activity because of their ability to interact with cell membrane[1]. Silver nanoparticles are used in a variety of human and animal medicines to treat skin infections, such as dermatomycosis [2]. Silver nanoparticles have unique

physical and chemical properties and is also use in various fields including health, food, medicine and industrial purpose. Nanoparticles plays an important role in cosmetics, air and water purification, household products, clothing, fpod products. Biosynthesized silver nanoparticles can also be use as antibacterial agent [3].

The majority of skin fungal infections occur in developed countries, particularly in tropical and sub-tropical locations where temperatures are high[4]. In Pakistan 27% of skin fungal infections are caused by *Trichophyton ruburm* whereas 41% of infections are caused by *Trichophyton violaceum* [4]. Most common fungal infection found in Pakistan are Tinea Mannum. Tinea Capitis, Tiea Cruris, Tinea Versicola and Tinea faciei. A wide range of topical applications are used to treat skin fungal infection.[5-6]. An antifungal drug called miconazole nitrate is used to treat fungal infections of the skin, such as ringworm, jock itch, athlete's foot, candidiasis, and Tinea versicolar[7]. Numerous fungal infections caused by *Aspergillus, Candida*, and *Cryptococcus gatti* are treated with a number of additional antifungal medications, including fluconazole, nystatin and amphotericin B.

Variety of fungal infections are treated with additional antifungal medicines such as fluconazole, nystatin, sertaconazole, and amphotericin B [8-11]. Sometimes these medications show side effects, due to excess use of these medicines the fungi becomes resistant, therefore different and latest modern technologies help us in treating and preventing fungal infections. An alternate approach to treat microbial illnesses is offered by nanotechnology. Various chemical, physical, and biological methods can be used to synthesize nanoparticles. Reagents used in chemical processes are hazardous and harmful to the environment [12], Most renewable environmentally friendly sources, including plant extracts, are used in the green synthesis approach, this technologies have advantages as compared to the toxic solvents used in physical and chemical method [13].In biological synthesis method various molecules like proteins, peptides, and polysaccharides act as capping and reducing agents, and they play important role in regulating the size and shape of the nanoparticles. Silver has long been recognized as an antibacterial since ancient times. Plants provides better platform for the synthesis of nanoparticles among various biological methods [14]. Additionally, plants include a wide range of phytochemicals that are involved in salt reduction, which enables them to decrease a variety of salts and synthesize diverse types, sizes, and shapes of nanoparticles.These elements are needed for nanoparticle synthesis: solvent medium, stabilizing agent and reducing agent [15] [16].

Emulsions are mixtures which are formed when two immiscible liquids are mixed by using emulsifier. Emulsions are used in the treatment of numerous skin conditions and are found in a variety of industries, including petroleum, food, cosmetics, and agriculture. Many benefits come with using topical dose to treat fungal infections, including the ability for the medication to function at the application site, improved therapeutic efficacy, and patient acceptance [17]. Emulsions provide an alternative to antifungal medications by enhancing the local or systemic effect of pharmaceuticals [18]. Cosmetic applications such as skin moisturizing and laxative preparation include the use of several emulsions [19]. In this study the AgNPs formulation is made using an oil in water emulsion.The goal of the current study was to synthesize silver nanoparticles utilizing *Phyllanthus Emblica* leaves. Different characterization techniques such as X-ray diffraction, UV-visible spectroscopy, EDX spectrum analysis and scanning electron microscopy were used to produce nanoparticles. These nanoparticles were optimized and toxic level was checked in mice model. After characterization the final formulation, an oil in water emulsion was prepared and different concentrations of silver nanoparticles were added. Formulation was then used to assess the *in vitro* and *in vivo* capabilities of AgNPs and their formulation efficacy against a wide range of fungal diseases, such as Tinea versicolor, Tiea cruris, Tiea mannum, and Tinea capitis. Therefore, this study is the first study that reveal the *Invivo* and *Invitro* antifungal potential of formulation thus highlighting the novel work of this research.

The objective of this research is to develop an environmentally friendly method for synthesizing silver nanoparticles using biological processes. This involves comprehensive characterization to understand their properties and evaluate their antifungal efficacy. The study also focuses on creating a practical formulation to enhance the stability, delivery, and effectiveness of these nanoparticles in antifungal

applications. Despite the extensive research on chemically synthesized silver nanoparticles, there is a significant gap in exploring biological synthesis, which is more sustainable and cost-effective. Furthermore, detailed characterization and standardization of biologically synthesized nanoparticles remain limited, hindering reproducibility. While the antimicrobial properties of silver nanoparticles are well-documented, studies specifically targeting their antifungal effects and incorporating them into functional formulations are scarce. This research aims to address these gaps and contribute to the development of innovative antifungal solutions.

