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Research Article

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Evaluation of antimicrobial and anticancer efficacy of silver nanoparticles phytofabricated by *Nyctanthes arbor-tristis* L. leaf extract

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ABSTRACT

Nyctanthes arbor-tristis L. (Oleaceae), commonly known as harsingar, is a plant with potential medicinal properties. The plant was used in traditional folk medicine and as a pharmacological activity. The present study reports a rapid and eco-accommodating technique to synthesize silver nanoparticles AgNPs with low cost and with no need to heat, using aqueous extracts of *N. arbor-tristis* L. Phytochemical analysis was conducted to detect the existence of alkaloids, flavonoids, saturated sugar, saponins, glycosides, steroids, tannins, terpenoids, and proteins in the plant. A color change observed in the test confirmed the synthesis of AgNPs. SEM, XRD, EDX, FTIR, and UV – Visible spectrophotometer (DLS) were used to characterize the synthesized AgNPs. The antibacterial potential of AgNPs was further tested against different bacterial strains such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Klebsiella pneumonia* displayed by finding the maximum zone of inhibition observed against *S. typhi* of 24.6 mm, *S. aureus* 21.6±0.57 mm, *P. aeruginosa* 19 mm, *K. pneumonia* 17 mm and *E. coli* 15.3±0.57 mm. Furthermore, the synthesized AgNPs were also exhibited as anticancer properties against MDA-231 cell line Human Breast cancer cell line which was determined dose dependent manner and their concentration of 2.5 to 30 µg/mL respectively, and noted the inhibitory range at 30 µg/mL of its concentration, which was further tested in high inhibitory effect on their leaf extract high concentration in cytotoxic, bactericidal experiments.

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INTRODUCTION

Nanotechnology stands at the forefront of modern scientific research, focusing on developing experimental processes for synthesizing nanoparticles of various sizes and shapes (Santosh & Manojkumar, 2002). The utilization of nanoparticles, typically ranging from 1 to 100 nm, represents a dynamic and evolving aspect of nanotechnology (Ankita *et al.*, 2014). Silver Nanoparticles (AgNPs) exhibit potent antimicrobial properties, capable of disrupting bacterial biofilms and preventing their adhesion to surfaces. The biological synthesis of nanoparticles, possessing antimicrobial, antioxidant, and anticancer attributes, reflects collaborative efforts across multiple scientific disciplines (Hsin *et al.*, 2008). These advancements in nanotechnology hold promise for creating novel resources to assess and formulate safer and more effective drug treatments (Chung *et al.*, 2016). Silver nanoparticles (AgNPs), among various metal nanoparticles, have garnered significant attention due to their effectiveness as antimicrobial agents, low toxicity, and wide array of *in vitro* and *in vivo* applications (Dada *et al.*, 2018). Nanoparticles are broadly classified into organic and inorganic categories. AgNPs stand out as unique inorganic nanoparticles due to their exceptional properties and functional versatility, which continues to pique researcher's interest (Shankar *et al.*, 2004). Nanoparticles exhibit partially or completely unique properties, such as size within various ranges, distribution, and morphology, contributing to their potential benefits in biomedical and industrial applications for human health and the environment, as extensively documented (Lanone & Boczkowski, 2006). Metal nanoparticles, in particular, hold special significance for their ease and cost-effectiveness of synthesis, coupled with promising applications (Juan *et al.*, 2010). *Nyctanthes arbor-tristis* L., commonly known as 'Night-flowering jasmine' and belonging to the family Oleaceae, is a shrub or small tree reaching up to 10m in height, with flaky grey bark (Dib *et al.*, 2021). *N. arbor-tristis* possesses a wide range of medicinal benefits for mankind (Orwa *et al.*, 2009). Tribal communities in central India utilize various parts of *N. arbor-tristis* to alleviate coughs, hiccups,

dysentery, snakebites, and sores (Jain *et al.*, 2005). Moreover, it is recognized in Indian traditional medicine for its immunotoxic, anti-allergic, anti-histaminic, purgative, anti-bacterial, and ulcerogenic properties (Saxena *et al.*, 2002). A combination of *N. arbor-tristis* leaves with those of *Hygrophila auriculata* and *Achyranthes aspera*, when crushed and consumed daily, is believed to alleviate spleen enlargement (Sen & Behera, 2020). *N. arbor-tristis* leaf extracts in various forms have been employed for treating acute and chronic intermittent fevers (Chopra *et al.*, 1994). Additionally, *N. arbor-tristis* exhibits high antispasmodic and anthelmintic activities (Das *et al.*, 2010). The aim of this study is to synthesize AgNPs from aqueous extracts of *N. arbor-tristis* and assess their enhanced potential applications in both antibacterial and synergistic cytotoxic effects. Furthermore, the green-synthesized AgNPs underwent characterization using scanning electron microscopy (SEM), energy-dispersive X-ray analysis (EDX), Fourier-transform infrared spectroscopy (FTIR), and UV–Vis spectroscopy to elucidate the compound(s) of *N. arbor-tristis* responsible for reducing Ag+ ions into AgNPs.

