

# Biosynthesized Silver Nanoparticles (AgNPs) from *Trapa natans* Peel Extract Exhibits Anti-Metastasis and Anti-Biofilm Potentials

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## ABSTRACT

**Background:** This study deals with the anti-metastasis and anti-biofilm activities using silver nanoparticles (AgNPs) against human breast cancer cells (MCF-7) via *in vitro* methods. Different studies have been reported that biofilms formed by *Klebsiella pneumoniae*, *Escherichia coli* and *Staphylococcus aureus* are resistance to most of the recently used antibiotics due to MDR effect. **Materials and methods:** The characterization of biosynthesized particles was done by UV-vis spectroscopy, FTIR, SEM and XRD techniques. Cytotoxicity analysis was done by MTT assay and Neutral Red Uptake (NRU) assay. Anti-biofilm activity was carried out by using the bacterial strains of *Klebsiella pneumoniae*, *Escherichia coli* and *Staphylococcus aureus* respectively. **Results:** AgNPs exhibit strong absorption peak in the 420–500 nm range due to Surface Plasmon Resonance in UV-Vis spectroscopy. In FTIR analysis, we found phenol and alcohol compounds (at 3447  $\text{cm}^{-1}$ ), carbonyl groups (at 1631  $\text{cm}^{-1}$ ), alcoholic groups (at 1384  $\text{cm}^{-1}$ ) and polysaccharides (at 1093  $\text{cm}^{-1}$ ). In XRD that AgNPs formed were highly crystalline in nature. In SEM the diameter of AgNPs ranges from 30-90 nm and TEM depicts the size of AgNPs to be  $15 \pm 2$  nm. *T. natans*-AgNPs exhibits strong cytotoxic activity against MCF-7 breast cancer cell line. **Conclusion:** The presence of different bioactive molecules in *T. natans* shows that, green synthesized AgNPs significantly inhibited proliferation of MCF-7 breast cancer cells by inducing apoptosis as well as antibiofilm activity using different bacterial colonies. For the very first time we are reporting the anticancer drugs repositioned as a potential antibiofilm agent in the *Klebsiella pneumoniae*, *Escherichia coli* and *Staphylococcus aureus* organisms.

**Key words:** *Trapa natans*, Silver Nanoparticles (AgNPs), Human Breast Cancer Cells (MCF-7), Anti-biofilm, Anti-metastasis, Cytotoxicity.

## INTRODUCTION

The use of medicinal plants as a source of relief from diseases can be traced back over five millennia to the written records of the early civilizations in India for a long period of time. Plants are the valuable source of natural products for the maintenance of human health, particularly in the last decade, with more extensive studies on natural therapies. According to World Health Organization, medicinal plants are the best source and safest way to acquire a range of drugs. Approximately 80% of individuals in developed countries use traditional medicines with compounds

derived from medicinal plants. Research in anti-cancer activity of higher plants in India started seriously in the sixties and gained momentum in the seventies.<sup>1</sup> Medicinal herbs constitute indispensable components of the traditional medicines practiced worldwide due to low cost, easy access and ancestral experience.<sup>2</sup>

According to the World Health Organization, cancer is a leading cause of death worldwide, and the number of deaths is projected to continuously rise in the coming years.<sup>3</sup> Worldwide, the most commonly diagnosed tumor types are lung, breast, and

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colorectal cancer.<sup>4</sup> Cancers can be developed in one of three ways, including incorrect diet, genetic predisposition, and by the environment. Chemotherapy is usually given in repeated cycles of treatment. Natural products have an important role as anticancer agents, and many of the cytotoxic drugs used clinically today are derived from plants.<sup>5</sup>

The use of silver and silver salts is extremely old as human civilization but the manufacture of silver nanoparticles (AgNPs) has been recently recognized. They are particularly used in medicine as antibacterial, antifungal and antioxidants and in agriculture. It is demonstrated that AgNPs stops the growth and replication of many bacteria such as *Bacillus cereus*, *Citrobacter koseri*, *Staphylococcus aureus*, *Salmonella typhi*, *Vibrio parahaemolyticus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and fungus *Candida albicans* by binding Ag/Ag<sup>+</sup> to the biomolecules present in the microbial cells. NPs have attracted increasing interest from each branch of medicine in their ability to deliver drugs in the optimal dosage range, often resulting in enhanced therapeutic efficiency of the drugs, weakened side effects and improved patient compliance.<sup>6</sup> In recent days green synthesis have received a huge attention due to its eco-friendly therapeutic approaches and advantages such as economic viability, less waste generation and simple in process.

In this study we used a simple green process to synthesize AgNPs using *Trapa natans* peel extract. The *Trapa natans* extract has been used as an efficient reducing and capping agent. The extract contains several polyphenolic compounds that possess a wide range of biological activities, including antioxidant, anticancer, antibacterial, antifungal effects, etc. *Trapa natans* peel extract derived on MCF-7 cells has not been established practically so far. From the treatment options for breast cancer, chemotherapy and radiation are plagued by side effects that include lumps in breast, hair loss, fatigue, bleeding and infections, whereas other treatments are effective in some patients only. For these reasons, more effective alternative cancer therapeutic compounds with minimal side effects are desperately being sought.

