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Synthesis, characterization and antimicrobial study of silver nanoparticles using methanolic fraction of *Artemisia vulgaris* leaf

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Abstract

Rational selection of active biomolecules in the synthesis of nanoparticles for reducing the precursor and functionalizing the nanoparticles (NPs) can offer remarkable comeback of biocompatibility and biological applicability. This work aimed at the synthesis of a cost-effective, ecofriendly, and a facile approach of silver nanoparticles (AgNPs) using methanolic leaf extract of Artemisia vulgaris. The phytochemical constituents present in the methanolic extract were characterized by qualitative chemical tests and spectroscopic measurements and employed for the reduction of silver nitrate into silver nanoparticles. Formation of AgNPs was monitored by UV-visible spectroscopic measurement. Fourier transform infrared (FTIR) spectroscopy reflected the presence of characteristic functional groups associated with the phytochemical constituents involved in the formation of nanoparticles. The crystalline phase and morphology of the NPs were assessed form X-ray diffraction (XRD) spectra and field emission scanning electron microscopy (FESEM), respectively. XRD pattern revealed the crystalline nature of nanoparticles with grain size of ~ 28 nm based on the Debye Scherer formula. Study of antimicrobial activity of AgNPs against Gram-positive bacteria Bacillus subtili, Gram-negative bacteria Escherichia coli, and fungus Candida albicans exhibited good potential to control the bacterial and fungal growth.

Keywords

Artemisia vulgaris, leaf extract, green chemistry, AgNPs, antibacterial activity, antifungal activity

Article information

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1 Introduction

An unchecked use and misuse of antibiotics has led to the emergence of a number of antibiotic-resistant bacterial strains which is a global health concern with serious implications. Development of new antibiotics has not been sufficient to cope with the raising antibiotic-resistant strains and this poses a significant challenge in the health sector. In the realm of growing threats of antibiotic resistant, it is imperative to cut down the uses of antibiotics to diminish the potential risk or to develop new class of materials with good antibacterial properties [1]. In this context, the nanoparticles' antibacterial capability may be useful in creating antibacterial medications to fight against harmful pathogenic strains.

Synthesis of silver nanoparticles have gained significant interest over the years especially due to their remarkable antimicrobial properties, outstanding electrical and thermal conductivities, optical and high catalytic activities, surface enhanced Raman scattering, chemical stability, antibacterial, antibiofilm, wound healing and other therapeutic abilities on a par with its cost-effective production [2]. Upon entering into the bacterial cell, silver ions cause to transform the DNA molecules into an intensive forms and losses its reproduction power resulting into the bacterial cell death [3]. As silver displays a specific roles in catalytic, antimicrobial and biological system, synthesis of AgNPs has been grown as an important antimicrobial agent in contrast to the ever increasing threats posed by antibiotic resistant microbes [4].

Though AgNPs can be synthesized by physical, chemical and biological method, the uses of toxic materials, tedious methods and high production costs of synthesis have turned the researchers towards the green synthesis method as an alternative approach for its cost-effective and biocompatible nanomaterial synthesis routs [5]. In this context, green method of nanomaterial synthesis for antibacterial applications can be the best alternative approach which uses non-hazardous, renewable, and low-cost materials as a precursor. Green synthesis of nanomaterials involves the uses of secondary metabolites present in plant extracts deploying their excellent reducing, capping and stabilizing properties [6].

Extensive researches are going on for the optimization of shape, size and stability of NPs using biomolecules extract from the different parts of plants. Recently, biosynthesis of silver nanoparticles have been carried out using Artemisia absinthium, Thymus vulgaris [7], Zingiber officinale [8], Premna integrifolia L. [9], Allium cepa L. [10], Salvia vertilillata [11], Moringa olifera [12], etc. Different parts of plant have been used to extract biomolecules for the preparation of nanoparticles. Furthermore, different types of extract such as aqueous extract, methanolic extract and so on are being used for the extraction of biomolecules. Methanol, being polar solvent, can extract many polar phytochemical constituents from plant which could be effective for the preparation of nanoparticles by green synthesis method. It was found that the AgNPs synthesis using papaya fruit showed a notable antibacterial activity against *E. coli* and *P. aeruginosa* [13]. In this research work, researchers used well diffusion technique for the study of antibacterial activities of AgNPs.