2 Material and Methods

The current study aimed to determine the antifungal effects of a formulation containing AgNPs on fungal skin infections. Before starting research, approval was taken from Hazara University's ethical committee Before applying the formulation human volunteers were used to apply the formulation, and before treatment, each patient's written consent was obtained. The goal of this investigation was to examine the antifungal properties of silver nanoparticles.

2.1 Preparation of Nanoparticles

The green method was used to prepare the AgNPs. *Phyllanthus emblica* (Amla) leaves were used in the synthesis of silver nanoparticles. First, a stock solution of silver nitrate and leaf extract were prepared. To prepare the leaves extract, 30g of leaves were combined with 100ml of distilled water, boiled, and constantly agitated for 30 minutes until a brownish-green extract formed. After filtering, the extract was kept in a flask for further use. To make stock solution 10 grams of silver nanoparticles was added to 200ml of deionised water, and the mixture was heated for 30 minutes while being continuously stirred, until the solution turned black. Two concentrations of AgNPs were prepared by adding 15 milliliters and 25 milliliters of stock solution, respectively, to 5 milliliters of leaf extract. Every solution was heated for 30 minutes. Centrifugation was carried out at 3000 rpm for 5 minutes. After removing the supernatant, the pellet was washed with ethanol and tap water and centrifuged. The pellets were gathered into petri dishes. Particles were then adequately dried in an oven at 100–120˚C for 30 minutes while the petri plates were covered with aluminum foil.

2.2 AgNPs shape and size identification

The characterization of silver nanoparticles was done to confirm shape, size and impurities. The following techniques are used for the characterization of silver nanoparticles:

2.2.1 UV. Visible spectroscopy

Visible spectroscopy was used to identify impurities present in nanoparticles. UV-visible spectroscopy of AgNP's standard falls between 195 and 250 nm.

2.2.2 EDX spectrum

The technique used for analyzing the elements in samples is called energy dispersed X-ray analysis. The sample's constituent elements have different atomic structures, which gives distinctive peaks on X-ray emission spectra.

2.2.3 XRD analysis

The crystal structure of AgNP's was examined by using X-ray diffraction. Dry silver nanoparticle powder was utilized for X-ray diffraction. When an X-ray beam strikes the sample's crystal structure, the beam diffracts in specific direction. By measuring the intensities and angles of diffracted rays, a crystallographer can create a three-dimensional image of the crystal.

2.2.4 SEM

SEM is used to examine the morphology and form of particles at the nanoscale. The electron beam in a SEM uses a raster pattern to examine the material. Two types of electron beams are created in SEM, which are detected by detectors to provide a three-dimensional image of the material.

2.3 Toxic effect of silver nanoparticles on mice

In order to assess potential toxicity of AgNPs nanoparticles, mice were given varying oral dosages such as150 mg/kg, 300 mg/kg, 400 mg/kg, and 500 mg/kg of 2.5, 5, and 10% AgNP concentrations. The mortality rate and physical behavior of the mice were observed.

2.4 Emulsion Preparation

2.4.1 Oil Phase

In the preparation of oily phase 0.2 g of propylene and 0.3 g of tween-80 were mixed, then 2.4 g of cetosteryl alcohol and 0.6 g of cetomacrogal were added to 2.4 ml of liquid paraffin. This mixture was then heated for 15 minutes at 70˚C.

2.4.2 Aqueous Phase

The aqueous phase was prepared by adding 4 g (methyl parabin), 0.16 g (sodium acid), and 0.02 g of propyl parabin sodium to 21.06 ml of distilled water, stirring continuously heated for 15 minutes at 30°C.

2.4.3 Water in oil emulsion

To prepare 20 gm base the oil phase, and aqueous phase were mixed in a 1:1 ratio and constant stirring was done at 3000 rpm for 30 min, until the mixture was fully homogenized. For 30 grams of emulsion different concentration of oil and liquid phase component were mixed.

2.4.4 PREPARATION OF FORMULATION

After preparations of base, various silver nanoparticles concentrations (2.5%, 5% and 10%) were made in distilled water and added separately with base. Then, variety of formulations based on varying concentrations of silver nanoparticles were made.

