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Synthesis of Silver Nanoparticles Using *Pelargonium graveolens* Essential Oil and Anti-Fungal Activity

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Abstract

Biosynthesis of nanoparticles is under exploration is due to wide biomedical applications and research interest in nanotechnology. Bioreduction of silver nitrate ($AgNO_3$) used for the synthesis of silver nanoparticles respectively with the plant extract, *Pelargonium graveolens* leaf oil crude extract. The plant extract is mixed with $AgNO_3$, incubated and studied synthesis of nanoparticles using UV–Vis spectroscopy. The nanoparticles were characterized by X-ray diffraction (XRD), FTIR, TEM and SEM equipped with EDS. The silver nanoparticles synthesized were generally found to be spherical in shape, while some of the NPs were found to be having structures of irregular shape with diameter of 164 nm and 122 nm. The results showed that the *Pelargonium graveolens* leaf oil extract is very good bio reductant for the synthesis of silver nanoparticles and synthesized nanoparticles active against clinically isolated human pathogens against *Candida albicans, Candida tropicalis* and *Candida kefyr*.

Keywords
Nanoparticles, fungus, *Pelargonium graveolens* extract.

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INTRODUCTION:

The 'green' environment friendly processes in chemistry and chemical technologies are becoming increasingly popular and are much needed as a result of worldwide problems associated with environmental concerns [1]. Silver is the one of the most commercialized nano-material with five hundred tons of silver nanoparticles production per year [2] and is estimated to increase in next few years. Including its profound role in field of high sensitivity biomolecular detection, catalysis, biosensors and medicine, it is been acknowledged to have strong inhibitory and bactericidal effects along with the anti-fungal, anti-inflammatory and anti-angiogenesis activities [3,4].

Gold nanoparticles with a size range of 2-20 nm have been synthesized using the live *Alfa alfa* plants [5]. Nanoparticles of metals such as silver, nickel, cobalt, zinc and copper were synthesized inside the live plants of *Brassica juncea* (Indian mustard), *Medicago sativa* (*Alfa*) and *Heliantus annus* (Sunflower). Certain plants are known to accumulate higher concentrations of metals compared to others and are

termed as hyper-accumulators. Of the plants investigated, *Brassica juncea* had better metal accumulating ability and later assimilating it as nanoparticles [6]. The main water soluble phytochemicals are flavones, organic acids and quinones which are responsible for immediate reduction. The phytochemicals present in *Bryophyllum sp*. (Xerophytes), *Cyprus sp.* (Mesophytes) and *Hydrilla sp*. It was suggested subsequent incubation resulted in the activation of quinones leading to particle size reduction. It was reported that catechol under alkaline conditions gets converted into protocatech aldehyde and finally into protocatecheuic acid. Both these processes liberated hydrogen and it was suggested that it played a role in the synthesis of the nanoparticles. The size of the nanoparticles synthesized from xerophytes, mesophytes and hydrophytes were in the range of 2-5nm [7]. Recently some gold nanoparticles have been synthesized using the extracts of *Magnolia kobus* and *Diopyros kaki* leaf extracts. The effects of temperature on nanoparticle formation within size range of 5- 300nm were obtained at lower temperature while a higher temperature supports the formation of smaller and spherical particles [8].

Biological organisms can be used to control the nucleation and development of the inorganic nanostructures [9]. Preparation of gold and silver nanoparticles by living plants was firstly reported. Previously it was reported that plant oil compounds that contain different constitutes like phenolic compounds and flavonoids [10]. Have been used for the biosynthesis of nanoparticles. The bioreduction of Ag+ to silver nanoparticles involves plant extracts and plant oil compounds [11]. It has been shown that variety of plant extracts served as green reactants in silver nanoparticles synthesis [12]. A recent study has demonstrated that the synthesized silver nanoparticles using plant oil compound citronellal displayed moderate anti-plasmodial activity [13].

Essential oils are volatile, natural, complex compounds that are produced by plants as secondary metabolites for protection against bacteria, viruses, fungi and pests [14]. They also have an important role in dispersion of pollens and seeds by attracting some insects. In Middle Ages essential oils were used for preservation of foods and as flavoring antimicrobial, analgesic, sedative, anti-inflammatory, spasmolytic and locally anesthetic remedies [15]. But characterizing these properties in laboratory dated back to the early 1900s. At present, nanoparticles have been produced commercially such as pharmaceutical, agronomic, food, sanitary, cosmetic and perfume. Today, antioxidant, antitumor and antiviral, antifungal and antibacterial activity of *P.graveolens* essential oils and their constituents is widely studied [16]. On the other hand, Antimicrobial resistance (AMR) is now a global concern.