Uses of environmentally benign materials like phytochemical extracts, fungi, bacteria, and enzymes for the synthesis of nanoparticles offers numerous benefits like low-cost, availability, biocompatibility, ecofriendly, etc. Recently, green synthesis of metal nanoparticles using medicinal plant such as A. vulgaris has shown potential antimicrobial, antioxidant and anti-proliferative activities [14, 15]. An essential oil of A. vulgaris consists of caryophyllene, trans-caryophyllene, Thujone, thujone, 1,8 cineole, and linalool as major constituents [16]. Various nanoparticles have been prepared using the plant extract of A. vulgaris. It was found that the iron NPs synthesized using A. vulgaris extract were potentially useful for environmental remediation [17]. In addition, A. vulgaris is commonly found in the climate of Nepal and can be cultivated for commercial scale without tedious efforts. The secondary metabolites present in such plants have remarkable reducing properties which can be correlated with the ability of plants extract for the synthesis of nanoparticles with improved characteristics. The phytochemicals present in A. vulgaris leaf extract can be employed as reducing, capping and stabilizing agent in the synthesis of silver NPs. Therefore, it seems rational for its use in the synthesis of silver nanoparticles.

Alomari et al. synthesized AgNPs by green synthesis method using aqueous extract of A. vulgaris leaf and studied for antimicrobial activities. Silver NPs prepared using this extract did not exhibit any effect on *Candida albicans* [14]. In the similar work, Rasheed et al. synthesized AgNPs using methanolic extract of A. vulgaris leaf and applied for their potential biomedical applications. In both cases, phytochemical tests have not been carried out. Before one embarks on the green synthesis of NPs, it is imperative to carry out the phytochemical test to ensure the action of phytochemical constituents in the mechanistic path of nanoparticle synthesis. Rational selection of biomolecules in the synthesis of silver nanoparticles is a strategic approach to enhance their biocompatibility and broaden their biological applicability.

Keeping this in view, this research aimed at synthesizing the AgNPs by a green synthesis route using methanolic extract derived from Artemisia vulgaris leaf of Sainamaina, Rupendehi, Nepal. So far the knowledge obtained from literature, A. vulgaris of this locality has not been reported for the synthesis of AgNPs. At the beginning, the presence of phytochemicals in the methanolic extract of A. vulgaris was assessed and used for the synthesis of AgNPs. As-synthesized AgNPs were characterized by UV-visible spectroscopy, XRD, FTIR, FESEM and TEM. Finally, the antibacterial efficacy of assynthesized AgNPs were studied against the Grampositive (Bacillus subtilis) and Gram-negative (Escherichia coli) bacteria. Antifungal test was carried out against Candida albicans.

2 Experimental

2.1 Chemicals and reagents

Methanol (99.8% EMPARTA), silver nitrate (99.8% Qualigens) and matured sample leaves of *Artemisia vulgaris* were collected from Sainamaina Municipality ward No. 11 (27°4131.9 N and 83°1539.4 E), Rupandehi, Nepal in September, 2022. In this work, chemicals of analytical grades were used in as-received form without further purification.

2.2 Extract Preparation

Artemisia vulgaris leaves were washed with DW, dried in the shade for two weeks followed by crushing to fine powder using a Herbal Medicine Disintegrator (model FW177). The leaf extract was prepared by soaking 100 g of leaf powder in 750 mL methanol for 10 days. Then the content was filtered using cotton muslin to obtain the extract and it was finally filtered through Whatman 42.

2.3 Phytochemical test

To determine the presence of major chemical constituents present in the plant extracts, phytochemical tests were carried out as per the standard protocols [18, 19]. Briefly, the methods adopted for phytochemical tests are as follows.

Test for alkaloids

Mayer's test and Dragendorff's tests were performed for alkaloid test.

Mayer's test: 3 drops of Mayer's reagent was added into the 2 mL of methanolic extract followed by shaking well. Appearance of yellowish precipitate indicates the presence of alkaloid.

Dragendorff's test: 3 drops Dragendorff's reagent was added into 2 mL extract followed by well shaking. Appearance of yellowish precipitate indicates the presence of alkaloid.