2.5 Experimental Design

In this research patients with fungal infections on their skin were chosen from various areas of Hazara. Five diseases—Tinea capitis, Tinea versicolar, Tinea mannum, Tinea faciei, and Tinea cruris—were chosen to check the effect of base and formulation. Before applying formulation, a patch test was done on the volunteer to see any allergic reaction. Then formulation was given to patients to apply on infection for 20 days. Follow up of patients was taken after 20 days and effect of formulation was noted.

2.6 Identification of fungal infections

The identification of fungal strains was done by the dermatologist of Ayub hospital and patients data was collected by using a questionnaire. Before the application of formulation, The Strains were collected and the written consent from patients were taken.

2.7 AgNPs *Invitro* Antifungal activity

2.7.1 sample collection

The patient's fungal strain was collected from hospital. To prevent contaimination, the affected skin area was cleaned before scraping. The disk diffusion method was used to assess the antifungal effect of AgNp's.

2.7.2 Media preparation

5 gram media was prepared by adding SDA (Sabrose dextrose agar) was dissolved in 150ml of water in a flask. Prepared media and petri dishes were autoclaved for at least half hour. After autoclave petri plates were placed under laminar flow hood to avoid contamination then autoclave media was poured in dishes. Solidification of media was done. To check contamination petri plates were incubated for 24 hours.

2.7.3 Inoculation of samples

The samples were dissolved in 1 ml of water. Then, for uniform growth 0.2 ml of samples were added to agar plates using sterile cotton swabs. After that petri plates were kept in incubator for 72 hours to observe the growth of fungi.

2.7.4 Serial dilution preparation

To prepare the stock solution 1gm of AgNP's were added in 1ml of distilled water. Then different serial dilutions of 20 µl, 30 µl, 40 µl and 50µl were prepared.

2.7.5 Antifungal activity of AgNPs

The disk diffusion method was used to test AgNPs' antifungal properties. Using a micropipette, serial dilutions of 20, 30, 40, and 50 µl were added on disks that were placed in media. Base was chosen as blank. Every plate was kept in an incubator for 2 days.

3 RESULTS

3.1 Nanoparticles Preparation

Emblica Phyllanthus were used to synthesized silver nanoparticles figure 3.1. After that, the identified particles were used for their antifungal potential both *in vitro* and *in vivo*

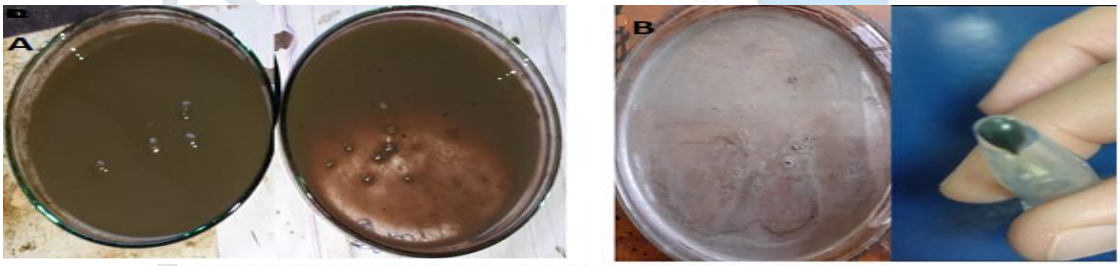


Figure 3.1: A show AgNPs before drying. (B) Shows AgNPs after drying.

3.2 AgNPs Characterization

3.2.1 U. V-Vis spectroscopy

UV-visible spectroscopy is used to find the impurities present in synthesized silver nanoparticles. AgNPs exhibit standard absorption in the UV-visible spectroscopic ranging in size from 195–250 nm. Therefore, in this study, the result of UV-visible spectroscopy was 199nm, as shown in (Figure 3.2 A). This observation thus confirmed that the synthesized nanoparticles were AgNPs.

3.2.2 XRD analysis

AgNPs were examined for size and crystalline structure using the XRD diffraction method. Dry powder silver nanoparticles were analyzed and compared with standards for X-ray diffraction, which verified that the sample included AgNPs, were nanocrystals. The diffraction lines for the sample were found at 2 theta angles of 27.5°, 29°, 32°, 38.5°, 46°, and 47.2° (Figure 3.2 B). For the synthesized Ag-NPs, the corresponding hkl values were 111, 111, 200, 211, 220, and 220.