Farnesol will enable nanoparticle biosynthesis methods to compete with other plant assisted biosynthesis routs for the formation of silver nanoparticles that are currently much more rapid and reproducible [7].

Plant oil compound assisted terpenoids (citronellol and farnesol) ketones, aldehydes and amides for anti-diabetic treatment and liver cancer treatment in albino Wistar rats [17].

Due to the non-toxic safe silver nanoparticles are being used for centuries and is capable of killing about 650 microorganisms that cause diseases. Silver has been described as being 'oligodynamic', that is, its ions are capable of causing a bacteriostatic (growth inhibition) or even a bactericidal (antibacterial) impact. Therefore, it has the ability to exert a bactericidal effect at minute concentration [18]. It has a significant potential for a wide range of biological application such as antibacterial agents for antibiotic resistant bacteria, preventing infections, healing wounds and anti-inflammatory [19]. Silver in ionic form strongly interacts with thiol groups of vital enzymes and inactivates them. That lead DNA loses its replication ability once the bacteria are treated with silver ions [20]. Silver nanoparticles destabilize plasma intracellular adenosine tri-phosphate (ATP) by targeting bacterial membrane resulting in bacterial cell death. Compounds of silver such as silver nitrate and silver sulfadiazine are used to prevent bacterial growth in drinking water, sterilization and burn care [21].

Silver nanoparticles interact with outer membrane of bacteria that leads to the death of the bacteria. Nano-sized silver have already provides a more durable antimicrobial protection, often for the life of the product. The antimicrobial property has opened a new era in pharmaceutical and medical industries both extracellularly as well as intracellularly. Silver is the metal of choice as they hold the promise to kill microbes effectively. Silver nanoparticles shows very strong bactericidal activity against gram positive as well as gram negative bacteria including multi-resistant strains [22].

MATERIAL AND METHODS

Materials

Silver Nitrate ($AgNO_3$) was purchased from Sigma Aldrich, India. All other reagents used in the reaction were of analytical grade with maximum purity. The plant leaf essential oils compounds were purchased from Commercial center Aromax Trading Company, Chennai, Tamil Nadu (India). The silver nitrate was purchased from HiMedia (Mumbai, India).

The Clinically isolated Vaginal Candidiasis fungus spp used for the test were *Candida albicans (C. albicans), Candida tropicalis (C. tropicalis) and Candida kefyr (C. kefyr)*. All the stock cultures were obtained from Microlab, Institute of Research and Technology, Vellore, Tamil Nadu, India. The microorganisms were grown overnight at 37°C in Mueller-Hinton Broth at p^H 7.4.

Biosynthesis of silver nanoparticles

Typical synthesis process of silver nanoparticles, 10 ml of plant leaf oil compound was added into 90 ml of 1 mM silver nitrate aqueous solution, biosynthesis reaction started after continuous stirring with magnetic stirrer for 15 minutes and the color reaction was observed in which clear $AgNO_3$ solution changed into dark brownish color which indicates that formation of corresponding silver nanoparticles. The UV–Vis spectra of silver nanoparticles synthesized by plant leaf essential oil *Pelargonium graveolens* are showed the broad peak observed at 420 nm was seen.

Characterization of nanoparticles

Several techniques are used for characterizing different nanoparticles. Here we have discussed the basic principles of few techniques that have been used for the characterized the silver and gold nanoparticles in this research work. They are Absorption spectrophotometer (UV-Vis). The particle size and surface morphology was analyzed using Transmission electron microscopy (TEM) operated at an accelerated voltage of 120 kV. Scanning electron Microscope (SEM), X-ray diffraction (XRD) measurements of the *Pelargonium graveolens* leaf oil broth reduced Ag nanoparticles were carried out on films of the respective solutions drop-coated onto glass substrates on a Phillips PW 1830 instrument operating at a voltage of 40 kV and a current of 30mA with CuKα1 radiation. Fourier transforms infrared spectroscopy and Fluorescence spectroscopy analysis. Characterization involved FTIR analysis of the dried powder of AgNPs, by scanning it in the range 350–3000cm−1 at a resolution of $4cm^{-1}$. These measurements were carried out on a Perkin–Elmer Spectrum-One instrument in the diffuse reflectance mode at a resolution of $4cm^{-1}$ in KBr pellets and the pellets was mixed with KBr powder and pelletized after drying properly.

