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Original Research Article

Anticandidal Effect of Green Synthesized Silver Nanoparticle from *Azadirachta Indica* Leaf Extract and Development of Ointment Against *Candida Albicans* Causing Vulvovaginal Candidiasis

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ABSTRACT

Background
Vulvovaginal candidiasis (VVC) is a condition that impacts women from various ethnic backgrounds and social strata. The precise epidemiology of the disease is difficult to determine due to inconsistent data; however, available information suggests that 70-75% of women will experience VVC at least once in their lifetime. Common symptoms include itching, vaginal discomfort, pain during intercourse, external pain during urination, and unusual discharge.
Methods
The current study investigates the anticandidal properties of neem (*Azadirachta indica*) leaf extracts. It compares the anticandidal effects of aqueous and methanolic leaf extracts, as well as green-synthesized silver nanoparticles (AgNPs/SNPs) derived from methanolic neem leaf extract, against *Candida albicans* isolated from clinical samples of patients with vaginal infection.
Results
The results showed that AgNPs exhibited a stronger inhibitory effect than both the aqueous and methanol extracts. The methanol extract demonstrated anticandidal activity against *C. albicans*, while the aqueous extracts had no effect. The anticandidal effect of AgNPs was evaluated using the agar well diffusion method. A topical formulation was created by incorporating the optimized AgNPs into an ointment base, and the in vitro anticandidal assay of this ointment was conducted using the same agar well diffusion method. The inhibitory effectiveness of the AgNPs-infused ointment was found to be comparable to that of a commercial product against *Candida albicans*.

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Conclusion
Overall, the findings suggest that this formulation has potential anticandidal effects against *Candida albicans* responsible for Vulvovaginal candidiasis.
Keywords: Candida albicans, Vulvovaginal candidiasis, anticandidal effects, women, AgNP.

INTRODUCTION

Candidiasis is one of the most prevalent and important opportunistic fungal infections, caused by *Candida* species. Different forms of candidiasis are common which affect various parts of the body, such as the oral and genital mucosa, skin, gastrointestinal tract and lungs.[(1)] A major issue of *Candida* infection is that it can cause severe, life-threatening blood stream infections also which may lead to colonization of *Candida* in internal organs (disseminated candidemia) and causes serious health problems in humans. Among the *Candida* species, *Candida albicans* is the most well documented one that causes majority of the infections, followed by *non-albicans Candida* (NAC) such as *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis* and *Candida krusei.*[(2)] In the next few years, medicinal plant extracts will gain importance in the field of treatment. Some of the common Indian medicinal plants used are neem (*Azadirachta indica*), and Moringa *(Moringa oleifera).* Neem (*Azadirachta indica*), whose antiviral, antifungal, antibacterial and insecticidal properties have been known for several years[(3)], is one of the mostly used medicinal plants in the traditional system because it contains many natural substances in its different parts. Leaves, seeds, and bark of neem tree have gained much attention because they have many biological activities against various disease-causing organisms[(4)]. There is still some controversy surrounding the efficacy of neem leaves.

The aim of this work was to green synthesize, characterize and evaluate the anticandidal effects of silver nanoparticles from methanolic extract of neem leaf and to develop a formulation from synthesized silver nanoparticle against *C.albicans* causing vulvovaginal candidiasis.

MATERIALS AND METHODS

A prospective laboratory-based study conducted during a period of 3 months starting from March 2024 to May 2024 in the Department of Microbiology, Dr Moopens medical college, Wayanad and Pazhassiraja college, Wayanad. The clinical samples of patients suffering from vaginal infection reaching the laboratory of Dr Moopens Medical college was used in this study. The colonies were identified based on the Gram stain characteristics, germ tube method, morphological characters on chrom agar and corn meal agar.

The plant Neem (*Azadirachta indica*) was selected for the study. The neem leaves were collected from Kozhikode district, Kerala, India. The completely shade dried leaves were coarse powdered using a mechanical blender and then subjected to cold methanol extraction and hot aqueous extraction[(5)]. 10g of dried neem leaves were dissolved in 100ml of methanol and left in the shaker incubator for two days. 10g of dried neem leaves were dissolved in 100ml of distilled water and boiled for 30 minutes. The crude extract thus collected was filtered and completely evaporated and stored at 4^0C until further use. Using the several methods outlined by Raman, 2006[(6)], a phytochemical analysis was conducted to ascertain the presence of different active components in aqueous and methanol extracts of *Azadirachta indica* leaves.