3.2.3 | EDX Spectrum

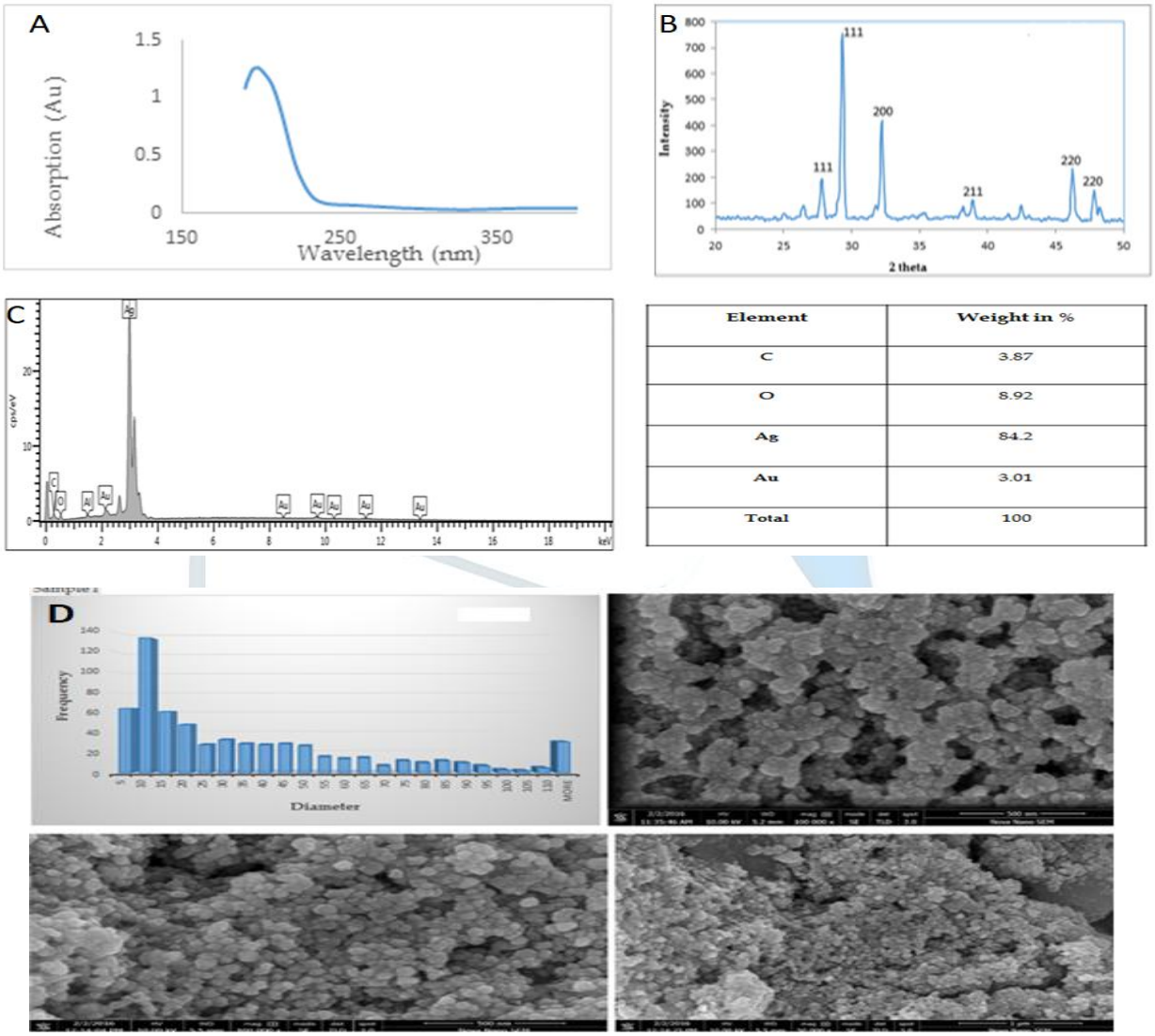
The EDX spectrum technique is used to analyze sample elements, as every element in a sample has a different atomic structure, the sample's X-ray emission spectrum will show a specific peak pattern (Figure

3.2 C) shows that the percentage of silver atoms in the sample was 84%, in comparsion to other elements including carbon, oxygen, and gold.

3.2.4 | SEM

SEM showed that the resulting nanoparticles are tiny, spherical in shape.

The various sizes of nanoparticles are shown in the graph (Figure 3.2 D). The large particles will be placed at top and small size at the bottom. The diameter-frequency bar graph is shown in (Figure 3.2D) Therefore in this study the sample's mean diameter is 27.5.