Gas Chromatography-mass spectrometry

GC-MS was done at South Indian Textile Research Association Coimbatore, Tamil Nadu (India) and analysis was carried out using a Hewlett-Packard 5890/5971A system fitted with a HP1 column (50 m x 0.20 mm fused silica capillary column; film thickness, 0.5µm). GC oven initial temperature was 60°C and was programmed to 220°C at a rate of 2°C/min and 220°C during 120 min under the following operation conditions: vector gas, He, injector and detector temperatures, 250°C; injected volume: 0.2 µl, with a ratio split of 1/100. Retention indices were determined with Hexane standards as reference. The mass spectra were performed at 70 eV of the mass range of 35 - 400 amu. Identification of the constituents was based on comparison of the retention times with those of authentic samples and on computer matching against commercial (Wiley, Mass Finder 2.1 Library, NIST98) and home- made libraries and MS literature data [23-26].

Antifungal assay

Agar well diffusion method

The antifungal activity of the synthesized AgNPs was carried out by well diffusion method. The fungal strains were grown in potato dextrose broth (PDB) for 72 h and used for the study. Microorganism was prepared by spreading 100µL of revived culture (containing 104 cells ml−1) on potato dextrose agar (PDA) (Hi Media) media with the help of spreader. 25µl volumes of Ag NPs and *Pelargonium graveolens* leaf oil crude were added to the respective well with 7 mm of dia. The ketoconazole and $AgNO_3$ were used as a positive and negative control for the antifungal assay. The Petri plates were incubated at 25 ◦C for 72 h in incubator during which activity was evidenced by the presence of a zone of inhibition (mm) surrounding the well.

RESULTS

UV spectrophotometry study

The UV–visible absorption spectra finding demonstrates a novel technique for the preparation of the Au NPs. The scale of wavelength was fixed between 200 and 700 nm, the surface Plasmon resonance (SPR) of the Ag NPs formed corresponded to 420 nm and there was an increase in intensity till 5hr as a function of time without any shift in the peak wavelength (Fig. 1). It can be observed that the reduction of Silver ions reaches saturation within 5hr of reaction, and after that, only slight variations can be noted in the intensity of SPR bands. This result indicates that the reaction is completed in 5hr.

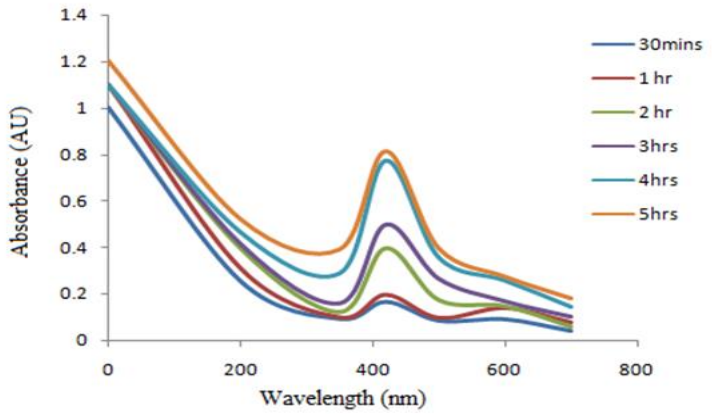


Figure 1 - UV–Vis spectrum analysis of silver nanoparticle reduced by plant leaf oil *Pelargonium graveolens*at 420 nm

Plate 1 - $AgNO_3$ solution

Plate 2-Synthesized silver nanoparticle

Biological synthesis of Silver Nanoparticles

X-ray diffraction analysis

The X-ray diffraction pattern of silver nanoparticle synthesized by plant essential oil *Pelargonium graveolens* was showed in (Figure 2). The XRD pattern thus clearly illustrates that the silver nanoparticles present green synthesis method are powdery in nature. The peaks indicated that the product have well crystalline. The XRD peaks at 38◦, 44◦, 64◦ and 77◦ can be indexed to the (1 1 1), (2 0 0), (2 2 0) and (3 1 1) Bragg's reflections of cubic structure of metallic gold respectively (JCPDS no. 04-0784). The broadening of Bragg's peaks indicates the formation of NPs. Nearly monodispersed Ag NPs with controllable size and uniform shape can be easily obtained in the simple aqueous reduction method. The mean size of Ag NPs was calculated using the Debye–Scherrer's equation by determining the width of the (111) Bragg's reflection [27].

