Int. J. Pharm. Investigation, 2020;10(2):146-150 *Original Article*

Antimicrobial and Anticancer Activity Studies on Green Synthesized Silver Oxide Nanoparticles from the Medicinal Plant *Cyathea nilgiriensis* Holttum

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ABSTRACT

Objectives: To synthesize silver oxide nanoparticles from the medicinal plant *Cyathea nilgiriensis* Holttum and to evaluate the percentage of inhibition of silver oxide nanoparticles against some selective bacterial species (three gram positive and three gram negative) and two fungal species. Anticancer activity was performed for Ag_2O nanoparticles using Trypan Blue Assay Method using Daltons lymphoma ascites cells. **Methods:** Silver oxide nanoparticles were synthesized by chemical precipitation method using the extract of the medicinal plant *Cyathea nilgiriensis* Holttum and Silver nitrate. Particle size Analyzer (PSA), X-ray Diffraction Analysis (XRD), Fourier Transform Infrared Spectroscopy (FT-IR), Scanning Electron Microscope Analysis (SEM) and Energy Dispersive X-Ray (EDX) studies studies were employed to confirm the size distribution, composition and crystallographic structure of the Silver oxide nanoparticles. **Results:** Particle size Analyzer and X-ray diffraction results proved the size and crystalline nature of Ag_2O nanoparticles. Fourier transform infrared spectroscopy studies has proved that biomolecules has acted as chapping and stabilizing agents in the synthesis of Ag_2O nanoparticle, Scanning electron microscope analysis has exhibited morphological appearance of Ag_2O nanoparticles and Energy Dispersive X-Ray analysis has proved the presence of both silver and oxygen in the synthesized Ag_2O nanoparticles. On comparing the zone of inhibition of three-gram positive bacterial species, *M. luteus* (20 mm) has shown larger zone of inhibition (20 mm) and when comparing the zone of inhibition of three-gram negative bacterial species, *K. pneumoniae* has shown larger zone of inhibition (21 mm). The increase of concentration from 10 µg to 200 µg of Ag_2O nanoparticles increases the anticancer efficiency of the biosynthesized Ag_2O nanoparticles. **Conclusion:** The Instrumentation results have proved the characteristic nature of the synthesized Ag_2O nanoparticles. Now it's clear that greener synthesized nanoparticles has proved to be better antimicrobial and anticancer agents.
Key words: *Cyathea nilgiriensis* Holttum, Ag_2O nanoparticles, Green chemistry, Characterization, antimicrobial and anticancer activity.

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DOI: 10.5530/ijpi.2020.2.27

INTRODUCTION

Metallic Nano particles obtained using plant extract could be reducing and capping agents.[1,2] Due to the unique properties silver oxide nanoparticle it is used in the fields such as sensing, optoelectronic, drug delivery,[3,4] oxidation catalysis,[5,6] sensors,[7] fuel cells,[8] photovoltaic cells,[9] all-optical switching devices, optical data storage systems[10] and in diagnostic biological probes.[11] The syntheses of nanoparticles through chemical methods are expensive, time consuming and are not ecofriendly. Hence, Greener methods of synthesis of Ag_2O nanoparticles have attracted many researchers wing to their cost-effectiveness and non-toxic nature.[12-14] The green synthesis techniques make use of nontoxic solvents such as water, biological extract, biological systems and microwave irradiation. The use of plant leaf extracts, bacteria, fungi and enzymes for the synthesis of silver nanoparticles offer numerous benefits of eco-friendliness and compatibility for pharmaceutical and other biomedical applications. Green synthesis does not require high pressure, energy, temperature and toxic chemicals. Silver has been recognized to have inhibitory effect on microbes present in medical and industrial process. The topical ointments having silver and silver nanoparticles are medicinally useful to prevent infections against burns and open wounds.[15] Thus synthesis of nanoparticles by greener methods gain importance. In the present study, the medicinal plant Cyathea *nilgiriensis* plant extract has been used to produce silver oxide nanoparticles. Based on the literature survey it is evident that the systematic study on the bio-synthesis and characterization of Ag_2O nanoparticles using *Cyathea nilgiriensis* Holttum extract has been carried out for the first time.