The green synthesis of silver nanoparticles was carried out following a modified procedure of Mahmoudi et al.[(7)] The formation of silver nanoparticle was observed using a UV-visible spectrophotometer (Schimadzu UV-1800). The absorption spectrum was observed in the range of 200- 800 nm. The Fourier Transform Infrared (FTIR) analysis of the silver

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nanoparticle (AgNP) for structural elucidation of the compounds was done from FIST lab, St.Marys College,Wayanad using ATR FTIR Spectroscopy(Perkin -Elmer).

The silver nanoparticles synthesised from Neem leaf extracts were added to the ointment base for the development of ointment against *C.albicans* causing Vulvovaginal candidiasis. The ointment base was prepared with glycerin (2ml), Shea butter(20ml), (potassium sorbate (0.30g), liquid paraffin (5ml), and gelatin (2ml) as components. All the components were mixed well to form the ointment base.[8,9]

Anticandidal assay of extracts and the prepared ointment was performed by agar well diffusion method in Muller Hinton Agar (MHA) plates.[10,9] The test organism cultures were adjusted to 0.5 Mc Farland standards giving a final inoculum of 1.5×108 CFU/ml. The cultures used were *Candida albicans* isolated from the clinical samples of patients suffering from vulvovaginal candidiasis and the ATCC strains of the organism. Different concentration of plant extracts (aqueous, methanol, and silver nanoparticles) and ointment were used for the study. The inhibition zones around the wells were measured with a ruler and recorded in millimeters. Fluconazole (25 mcg) served as the standard antifungal agent for comparison Candid cream was used as standard drug against the fungus. To determine the MIC of synthesized silver nanoparticles, broth microdilution test was performed using sterile 96-well plates.[11] The physical evaluations were carried out on the ointment by using the following parameters [12]:

Colour and Odor: Color and odor of ointment, examine by visual examination.

Irritancy: Mark a 1 cm^2 area on the left-hand dorsal surface. The cream was then applied to that area, and the time was recorded. Afterward, the area was monitored for irritancy, erythema, and edema for up to 24 hours, and the findings were reported.

Greasiness: Ointment was applied on the skin surface in the form of smear and checked if the smear was oily or grease-like

After feel: Emollience, slipperiness and amount of residue left after the application of fixed amount of ointment was checked.

Removal: The ease of removal of the ointment applied was examined by washing the applied part with tap water.

RESULTS

The preliminary phytochemical analysis of neem leaf extracts showed the presence of Alkaloids, Tannins, phenolic compounds, Coumarins, Proteins, and carbohydrates both aqueous and methanolic extract of neem leaves. Whereas Steroids and Terpenoids were only present in the methanolic extract.

Green synthesis of silver nanoparticles from methanol extraction of the neem leaves were carried out by photoirradiation method. The leaf extract colour changed from green to brown colour indicates the formation of silver nanopaticle. The solution showed absorption peak at 460nm in the UV-Vis spectrophotometer analysis, which indicates the successful formation of AgNPs. The synthesized nanoparticles displayed absorption peaks associated with CH, C=C and OH functional groups, as revealed by FTIR spectrophotometry.

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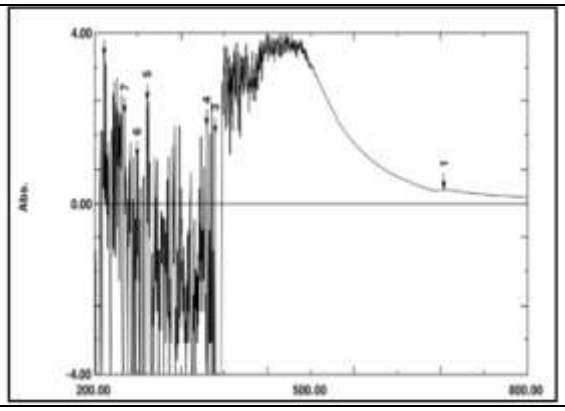