Fourier transforms infrared spectroscopy

The possible potential biomolecules responsible for the reduction of silver ions to silver nanoparticles was identified using FT–IR analysis. Figure 3 shows the FT–IR spectrum of plant essential oil *Pelargonium graveolens* assisted silver nanoparticles. The band at 3434.3 cm^{-1} represents N–H stretching groups of amides. The band at 1726.2 cm^{-1} corresponds to N–H groups of primary amines. The peak at 1379.1 cm^{-1} corresponding to amide II and amide III of aromatic rings either may by poly phenols associated with synthesized silver nanoparticles which is segregated by plant essential oil *Pelargonium graveolens*. The band at1, 056.5cm^{-1} shows that C–O stretching vibrations of alcohols, carboxylic and C–N stretching of aliphatic amines. The intense peak of 836.3, 742.1, 534.2, and 436.9 cm^{-1} shows that C–Cl, C–Br and C–I group of alkyl halides. The observed peaks at 1113 cm1 denote eCeOC- linkages, or eCeO- bonds. The observed peaks are mainly attributed to flavanoids

and terpenoids excessively present in plants extract [28].

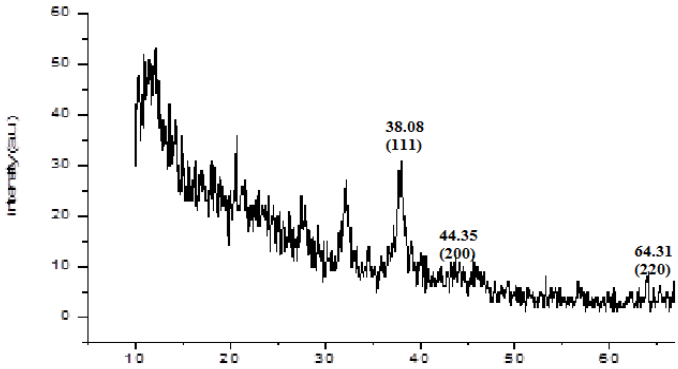


Figure 2 - XRD patterns of silver nanoparticles synthesized by plant leaf essential oil *Pelargonium graveolens*

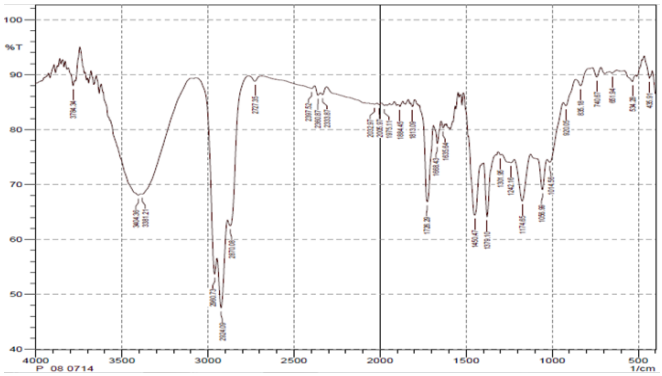


Figure 3 - FTIR spectrum of vacuum dried powder of silver nanoparticles synthesized by plant leaf essential oil *Pelargonium graveolens*.

Transmission electron microscope analysis

The TEM picture of silver nanoparticle synthesized by the plant essential oil *Pelargonium graveolens* was shown in (Figure 4). The microphotography image shows that the silver nanoparticles are with a diameter of 164 nm and 122 nm. Transmission electron microscopy (TEM) has been used to identify the size, shape and morphology of nanoparticles. It reveals that the silver nanoparticles are well dispersed and predominantly spherical in shape, while some of the NPs were found to be having structures of irregular shape as shown in (Figure 4).