MATERIALS AND METHODS

Plant materials collection and preparation

The pure and analar-grade Silver nitrate [$AgNO_3$] was used for this study. The pure and shade-dried leaves of *Cyathea nilgiriensis* (2 g) were powdered and subjected to extraction using deionized water. The extract obtained was filtered through Whatman No. 1 filter paper and stored in a refrigerator for further use.

Synthesis of Ag_2Onanoparticles

Exactly about 0.1 M of $AgNO_3$ was dissolved in50 mL of double-distilled water along with10 mL of *Cyathea nilgiriensis* aqueous extract under under magnetic stirring at 80°C for 4 hr. The complex formed was ultra-centrifuged at 10,000 rpm for 10 min, rinsed with water and further

centrifuged at 5,000 rpm for 10 min. The complex residue was dried in an oven at 40°C for 8 h and was then calcined in a muffle furnace at 600°C to obtain biosynthesized Ag_2O nanoparticles.

Instrumentation for Characterization of Nanomaterial

The size of the nanoparticle was measured using the particle size analyzer (Nanophox, Sympatec, Germany). The biosynthesized Ag_2O nanoparticles were analyzed for IR characteristics using a Nicolet 520P FT-IR spectrometer set to be in the range of 500 – 4,000 cm^{-1}. A powder XRD analysis was carried out on a PAN analytical X-ray diffractometer operated at 40 kV with a current of 30 mA under Cu-Kα radiation of a 2θ range of 10–80°.The SEM images were recorded using a JEOL JSM 6390 system to identify the crystallographic details of Ag_2O nanoparticles. To confirm the presence of the constituent elements – silver and oxygen, an energy dispersive spectroscopic (JED 2300, JEOL) study was done on Ag_2O nanoparticles.

Antimicrobial activity

The antimicrobial activity was studied applying the disc diffusion method.[16] Ciprofloxacin and fluconazole were taken as standards for antimicrobial activity. A panel of 6 common pathogenic bacteria consisting of three gram-positive type S. *aureus* (NCIM 2079), *B. subtilis* (NCIM 2063), *M. luteus* (MCIM2169) and three-gram negative type *E. coli* (NCIM 2065), *S. paratyphi* (NCIM 2501), *K. pneumonia* (NCIM2707) and 2 fungal strains *C. albicans* (MTCC 3100) *and A. niger* (MTCC 1344) was used. These microbial strains were obtained from the Kovai Medical College and Hospital (KMCH), Coimbatore, Tamil Nadu, India.

Anticancer activity

The *in vitro* cytotoxicity potentials of the biosynthesized Ag_2O nanoparticles were evaluated using Trypan blue dye assay method.[17,18] The cells were aspirated from the peritoneal cavity of tumor bearing mice. The cells were washed three times using PBS and the viability of the cells was checked using trypan blue. Different concentrations (10, 20, 50, 100 and 200 µg) of Ag_2O nanoparticles were prepared. In a test tube, 100µl of Ag_2O nanoparticles was mixed with 800µl of phosphate buffer saline and 100µl (1×10^6 in 1ml) of Dalton's Lymphoma Ascites (DLA) was added. All the test tubes were incubated at 37°C for 3 hr. About 100µl of trypan blue dye was added to each of the test tubes. Dead cells got stained by the trypan blue color while live cells did not absorb the dye. The numbers of stained and unstained cells were measured using hemocytometer. Percentage of cytotoxicity was calculated by the given formula.

$$\text{Cytotoxicity (\%)} = (N_d/N_d+N_l)\times100$$

Where Nd - No of dead cells

N_l - No of live cells

RESULTS

Particle Size Analyzer

The average size of the nanoparticles and the statistical distribution of the size were determined using the particle size analyzer. The results are shown in Figure 2. The particle size of silver oxide nanoparticles is found to be below 100nm.