Test for flavonoids

5 mL of dilute ammonia solution was percolated

into 2 mL of methanolic extract followed by addition of conc. Sulphuric acid from side of the testtube. Appearance of yellow color shows the presence of flavonoids.

Test for terpenoids

2 mL of $CHCl_3$ was percolated into 5 mL of methanolic extract followed by slow addition of 3 mL of conc. H_2SO_4 . Appearance of a reddishbrown color indicates the presence of terpenoids.

Test for saponins

20 mL of distilled water was added into 5 mL of extract followed by vigorous shaking. Appearance of froth indicates the presence of saponins.

Test for quinone

Few drops of concentrated sulphuric acid were added into 2 mL of extract. Appearance of yellow precipitate shows the presence of quinones.

Test for polyphenols

3 drops of 5 % FeCl₃ solution were added into 2 mL of extract followed by shaking well. Appearance of black color indicates the presence of polyphenol. **Test for glycosides**

3 drops of Molisch's reagent were added into 2 mL of extract followed by shaking well. Then few drops of conc. H_2SO_4 were added slowly from the side of test-tube and left to stand for few minutes. Appearance of violet ring at the junction of two layers indicates the presence of glycosides.

Test for proteins

Biuret test was performed for proteins. For this, 2 mL of 5 % NaOH was added into 2 mL extract followed by addition of $CuSO_4$ solution. The appearance of pink color indicates the presence of protein.

2.4 Synthesis of AgNPs

8.2 g of silver nitrate was dissolved in 500 mL distilled water to prepare 0.01 N silver nitrate solution. Silver nanoparticles were prepared by adding 10 mL and 6 mL of plant extract separately into a beaker with 30 mL of 0.01 N AgNO₃ solution (Extract precursor ratio: 1:3 AgNPs and 1:5 Ag-NPs, respectively) with constant stirring at room temperature. Thus, formed precipitate was filtered and washed with distilled water and ethanol. Then it was dried in vacuum oven at 60 °C. Finally pure AgNPs was collected.

2.5 Physicochemical characterization

The phytochemical assay was based on the visual changes of the solution after chemical treatment. Double beam UV visible spectrophotometer (Labtronics 2802) was used to determine the formation of AgNPs in the Department of Chemistry, Amrit Campus, Tribhuvan University, Kathmandu, Nepal. For this purpose, AgNPs was dispersed in ethanol by sonication for 15 min and taken in quartz cuvette for the measurement of absorbance within the window of 300-700 nm at a scan interval of 5 nm. Fourier Transform Infrared (PerkinElmer 10.6.2) Spectroscopy was used to identify the functional group associated with potential biomolecules attached with silver nanoparticles in the cut off range 500-4000 $\rm cm^{-1}$ with scan interval 4 cm⁻¹. The crystallinity and crystal phase of the obtained materials were probed by an X-ray diffraction (XRD, Rigaku diffractometer, Japan, Cu K, = 1.5406 Å) within the two theta angle of 10-80° at a scan rate of $5^{\circ}/\text{min}$ at 0.02° steps. The surface morphology of assynthesized material was studied using field emission scanning electron microscopy (FE-SEM, Hitachi, Tokyo, Japan) equipped with energy dispersive x-ray spectroscopy (EDX). The transmission electron microscopy (TEM) image of as-synthesized composite nanoparticles was studied using highresolution transmission electron microscopy (HR-TEM, JEM-2200, JEOL, Ltd, Japan).

2.6 Antimicrobial activity

Antibacterial and antifungal activities of 1:3 Ag-NPs, and 1:5 AgNPs were carried out using Agar well diffusion methods. Inhibitions of bacterial and fungal growth were tested in terms of zone of inhibition. Microbials culture was prepared in a tryptone soy broth medium at 37 °C for 10 h. The microbial cell suspension was diluted to obtain 10^7 colony forming unit per milliliter (CFU/mL). Then 100 μ L inoculum was spread over the entire tryptone soy agar plate. Sterilized AgNPs and control samples were placed over the agar plate containing corresponding bacteria. In this experiment, $5\mu L$ of standard kanamycin was loaded into respective sections with the help of micro pipette. Kanamycin was used as standard for antimicrobial test. The plates were incubated in bacteriological incubator for 12 hrs at 37 °C. Each plate was then observed for the zone of inhibition (ZOI) produced by antibacterial and antifungal activity. ZOI was measured by the use of ruler [20].

