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Clinical Evaluation of Antifungal Activity of Silver Nanoparticles for Treatment of Onychomycosis

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Abstract

Onychomycosis is the most common fungal infection of the nails. It results in discoloration and thickening of nails. Conventional methods of treatment include oral and topical use of antifungals like nystatin, amphotericin B, clotrimazole, fluconazole, terbinafine. Oral administration of antifungal agents requires prolonged use resulting in significant side effects. Topically applied antifungal agents fail to penetrate the nail plate. Onychomycosis does not resolve spontaneously and even if successfully treated, tend to recur. The other option left is removal of nail. The aim of the present study was to prepare silver nanoparticles using *Ocimum sanctum* leaf extract. The formation of silver nanoparticles (10-100nm) was confirmed by a strong absorption peak at 423nm. *In vitro* disk diffusion method showed a concentration dependent zone of inhibition with the prepared nanoparticles. In clinical studies [duly approved by the institutional Ethical Committee (IEC), IEC 068], 21 patients suffering from onychomycosis were enrolled. They were equally divided into control, standard and treatment group. Inclusion and exclusion criteria were kept as a minimum of 25% involvement of the nail area and maximum of 75% involvement of the nail area respectively. These studies revealed that 10-15% of infected area cleared upon topical application of cream (25 mg/cm^2) containing silver nanoparticles (0.04%w/w) on twice daily application for two weeks. Results were quite comparable with those of terbinafine antifungal cream. Supportive study involved taking a swab from the treated nails and mounting on a suitable nutrient media.

Keywords: Onychomycosis, silver nanoparticles, *Ocimum sanctum*, antifungal activity, green synthesis

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Introduction

Onychomycosis is a common fungal infection of the nail with very high incidence rate. Though it is known to affect both toenails and fingernails, toenail infections are more frequent (Baran et al., 2000). Its incidence rate in adults is approximately ten percent. The commonest symptom of onychomycosis is that the affected nails become thickened. Discolouration into various hues of white, black, yellow or green may also occur. [2] As the infection advances, the nails turn brittle. This leads to small pieces of the nails breaking off. If timely treatment is not done, the affected skin gets inflamed and painful. White/ yellow patches on the nail bed as well as scaly skin in the vicinity of nail are accompanied by a foul smell on further deterioration. At systemic level, however, there are no symptoms, unless the pathology becomes too severe to handle. [2]

Patients with onychomycosis also undergo psychosocial issues due to the appearance of the nail. This psychosocial aspect of the disease is more prominent in the onychomycosis of finger nails as compared to that in toe nails. The disease is principally of four types- distal subungual onychomycosis; white superficial onychomycosis (WSO); proximal subungual onychomycosis, and candida onychomycosis. The commonest form of *Tinea unguium* is distal subungual onychomycosis. The commonest cause of this is the dermatophyte *Trichophyton rubrum*, an infection that enters the nail bed and then proceeds beneath the nail plate leading to accumulation of subungual debris and nail discoloration. It is generally diagnosed by potassium hydroxide preparations and/or fungal culture. [3] Fungal invasion of the superficial layers of the nail plate, on the other hand, leads to WSO, which manifests in the form of “white islands”. [4] In Candida onychomycosis, the fingernails are invaded and it usually occurs in people who keep their hands immersed in water for long periods. This type of infection is normally preceded by an insult to the nail either by infection or trauma. Conventional methods of treatment of fungal infection include oral as well as topical use of anti-fungal drugs.

Fungal infections are quite common in immunocompromised patients. It has been known since ancient times that silver and its compounds have strong inhibitory and microbicidal effects against bacteria, fungi and virus. [5] Fungi are usually saprophytic. Once they enter the host cells it becomes parasitic. The transition of the fungi yeast to the mycelial growth in the host cell is said to be the dimorphic transition. This transition is responsible for pathogenicity in the host cells. The mycelial form of fungus is induced by temperature, pH and serum. [6] Silver nanoparticles attach to the fungal cell membrane and penetrate inside the cell. Silver nanoparticles release silver ions which attach to the respiratory sequence and stops cell division leading to cell death. Significant inhibition of formation and extension of serum-induced mycelia in the presence of silver nanoparticles has been reported. [7] This formed the basis for clinical evaluation of Antifungal Activity of Silver Nanoparticles for Treatment of Onychomycosis. Silver nanoparticles were prepared using *Ocimum sanctum* leaf extract as natural products are recently being used as safe and efficacious antimycotic agents.