Fourier Transform Infrared Spectroscopy Analysis

Figure 3 shows the FT-IR spectrum of bio-synthesized silver oxide nanoparticles. The peak around 574 cm^{-1}corresponds to Ag-O vibrations.[19-21] Other bands can be attributed to silver nitrate and the phytochemical constituents of the leaf extract. The O-H stretch appears in the spectrum at 3449 cm^{-1}. The band 1629 cm^{-1} corresponds to the carbonyl group of flavonoids. Strong band at 1383 cm^{-1} corresponds to phenolic stretching

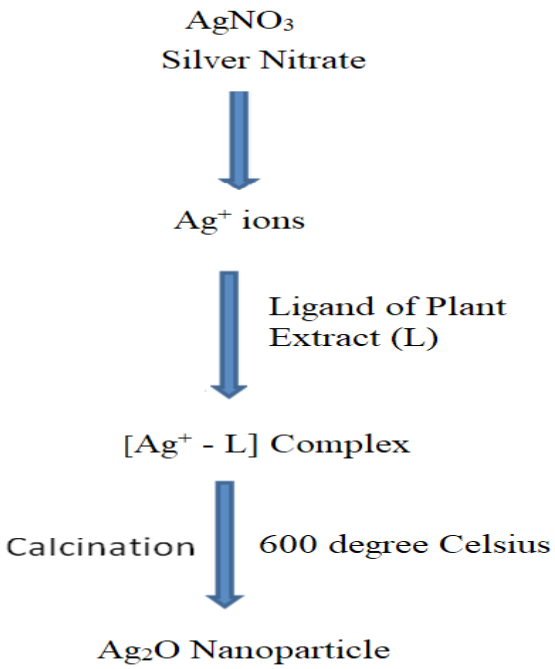

Figure 1: Scheme of Ag_2O Nanoparticle synthesis.

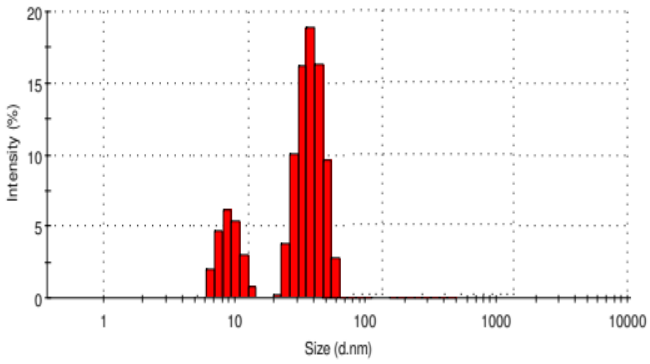

Figure 2: PSA of Ag_2O nanoparticle.

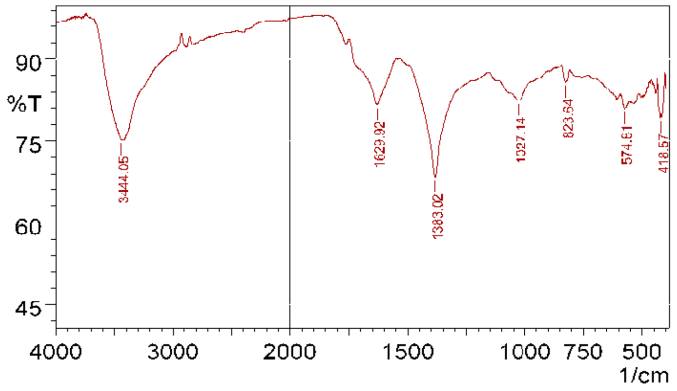

Figure 3: FT-IR spectra of Ag_2O nanoparticles.