MATERIALS AND METHODS

Nyctanthes arbor-tristis L Leaf Extract

The leaves of *Nyctanthes arbor-tristis* were collected from the natural habitats, carefully cleaned and left to dry at room temperature in the shade until all moisture was eliminated (for approximately 12-14 days). Once dried, the leaves were ground into a coarse powder. Subsequently, 20 grams of this leaf powder was boiled in 100 mL of double-distilled water for 15 minutes. After boiling, the aqueous extract of the leaves was allowed to cool, filtered using Whatman No.1 filter paper, and then stored at 4 °C for future use. The desiccated leaves were preserved in an airtight bag at 37 °C for further analysis.

Phytochemical Screening of Extract

A phytochemical screening of the leaf extract was conducted to identify potential biomolecules involved in the reduction of silver ions to metallic Ag. To prepare the crude extract, 10 grams of dried, finely powdered *N. arbor-tristis* leaves were soaked in 100 mL of double-distilled water. The mixture was then subjected to centrifugation at 5,000 rpm for 20 minutes at 4 °C, followed by filtration using Whatman No. 1 filter paper. Qualitative phytochemical characterization was performed to assess the presence of alkaloids, flavonoids, carbohydrates, saponins, glycosides, steroids, tannins, terpenoids and proteins (Harborne, 1984).

Synthesis of Plant- mediated AgNPs

A total of 10 mL of the leaf extract was combined with 90 mL of a 1 mM Silver Nitrate solution (0.0015 mg) and stirred continuously for thorough mixing at room temperature. Upon visual examination, the color of the solution transitioned from light yellow to dark brown, indicating the formation of AgNPs.

Characterization Techniques of Silver Nanoparticles

The synthesized AgNPs were analyzed using various techniques. Ultraviolet-visible (UV-Vis) spectroscopic analysis was employed to examine their characteristics. The shape and size of the nanoparticles were assessed using scanning electron microscopy (SEM), while the crystalline structure was determined using X-ray diffraction (XRD) method. Elemental composition and chemical states of the synthesized silver nanoparticles were characterized by energy-dispersive X-ray spectroscopy (EDX), while dynamic light scattering (DLS) was utilized to determine the average size and polydispersity index. Fourier transform infrared spectroscopy (FT-IR) was employed to analyze the phytochemicals responsible for nanoparticle synthesis.

Antibacterial Assay

The potential of AgNPs was evaluated against pathogenic bacteria including *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus* and *Klebsiella pneumoniae* using the well diffusion method (Wikler, 2007). Fresh cultures of each bacterial strain were evenly swabbed onto Petri plates containing pre-sterilized LB (Luria Bertani) agar. Sterile discs with a diameter of 6 mm were impregnated with AgNPs solution at various concentrations (50, 60, 70, 80, and 90 µL, respectively). These impregnated discs were then placed onto the plates and incubated for 24 hours at 37 °C. Ampicillin antibiotic discs were included as controls. After incubation, the zones of inhibition formed around the discs were measured at different levels.

MTT Assays

MDA-MB-231 cell lines were obtained from the cell repository of the National Centre for Cell Sciences (NCCS), Pune, India. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), along with 100 µg/mL of Penicillin and 100 µg/mL of Streptomycin to prevent bacterial contamination. The cell culture was kept in a humidified environment with 5% CO_2 at 37 °C. MDA-MB-231 viable cells were harvested and counted using a hemocytometer. They were then diluted in DMEM medium to a density of 1×104 cells/mL and seeded into 96-well plates, allowing attachment for 24 hours. After attachment, the MDA-MB-231 cells were treated with different concentrations of *N. arbor-tristis* AgNPs (ranging from 2.5 to 30 µg/mL) in each well. The cells were then incubated at 37 °C in a humidified atmosphere with 95% air and 5% CO_2 for 24 hours. Following the incubation period, the drug-treated cells were washed with fresh culture medium, and then MTT (5 mg/mL in PBS) dye was added to each well. The plates were further incubated for 4 hours at 37 °C. The purple Formosan precipitate formed was dissolved in 100 µL of concentrated DMSO, and the cell viability was measured by absorbance at 540 nm using a multi-well plate reader (Mosmann, 1983).