Element	Weight in %
C	3.87
O	8.92
Ag	84.2
Au	3.01
Total	100

FIGURE 3.2: (A) Shows result of UV-visible spectroscopy of AgNPs (B). shows result of XRD of AgNPs (C). Displays EDX spectrum analysis (D). shows scanning microscopy results.

3.3 Toxicity of AgNPs on mice

All mice were found to be alive after treating with dosages of 150, 300, and 400 mg/kg of silver nanoparticles at concentrations of 2.5, 5, and 10% (Figure 3.3). but when the dose was increased to 500mg/kg it causes toxic effect, resulting in mice death as shown in (Table 3.1). Similarly, mice were

administered with Phyllanthus emblica leaf extract with dosage of 250 mg/kg, and the toxic effect was determined.

Table 3.1: Shows the toxic effect of silver nanoparticles on mice.

AgNPs cocentration	Dosage mg (kg/w.b)	Rate of mortality
2.5%	150 300 400 500	Living Living Living Living
5%	150 300 400 500	Dead Dead Dead Dead
10%	150 300 400 500	Dead Dead Dead Dead

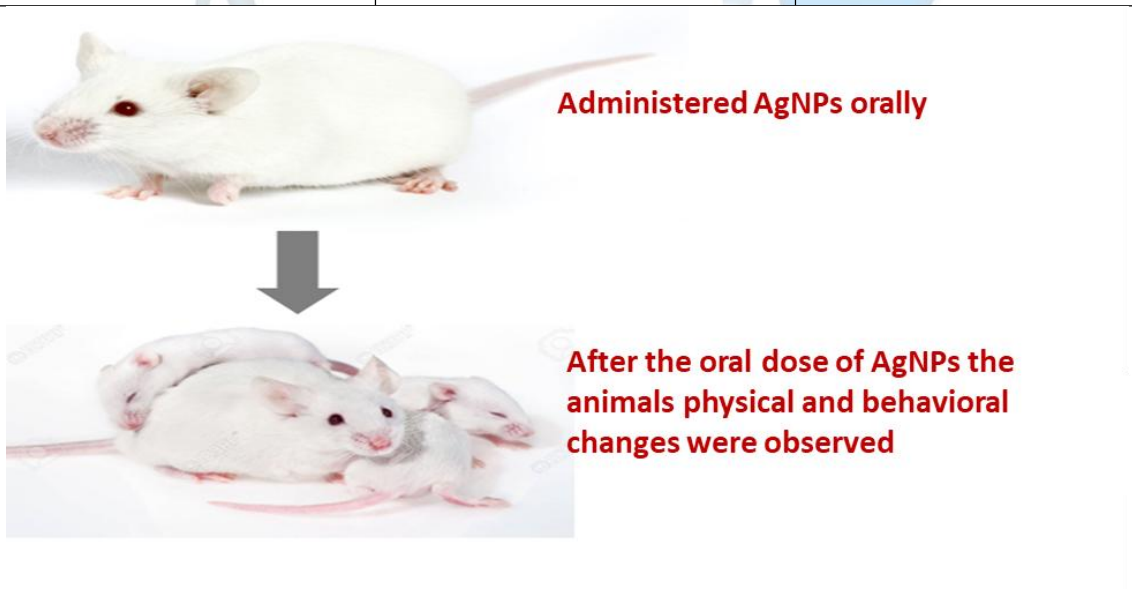


FIGURE 3.3: Effect of acute toxicity of AgNPs on mice.

3.4 Panel Test

A formulation with varying nanoparticle concentrations was applied to a patient who had a fungal skin infection. To check for allergic reactions, the formulation was applied as a control on normal skin. The base, the formulation, and any concentration of silver do not cause allergic reactions. Patients were chosen randomly. The formulation showed good spreadability. Among all the concentrations, 5% of silver nanoparticles was found to be the most potent and was chosen for further study.

3.5 General presence of Skin Fungal Infections

In this research total 140 patients with fungus infection of the skin were collected. Tinea versicolar was the most common disease, followed by Tinea mannum, Tinea capitis, Tinea cruris, and finally Tinea facie.

The graph in (Figure 3.5) showed that Tinea versicolar had a 30% presence, Tinea mannum, Tinea cruris, and Tinea faciei had a 20% presence, and Tinea capitis prevalence was 10%.