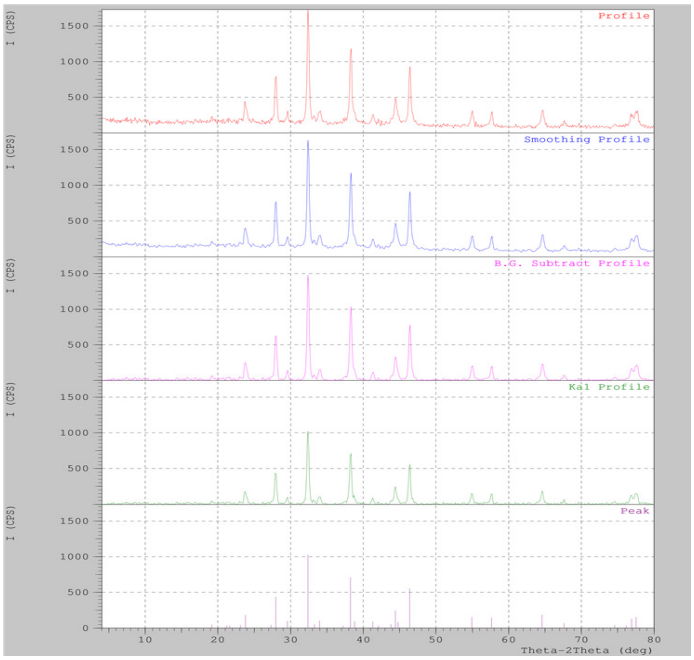

Figure 4: XRD spectra of Ag_2Onanoparticles.

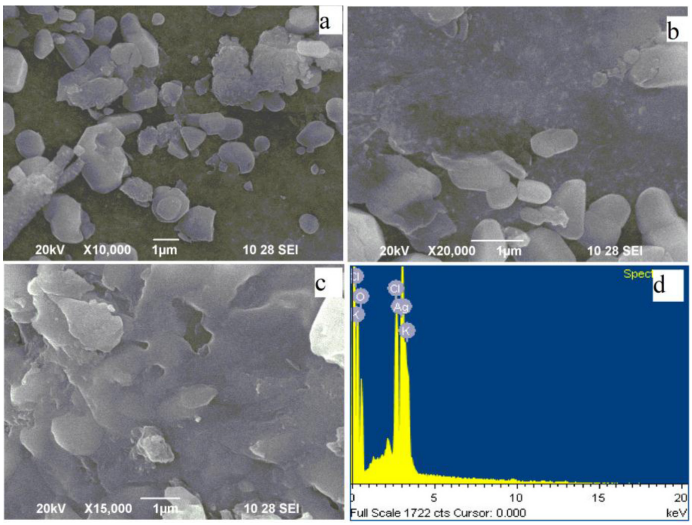

Figure 5: SEM (a, b & c) & (d) EDX spectra of Ag_2O nanoparticles.

vibrations. Strong band at 823 cm^{-1} corresponds to aromatic C-H bending. The results of FT-IR analysis indicated that phenolic type of compound has favored and stabilized the formation of metal nanoparticles minimizing the agglomeration. Figure 1 shows the scheme of the nanoparticle synthesis.

X-ray diffractometer

Figure 4 Shows the X-ray diffractometer (XRD) spectrum of the bio-synthesized silver oxide nanoparticles. It shows intense band peaks at 27.94, 32.27, 46.34,54.92 and 67.48 which corresponds to (110), (111), (211), (220) and (222) planes of face centered cubic silver respectively. All the obtained peaks are similar to standard pure Ag_2O.[22,23] The average size of the particles was determined by applying the Debye-Scherrer's formula.

Morphological analysis

The SEM and EDX images of synthesized silver oxide nanoparticles are shown in Figure 5 (a), (b), (c) and (d). The results showed that Ag_2O nanoparticles were formed in different sizes and below 100 nm. A closer look at the image shows the presence of several nanoparticle clusters. In Figure 5 (a), particles the particles appear to be agglomerated and some individual crystals are clearly visible and are hexagonal in shape. In Figure 5 (b) and (c) some particles appear to be spherical in shape. The EDX results shown in Figure 5(d) further confirm the presence of silver and oxygen.