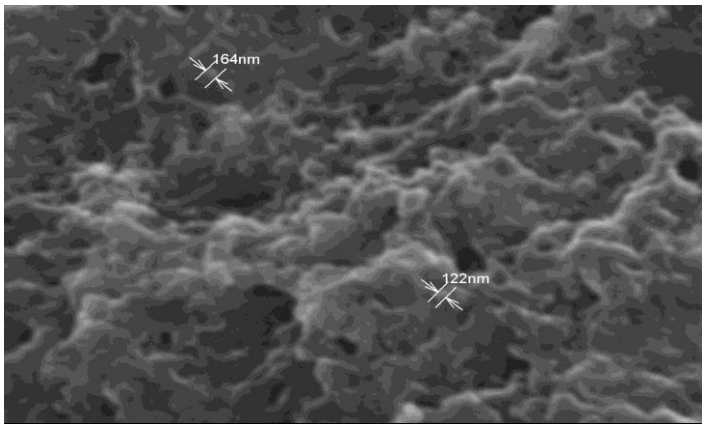


Figure 4 - Transmission electron microscope image of silver nanoparticle synthesized by plant leaf essential oil *Pelargonium graveolens*

Scanning electron microscopy analysis

The scanning electron microscopy the structure of synthesized silver nanoparticle *Pelargonium graveolens* was noted in which the silver nanoparticles is in agglomerated form (Figure 5). The results shown below proved that the nanoparticles are synthesized due to the action of plant essential oil *Pelargonium graveolens* which act as good biomedical properties to kill pathogenic microorganisms. The analysis of energy dispersive spectroscopy (EDS) of the silver nanoparticles the presence of elemental silver signal was confirmed (Fig 5A). SEM analyses of the synthesized silver nanoparticles were clearly distinguishable measured 35–60nm in size. It is clear that the triangles, pentagons, and hexagons structures with sizes of up to 45nm in (Figure 5). The co-existence of Ag NPs in smaller and larger size was due to the Ag NPs formed in early and later stages of the reaction, which shows that both nucleation to form new NPs and aggregation to form larger particles happened consecutively. Fig. 5A shows the spot-profile EDX of Ag NPs showed strong signals for silver atoms along with weak signals from carbon and oxygen. These weak signals could have been arisen from X-ray emission from macromolecules like proteins / enzymes bound to the NPs or in the vicinity of the particles.

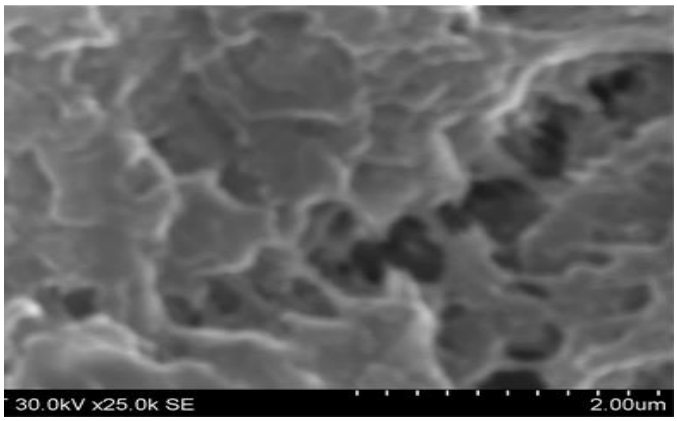


Figure 5- Scanning electron microscope image of silver nanoparticle synthesized by plant leaf essential oil *Pelargonium graveolens*

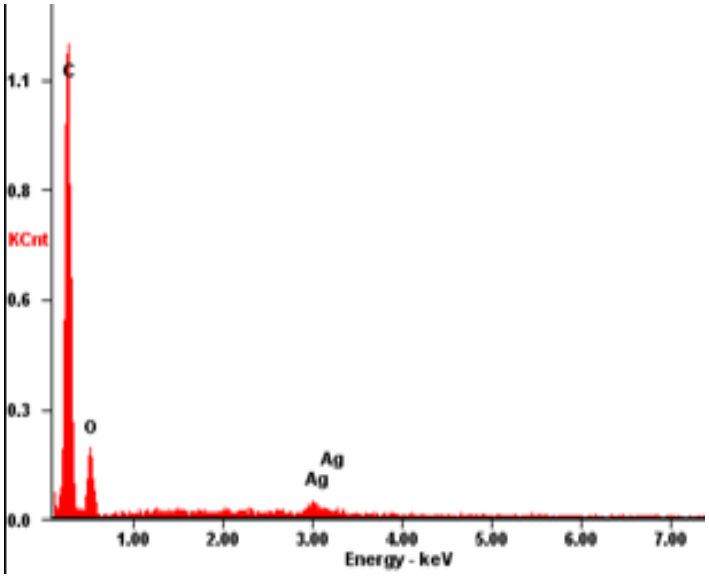


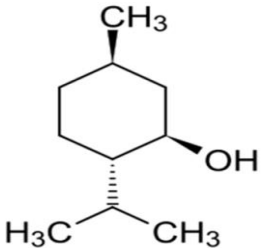
Figure 5A- SEM- EDS spectrum showed the presence of silver signal