Measurement of reactive oxygen species (ROS)

Dichlorodihydrofluorescein (DCF) reacts with various free radicals such as hydroxyl, peroxyl, alkoxyl, nitrate, and carbonate, resulting in the formation of a fluorescent molecule

with excitation at 530 nm and emission at 485 nm. DCF remains unoxidized by hydrogen peroxide or superoxide radicals. After exposure to *N. arbor-tristis* AgNPs (10 & 20 µg/mL), pre-seeded MDA-MB-231 cells (2×106 cells/well) were incubated for 24 hours at 37 °C (5% CO2), with untreated cells maintained as controls. Subsequently, the cells were washed with PBS and loaded with 25 µM DCFH-DA in DMEM for 30 minutes at 37 °C. Following this, the treated cells were washed with DMEM, and fluorescence was recorded every 5 minutes over a 30-minute period (excitation at 485 nm, emission at 535 nm) using a spectrofluorometer at 37 °C. The increase in reactive oxygen species (ROS) was calculated by the mean slope per minute and normalized to the unexposed control.

Measurement of mitochondrial membrane potential (MMP)

MDA-MB-231 cells were seeded in 6 well plates with a cover slip and treated with different concentrations of *N. arbor-tristis* (10 & 20 µg/mL). The cells were stained with Rh-123 dye and incubated for 15 min. PBS wash was engaged (twice) and allowed to fix in plates. The fluorescence intensity was measured at 535 nm and the percentage of MDA-MB-231 cells reflecting pathological changes was calculated (Bhosle *et al.*, 2005).

Measurement of apoptotic induction using Ao/EB dual staining method

MDA-MB-231 cells were seeded at a density of 5 x 104 cells per well in a 6-well plate and allowed to incubate for 24 hours. Following exposure to AgNPs for the specified duration, the cells were detached, washed with cold PBS, and then stained with a mixture of acridine orange (AO) and ethidium bromide (EB) in a 1:1 ratio at room temperature for 5 minutes. The stained cells were examined under a fluorescence microscope at 40x magnification. The number of cells displaying characteristics of apoptosis was determined by counting the total number of cells present in the field.

RESULTS AND DISCUSSION

The results of the phytochemical examination revealed the absence of amino acids, while flavonoids, alkaloids, glycosides, steroids, phenols, saponins, terpenoids, cardiac glycosides, and tannins were present (Table 1). The findings indicated the presence of various phytochemicals, particularly glycosides such as anthraquinone and cardiac glycosides. Previous research studies have also documented the presence of different types of glycosides, including flavanol glycosides like astragaline (kaempferol 3-glucoside) and nicotiflorin (kaempferol 3-rhamnoglucoside) (Singh *et al.*, 1965), as well as iridoid glycosides like arborsides A, B, and C (Srivastava *et al.*, 1990). Additionally, triterpenoids (friedeline, lupeol tannic acid, ascorbic acid, methyl salicylate, nyctanthic acid, oleanolic acid, and an amorphous glycoside) (Anjaneyulu & Murty, 1981), iridoid glycosides (6,7-di-O-benzonylnyctanthoside (I) and 6-O-trans-cinnamoyl-6-b-hydroxyloganin (II) and 7-O-trans-cinnamoyl-6-b-hydroxyloganin) (Stuppner *et al.*, 1993), phenyl propanoid glucoside (desrhamnosyl verbascoside 5) (Mathuram

Table 1: Phytochemical analysis of *N. arbor-tristis*

S. No.	Test	Results
1	Carbohydrates	+
2	Saponins	+
3	Steroids	+
4	Carotenoids	+
5	Alkaloids	+
6	Flavonoids	+
7	Cardiac Glycosides	+
8	Anthraquinone glycosides	+
9	Starch	+
10	Tannins	+
11	Phenol	+
12	Terpenoids	+
13	Gum & mucilage	+
14	Amino acids	-
15	Proteins	+

et al., 1994), iridoid glucoside (arborside D) (Singh *et al.*, 1995), poly acetylenes and flavanol glycoside (quercetin-3,30-dimethoxy-7-O-rhamnoglucopyranose) (Kannan & Singh, 2010) were identified. Additionally, miscellaneous compounds such as mannitol, amorphous resin, glucoside, glucose, and essential oil (Agrawal & Pal, 2013), as well as Vitamin C and carotene (Basu *et al.*, 1947), mannitol, β-amyrin, β-sitosterol, hentriacontane, and benzoic acid (Khanapur *et al.*, 2014), calceolarioside A (Poddar *et al.*, 2008), octacosane and 10-hydroxyl-30,4-dimethyl-1,10-bi(cyclohex-3-en)-2-one (Rahman *et al.*, 2011), and β-sitosterol (Nirmal *et al.*, 2012) were also detected.