Antimicrobial activity of Ag_2O nanoparticles

In this study antimicrobial activity of Ag_2O nanoparticles synthesized using the extract of the medicinal plant Cyathea nilgiriensis was found to be significant. Figure 6 shows the antibacterial activity of the biosynthesized Ag_2O nanoparticles. Antibacterial activity was measured against three grams of positive bacteria (*S. aureus*, *B. subtilis, M. luteus*) and three gram of negative bacteria (*E. coli*, *S. paratyphi, K. pneumoniae*). Anti-fungal activity was carried out against *C. albicans* and *A. niger*, Ciprofloxacin is taken as the standard for all microbial strains. Table 1. Shows inhibitory effect of the Ag_2O nanoparticles. The zone of inhibition for standard ciprofloxacin for three gram positive bacteria was found to be 34 mm (*S. aureus*), 27 mm (*B. subtilis*) and 36 mm *M. luteus.* The zones of inhibition of Ag_2O nanoparticles for three gram positive bacteria are found to be 14 mm (*S. aureus*), 12 mm (*B. subtilis*) and 20 mm *M. luteus.* When we compare the above zone of inhibition of Ag_2O nanoparticles for three gram positive bacteria, *M. luteus* (20 mm) shows a larger zone of inhibition. The zone of inhibition of standard ciprofloxacin for three gram negative bacteria ranges from 36 mm (*E coli)*, 35 mm (*S. paratyphi)* and 32 mm (*K. pneumoniae*).The zones of inhibition of Ag_2O nanoparticles for three gram negative bacteria are found to be 15 mm (*E coli*), 17 mm (*S. paratyphi)* and 21 mm (*K. pneumoniae*). On comparing the zone inhibition values of Ag_2O nanoparticles for three-gram negative bacterial species *K. pneumoniae* (21 mm) shows a larger zone of inhibition. Table 2. Shows antifungal results for the biosynthesized Ag_2O nanoparticles. The antifungal images of the Ag_2O nanoparticles protecting against two strains are shown in Figure 7. The zone of inhibition for the standard fluconazole of two fungal species was found to be C. *albicans* (35 mm) and *A. niger* (09 mm). The zone of inhibition of Ag_2O nanoparticles for two fungal species is found to be C. *albicans* (13 mm) and *A. nige* (10 mm).

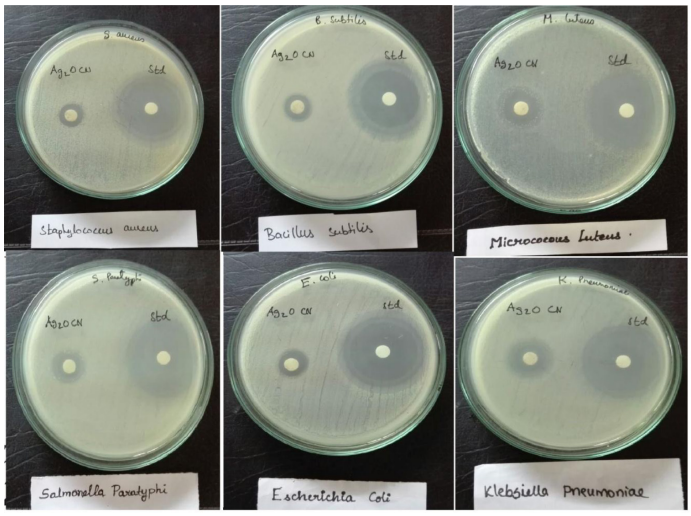

Figure 6: Antibacterial images of Ag_2O nanoparticles a) *S. aureus* b) *B. subtilis*, c) *M. luteus*d) *S. Paratyphi* e) *E. coli* f) *K. pneumonia*

Table 1: Zone inhibition values of gram positive and gram negative bacterias.

Name of organisms	Zone of Inhibition (mm)	
	Std Ciprofloxacin (10µg/disc)	Samples (100µg/disc)
		CN – Ag_2O
S. aureus	34	14
B. subtilis	27	12
M. luteus	36	20
E. coli	36	15
S. paratyphi	35	17
K. pneumoniae	32	21

Table 2: Zone inhibition values of fungal species.

Name of organisms	Zone of Inhibition (mm)	
	Std Ciprofloxacin (10µg/disc)	Samples (100µg/disc)
		CN – Ag_2O
A. niger	09	10
C. albicans	35	13

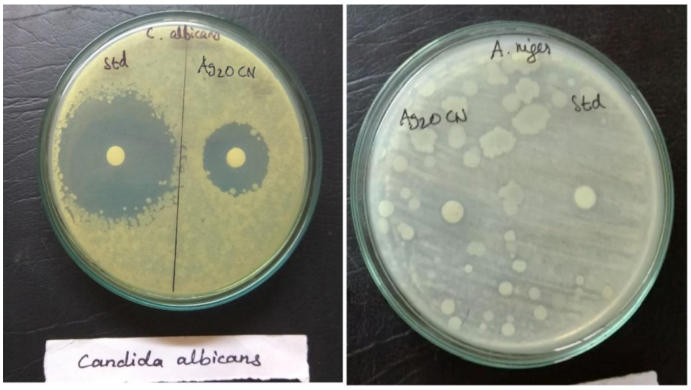

Figure 7: Antifungal images of Ag_2O nanoparticles a) *C.albicans* b) *A.niger*

From the above result it's clear that *C.albicans* (13 mm) presents a larger zone of inhibition compared to those of the other fungal species *A. niger* (10 mm) for the synthesized Ag_2O nanoparticles. Thus, nanoparticles can be used as antifungal agents and can help to overcome hurdles of fungal diseases in human beings.