## MATERIALS AND METHODS

### Preparation of plant extract

The fresh *Trapa natans* (water chestnut) plant fruits were purchased from a local market in Balasore, Odisha, India and washed using distilled water to remove impurities. The outer hard cover peel was removed from the fruit and air dried. The dried samples were crushed

ground into powder using a mortar/pestle and stored in airtight bottles. Thereafter, 5 gms of *T. natans* powder was added to 100 ml of distilled water in steam bath for 15-20 mins at 80°C. The extract obtained was cooled to room temperature and filtered through Whatman No.1 filter paper. The solution was treated as source extract and was stored for further analysis.

### Biosynthesis of silver nanoparticles

For preparation of AgNPs, 10 mL of the prepared *T. natans* peel extract was added drop wise to 90 mL of 0.1 mM silver nitrate (AgNO<sub>3</sub>) aqueous solution. The mixture obtained was then stored under dark condition in room temperature to avoid auto-oxidation for 24hr and the color of the solution was then changed to yellowish-brown (indicate the formation of NPs). The solution was then transferred to round bottom flask and heated at 90°C with continuous stirring up to 15mins. Solution was centrifuged at 900rpm in room temperature and the pellet form of silver nanoparticles was then dried up at 60°C in hot air oven (forms blackish powder) and was finally diluted in 1% dimethyl sulfoxide (DMSO). The biosynthesized AgNPs was then stored and employed for further characterization using UV-Vis spectroscopy, FTIR, SEM and TEM techniques, etc.

### Characterization of biosynthesized silver nanoparticles

#### UV-Vis Spectral Analysis

Spectral analyses of synthesized NPs at different reaction concentration were observed. The absorption spectra of the mixture were recorded with a Shimadzu UV-visible spectrophotometer (UV-1800) from 200 to 800 nm and resolution of 1 nm.

#### FTIR Spectroscopy

Fourier transform infrared spectroscopy (FTIR) was utilized to identify the presence of possible biomolecules of *T. natans* extract involved in formation and stabilization of AgNPs. For this, purified *T. natans* AgNPs (in powder form) was grinded manually with KBr pellets and then used for FTIR measurement. The FTIR of particles were recorded at a wavelength of 400-4000 cm<sup>-1</sup> using FTIR, Shimadzu.

#### XRD Analysis

The phase purity and particle size of *T. natans* AgNPs were determined by using the X-ray diffractometer (XRD-Bruker AXS). The X-ray diffraction (XRD) pattern

was carried out at a current of 30mA and voltage of 40 kV with Cu K $\alpha$  radiation (1.5405 Å).

### Microscopy Study

Morphological analysis of *T. natans* AgNPs was conducted by using transmission electron microscopy (TEM; HITACHI H-7650, Tokyo, Japan) at voltage 120 kV. To observe the surface morphology and size distribution of AgNPs, Scanning electron microscope is used as the most powerful diagnostic devices. For SEM observation, the sample was coated with thin layer of platinum and SEM image has been taken by SEM (Philips XL-30) at 26 KV.

### Determination of *in vitro* Anticancer Activity of Synthesized AgNPs

#### Cell Culture

Breast cancer cell line (MCF-7) was obtained from the National Centre for Cell Science (NCCS), Pune, India. The MCF-7 cells were freshly cultivated as monolayer in Dulbecco's Modified Eagles's Medium (DMEM), supplemented with 10% FBS, 1% glutamine, and 100 U/mL penicillin/streptomycin and incubated at 37°C in 5% CO<sub>2</sub> atmosphere.

#### Cytotoxic assay

The cytotoxic effects of *T. natans* AgNPs on MCF-7 cell line was measured using MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium]. The MCF-7 cells were seeded into 96-well plates and in DMEM supplemented with 10% FBS for 24 hr at 37°C in 5% CO<sub>2</sub> incubator. After 24 h of the culture, cells were treated with different doses of AgNPs in the growth medium for another 24 hr under the same culture conditions. Wells with only cells (media as a vehicle only) without nanoparticles were considered as negative control, whereas wells with only MTT, considered as positive control. Cell viability was determined by adding MTT dye (100  $\mu$ l of 0.1 mg/mL stock) to respective well and incubated for 4 hr at 37°C with 5% CO<sub>2</sub> in the dark. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) a yellow tetrazole reduced to purple formazan in living cells by the action of NAD(P) H-dependent cellular oxidoreductase enzymes. The insoluble formazan crystals were dissolved in isopropanol for 1 hr at 37°C, into a colored solution. The quantification of formazan was determined by measuring absorbance at 570 nm. The degree of light absorption depends on the solvent.<sup>7,8</sup>The percentage of cell viability was calculated according to the following formula.<sup>9</sup>

$$\% \text{ cell viability} = \frac{([\text{OD treated cell} - \text{OD blank}] / [\text{OD control cell} - \text{OD blank}]) \times 100}{}$$

### Neutral Red Uptake Cytotoxicity Assay

MCF-7 cells were treated with 1–100  $\mu$ g/ml *T. natans*-AgNPs for 24 h. The cells were then washed with PBS twice and incubated in with 50  $\mu$ g/ml of neutral red containing medium for 3 h. The cells were washed off with a solution (1% CaCl<sub>2</sub> and 0.5% formaldehyde). The dye was extracted in 1% acetic acid and 50% ethanol mixture. The plates were measured at 550 nm. The results were expressed in percentage of control.