The unique optical properties of nanosized materials, allowing them to exhibit a wide range of colors when synthesized into nanoparticles, have garnered significant interest. One primary indicator of the presence of phytochemicals in a plant extract, capable of reacting with silver nitrate to form silver nanoparticles, is the observed color shift in the reaction mixture from pale yellow to dark reddish-brown, typically occurring within an hour. This color transition ceased after a 24-hour reaction period, accompanied by precipitation, indicating the completion of nanoparticle synthesis (Figure 1). The absorption spectra of the generated silver nanoparticles in the reaction mixture, obtained through UV analysis within the 200-800 nm range, exhibit a sharp absorbance with the highest peak occurring between 224.25 and 281.10 nm (Figure 2).

FTIR identify the biomolecules responsible for capping, reducing and stabilizing the AgNPs present in the leaf extract of *N. arbor-tristis*. Figure 3 illustrates the spectrum which clearly shows peaks at 3422- O-H stretching of hydroxyl groups. 2924-Methylene C-H asym/sym. Stretch methylene group 1621-Alkenyl C=C stretch. Olefinic (alkene). 1270-Aromatic ethers, aryl-O stretch. Ether and oxy compound and 1385-gem-Dimethyl o "iso"- (doublet) methyl(-CH3) compound, 1105-organic siloxane or silicone (si-o-c) simple hetero-oxy compounds 692-C-Br stretch in Aliphatic organohalogen compound 629-Algne C-H bend in Acteyleic (Alkyne) compound and 583-Disulfides (C-S stretch) in Thiols and thio-substituted compounds and 779-C-H 1, 3-Disubstitution (meta) in Aromatic ring (aryl).

The obtained results suggest the presence of various functional groups in the leaf extract as well as in synthesized AgNPs (Nandiyanto *et al.*, 2019).

In XRD analysis, AgNPs were subjected under XRD diffraction peaks at the 111, 200, 220, and 311 planes. The patterns of the dried synergistic powder sample of AgNPs had shown distinct four diffraction peaks at 2 hrs angles of 38°, 44°, 64° and 77°, which can be endorsed to the reflections from lattice planes indexed to the (111), (200), (220) and (311) planes which was reflected the crystal structures (Figure 4). Thus, XRD used to newly synthesized product is purely nano silver with high crystalline. XRD shows crystalline nature of FCC silver with the planes (111), (200) and (220). SEM analysis revealed that the uniform distribution of AgNPs was observed on the surfaces of the cells (Figure 5). AgNPs are spherical in shape with smooth morphology and particle size ranging from 59.83 to 78.01 nm. The range was supported as 50 to 80 nm of the size particle was observed with the uniform spherical shaped (Basu *et al.*, 2016). Presence of the larger sized AgNPs was observed during SEM analysis may be attributed to the aggregation of the smaller silver nanoparticles (Jasim *et al.*, 2017). EDAX spectroscopy confirmed the presence of the signal characteristic of elemental silver which showed in the peaks (Figure 6). Ag Nano crystallites showed optical absorption band peak at approximately 3 keV which is a characteristic feature for the absorption of metallic silver nano crystalline due to interference of O_2, Na, Si, Ag elements. DLS technique makes use of particle size analysis of colloidal solution upon irradiating with the light source. This measurement condition was upheld at 25 °C. From the DLS histogram (Figure 7), the average particle size is estimated to be 70.4 nm with a polydispersity index 0.358 and diffusion Const. (D): 6.991e- (cm^2/sec) respectively.

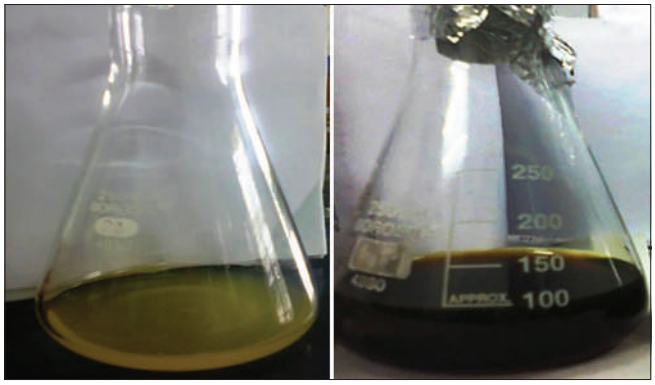

Figure 1: Leaf extracts of *N. arbor-tristis* AgNPs

In regards with antibacterial activity, AgNPs was evaluated with the pathogenic microorganisms of *P. aeruginosa*, *S. typhi*, *S. aureus*, *E. coli*, and *K. pneumonia*. Maximum zone of inhibition was observed against *S. typhi* (24.6±0), *S. aureus*