Anticancer activity

Several in vitro studies have indicated that silver nanoparticles can enter cells by endocytosis.[24] Figure 8. [a-f] shows anticancer images of Ag_2O nanoparticles at different concentrations. DLA (Daltons lymphoma ascites) bearing mice cell lines is used for finding cancer activity using different concentrations (10, 20, 50, 100 and 200µg) of Ag_2O nanoparticles. The cyclophosphamide drug is taken as a standard for the DLA cell line. Figure 8a shows a control image which has no inhibition. The cyclophosphamide drug is taken as a standard for the DLA cell line. Figure 8a shows a control image which shows no inhibition. Figure 8 [b-f] shows clear that Ag_2O nanoparticles have increasing percentage of inhibition over higher concentration of the test sample. Table 3 shows the anticancer activity of Ag_2O nanoparticles.

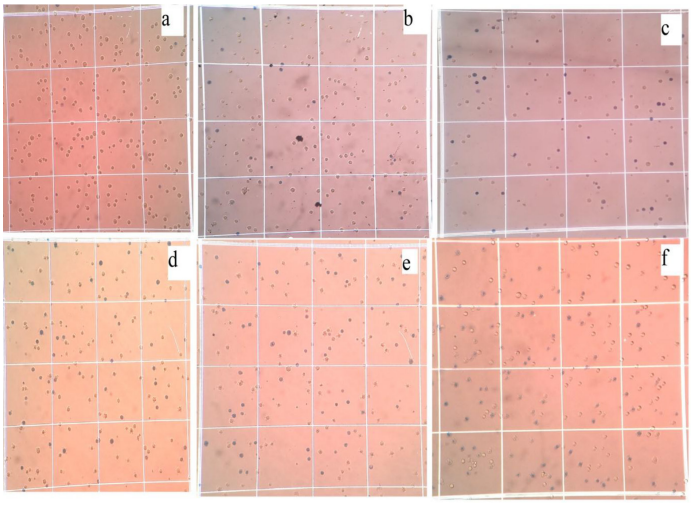

Figure 8: Anticancer activity of Ag_2O nanoparticles a) Control b) 10mg c) 20mg d) 50mg e) 100mg f) 200mg

Table 3: Anticancer activity results of Ag_2O nanoparticles.

Cell line	Name of sample		Anticancer Results					
DLA	Ag_2O nano-particles	Concentration µg/ml	Control	10	20	50	100	200
		% of inhibition	100	12	26	30	42	56

DISCUSSION

The scheme of the possible mechanism for the formation of silver oxide nanoparticles is given in Figure 1. Suresh *et al.* performed Preliminary phytochemical screening for the ethanolic extract of C. *nilgiriensis* and confirmed the presence of Tannin, Saponnin, Flavonoids, Steroids, Terpenoids, Triterpenoids, Carbohydrate, Protein, Anthroquinone, Polyphenol, Glycoside and Coumarin.[25] Flavonoids are a group of polyphenolic compounds comprising anthocyanins, isoflavonoids, flavonols, chalcones, flavones and flavanones. The metal ion reduction may be due to the internal mechanism which leads to the conversion ketone to carboxylic acid in flavonoids. Some flavonoids have the ability to chelate with metal ion with their carbonyl group or p-electrons. Moreover, the functional group of water-soluble compounds such as flavonoids, terpenoids and phenolic groups present in the aqueous extracts of *C. nilgiriensis* appears to be responsible for the synthesis of Ag_2O nanoparticles and their stabilization. Johnson *et al.*[26] reported the reduction of Ag ions and the stabilization of Ag nanoparticles due to the presence of flavonoids in Cyathea nilgiriensis extracts. In this work the flavonoid type of compounds could have formed complex with silver (I) ion of Silver Nitrate solution. Then this complex solution was then heated in hot air oven for 8 hr to form silver hydroxide further it was calcinated at 600°C to form stable silver oxide nanoparticles.