3 Results and discussion

3.1 Phytochemical analysis

In this study, initially, presence of some phytochemicals were tested by chemical test. The results obtained in this test are presented in table 1.

Result shows that the leaf extract did not show the presence of alkaloids but consists of bioactive substances including flavonoids, saponins, terpenoids, quinones, polyphenols, proteins, and glycosides present. For the preparation of nanomaterials, these bioactive phyto-constituents were thought to function as stabilizing and reducing agents. The study by Thangjam et al., revealed that the leaves extract of *Artemisia vulgaris* included phytochemicals such flavonoids, triterpenoids, glycosides, polyphenols, Saponins, and proteins [21].

3.2 UV-Visible spectroscopy

The absorbance recorded of silver nanoparticles in double beam UV-vis spectrophotometer is shown in figure 1(a). The UV-Vis spectra of silver nanoparticles showed a characteristic surface plasmon resonance (SPR) peak at ~ 430 nm, indicating the finely dispersed AgNPs. In the similar experiment, Ag-NPs synthesis in aqueous solution was monitored by recording the absorption spectra at a wavelength range of 300-600 nm [22]. The peak at 430 nm was found to be a characteristic absorbance peak of the Artemisia vulgaris variant metabolites and proteins, which play an important role in the reduction of silver ions into synthesized NPs. Our findings are in good agreement with previous reports [23–25]. In the similar work of Rasheed et al., UV-vis absorbance peak of AgNPs synthesized using aqueous extract of A. vulgaris L. was detected at 420 nm [15]. According to the study done by Alomari, prominent peak of AgNPs was observed at 431 nm [14]. In the same way, the AgNPs developed from Acorous calamus rhizome extract displayed broader peak around 400 nm [26]. Similarly, several researchers have reported that the peak of AgNPs appears to be around this region [27, 28]. Comparing with these reports, the UV-vis absorbance peak observed for AgNPs of this work seems to be appreciable.

Phytochemicals	Presence/absence
Alkaloids	Absent
Flavonoids	Present
Saponins	Present
Terpenoids	Present
Quinones	Present
Polyphenols	Present
Proteins	Present
Glycosides	Present

Table 1: Showing the results obtained by phytochemical test



Figure 1: (a) UV-Vis spectra of 1:3 AgNPs and 1:5 AgNPs, (b) FTIR spectra of *A. vulgaris* leaf extract only and green synthesized AgNPs and (c) XRD patterns of green synthesized AgNPs.

3.3 FTIR spectroscopy

The functional groups present in as-synthesized Ag-NPs were investigated using FTIR Spectroscopy. The FTIR spectra of plant extract and assynthesized nanostructure are shown in figure 1(b). In the spectra of leaves extract, a board spectrum at 3322 cm^{-1} was due to the O-H bond stretching. The C–H stretching of alkane has resulted in a band at 2945 cm^{-1} and 2835 cm^{-1} . The absorption band at 1655 cm^{-1} is due to C=O or C=C stretching of carbonyl compounds [29]. The C-H bending of alkane gives rise to band at 1449 cm⁻¹ and 1413 cm^{-1} . An absorption peak at 1116 cm^{-1} was ascribed to C-N stretching of amine, and band at 1022 cm^{-1} was attributed to ester and tertiary alcohol. These assigned peaks were carried out in accordance with spectrometric identification of organic compounds [30]. The functionalities present in methanolic extract of leaves of Artemisia vulgaris were found to be well indexed with some previously published report [31].

3.4 XRD

The crystallite nature and size of the as-synthesized nanoparticles were carried out by using X-ray diffraction spectroscopy. The XRD patterns of the green synthesized AgNPs using A. vulgaris leaves extract is shown in figure 1(c). The XRD patterns of AgNPs were appeared at 2θ values of 27.78° , 32.35°, 38.09°, 45.53°, 54.78°, 57.53, 64.45°, 67.37° and 76.74°. The four distinct diffraction peaks at 2θ values of 38.09°, 44.53°, 64.45°, and 76.74° were well indexed to the (111), (200), (220), and (311) reflection plane of cubic structure of silver, respectively, with JCPDS card no: 90-13050, space group: Fm-3m [8, 32]. These data are in good agreement with those of Elemike et al. on their work on AgNPs using A. afra [33]. Furthermore, along with these representatives peaks of silver, some additional peaks values of 27.78°, 32.35°, 46.36°, 54.78°, 57.53°, and 67.37° were also observed at 2θ value which are well matched to the peaks from the JCPDS card no: 76-1393 for silver oxide. Presence of some of these peaks was due to the oxidation of silver during the long term storage before the characterization. Similar studies of XRD patterns for Silver nanoparticles have been reported in elsewhere [34, 35]. The average crystallite size of the green synthesized silver nanoparticles was calculated by using Debye-Scherrer formula,