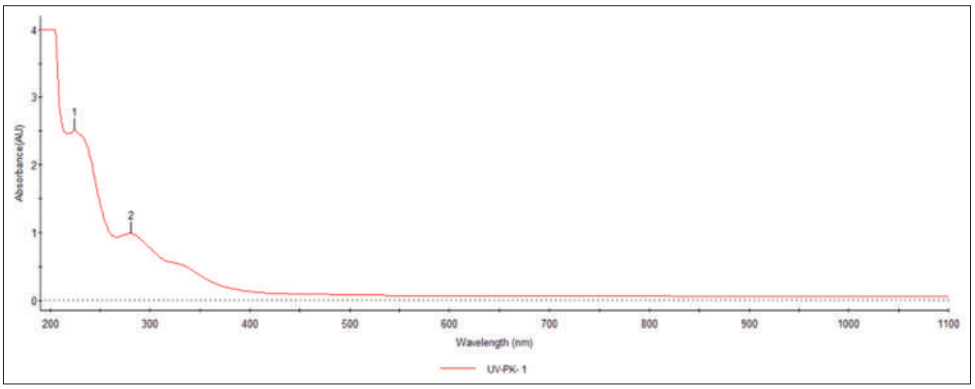

Figure 2: UV-Visible absorption spectra of biosynthesized AgNPs from *N. arbor-tristis*