The results of FTIR analysis confirmed that phenolic compounds in flavonoids have a stronger ability to bind with metal, indicating that phenolic group could form metal nanoparticles to prevent agglomeration and thereby stabilize the medium. This suggests that biological molecules could do dual functions of formation and stabilization of silver oxide nanoparticles in aqueous medium.

From the antibacterial report it's clear that the inhibition created of biosynthesized Ag_2O nanoparticles is due to disruption of the membrane with the generation of surface oxygen species, which play an important

role and finally lead to the death of pathogens. Interestingly, the inhibition zone was different according to the type of pathogens and the concentrations of Ag_2O nanoparticles employed. From the antimicrobial result its clear that by increasing the concentration of Ag_2O nanoparticles in the discs, the growth inhibition has also been increased consistently because of proper diffusion of nanoparticles in the agar medium. Another impact is due to the small size of nanoparticle than the pore size of bacteria it crosses the cell membrane of the bacteria and inhibits its growth[27]

The antifungal activity of Ag_2O is probably derived, through the electrostatic attraction between negatively charged cell membrane of microorganism and positively charged nanoparticles and the orientation of Ag_2O nanoparticles.[28] Ag_2O nanoparticles synthesized using green method was found to have good antifungal activity against the tested fungi. Thus, nanoparticles can be used as potential antifungal agents and help overcome the hurdles in fungal disease management posed by development of resistance to conventional fungicides.

From the Anticancer activity report it's clear that the increase of concentration from 10 µg to 200 µg increases the inhibition. At 200 µg the maximum percentage of inhibition was observed for Ag_2O nanoparticles. So it is concluded that green synthesized silver oxide nanoparticles have shown good antimicrobial and anticancer behavior at different concentrations.

CONCLUSION

Ag_2O nanoparticles have been successfully synthesized via novel greener procedure using only silver nitrate and the aqueous extract of the leaves of *Cyathea nilgiriensis* Holttum. The biosynthesis of Ag_2O nanoparticles was done via hydroxide precipitation followed by calcination at 600°C. The Ag_2Onanoparticles were analyzed through PSA, XRD, FT-IR, SEM and EDX to prove the characteristics of the nanoparticles. The PSA result confirmed the produced Ag_2O nanoparticles are below 100 nm in size. Biosynthesized Ag_2O nanoparticles were found to have significant antimicrobial and anticancer activities. The Ag_2O nanoparticle can be studied further to be the composite of a drug for commercial biomedical applications.

ACKNOWLEDGEMENT

The authors are extremely grateful to the SNS College of technology, Coimbatore, Tamil Nadu, India for providing financial support (faculty research seed money) and research facilities to accomplish this study.

CONFLICT OF INTEREST

There is no conflict of interest with this research.

ABBREVIATIONS

PSA: Particle Size Analyzer; **XRD:** X-ray Diffraction Analysis; **FT-IR:** Fourier Transform Infrared Spectroscopy; **SEM:** Scanning Electron Microscope; **EDX:** Energy Dispersive X-Ray; ***S. aureus:*** Staphylococcus aureus; ***B. subtilis:*** Bacillus subtilis; ***M. luteus:*** Micrococcus luteus; ***E. coli:*** Escherichia coli; ***S. paratyphi:*** Salmonella paratyphi; ***K. pneumonia:*** Klebsiella pneumoniae; ***C. albicans:*** Candida albicans; ***A. niger:*** Aspergillus niger.

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Article History: Submission Date : 27-03-2020; Revised Date : 16-04-2020; Acceptance Date : 09-05-2020.
Cite this article: Pradheesh G, Suresh S, Suresh J, Alexramani V. Antimicrobial and Anticancer Activity Studies on Green Synthesized Silver Oxide Nanoparticles from the medicinal plant *Cyathea nilgiriensis* Holttum. Int. J. Pharm. Investigation. 2020;10(2):146-50.