2. Material and Methods

2.1 Biosynthesis

Aqueous *Ocimum sanctum* leaf extract was prepared. This extract was added to 5mM silver nitrate solution at 60˚C and maintained to reduce the silver. The bioreduction of the silver ions was checked intermittently by measuring the UV- Visible spectra (200-800 nm) of the

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colloidal mixture. The appearance of reddish-brown coloured solution pointed at the formation of the silver nanoparticles. [8] The nanoparticles so obtained in were subjected to repeated centrifugation cycles at 12,000 rpm for 15 minutes each. The pellets obtained were redispersed in triple distilled water to remove any water-soluble substances like proteins and secondary metabolites. The nanoparticles were then kept at a temperature of -20˚C overnight and then lyophilized to dry the nanoparticles. The freeze-dried purified silver nanoparticles, were characterised by means of UV-Vis spectroscopy, FTIR, Zetasizer, Transmission Electron Microscopy and X-ray diffraction analysis. After the preparation of nanoparticles, they were weighed, resuspended in deionised water and stored in a refrigerator. [9]

2.2. Evaluation of Antifungal activity

Antifungal activity of the prepared AgNPs was determined using the agar well diffusion assay method. [10] Stock cultures of *Candida albicans* were prepared and maintained in Yeast Extract Peptone Dextrose medium at 37ºC. The antifungal activity was evaluated by seeding 0.1 ml of the culture on nutrient agar plates. As per standard method, 6 mm wells were made and 20 µl mixture containing silver nanoparticles was added to the wells in concentrations of 250µg/ml, 500µg/ml, 750 µg/ml and 1000 µg/ml. These were then kept for incubation at 37°C. After 24 h, the diameters of zone of inhibition were measured using a meter ruler. The experiment was performed in triplicate and an average of three readings was considered. [11]

2.3 Formulation of cream containing silver nanoparticles

An o/w cream of silver nanoparticles was prepared using indigenously developed method. The oil soluble components such as stearic acid, glycerol, lanolin, propyl paraben was mixed. The temperature of the mixture was slowly raised to 65˚C. Meanwhile, the water-soluble components i.e. triethanolamine and methyl paraben were dissolved in water and heated to 65˚C. The aqueous phase was added to oily phase slowly with constant trituration. Weighed quantity of silver nanoparticles were added to the mixture at 30˚C to avoid any degradation of natural components of Ocimum Sanctum leaf extract adsorbed on to the surface of silver nanoparticles.

2.4 Evaluation of antifungal activity of silver nanoparticles in patients with onychomycosis

Inclusion and exclusion criteria include at least 25% involvement of the toenail area and not more than 75% involvement of the toenail area respectively. This clinical study was approved by The Institutional Ethical Committee (IEC) under the reference IEC No. 068. The procedure includes three groups of 21 patients with onychomycosis. volunteers with control group, standard group, and treatment group. Measured amount of prepared topical formulation of silver nanoparticles was applied (25mg/cm^2) to the infected surface of nail twice a day for two weeks. The volunteers were examined for any residual fungal infection on nails. The results were further confirmed by taking a swab of the nails and mounting on a suitable media. The results were analysed statistically.

3.Results and Discussion

3.1 In vitro antifungal evaluation of silver nanoparticles

The results of antifungal activities of prepared formulation are given in Table 1. The formulation showed better antifungal activity against *Candida albicans* (MTCC 183) fungal strains as compared to the standard i.e. Terbinafine.

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Table 1: Zone of inhibition of prepared formulation and standard (Terbinafine)

Concentration (µg/ml)	Zone of inhibition prepared formulation (mm)	Zone of inhibition of standard (mm)
250	10±0.288	6±0.115
500	15±0.577	9±1.154
750	20±1.154	11±0.577
1000	24±0.577	15±0.000

*Control (Distilled water) – No inhibition.

The comparison between the developed formulation and standard is shown in Figure 1.