### Measurement of ROS

Intracellular reactive oxygen species (ROS) levels were identified by the fluorescent probe 2', 7'-dichlorofluorescein diacetate (DCFH<sub>2</sub>-DA) (Sigma). In 1 mL stock 10  $\mu$ M prepared in DMSO was added to each plate and incubated for 30 min at 37°C. Samples were then measured with a fluorescent plate reader (Biotek- FL  $\times$  800). DCF fluorescence was measures at 485 nm of excitation and 520 nm of emissions. ROS production was determined from an H<sub>2</sub>O<sub>2</sub> standard curve (10–200 nM).<sup>10</sup>

### Apoptosis Assay

The apoptosis/necrosis induced by *T. natans* AgNPs in MCF-7 cells was analysed using Annexin-V- FITC/ propidium iodide (PI) apoptosis detection kit (Sigma-Aldrich Co.). In brief, cells treated with *T. natans* AgNPs for 12, 24, or 48 h were harvested, washed with PBS twice, resuspended in binding buffer and incubated with Annexin V-FITC and propidium iodide solutions in the dark for 30 min at room temperature. Apoptosis was determined by flow cytometry.

### Determination of Anti-Biofilm Activity of Synthesized AgNPs

The anti-biofilm activity of biosynthesized *T. natans* AgNPs was observed with or without *T. natans* AgNPs-against the biofilm producing standard bacterial strains. Biofilm generation of the concerned pathogenic bacteria was carried out using the tissue culture plate (TCP) technique as explained by Balasamy *et al.*<sup>11</sup> which is commonly used worldwide and is considered as standard test for detection of biofilm production with some modifications. The bacterial strains of *Klebsiella pneumoniae*, *Escherichia coli* and *Staphylococcus aureus*, were grown on blood agar plate for overnight at 37°C and the next day a single colony of all bacteria was inoculated into different conical flasks containing the 100 mL TSB at 37°C for 6–7 hr with shaking at 100 rpm until to obtain approximately 2.5  $\times$  10<sup>8</sup> CFUs/mL. These bacterial inoculums were further diluted (1:100) with

fresh TSB medium to approximately  $10^6$  CFUs/mL and transferred it to 96 wells flat bottom TCPs and incubated at 37°C for overnight. After completion of the incubation, the old culture medium was changed with a fresh TSB medium including different concentrations of *T. natans* AgNPs (6.5–100 µg/mL), without disrupting the biofilm. Samples with AgNPs were further incubated at 37°C for 24 h. The medium was then discarded; each well were washed gently two times with sterile 1x Phosphate buffer saline (PBS) to eliminate the planktonic state or free floating bacteria and dried it at room temperature for 20 min. 0.1% crystal violet solution was added to each well for 15 min to stain the biofilms. The excess stain was eliminated by washing three times with sterile 1% PBS, and dried at room temperature for 30 min. 100 mL of 95% ethanol was then added to each well. The optical density (OD) of stained acquired biofilm was observed at wavelength 595 nm by a micro ELISA reader. Experiments were repeated in triplicate. Average OD values of sterile medium were measured and deducted from the all test values.<sup>12</sup> The Optical Density of the sample was converted to biofilm inhibition percentage and calculated as follows:

$$\text{Percentage of Biofilm} = \frac{\text{OD of the test}}{\text{OD of the control}} \times 100$$

### Statistical Analysis

The data obtained from each experiment were presented as mean  $\pm$  standard error values. Difference between the control and the test were evaluated using the Student's *t*-test or otherwise stated in their respective legends. In this study, One-way ANOVA (post-hoc Tukey test) was used to analysis of differences between groups. Results with  $P < 0.05$  were considered to be statistically significant. The half maximal inhibitory concentration ( $IC_{50}$ ) value was calculated by 4-parameter curve fitting using the Sigma Plot program (version 12, SPSS, Inc., Chicago).

## RESULTS AND DISCUSSION

### Characterization of AgNPs by UV-visible spectroscopy

UV-vis spectroscopy is one of the most widely used techniques to characterization of silver nanoparticles in the size range of 2–100 nm. It is well known that AgNPs exhibit strong absorption peak in the 420–500 nm range due to the collective oscillation of free conduction band electrons in particles, phenomenon known as the Surface Plasmon Resonance. The AgNPs synthesis was initially identified by appearance of yellowish-brown

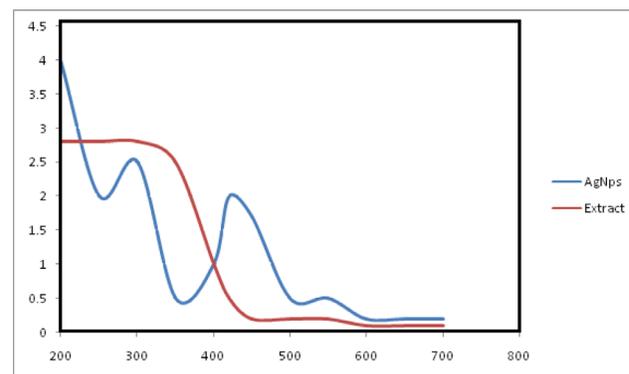
color in the reaction mixture. The SPR band centered at 420 nm confirms the formation of Ag nanoparticles (Figure 1). While, the UV-vis spectra of *T. natans* extract didn't show any peaks in the visible range. It was considered, as the incubation time of silver nitrate solution with the plant extract increases the intensity of the absorption peaks also increased progressively, which indicated the increased reduction of silver nitrate by *T. natans* extract solution and increased AgNPs concentration in reaction mixture.

