International Journal of Advance Interdisciplinary Research
Vol. 02 Issue 1, Part A (Jan-March) 2026, pp. 279-295, e-ISSN: 3107-913X
DOI: https://doi.org/10.66095/ijair.2026.v2.i1.a.18

Green synthesis of silver nanoparticles using Citrus Orange peels and evaluation of their anticancer Property

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Abstract

This work aimed to synthesis silver nanoparticles (AgNPs) using biological waste products Citrus orange peels, its characterization, and their cytotoxic effect. SEM and TEM analysis confirmed the formation of spherical and uninform nanoparticle. The average size of the synthesized nanoparticle was 10-11 nm as measured by DLS technique. FTIR analysis of the green synthesized AgNPs showed the presence of alcohols, phenolics, alkynes, aliphatic primary amines, and amino acid groups. Finally, the evaluation of the cytotoxic effect of the green synthesized AgNPs were done using human colon carcinoma cell line (HCT-116). The results revealed the concentration has a direct correlation with cell viability. The 50% inhibitory concentration (IC50) of the HCT-116 cell line was expected as 8-10 µg/ml.

Keyword: Citrus orange peel, silver nanoparticle, characterisation, anticancer agent

Introduction

Nanoparticles (1 to 100 nm) have significant role in biotechnology sector with commercial, industrial, agricultural and medicinal applications. Silver (Ag) have biocompatibility, bactericidal properties, and therapeutic abilities. Therefore, they are preferred for medical treatment, cometic agent and management of various diseases. Silver is routinely used in the form of silver nitrate for antimicrobial activity.

A number of benefits, including ease of use, environmental friendliness, and ease of scaling, have generated considerable interest in green nanotechnology. Many plant extracts have shown anticancer activity against several cancer cells. Silver nanoparticles have shown to be able to penetrate microbial membranes, and altered cell function, cellular structure and potentially inducing cell death [1]. One study used a nanocrystalline silver structure and examined that it suppressed the expression of tumour necrosis factor alpha and IL-12, both cytokines that promote an immune response once recognized by immune cells [2]. Mukherji and Agnihotri synthesised silver nanoparticles using $AgNO_3$, as a precursor. It has been reported that ethanol is a good reducing agent for the synthesis of silver nanoparticles having a size range of 5-20 nm. Flavonoid compounds isolated from citrus peel have been identified as agents with utility in the treatment of cancer. This work provides the anticancer potential

found within the citrus peel. Historical studies have identified a number of cellular processes that can be modulated by citrus peel flavonoids including cell proliferation, cell cycle regulation, apoptosis, metastasis, and angiogenesis. More recently, molecular studies have started to elucidate the underlying cell signalling pathways that are responsible for the flavonoids' mechanism of action. These growing data support further research into the chemo preventative potential of citrus peel extracts, and purified flavonoids in particular [3]

In this study, we aimed to investigate the green synthesis of silver nanoparticles using orange peel extract, and evaluate their cytotoxic effects.

Method and Methodology

2.1. Collection of Citrus orange peels and preparation of extract:

Citrus orange peels were collected and cleaned thoroughly using distilled water to remove the dust particles adhering to the surface of the fruit peel. Ten grams of peels were transferred into **2**5 mL of boiled distilled water and left to boil for 10 min. The extract obtained was filtered through filter paper, and then, it was stored at 4 ℃ for further use.

2.2. Green synthesis of AgNPs using Citrus orange peel extract:

Green synthesis of AgNPs involved the addition of 0.008 g (1 mM) silver nitrate (AgNO3) to 50 mL of distilled water, and then, the solution was stirred with magnetic stirrer for 15 min at 45 C at 1100 rpm. Then, 5 mL of the **Citrus orange** peels extract (LPE) was added.

2.3. Characterization of silver nanoparticles

2.3.1. Visible observation:

Colour change of the mixture to brown colour had confirmed the formation of AgNPs.

2.3.2. UV–Visible spectrophotometry:

Green synthesized AgNPs were analysed with the help of UV–Visible spectrophotometer with typical optical spectrum for AgNPs is in the range 350–550 nm [4]

2.3.3. Dynamic light scattering evaluation:

For the size evaluation, the instrument used was Zetasizer Nano ZS (Malvern Instrument Limited, UK). Three millilitres of the AgNPs from the LPE were filtered through 0.20 mm pore sized syringe, and then, the solution was analysed.