Figure 1a: UV-visible spectra of neem leaf - AgNPs

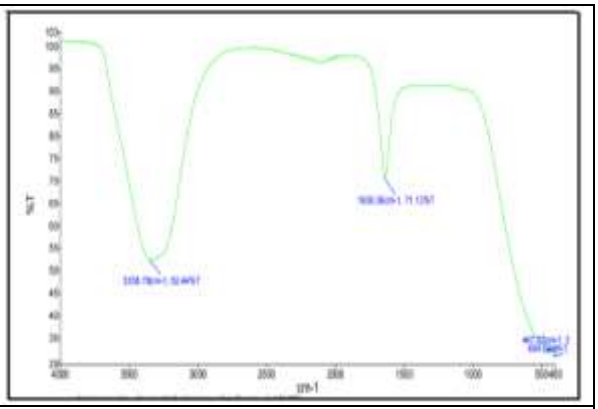

Figure 1b: FTIR analysis of neem leaf - AgNPs

The anticandidal anctivity of neem leaf extract and synthesized silver nanoparticles were carried out using agar well diffusion method. The green synthesized nanoparticles of *A. indica* showed better inhibitory effect on *C.albicans* than the aqueous and methanolic leaf extracts. Aqueous ectract showed least or no inhibitory effect compared to the others. The activity index was found to be maximum in the methanolic leaf extracts than the aqueous extract. The minimum inhibitory concentration of synthesized silver nanoparticle from neem leaf extract is 0.002 mg/ml.

Anticandidal avtivity of the ointment developed by incorporating neem leaf-AgNP was evaluated by agar well diffusion method. The ointment developed from the neem leaf-AgNP showed better anticandidal activity against *C.albicans*. The anticandidal activity of the developed ointment was compared with commercially available ointment (Candid ointment) and anticandidal activity of developed ointment is almost similar to that of candid cream. The physical properties of the ointment were also evaluated. The developed ointment was of light peach colour in appearance and was having a pleasant aroma. The ointment was non- greasy and non-irritant. The ointment applied was smooth and left no residue after application. Also, the ointment applied on skin was easily removable by washing with tap water.

Test Organism	**Inhibition zone of aqueous extract in mm concentration (mg/ µl)**				**Inhibition zone of Fluconazole (25 mcg) in mm**	**Inhibition zone methanolic extract in mm concentration (mg/ µl)**				**Inhibition zone of Fluconazole (25 mcg) in mm**
	20	**40**	**60**	**80**		**20**	**40**	**60**	**80**	
ATCC of *C.albicans*	R	R	R	R	24	12	14	14	16	26
C.albicans	R	R	R	R	23	12	14	15	16	23

Table 1: Anticandidal activity of extraction of A.indica

Test organism	**Inhibition zone of silver nanoparticle in mm concentration (mg/ µl)**				**Inhibition zone of Fluconozole (25 mcg) in mm**
	20	**40**	**60**	**80**	
ATCC of *C.albicans*	13	16	17	19	26
C.albicans	12	14	15	17	25

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Table 2: Anticandidal activity of silver nanoparticle of neem

Organism	Inhibition zone of OB	Inhibition zone of developed ointment from neem AgNP			
		20	40	60	80
C.albicans	6	6	10	16	18

Table 3: Anticandidal activity of developed ointment from neem AgNP

Organism	Inhibition zone of OB	Inhibition zone of neem ointment	Inhibition zone of Candid ointment
C.albicans	15	34	38

Table 4: Comparison of anticandidal activity of developed ointments with candid ointment

DISCUSSION

Antibiotic resistance is a major concern and development of new agents from plants could be useful in meeting the demand for new antimicrobial agents with improved safety and efficacy. *Azadirachta indica* was used in this study to determine their anticandidal effect, the methanolic extract of neem leaves shows anticandidal activity against *Candida albicans* while aqueous extract has no activity. Similar results were found in a study conducted by Adil Rasool et al., 2018[13]. Similar zone of inhibition for methanolic extract of neem were found in another study conducted by Oscar et al., 2019[14].