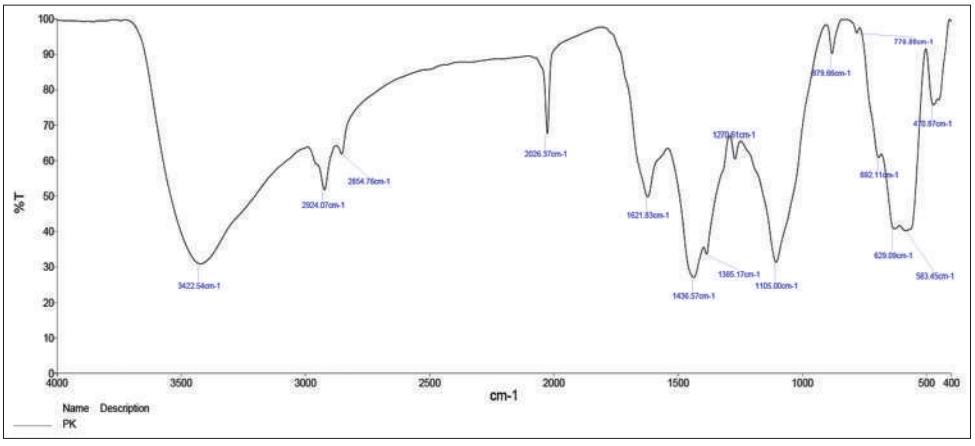

Figure 3: Fourier Transforms Infra-Red Spectroscopy of AgNPs

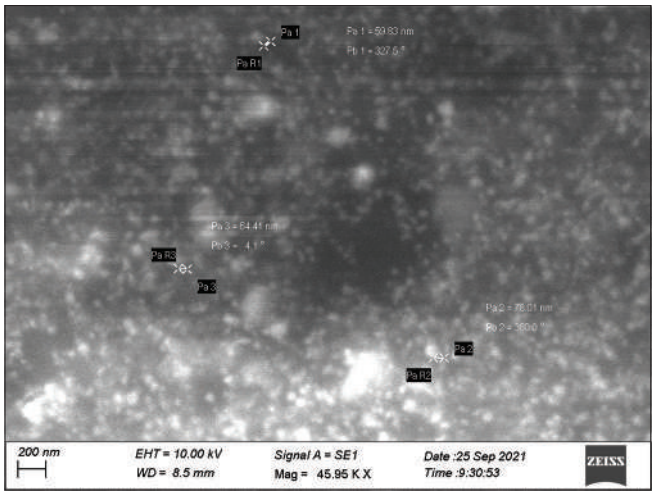

Figure 4: X-ray Diffraction spectrum (XRD) analysis

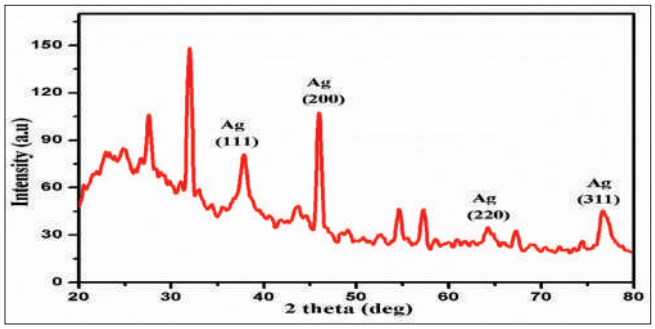

Figure 5: Scanning electron microscope (SEM) analysis of AgNPs

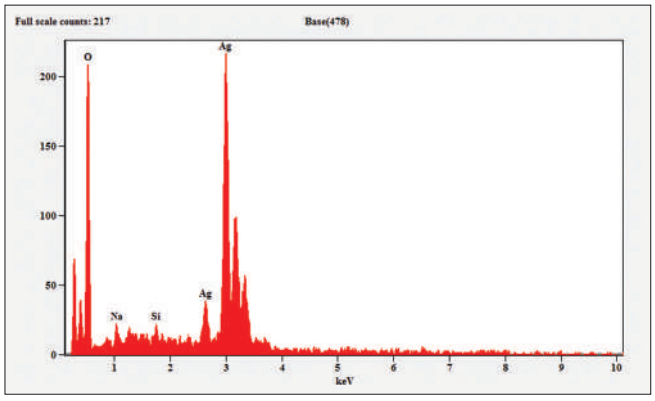

Figure 6: EDAX of derived AgNPs

(21.6±0.57), *P. aeruginosa* (19±0), *K. pneumonia* (17±0) and *E. coli* (15.3±0.57) with the triplicate reading (mean±S.D) (Figure 8). AgNPs shows maximum activity against the *S. typhi* and *S. aureus*. Nano silver material is more effective towards Gram positive strain compared to Gram negative and it was reasoned that factors other than membrane structure might be playing the role (Ruparelia *et al.*, 2008). AgNPs using *N. arbor-tristis* aqueous leaf extract demonstrated significant antibacterial potential against both Gram negative (*E. coli*) and Gram positive (*S. aureus*). The ZoI (Zone of Inhibition) increased in a dose dependent manner with increase in concentration of AgNPs (Kaur & Kaushal, 1970). The efficacy

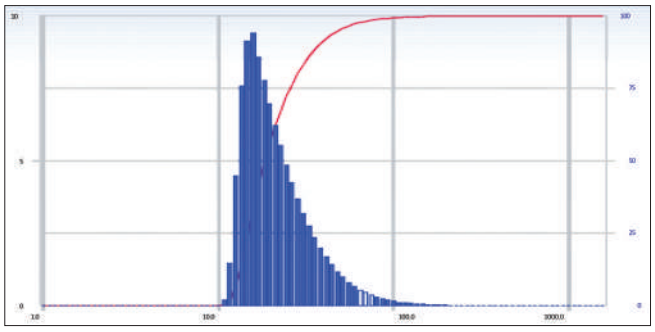

Figure 7: Dynamic Light Scattering (DLS) plot for green synthesized AgNPs

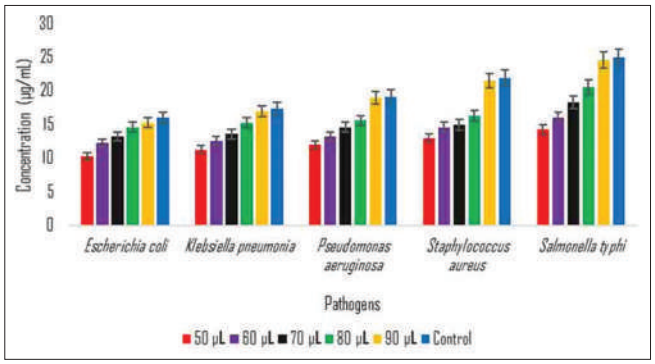

Figure 8: Antibacterial activity of *N. arbor-tristis* AgNPs

of AgNPs against *E. coli* and community associated methicillin-resistant *S. aureus* was established (Qais *et al.*, 2019). Aqueous, Methanol, Aqueous and Methanol, Ethanol leaf extracts hold antibacterial potential (Darokar *et al.*, 1998; Chatterjee *et al.*, 2007; Mahida & Mohan, 2007; Sathiya *et al.*, 2008). Ethanolic extract of the stem and leaves had antibacterial activity against *S. aureus*, *S. epidermidis*, yeast and *C. albicans* (Bhatt *et al.*, 2005), whereas, the aqueous extract had shown moderate active against *P. testosteroni* (Nair *et al.*, 2005). Likewise, leaves revealed its activity against drug-resistant bacteria, *S. aureus* and *S. paratyphi*, aqueous and methanol extracts of the leaves possess bactericidal activity against *S. aureus*, *B. subtilis* and *E. coli* (Ahmad & Beg, 2001). Further, methanolic extract of the leaves was also active against multi-drug resistant bacterial strain such as *S. aureus*, *S. epidermis*, *S. typhi* and *S. paratyphi* (Mahida & Mohan, 2007). In more specific, both ethyl acetate and chloroform were used to extract dried leaf, flowers, fruits and seed which exhibited significant activity against Gram negative bacteria as compared to Gram positive through preliminary antibacterial assay (Priya & Ganjewala, 2007) through disc diffusion assay. AgNPs possess high antibacterial activity against Methicillin – resistant *S. aureus* which showed the zone of inhibition (5-10 mm) (Mishra *et al.*, 2020). Ethanolic leaves extract showed antibacterial activity against *P. aeruginosa* and *S. typhi* (Singh & Vyas, 2018).