2.3.4. Transmission electron microscopy (TEM):

The morphology of the green synthesized AgNPs was examined using TEM. A drop of AgNPs solution was loaded on carbon-coated copper grid and the solvent was allowed to evaporate. The TEM micrograph images were captured using JEM-1400(Jeol Ltd, Japan) with an accelerating voltage of 100 kV.

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2.3.5. Energy dispersive x-ray equipped with TEM:

A drop of the sample was loaded on the carbon-cupper coated grid and left to dry. The elemental composition of the synthesized AgNPs was evaluated using the energy dispersive x-ray analyser (JEM-2100F 200 kV, Joel Ltd, Japan).

2.3.6. Fourier-transform infrared spectroscopy:

FTIR analysis was conducted using Thermos 6700 (Thermos Fisher Scientific, USA) to determine the functional groups found in the LPE that led to the formation of AgNPs. The absorption bands were observed in the regions of 500–4000 cm1.

2.3.8. Analysis of cytotoxic effect of the AgNPs:

HepG2 cell lines were treated with optimum concentration of crude extract of orange and its silver nanoparticle. The dilutions were prepared in DMSO. After treatment the cells were incubated for 24 hours in CO_2 incubator. After 24 hours, 20 µl of MTT reagent was added and incubated for 4 hours at 37^{o}C. After incubation, the formazan produced was solubilized by the addition of 100 µl of DMSO and the absorbance was recorded at 570 nm by ELISA reader. Cells without treatment were used as a positive control. Effective dose is required for inhibiting cell growth by 50 % (IC_{50} micrograms per mil-litre) was determined.

Cell viability was calculated as follows:

[Mean OD (Treated sample) – Blank OD / Mean OD (Control) – Blank OD] ×100

The cytotoxicity test was conducted according the method described by [5].

2.4. Statistical analysis

The standard deviation of the mean was calculated according to [6]

3. Results:

3.1. Visible observation and UV–visible spectrophotometry results:

The formation of the green synthesized AgNPs from the LPE was visually confirmed via color change after 15 min. Analysis with UV–visible spectrophotometer at wavelengths of 350–550 nm showed the formation of AgNPs as shown in Fig. 1.

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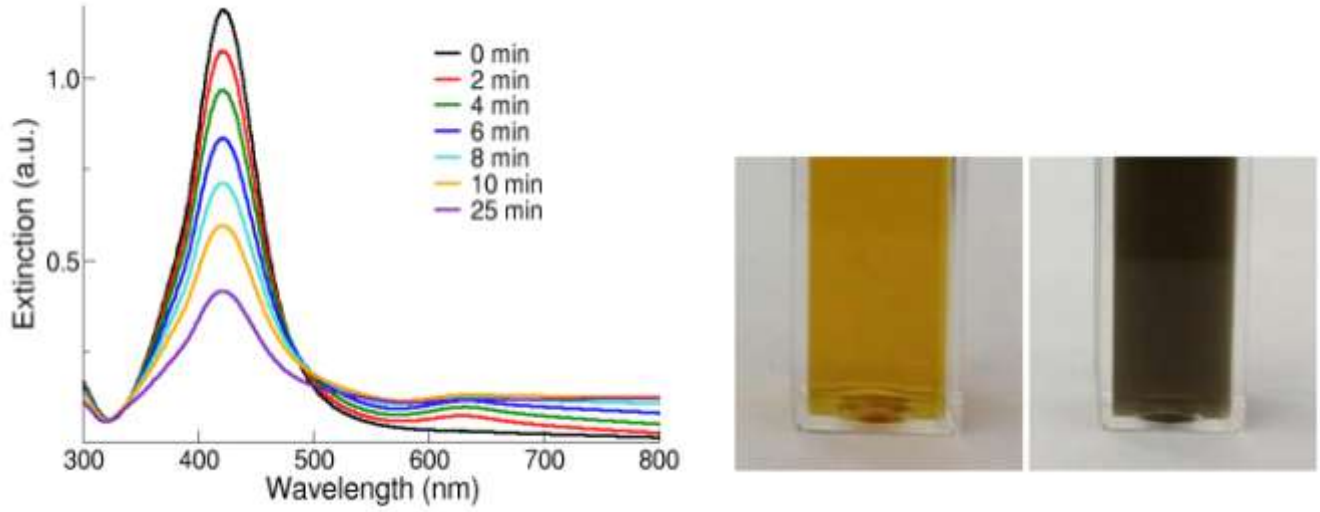

Fig. 1. UV–Vis spectra and colour change during silver nanoparticle formation

Ref. Prashant K. Jain, Kyeong Seok Lee, Ivan H. El-Sayed, Mostafa A. El-Sayed, 2006. Calculated Absorption and Scattering Properties of Gold Nanoparticles of Different Size, Shape, and Composition: Applications in Biological Imaging and Biomedicine

3.2. Dynamic light scattering results:

The average size of AgNPs synthesized using LPE was found to be 59.74 nm as measured by DLS technique.