The present study also aimed at the qualitative analysis of phytochemicals in the methanolic and aqueous extract of *Azadirachta indica* leaves. Alkaloids, Tannins,phenolic compounds, Coumarins, Proteins, and carbohydrates were present in both aqueous and methanolic extract of neem leaves. Bharat *et al*[15] revealed the presence of phytochemicals such as alkaloids , flavonoids, phenols, glycosides, saponins and tannins in leaves extract of *Azadirachta indica* . Presence of these compound in the plant extract may be responsible for the investigated antibacterial activities.

Previously, numerous studies were performed for synthesize AgNPs with significant antimicrobial activities from plant extracts like leaf extracts of *Azadirachta indica*[16] and on various other plants also. In these studies, phytocompounds in the plant extract serve as reducing and/or capping agent in the reaction with silver nitrate (AgNO3), a commonly used precursor in silver nanoparticle synthesis[17]. The results obtained from the UV-Vis spectra indicate that silver nanoparticles were formed. In this study, the plasmon resonance band of the biosynthesized AgNPs was observed at 460 nm *A.indica* respectively, compared to certain other reports, where the bands were mostly shown in the range of 435 to 445 nm. This effect may be due to the concentration of the leaf extract being low in our sample, and such effects have been reported by Ahmed et al 2016[18], where increasing the concentration of the plant extract was shown to increase the intensity of absorption. The green synthesized AgNPs of *A.indica* showed anticandidal activity against *Candida albicans* than methanolic leaf extracts. Similar zone size of inhibition of green synthesized silver nanoparticle from neem leaves were found in a study conducted by Aditya et al., 2022[19].

Fourier-transform infrared (FTIR) spectroscopy studies of AgNPs were made to find out the possible compounds responsible for the efficient stabilization and capping of AgNPs synthesized with the leaf extracts. In this study, the synthesized nanoparticles displayed absorption peaks associated with CH, C=C and OH functional groups, as revealed by FTIR spectrophotometry. A similar interpretation was reported by Mehwish *et al*[20], Moodley *et*

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al[17], and Bindhu *et al*[21]), who showed functional groups with slight peak shifts were presented in AgNPs, and this indicated the reduction and stabilization of AgNPs by the extract of the *Moringa oleifera* leaves.

Green synthesized AgNPs of *A.indica* were used for the development of topical herbal ointment for Vulvovaginal candidiasis and it shown better anticandidal activity against *Candida albicans* by agar well diffusion method. The result from a study conducted by Stephano et al., 2023[22] correlates with the present study. The similar anticandidal activities of the developed herbal ointment were found in another study conducted by Chukwuemeka et al., 2015[23]. Also, the anticandidal activity of developed ointment is little similar with the activity of commercially available ointment "Candid". Thus the herbal ointment developed from green synthesized AgNPs of *A.indica* can be applied for treatments of vulvovaginal candidiasis if the results of this studies are scientifically proven in-vivo.

CONCLUSION

The study demonstrated that silver nanoparticles synthesized from Neem leaf extract exhibit substantial anticandidal activity against Candida albicans. The green synthesis method proved effective in producing nanoparticles with promising antifungal properties. Both the methanolic extract of Neem leaf and the silver nanoparticles showed significant inhibition of Candida albicans, with the latter outperforming the aqueous extract in terms of efficacy. The formulation of a topical ointment incorporating these silver nanoparticles further enhanced the antifungal activity, highlighting its potential as a viable treatment option for vulvovaginal candidiasis. Overall, the findings underscore the effectiveness of combining natural antimicrobial agents with nanotechnology in developing advanced therapeutic solutions for fungal infections. Furthermore, the silver nanoparticles exhibited superior inhibitory effects against *Candida albicans*, and the efficacy of the formulated ointment was assessed against a commercially available product, showing that the ointment containing nanoparticles demonstrated comparable effectiveness in combating Candida albicans. These results indicate that the formulation holds considerable promise for inhibiting the yeast responsible for vulvovaginal candidiasis.

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