In connection with cytotoxic assays, AgNPs showed the effects in dose dependent manner. Histogram very precisely demonstrated that with the increase in concentration of AgNPs, there is progressive decrease in percentage cell viability

(Figures 9 and 10). The concentration necessary to produce 50% of MDA-231 cell-lines death. Photomicrograph (20X) represents morphological changes in cancer cells such as shrinkage, detachment, membrane blebbing, and distorted shape induced by *N. arbor-tristis* AgNPs treatment (10 and 20 µg/mL for 24 h) as compared with control. Control showed normal intact cell morphology and their images were captured by light microscope.

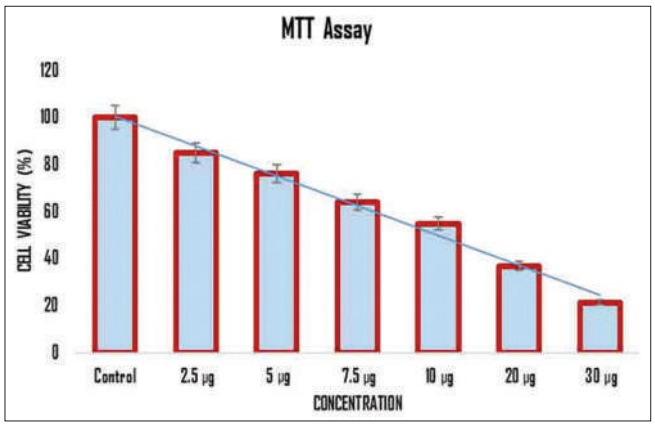

Figure 9: MTT assay for cytotoxicity of AgNPs

Hydroalcoholic extract of *N. arbor-tristis* leaves has chemo preventive against dimethyl benzanthracene (DMBA) which induced skin tumorigenesis (Dinamani *et al.*, 2009). Ethanolic leaf extracts of *N. arbor-tristis* possess and anticancer properties on human cancer cell lines (Benwefit, 2019). A benzofuran derivative 4-hydroxy hexahydrobenzofuran-7-one isolated from the leaves was evaluated on Ehrlich ascites carcinoma cells at 20 mg/kg which inhibited the cell growth by 43.27% and did not have any cytotoxic effect (Khatune *et al.*, 2003). *N. arbor-tristis* was not engaged mostly for its anticancer properties. However, two iridoid glycosides Arbortristoside A and B have been reported to have anticancer activity against methyl-cholanthrene induced fibrosarcoma at 2.5 mg/kg in mice (Das *et al.*, 2008). Live cells show green fluorescence with a normal nuclear appeared. Early apoptotic cells with fragmented nuclear show yellow fluorescence with condensed chromatin (Figure 11). Late apoptotic cells show orange fluorescence with chromatin condensation or fragmentation (uniformly red/orange-stained cell nuclei). Intracellular ROS generation was observed in AgNPs treated cells (10 & 20 µg/mL) which were compared with control (dull green fluorescence) which was captured by fluorescent Microscope (Figure 12). In concern to MMP, a gradual decrease of green fluorescence indicates a decrease in MMP