$$D = \frac{K\lambda}{\beta cos\theta} \tag{1}$$

where,

D = Crystallite size of materials

 $\lambda =$ Wavelength of Cu K radiation (0.15406 nm)

 $\theta = \text{Bragg's angle}$

 $\beta =$ Corrected half width of the diffraction peak (in radian)

K= Shape factor which usually equals to 0.94

The most intense peaks observed in the XRD spectra are 38.09° and 45.53°. The average beta factor was used in grain size calculation. The average crystallite size of green synthesized AgNPs was found at around 28 nm.

3.5 FESEM and EDX analysis

The surface morphology of as-prepared silver nanoparticles was investigated via field emission scanning electron microscopy (FESEM) coupled with energy dispersive X-rays spectroscopy (EDX) and elemental mapping. The surface morphology of silver nanoparticles with different magnifications are shown in figure 2. The images demonstrate the homogeneously generated, narrow size distribution of the silver nanoparticles. The formation of silver nanoparticles occurs at the nanoscale range which is facilitated by the plant metabolites in their synthesis and stabilization. Similar results were also reported in the synthesis of silver nanoparticles using other phytochemical constituents [36, 37]. Successful synthesis of silver nanoparticles in nanometric range is also supported by transmission electron microscopy (TEM) as shown in the figure 4. The TEM image clearly shows the nanosized AgNPs.

Additionally, energy dispersive X-ray with elemental mapping was carried out to ensure the synthesized product, as seen in Figure 3. EDX spectral analysis demonstrated higher silver counts at 3 keV and hence confirming the predominant presence of silver in the nanoparticles. Silver makes up the bulk of the nanoparticle's composition (91.31) mass % and 61.84 atom %). Observation of some other peaks such as peaks of carbon and oxygen in vicinity of silver major peaks corresponds to that C and O elements are characteristic of plant extract. The outcome reveals that the nanoparticles are mostly made of silver, with a little carbon and oxygen. Small amount of carbon could be due to the effect of carbon tape used during FE-SEM/EDX characterization. In addition, the carbon and oxygen could be associated with phytochemicals capped onto the silver nanoparticles as methanolic extract of *Artemisia vulgaris* leaf was used as capping and reducing agent. Some amount of oxygen in EDX could be associated with the oxidized silver nanoparticles upon long storage [15,38]. Figure 3 represents the elemental mapping of assynthesized product and revealed the presence of C, O, and Ag.

3.6 Antimicrobial activities

In this study, the antimicrobial activity of green synthesized AgNPs using AVLE was studied against Gram-positive bacteria (*Bacillus subtilis*), Gramnegative bacteria (*Escherichia coli*), and fungi (*Candida albicans*) by agar well diffusion method. Thus obtained results are shown in Table 2. *Kanamycin* was used as a positive control to compare with the obtained results of as-synthesized Ag-NPs to observe their antimicrobial efficiencies.

Green synthesized AgNPs showed the potential antimicrobial activity against Gram-positive bacteria (Bacillus subtilis), Gram-negative bacteria (Escherichia coli), and fungi (Candida albicans). Ag-NPs of 1:5 ratio showed best efficiency as compared to 1:3 AgNPs and leaves extract alone. The zone of inhibitions of 1:5 AgNPs for Bacillus subtilis, Escherichia coli, and Candida albican were found as 5 mm, 6 mm and 6 mm, respectively and are comparable to the positive standard control Kanamycin (9 mm zone of inhibition). However, the zone of inhibitions exhibited by 1:3 AgNPs for Bacillus subtilis, Escherichia coli, and Candida albican were 4 mm, 5 mm and 4 mm, respectively. These values are almost comparable to those exhibited by Artemisia vulgaris extract. The result showed that as-prepared silver nanoparticles exhibited significant inhibition activities against the tested microbial. The zone of inhibition exhibited by extract, AgNPs and control (kanamycin) are shown in the histogram (figure 5).