### FTIR Analysis

The FTIR spectrum of bio-synthesized AgNPs was used to identify the functional groups present on the surface of the particles. As shown in Figure 2, the broad peak centered around  $3447\text{ cm}^{-1}$  represent O-H stretching vibration in phenols and alcohols compounds.<sup>13</sup> The distinct peak located at  $1631\text{ cm}^{-1}$  represent the C=O stretching vibrations in carbonyl groups of proteins.<sup>14</sup> The peaks at  $1384\text{ cm}^{-1}$  corresponds to C=O stretching of the alcoholic groups.<sup>13</sup> Another peak emerged at  $1093\text{ cm}^{-1}$  attributed to C-O-C stretching vibrations of polysaccharides.<sup>15</sup> Hence, FTIR study showed some of the bioactive compounds present in the *T. natans* extract like proteins, polysaccharides and phenolic compounds formed a strong coating on the biosynthesized silver nanoparticles.

### X-Ray Diffraction Analysis

XRD is a powerful technology used for characterization of crystalline materials.<sup>16</sup> The peaks of different nanoparticles formed show a characteristic pattern. XRD pattern obtained confirms the formation of nanoparticles. XRD of the AgNPs gave four characteristic peaks in the 2 $\theta$  spectrum, which range from 20° to 80°. The XRD spectrum pattern of dried *T. natans* AgNPs have shown distinct diffraction peaks at 2 $\theta$  angles of 38.91°, 45.73°, 66.75° and 79.89° which can be indexed



**Figure 1: UV-Vis absorption spectrum of *T.natans* AgNPs and *T.natans* extract.**

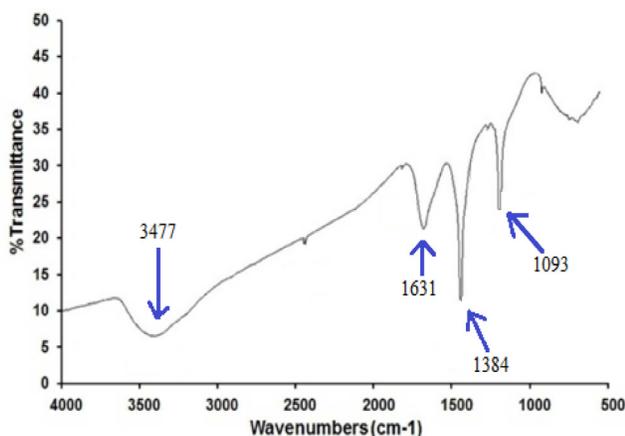


Figure 2: FTIR spectra of the biosynthesized silver nanoparticles from *T.natans*.

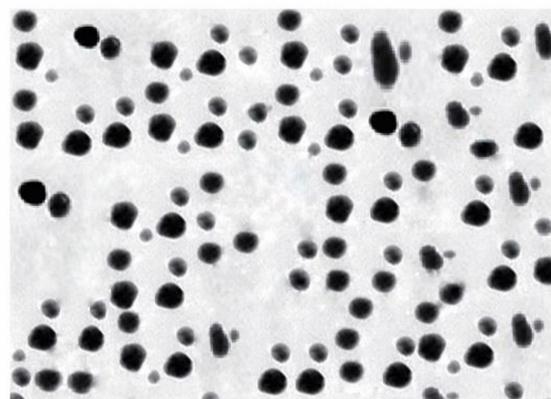


Figure 4: Scanning electron microscopy image of silver nanoparticles synthesized from *T.natans*.

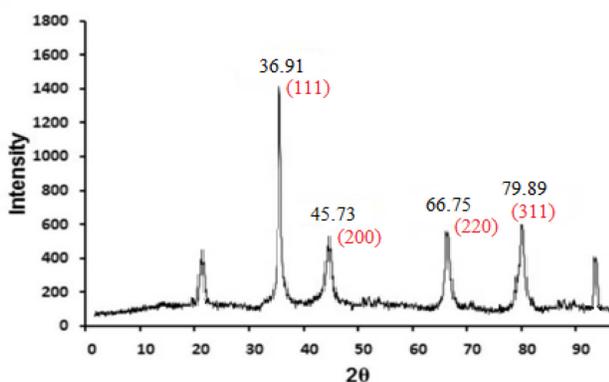


Figure 3: X-ray diffraction pattern of *Trapa natans* silver nanoparticles.

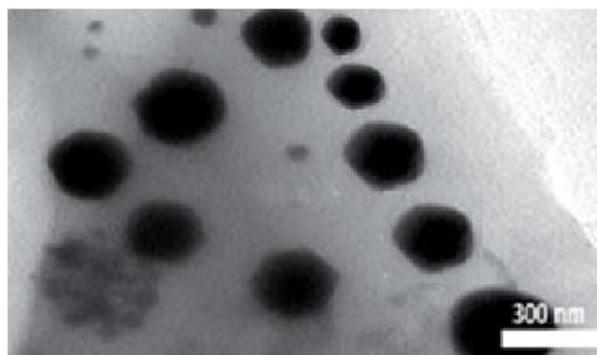


Figure 5: Transmission electron microscopy image of silver nanoparticles synthesized from *T.natans*.

to the (111), (200), (220) and (311) Bragg's reflection of the face-centered cubic structure of silver crystals (Figure 3). Thus, the XRD results clearly demonstrated the silver nanoparticles formed in our experiment were highly crystalline in nature.