Gas Chromatography-mass spectrometry

The chemical compounds of *Pelargonium graveolens* oil is given in Table 1

Sl. No.	COMPOUND NAME
1.	9-Octadecenoic acid (Z)- (CAS)
2.	6,7-Epoxy-7-methyl-1,9-decadien-5-one
3.	8-exo-Bromo-4-endo,7-endo-dihydroxy-2-oxabicyclo[3.3.0]octane-3-one
	(1R)-7,7-Dimethyl-2,3-dioxobicyclo[2.2.1]hept-1-yl
4.	Germacrene-D
5.	1-(1,5-Dimethyl-1-vinyl-4-hexenyl)cyclohexanol
6.	(-)-Menthyl p-(2-Hydroxyethyl)benzenesulfinate
7.	3,5,5-Trimethyl-3-butylcyclohexanone
8.	(2S,4R)-2-(2-methyl-1-propenyl)-4-methyl-tetrahydropyran
9.	4-Octen-3-one, 2-methyl-4-propyl- (CAS)
10.	(3E)-Dec-3-en-2-one
11.	Geranium

The chemical structure of *Pelargonium graveolens* oil is given in Figure 6



***In vitro* antifungal assay**

The plant essential oil *Pelargonium graveolens* showed notable antifungal activity against *Candida tropicalis* and *Candida kefyr* in Table 2. The essential oil *Pelargonium graveolens* was very highly active against *Candida tropicalis* (16.52±0.22). Silver nitrate solution was least active against *Candida albicans* (0.00±0.30).The silver nanoparticle *Pelargonium graveolens* was also highly active against *Candida tropicalis* (13.89±0.38) and least against *Candida albicans* (6.12±0.45). All fungi were found to be sensitive to all test essential oil *Pelargonium graveolens* and synthesized silver nanoparticle *Pelargonium graveolens* and mostly comparable to the standard reference antifungal drug ketoconazole to some extent. The results are shown in figure 7 and Plate 1.

Table 2- Antifungal activity of plant *Pelargonium graveolens* synthesized silver nanoparticle.

Microorganisms	*P.graveolens*	$AgNO_3$ solution	*P.graveolens* + AgNP	Control (Ketoconazole)
Candida albicans	9.79± 0.14[a]	0.00±0.30[a]	6.12±0.45[a]	16.31±0.07[b]
Candida tropicalis	16.52±0.22[c]	1.36± 0.44[a]	13.89±0.38[c]	14.37± 0.15[a]
Candida kefyr	14.77±0.39[b]	0.00±0.31[a]	11.22±0.88[b]	17.69±0.24[b]

The values are represented as the Mean ± SD of five replications. These plant essential oil *pelargonium graveolens* and with the synthesized silver nanoparticle have significant effect at $p<0.05$ levels.

Figure 7- Antifungal activity of plant *Pelargonium graveolens* synthesized silver nanoparticle.

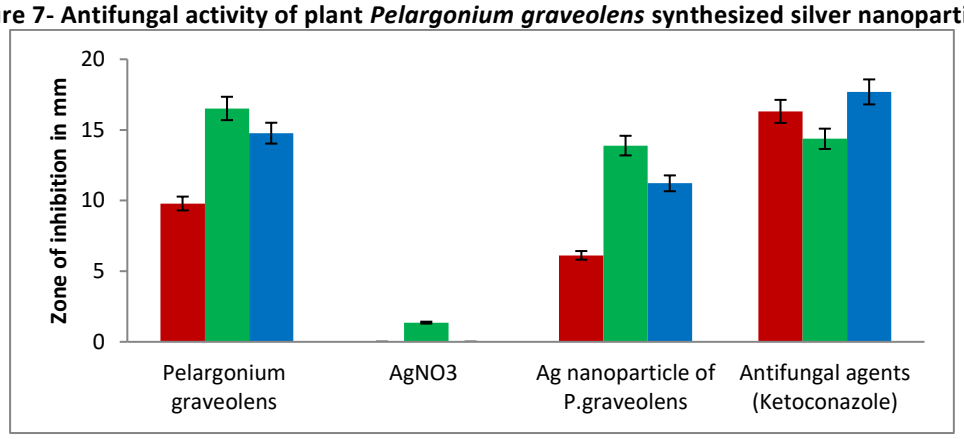


Plate1a-c: Anti-fungal activity of synthesized silver nanoparticle from plant *Pelargonium graveolens* oil against selected Fungi