In the similar work carried out by Rasheed et al., the antibacterial activities of NPs were investigated using the disc diffusion method against 5 pathogens, namely Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumonia and Haemophilus influenza [15]. The highest value was recorded against S. aureus (18 \pm 0.27 mm) and the lowest value was recorded against V. cholera (12 \pm 0.18 mm) by AgNPs. However, they have not mentioned the ZOI of control. Similarly, in the study done by Alomari [14], green synthesized AgNPs showed significant activity against Grampositive bacteria (Bacillus subtilis, Staphylococcus aureus), and Gram-negative bacteria (Escherichia coli, Pseudomonas aeruginosa), whereas the fungi (Aspergillus flavus and Candida albicans) showed highest resistance towards AgNPs with 0.0 inhibition zone. In the work, Alomari synthesized the AgNPs using aqueous fraction of leaf extract of A. *vulgaris*. Meanwhile, silver nanoparticles prepared in our work exhibited inhibition activities against

fungal species, *Candida albicans*. This could be associated with the effect of size of nanoparticles, active ingredient and phytochemicals functionalized NPs.



Figure 2: FESEM images of 1:3 AgNPs (a, b and c) and 1:5 AgNPs (d, e and f) at different magnification.



Figure 3: Elemental mapping of silver nanoparticles (a) morphological view, (b-e) elemental mapping:(b) carbon, (c) oxygen, (d) silver, (e) carbon, oxygen and silver, (f) EDS spectra (g) mass percentage and atom percentage of as-synthesized 1:5 AgNPs.



Figure 4: Transmission electron microscopy image of as-synthesized 1:5 AgNPs under different scale bar.



Figure 5: Histogram representation of antimicrobial activities of leaf extract and AgNPs compared to control Kanamycin.



Figure 6: Antimicrobial activity of green synthesized AgNPs against (A) B. subtilis, (B) E. coli, (C) C. albicans, and of AVLE against D, E, F (B. subtilis, E. coli and C. albicans, respectively). Where, C+=positive control, S1= 1:3 AgNPs, S2= 1:5 AgNPs, C-= Negative control, and S= plant extract.

Table 2: Antimicrobial activity of as-synthesized AgNPs using Artemisia vulgaris leaves extract

Samples	Zone of inhibition (mm)	Bacterial Species	Fungal Species
Bacillus subtilis	5	G+	-
Escherichia coli	5	G-	-
Candida albicans	4	-	-
Extract (100 μ L)			
1:3 AgNPs (5 mg)	4	5	4
1:5 AgNPs (5 mg)	5	6	6
Kanamycin (5 $\mu {\rm g/mL})$	9	9	9

4 Conclusion

In this work, silver nanoparticles were successfully prepared by green synthesis method using the methanolic extract of Artemisia vulgaris leaf. Phytochemical screening of the methanolic extract ensured the presence of flavonoids, saponins, terpenoids, quinones, polyphenols, proteins and glycosides. As-prepared silver nanoparticles was characterized using UV-vis, FTIR, XRD, FESEM, TEM, EDX and elemental mapping. The AgNPs exhibited the surface plasmon resonance at ~ 430 nm. The average particle size of the AgNPs was $\sim 28~\rm{nm}$ according to Debye Scherer equation. This value is in agreement with those exhibited in TEM image. To the biomedical point of view, the synthesized nanoparticles were found to act as good antimicrobial agents. It was confirmed that biosynthetic silver nanoparticles showed excellent antibacterial performance against Gram-positive bacteria (*Bacillus subtilis*) and Gram-negative bacteria (*Escherichia coli*), and also showed antifungal activity against *Candida albicans*. The extract from *A. vulgaris* has potential value for various biomedical and pharmaceutical applications. All in all, the finding suggests a successful synthesis of silver nanoparticles by green synthetic route which is good enough to act as antibacterial and antifungal agents for biomedical applications.

Conflict of Interest

Authors declare no conflict of interest.

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