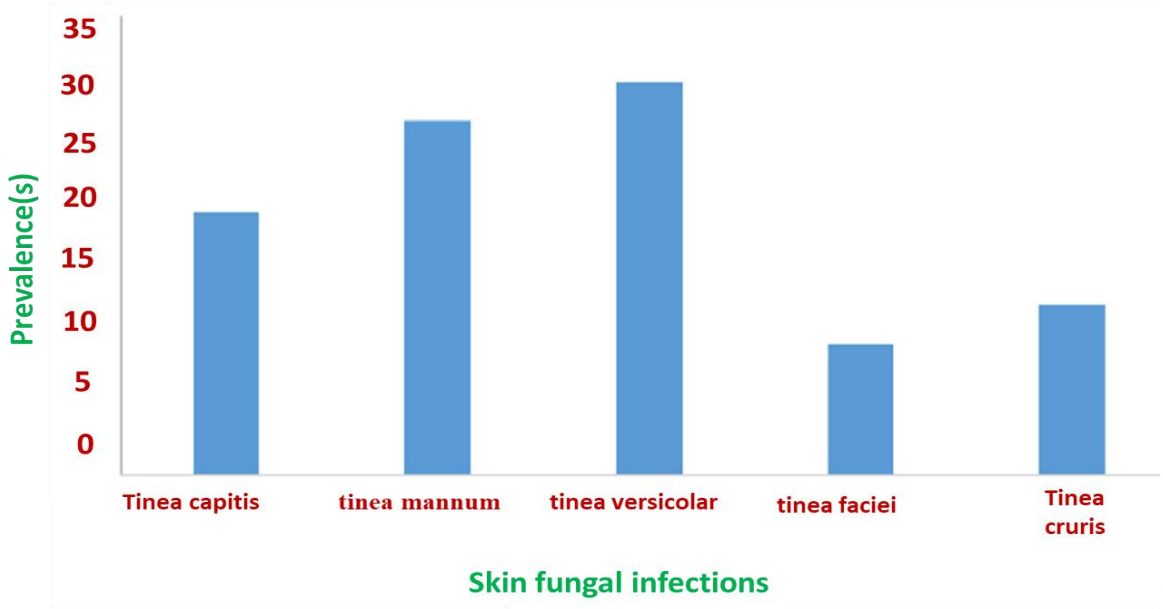


FIGURE3.5: General prevalence of Skin fungal infections

3.6 Age wise prevalence of skin fungal infections

The majority of patients with fungal infections on their skin belonged to the age between (1 -40). People suffering from tinea capitis, 71% were found to be between the ages of (11- 20). In a similar vein, tinea versicolar affected 91% of individuals aged (1 – 40). Similarly 90 percent of patients with tinea mannum were of the age 30 to 40. Patients with tinea faciei were mostly between the ages of (21-30). Similarly, it was found that individuals between the ages of (31-40), 60% were of Tinea cruris infections.

3.8 Gender-wise prevalence of skin fungal infections

3.8.1 formulation antifungal effect against Tinea versicolar

In this research 12 people with Tinea versicolar used formulation. Among 12 patients male population was 83% and 17% were female. Patients with tinea versicolar showed a 100% recovery rate after using the formulation for 20 days, as illustrated in (Figure 3.6 A).

3.8.2 formulation antifungal effect against Tinea capitis

9 patients with Tinea capitis were chosen for this research and were given 5% formulation as treatment. Out of 9 patients 33% were female and 67% of patients were male.Every patient showed 100% recovery. After 20 days of follow up no hair loss and infection reoccurrence was noted as shown in (Figure 3.6 B).

3.8.3 Formulation antifungal effect against Tinea cruris

To check the effectivness of formulation 4 patients having Tinea cruris were treated. Every patient was a female. The disease was mostly observed in female with age group of 11-60. Patient were treated with formulation for 20 days.formulation effect were seen significant and no reappearance of infection were observed in follow up. The 100% recovery was illustrated in (Figure 3.6 C).