3.3. Scanning electron microscopy (SEM)

The topography and morphology of nanoparticles can be observed by SEM, which is also used to calculate the size of various nanoparticles at the micro- (10^{-6}) and nano (10^{-9}) scales [7,8]. A high-energy electron beam, produced by SEM, is directed at the surface of the sample nanoparticles. and the backscattered electrons produced give the characteristic features of the sample [9]. Electron microscopy analysis is used to examine the changes in the morphology of the cell before and after nanoparticle treatment. Several studies have reported that the visible modifications in cell shape and perforations of nanoparticles in the cell wall have been used as indicators of the antimicrobial action of nanoparticles [10,11]. Using SEM control bacterial cells exhibited smooth and undamaged structures, while cells treated with silver nanoparticles for 60 min were significantly damaged, with clear morphological changes to the cell membrane leading to loss of membrane integrity [12].

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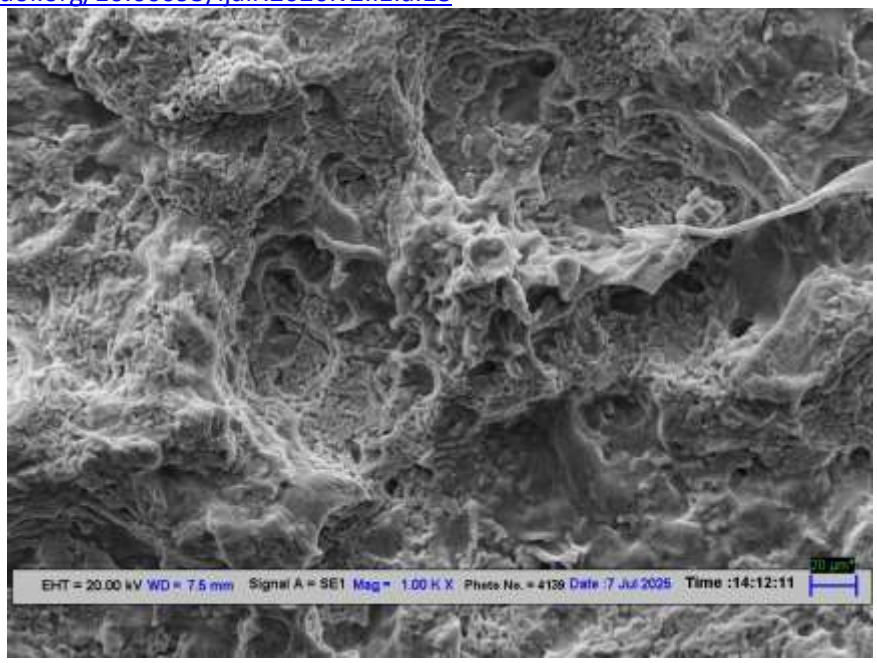

Fig2. Morphological analysis of orange peel extract by SEM Microscopy

Ref. IIT Delhi, SEM Test.

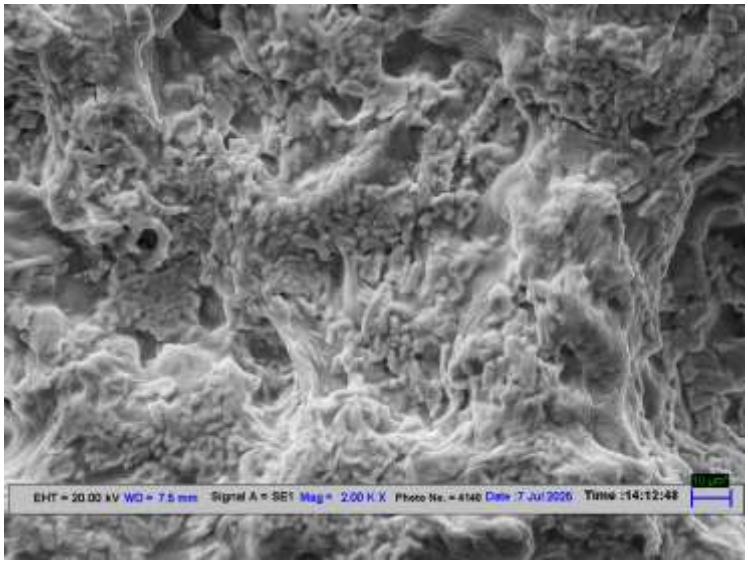

Fig3. Morphological analysis of nanoparticle of orange peel extract by SEM Microscopy

Ref. IIT Delhi, SEM Test.