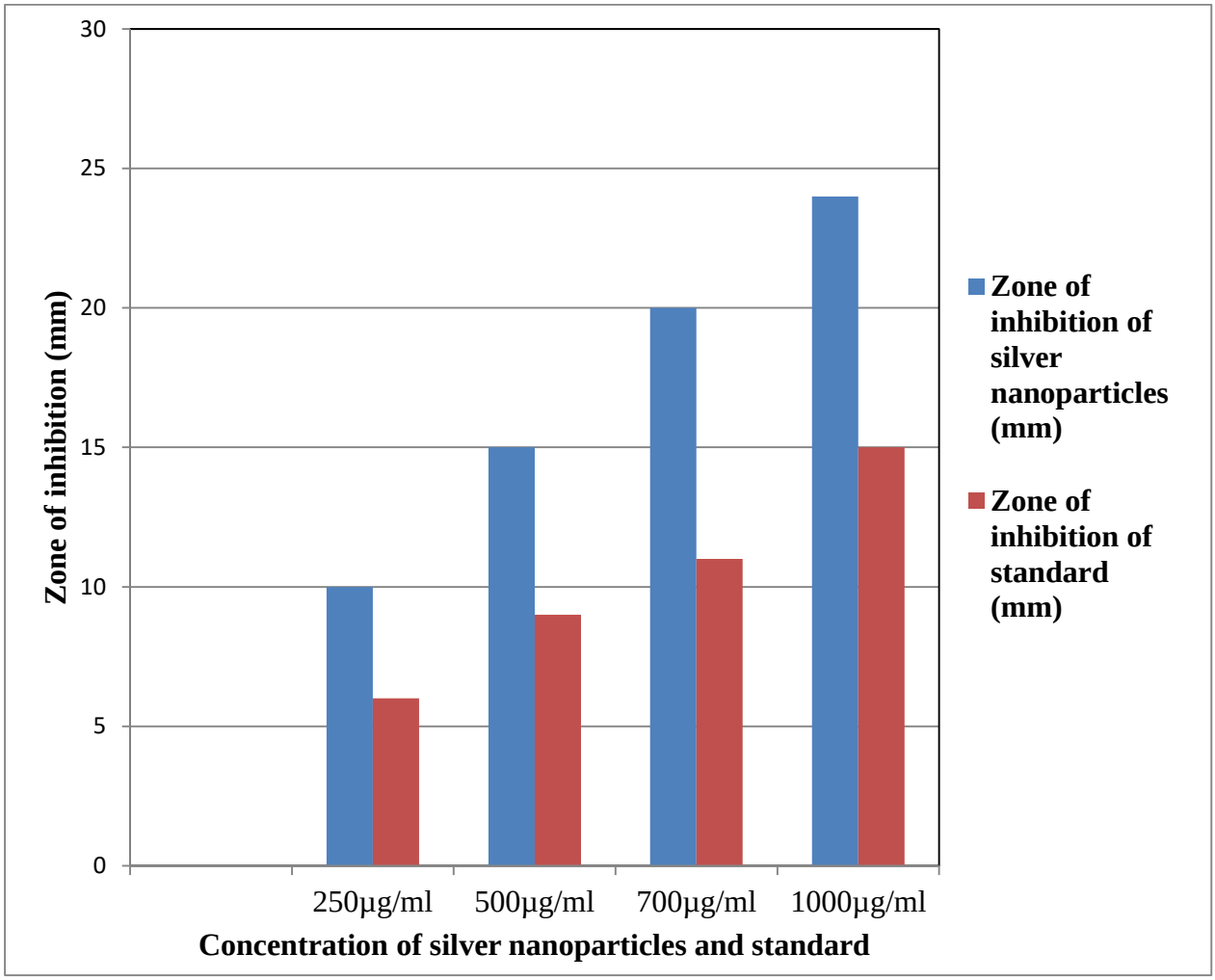

Figure 1: Graphical representation of zone of inhibition of silver nanoparticles and standard

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3.2 *In vivo* clinical evaluation of antifungal activity of silver nanoparticles for treat

3.2.1 Area of clear nail

Sequential measurements of the distance from proximal nail fold to a predefined distal mark, such as the most proximal edge of the nail give a fairly good idea for the efficacy of the treatment. When the treatment used allows normal nail to replace the affected nail, it leads to progressive increase in the clear distance from the proximal nail fold to the most proximal portion of the observed change. The results of the two weeks (bid) application of the prepared formulation are shown in table 2.

Table 2: Mean total surface area of nail, infected area of nail and treated area of nails.

Patient	Total surface area of nails (mm^2) (Mean)	Infected area (mm^2) (Mean)	Treated area (mm^2) (Mean)
1 (Toe nail)	122±2.516	70±1.732	11±1.732
2 (Toe nail)	130±1.154	73±1.154	7.0±0.577
3 (Toe nail)	110±1.154	60±1.000	6.0±0.115
4 (Toe nail)	120±1.154	60±0.577	4.0±0.173
5 (Toe nail)	122±2.000	64±1.000	5.0±0.115
6 (Toe nail)	130±1.154	71±1.154	6.0±0.577
7 (Toe nail)	151±1.527	71±1.154	4.0±0.230
8 (Toe nail)	130±0.577	70±0.577	5.0±0.057
9 (Toe nail)	120±0.577	67±0.577	4.0±0.115
10 (Toe nail)	121±1.000	70±1.000	10±0.173
11 (Toe nail)	100±0.577	71±1.527	4.0±0.114
12 (Toe nail)	121±1.577	63±1.154	5.0±0.577
13 (Toe nail)	100±1.154	68±1.732	6.0±0.111
14 (Toe nail)	120±0.577	60±0.577	3.0±0.577
15 (Toe nail)	130±1.154	71±1.154	4.0±0.577

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Conclusions

Silver nanoparticles were prepared using *Ocimum sanctum* leaf extract, which combined the inherent antifungal activities of silver metal and Ocimum sanctum extract for enhanced antifungal activity. The results obtained from antifungal activity of silver nanoparticles including in vitro disk diffusion method clearly showed a concentration- dependent inhibition of fungal growth. Also, the AgNPs exhibited a greater inhibitory effect in comparison to the standard antifungal agent against *Candida albicans*. In vivo clinical studies also revealed that 5-15% of infected area of nails cleared upon application of cream containing silver nanoparticles.

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