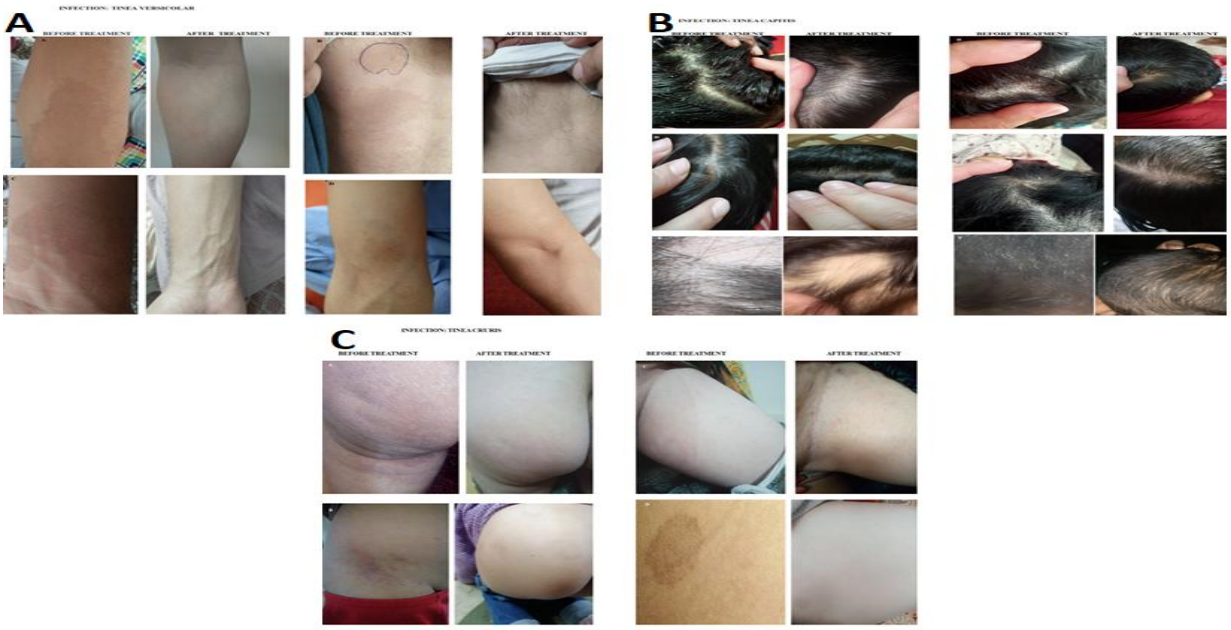


Figure 3.6 (A) Before and after effect of formulation on patients suffering from tinea versicolar (B)Before and after effect of formulation on patients suffering from tinea capitis (C) Before and after effect of formulation on patients suffering from tinea cruris.

3.8.4 formulation antifungal effect against Tinea mannum

To check the effectiveness formulation was applied on 10 patients suffering from Tinea mannum 30% patients were female and 70% were male. Patients had 100% recovery rate after using formulation as shown in (Figure 3.7 A).

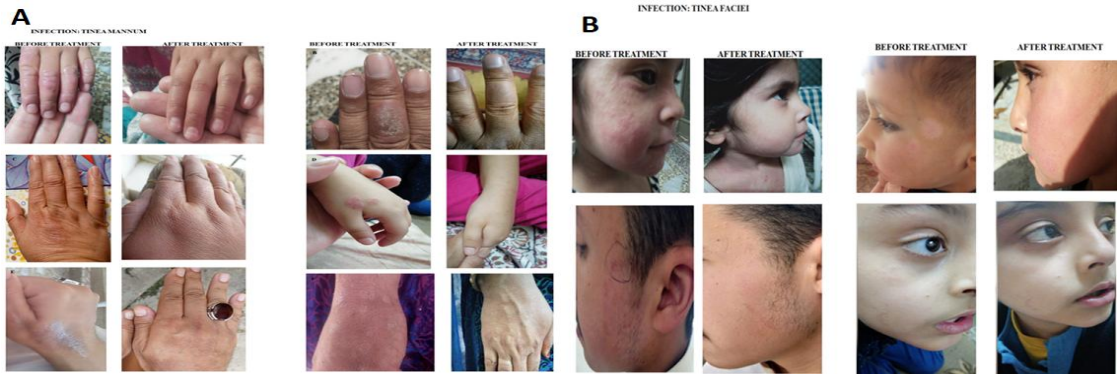


Figure 3.7 (A) Before and after effect of formulation on patients suffering from tinea mannum (B) Before and after effect of formulation on patients suffering from tinea facial.

3.9 Invitro antifungal assay of silver nanoparticles and formulation

The diameter of inhibition and the inhibition zone are shown on the graph in the(Figure 3.8 A). The leaves extract of *Phyllanthus emblica* significantly inhibited the growth of skin fungal strains. The mean inhibition zone for fungal strains ranges between 15nm. The concentration of nanoparticles affects the antimicrobial activity therefore in the graph of (Figure 3.8 B) a greater inhibition zone was seen in 50uL dilutions as compared to other dilutions.