a.Candida albicans

b. Candida tropicalis

c. Candida kefyr

DISCUSSION

Nanoparticles (NPs) are being viewed as fundamental building blocks of nanotechnology. Biosynthesized Ag NPs are used in antimicrobial [29], anti-viral and anti-human immunodeficiency virus (anti-HIV) studies [30]. [31] reported that Ag NPs showed the in vitro and in vivo inhibitory effect on *Leishmania infantum*. Using plants for NP synthesis can be advantageous over other biological processes because it eliminates the elaborate process of maintaining cell cultures and can also be suitably scaled up for large-scale NP synthesis[16]. Synthesis of NP using microorganisms or plants can potentially eliminate this problem by making the NPs more biocompatible. In addition, a large number of previous studies have dealt with the adverse effects of silver and silver NPs on microorganisms. It has been established that extracts from algae, leaf, plants, and yeast acted both as reducing-, capping- and shape directing-agents in the green synthesis of Ag and Au-nanoparticles [32,33]. A reported simple and fast biosynthetic method to the formation of gold and silver nanoparticles using living plants and/or its extract as reducing agents for the second time [34]. Very recently, [35] have demonstrated that the presence of halide ions and modulation of temperature can control the morphology of biologically synthesized silver triangles using lemongrass leaf extract. It has also been shown that the size of nanoparticles using this technique can be controlled through p^H adjustment, exposure time, and temperature reaction.

Nam *et al*. [36] have reported that the development of an antimicrobial gel formulation containing silver nanoparticles in the size range of 7–20 nm synthesized by a proprietary biostabilization process exhibited good antifungal activity against *A. niger and C. albicans.* The modified denture base acrylic combined with silver nanoparticles displayed significant antifungal properties against *C. albicans* strain [37]. The antimicrobial activity of N-cholyl amino acid capped Ag NPs reported effective antifungal agent against *C. albicans, Candida krusei* and *Candida tropicalis* using RPMI broth were determined by MIC studies [38]. The majority of human infections by *Candida* occur at the mucosa

[39]. Several epidemiological studies have documented that vulvovaginal candidiasis is a widespread, common disease affecting up to 75% of healthy women, with some of them affected by recurrent, often intractable forms of the disease. Recurrent vulvovaginal candidiasis (RVVC) is a much more serious clinical condition due to the recurrences of symptoms (four or more episodes per year) and for its refractoriness to successful treatment. Long-term maintenance therapy with fluconazole may help lengthen the asymptomatic periods between recurrences, but does not provide a long-lasting cure [40]. Recent epidemiological investigations have suggested that the prevalence of RVVC may be higher than previously estimated and can be as high as 7%–8% of women who experience a first episode. In these cases, the quality of life is devastated, and the associated cost of medical visits is high. Anti-fungal therapy is highly effective for individual symptomatic attacks, but does not prevent recurrences. In fact, maintenance therapy with an efficacious anti-Candida drug lengthens the time to recurrence, but does not provide a long-term cure.

CONCLUSION

A simple one-pot green synthesis of stable silver nanoparticles using *P. graveolens* oil leaf extract at room temperature was reported in this study. Synthesis was found to be efficient in terms of reaction time as well as stability of the synthesized nanoparticles which exclude external stabilizers/reducing agents. It proves to be an eco-friendly, rapid green approach for the synthesis providing a cost effective and an efficient way for the synthesis of silver nanoparticles. Therefore, this reaction pathway satisfies all the conditions of a 100% green chemical process. The synthesized silver nanoparticles showed efficient antimicrobial activities against *Candida tropicalis, Candida kefyr* and *Candida albicans*. Benefits of using plant extract for synthesis is that it is energy efficient, cost effective, protecting human health and environment leading to lesser waste and safer products. This eco-friendly method could be a competitive alternative to the conventional physical/chemical methods used for synthesis of silver nanoparticle and thus has a potential to use in biomedical applications and will play an important role in opto-electronics and medical devices in near future.

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