### SEM Analysis of Silver Nanoparticles

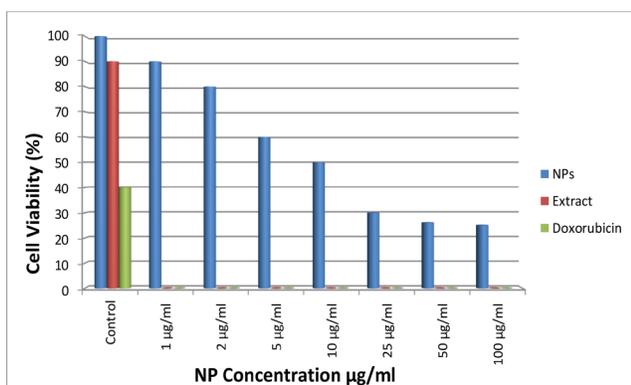
The Scanning Electron Microscopy (SEM) technique was used to identify the morphology and size of the bioreduced AgNPs. The SEM image of *T.natans*-AgNPs is shown in Figure 4. The nanoparticles were almost spherical in shape with a narrow size distribution. The diameter of the silver particles was found to be in the range of 30-90 nm and a few particles were agglomerated due to the binding of some capping agents present in the *T.natans* extract or due to the particle aggregation in SEM measurement.

### TEM Analysis of Silver Nanoparticles

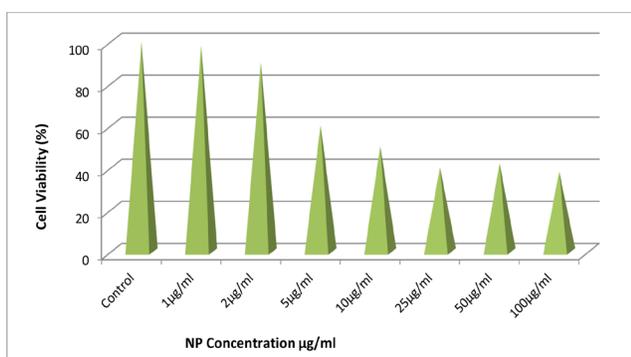
The Transmission Electron Microscopy (TEM) image of synthesized AgNPs is depicted in Figure 5. The TEM image indicates that AgNPs are polydispersed particles and are mostly spherical in shape. The sizes of AgNPs were  $15 \pm 2$  nm. With increasing concentrations, NPs became spherical.

### Cytotoxicity Analysis

NPs are emerging as promising agents in cancer therapy due to their different physiochemical characteristics.<sup>17,18</sup> Since metallic NPs have various features of metals, it has many advantages over simple plant extract. Earlier studies indicated that NPs smaller than 100nm size could easily and efficiently enter into cells.<sup>19,20</sup> To determine the potential of *T.natans*-AgNPs as therapeutic agents for the treatment of human breast cancer (MCF-7), their cytotoxicity were evaluated by MTT assay and neutral red uptake assay (NRU). The key results obtained by MTT and NRU assays in MCF-7 cells exposed to



**Figure 6(a):**Effect of silver nanoparticles synthesized from *Trapa natans* cell viability of MCF-7 cells. Cells were treated with nanoparticles at various concentrations for 24h and cytotoxicity was determined by the MTT assay.



**Figure 6(b):**Effect of silver nanoparticles synthesized from *Trapa natans* cell viability of MCF-7 cells. Cells were treated with nanoparticles at various concentrations for 24h and cytotoxicity was determined by the NRU assay.

1 µg/ml to 100 µg/ml for 24 hr are summarized in Figure 6(a) and 6(b). The results exhibit a concentration dependent decrease in the viability of MCF-7. However, at concentrations 25, 50 and 100 µg/ml cell death increases. (Table 1 and Table 3) Incubation with AgNPs synthesized from *T.natans* at high concentration that is 25, 50 and 100 µg/ml, lead to a reduction in cell viability. Like MTT assay, a concentration-dependent decrease in cell viability of MCF-7 cells exposed to *T.natans*-AgNPs was also observed by NRU assay. The cell viability recorded at 100 µg/ml of *T.natans*-AgNPs shows maximum decrease in cell viability.