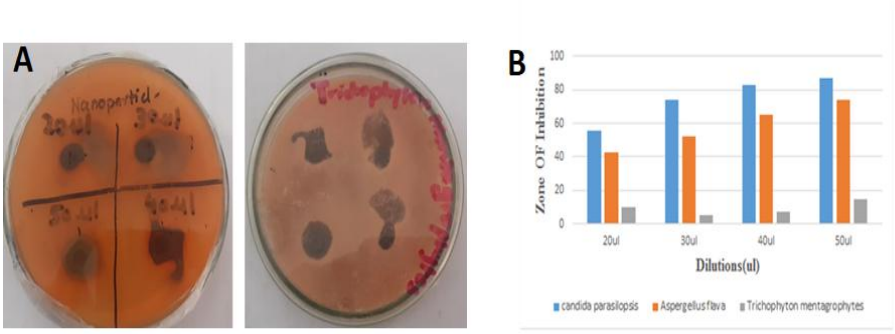


Figure 3.8: (A) Invitro antifungal effect of AgNPs against fungal strains (B) Inhibition zone of AgNPs dilution against fungal strains.

4: DISCUSSION

There are various functional platforms for nanoparticles. For many years AgNPs have been used as antimicrobial agents.

According to this research, AgNPs showed strong antifungal properties and were very effective against infections. The shape, size, and concentration of nanoparticles determine their antimicrobial activity (Rong et al., 2013). According to Rong et al. (2013), most of the nanoparticles with a size range between 10–15 nm shows strong anti-microbial effect. In this research nanoparticles size falls in the range of (27-33nm). Tinea versicolar is a skin fungal infection of stratum corneum The common causes of Tinea versicolar are environmental factors and host sensitivity. Tinea versicolar is found in 20–50% of the world's population, and it occurs more commonly in high temperatures (Mellen et al., 2004). The causative agent of Tinea versicolar is Malassezia furfur which are normal skin flora. In our study majority of people having Tinea vesicular infection were between the age 21-35, it may be because to high sebum secretion. In this study most of the people suffer from this infection in the summer season.

According to Nenoff et al. (2014), Trichophyton violaceum, which is frequently found in tropical regions, is the common. dermatophyte that causes tinea capitis. Geographical location, a wide range of environmental and cultural factors, and the prevailing pattern of infection all affect the pattern of dermatomycoses and their etiological agent (Macura et al., 2008).

In this research it was found that Tinea capitis was more common in children.The reason for the presence of Tinea capitis in children may be due to poor health condition, hairl cut by contaiminated instruments, small hair that help in transfer of infection from one child to another. In this investigation, Tinea capitis equally affected males and females. Conflict over gender preponderance was discovered in earlier investigations. According to some research, boys are more susceptible because of their short hair, which facilitates the spores' easy transmission (Friedlander et al., 2003). In a few studies due to tightly braided hair, this infection was reported to be more frequent in girls. The majority of cases of Tinea capitis in our study were in children whose family members also had this illness; this could be because there was a greater likelihood of regular family interaction and exchanging personal items with an infected individual.

The infection of the face, excluding the male beard area, is known as tinea faciei. In this study both male and female were effected with this infection., One factor that contributes to the infection is the use of topical steroids in cream form, which are harmful to the skin of the face (Nicola et al., 2010). The hands fungal infection is called tinea mannum. In our study it was found that both genders (males and females) were equally effected from this disease. Previous literature indicates that there has been little to no research on the epidemiology of tinea cruris.

5 Conclusion

The synthesis, characterization, and antifungal effects of biogenic silver nanoparticles (AgNPs) highlight their potential as effective agents against fungal infections, especially in the face of rising multidrug

resistance. Recent studies demonstrate that AgNPs, produced through environmentally friendly biological methods, exhibit significant antifungal activity against various pathogenic fungi, including Aspergillus and Candida. Their mechanisms of action involve disrupting fungal cell walls and membranes, leading to cell lysis. The biogenic synthesis not only enhances the antimicrobial efficacy of AgNPs but also ensures their biocompatibility. Given these promising results, further research is essential to optimize synthesis conditions and explore their clinical applications, while also addressing the mechanisms behind fungal resistance. Overall, biogenic silver nanoparticles represent a promising alternative to traditional antifungal agents, with the potential to significantly impact both medical and agricultural practices.

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