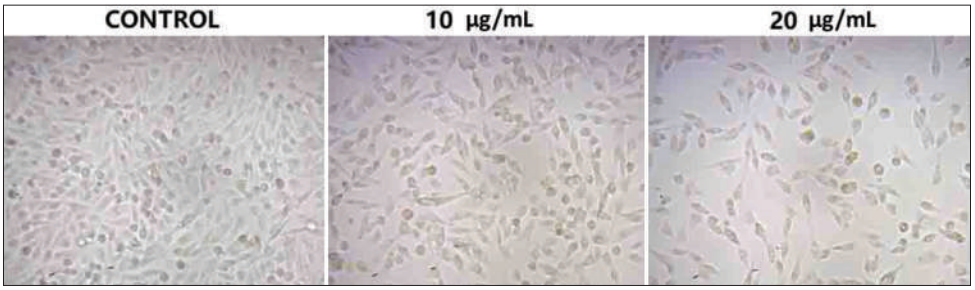

Figure 10: Inhibitory effects of *N. arbor-tristis* treated MDA-MB-231

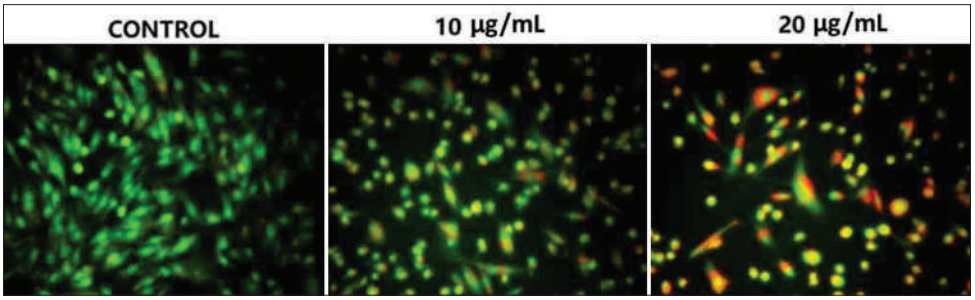

Figure 11: Effect of *N. arbor-tristis*on the apoptotic incidence in MDA-MB-231 cells

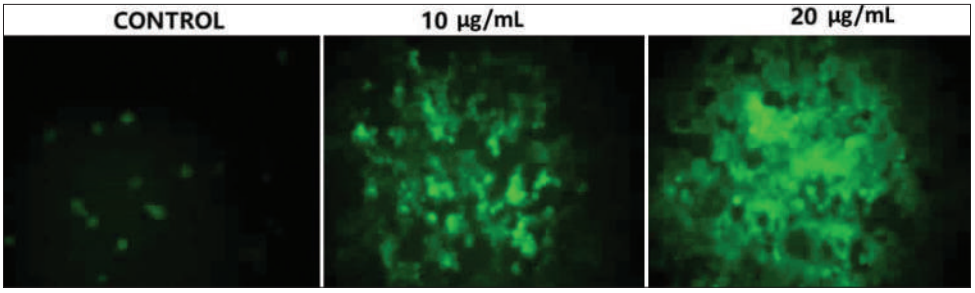

Figure 12: Effect of *N. arbor-tristis* on the intracellular ROS generation in MDA-MB-231 cells by DCFH-DA staining

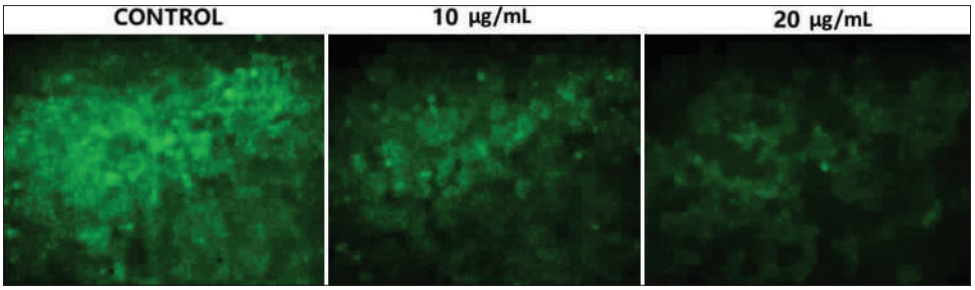

Figure 13: Effects on Mitochondrial Membrane Potential of MDA-MB-231 cells

(Mitochondrial Membrane Potential) which clearly indicates the activity of AgNPs of *N. arbor-tristis* which was analysed by fluorescent microscope at 20X magnification (Figure 13). The fluorescent image shows control (Rh accumulation) and *N. arbor-tristis* (No Rh accumulation).

CONCLUSION

We developed an environmentally friendly, quick biological technique for the production of AgNPs utilizing *N. arbor-tristis* plant leaf extracts, which is simple, cost-effective, and efficient. Furthermore, this technology will give an alternate method for producing biocompatible and size-controlled AgNPs based on plant extracts (with the potential for greater consistency and less variance). This method addresses the current issue of disparity to a greater extent and offers the option of synthesizing smaller, controlled, and uniform AgNPs particles using a green biosynthesis approach for medical applications such as antimicrobial agents, bio-scaffolds, therapeutic purposes, and controlled released and targeted drug delivery. The cytotoxic effect of AgNPs was assessed using the percentage of decrease, zone of inhibition, and lowest inhibitory concentration on five distinct bacteria strains. As a result, these promising lead phytotherapy compounds may be the most promising in pharmaceutical applications. Furthermore, in-depth and optimization research will expand on this possibilities.

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