In this study, the cytotoxicity assessments were performed using two independent end points (MTT and NRU) assays.<sup>21</sup> The MTT, a colorimetric assay is based on the mitochondrial dehydrogenase enzyme of viable cells;<sup>22</sup> however, NRU assay is based on the lysosomal integrity of viable cells.<sup>23</sup> The cytotoxic responses of the *T.natans*-AgNPs, suggesting that biosynthesized AgNPs

**Table 1: Calculation of MTT Assay.**<sup>22</sup>

	NP Concentration µg/ml of AgNPs							
	Control	1 µg/ml	2 µg/ml	5 µg/ml	10 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml
Cell Viability (%)	100	90	80	60	50	30	26	25

**Table 2: The IC<sub>50</sub> of *T.natans* AgNPs, *T.natans* Extract and Doxorubicin.**<sup>22</sup>

Cell line	IC <sub>50</sub> µg/mL		
	<i>T.natans</i> AgNPs	<i>T.natans</i> Extract	Doxorubicin
MCF-7	26 ± 3.14 µg/mL	34 ± 2.04 µg/mL	47 ± 1.09 µg/mL

Notes: Values are expressed as Mean ± (SD= 0.001) of three replicants

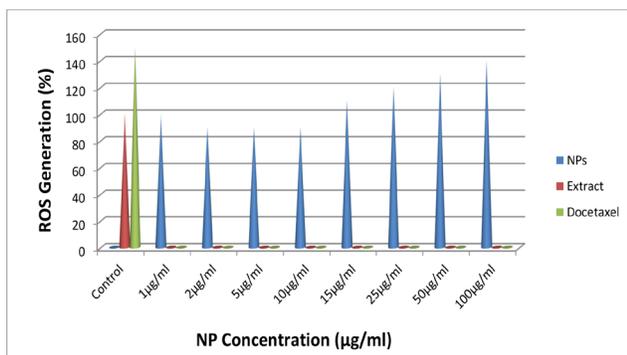
**Table 3: Calculation of NRU Assay.**<sup>21</sup>

	NP Concentration µg/ml of AgNPs							
	Control	1 µg/ml	2 µg/ml	5 µg/ml	10 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml
Cell Viability (%)	100	98	90	60	50	40	42	38

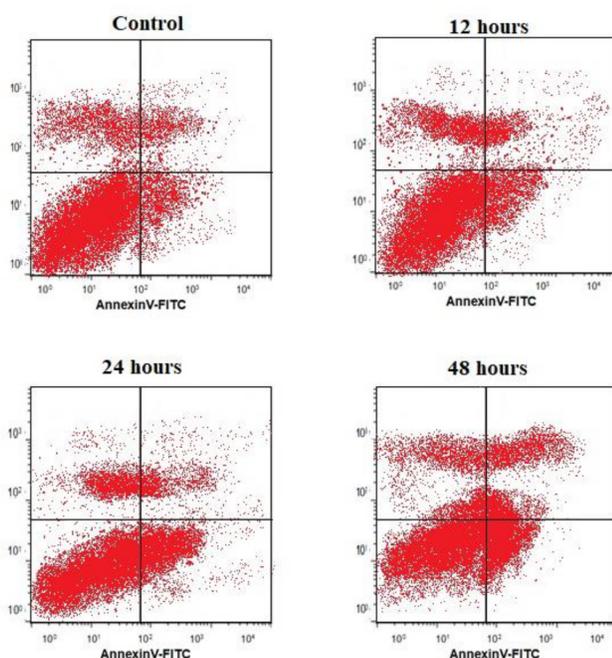
could contribute in search of alternative chemotherapeutic agent. (Table 2) Our results showed more than 50% of cell death even at 5 µg/ml of *T.natans*-AgNPs. The cytotoxic effects induced by *T.natans*-AgNPs at lower concentrations could be due to the plant components attached to the AgNPs.<sup>24</sup> The results obtained from this study are also very well supported with various evidences for the cytotoxic effect of biosynthesized AgNPs using *T.natans peel* extract against the breast cancer MCF-7 cell line.

### Determination of Intracellular Reactive Oxygen Species (ROS)

The result obtained from ROS generation in MCF-7 cells exposed to *T.natans*-AgNPs for 24 hr is shown in Figure 7.A statistically significant induction in ROS generation was measured in MCF-7 cells exposed to *T.natans*-AgNPs at 10, 25, 50 and 100 µg/ml concentrations. The ROS levels generated in responses to AgNPs were significantly higher than in AgNPs treated cells than control. Nanoparticles are expected to induce their toxicity via oxidative stress by generating reactive oxygen species (ROS) involved in different cellular processes ranging from apoptosis and necrosis to cell proliferation and carcinogenesis.<sup>25</sup> It has been documented that nanoparticles increase the ROS generation at cellular level. Taken together, all of these findings indicate that cell death is mediated by the production



**Figure 7: Reactive oxygen species generation in silver nanoparticles synthesized from *Trapa natans* treated MCF-7 cells.**



**Figure 8: Annexin V-PE assay. The scatter plots show early apoptotic, late apoptotic and necrotic cells following 12, 24, 48 hr treatment.**

of ROS, which is likely to alter the cellular redox status and it is a potential cause for cell death.

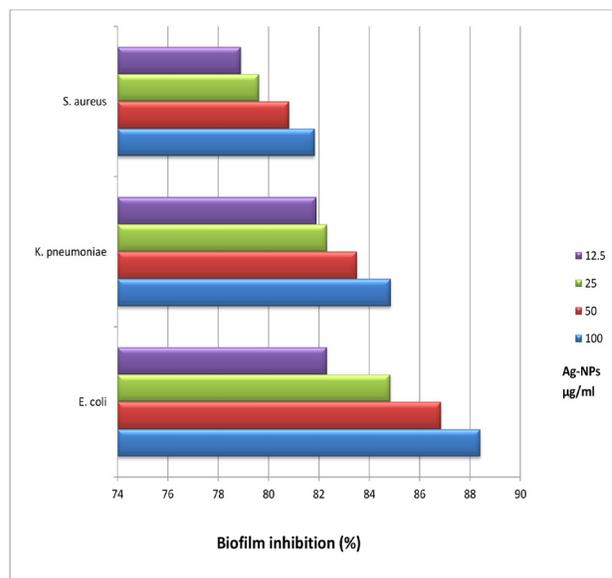
**Apoptosis/Necrosis Assessment Using Annexin V**

Annexin-V/PI double-staining assay showed that MCF-7 cells treated with *T.natans*-AgNPs became apoptotic gradually, with concomitant decrease in viable cells in a time-dependent manner. After 12-h treatment, MCF-7 cells were primarily in the early phase of apoptosis and began to enter the late phase of apoptosis from 24 h onward. (Table 4) The results obtained from the induction of apoptosis/necrosis using flow cytometry are summarized in Figure 8. The MCF-7 cells exposed to *T.natans*-AgNPs

**Table 4: Flow cytometry analysis of FITC conjugated annexin V/ PI staining.<sup>26</sup>**

Cell Stage	Cell %			
	Control	12hr	24hr	48hr
Viable	94.4± 0.35	80.55± 0.67	78.25± 0.25	74.3± 0.35
Early Apoptosis	5.55± 0.75	7.57*± 0.95	8.85*± 0.35	11.7*± 0.45
Late Apoptosis/ Necrosis	3.92± 0.20	8.85*± 0.95	10.85*± 0.75	12.3*± 0.20

Notes: Values are expressed as mean ± SD of triplicate experiments. Data has been analyzed using one-way ANOVA. \* indicates significant value (P - 0.05) different from control.



**Figure 9: Anti-biofilm activity by using different concentrations (12.5, 25, 50, 100 µg/mL) of silver nanoparticles synthesized from *T.natans* peel extract against different standard bacterial strains.**

significantly increases the late apoptotic and necrotic cells as compared to untreated control cells. It is well known that high amount of ROS generation could lead to apoptotic and necrotic cell death.<sup>26</sup> The excessive ROS generation has been linked with the substantial DNA damage and apoptosis/necrosis.<sup>27</sup> Our results are consistent with the recent reports of apoptosis cell death due to the exposure of nanoparticles<sup>28</sup> including the exposure to plant synthesized silver nanoparticles.<sup>29</sup>

**Antibiofilm Activity**

The results of tissue culture plate method indicates that antibiofilm activity of the biosynthesized AgNPs from *T.natans* peel extract was 82.32% for *E. coli*, 81.90% for *Klebsiella pneumoniae*, and 78.90% for *Staphylococcus aureus*, respectively at 12.5 µg/mL (Figure 9 and Table 5).

**Table 5: Antibiofilm activity of the biosynthesized AgNPs.<sup>31</sup>**

Bacteria	Concentration of Ag-NPs (µg/ml)			
	12.5 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml
<i>Escherichia coli</i>	82.32	84.84	86.86	88.42
<i>Klebsiella pneumoniae</i>	81.90	82.32	83.52	84.86
<i>Staphylococcus aureus</i>	78.90	79.62	80.82	81.84

These findings indicate that the uptake of the biosynthesized *T.natans*-AgNPs may reduce the biofilm forming abilities of tested pathogens. Similar studies were documented as results of biofilm inhibition at 100 µg/mL AgNPs.<sup>30</sup> In addition, the antibiofilm action of AgNPs has been attributed to the diffusion through biofilms imparting antimicrobial activity.<sup>31</sup>

## DISCUSSION

Green synthesis of silver nanoparticles is a promising approach for overcoming the limitations of conventional physicochemical routes. Bio-molecular reducing agents present in plant extracts such as proteins, reducing sugars, alkaloids, terpenoids, and phenolic compounds act as reducing agents as well as capping agents for the synthesis of silver nanoparticles.<sup>32</sup> However, depending on the applied green extract, the biological activity, or toxicity profiles of the green synthesized AgNPs may differ considerably.<sup>33</sup> Therefore, the present study reported the green synthesis of silver nanoparticles using *T.natans* leaf extract and testing their anti-proliferative activity against human skin cancer cells. AgNPs have generated a great deal of interest in biomedical field due to their potential therapeutic effects for variety of diseases such as acquired immunodeficiency syndrome (AIDS), retinal neovascularization, various infectious diseases and cancers.<sup>34,35</sup>

N. Ahmad *et al.* reported the efficiency of green synthesized Ag-Au NPs using *Trapa natans* peel extract as a cytotoxic agent against HCT116, MDA-MB-231 and HeLa cells. Their results showed that, the biosynthesized Ag-Au NPs had a clear cytotoxic activity on cells. The anticancer activity of this biosynthesized Ag-Au NPs was determined to be highly related to apoptosis induction in HeLa, HCT116, MDA-MB-231 cells through the significant increase in ROS production. In another study by M.M. Saber *et al.* they used leaf extract of *Trapa natans* plant to biosynthesize silver nanoparticles as an antitumor agent against A431 skin cancer cell line. Their results showed that the presence of different bioactive molecules in *T.natans* extract bond to AgNPs surface like glycosides, amino acids,

flavonoids, and polyphenols as well as nano-sized silver particles may be responsible for anticancer activity of biosynthesized AgNPs.

In our study, *T. natans*-AgNPs exhibited strong cytotoxic activity against MCF-7 breast cancer cell line. The presence of different bioactive molecules in *T.natans* shows that, green synthesized AgNPs significantly inhibited proliferation of MCF-7 breast cancer cells by inducing apoptosis as well as antibiofilm activity using different bacterial colonies. UV-vis spectroscopy, FTIR, SEM, TEM and XRD techniques were used to characterization of biosynthesized particles. In UV-vis spectroscopy we found that AgNPs exhibit strong absorption peak in the 420–500 nm range due to Surface Plasmon Resonance. In FTIR analysis the functional groups are identified by distinct peaks and we found phenol ansalcohol compounds (at 3447 cm<sup>-1</sup>), carbonyl groups (at 1631 cm<sup>-1</sup>), alcoholic groups (at 1384 cm<sup>-1</sup>) and polysaccharides (at 1093 cm<sup>-1</sup>). In XRD, the results clearly demonstrated that AgNPs formed in our experiment were highly crystalline in nature. In SEM we found the diameter of AgNPs to be in the range of 30-90 nm and TEM depicts the size of AgNPs to be 15 ± 2 nm. We found the IC<sub>50</sub> value of AgNPs (26 ± 3.14 µg/mL), Extract (34 ± 2.04 µg/mL) and Doxorubicin (47 ± 1.09 µg/mL). Primarily MCF-7 cells after 12 hr treatment were in early apoptosis phase and proceed to late apoptosis from 24 hr onwards. This study showed that *Trapa natans*-AgNPs have the capacity of inducing apoptosis and necrosis cell death of MCF-7 cells. *Trapa natans*-AgNPs were also found to induce oxidative stress as observed by the increase in ROS level. Thus, our findings suggest the anticancer potential of biosynthesized *Trapa natans*-AgNPs against human breast cancer cells and could play an important role in the development of new therapeutic agent for the treatment of cancer. The biologically synthesized particles exhibits antimicrobial effect and the inhibition of biofilm formation against *E. coli* (82.32%), *K. pneumonia* (81.90%) and *S. aureus* (78.90%) at 12.5 µg/mL concentration of AgNPs. Hence our full investigation shows the biosynthesis of silver nanoparticles using *Trapa natans* plant and its anticancer with antibiofilm activities.

## CONCLUSION

This investigation demonstrates the biosynthesis of silver nanoparticles (AgNPs) via reduction of silver ions using *Trapa natans* plant and its anticancer potential against human breast cancer (MCF-7) cells and also antibiofilm potentials against biofilms. Our results suggested that water chestnut extracts have antibiofilm

and antiproliferative activities and can be utilized as effective and safe antioxidant sources. Our results showed that biosynthesized AgNPs (*Trapa natans* AgNPs) induced a concentration-dependent cytotoxicity in MCF-7 cells. The biologically synthesized particles also exhibited antimicrobial effect and the inhibition of biofilm formation against *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*.

Our findings indicate a cost-effective and eco-friendly approach for *Trapa natans* AgNPs synthesis which may support the development of potential biomedical applications and alternative therapy involving the design of new antibiofilm agents and multi drug resistance bacterial infections, and could be further developed as a template for other anti-cancer actions. Further characterization of their chemical composition, structure of the active compounds, and mechanism of antibiofilm and antiproliferative activity is required so that it can be used in fields of medicine, cosmetology and pharmacy.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ABBREVIATIONS

**AgNPs:** Silver Nanoparticles; **FTIR:** Fourier Transform Infrared Spectroscopy; **XRD:** X-ray Diffraction; **SEM:** Scanning Electron Microscopy; **TEM:** Transmission Electron Microscopy; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **ROS:** Reactive Oxygen Species.

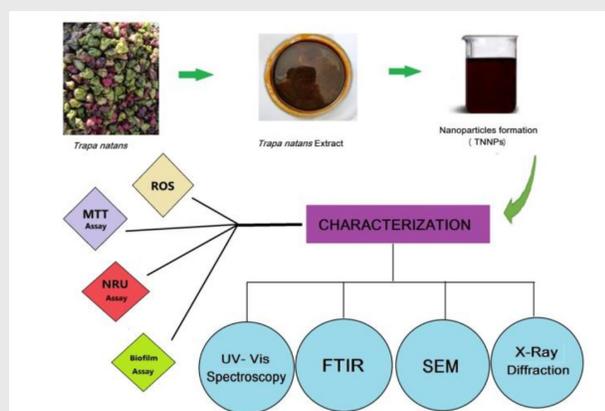
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## PICTORIAL ABSTRACT



## SUMMARY

The objective of the present study is to prepare and evaluate silver nanoparticles (AgNPs) of *T.natans* which can show anti-metastasis and anti-biofilm activities via *in vitro* methods. The characterization of *T.natans* AgNPs was done by UV-vis spectroscopy, FTIR, SEM and XRD techniques. Cytotoxicity analysis was done by MTT assay and Neutral Red Uptake (NRU) assay. Antibiofilm activity was carried out by using the bacterial strains of *Klebsiella pneumoniae*, *Escherichia coli* and *Staphylococcus aureus* respectively. The *T. natans* silver nanoparticles prepared inhibits the proliferation of MCF-7 breast cancer cells by inducing apoptosis as well as antibiofilm activity using different bacterial colonies.

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