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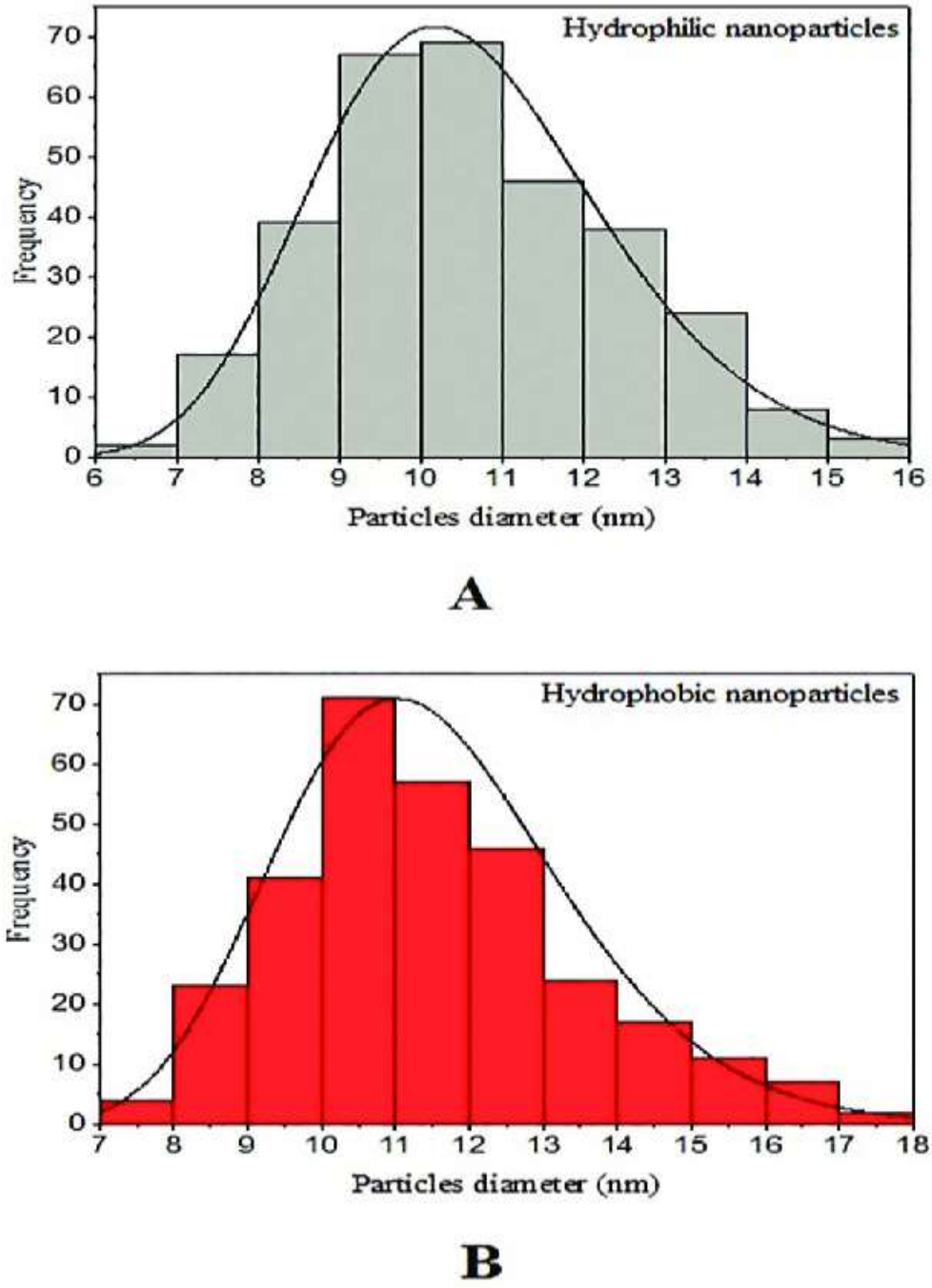

Fig.4

3.3. Transmission electron microscop results:

As shown in the TEM images shown in Fig. 3a-d, most of the AgNPs were observed to be spherical, and few agglomerated AgNPs were also observed.

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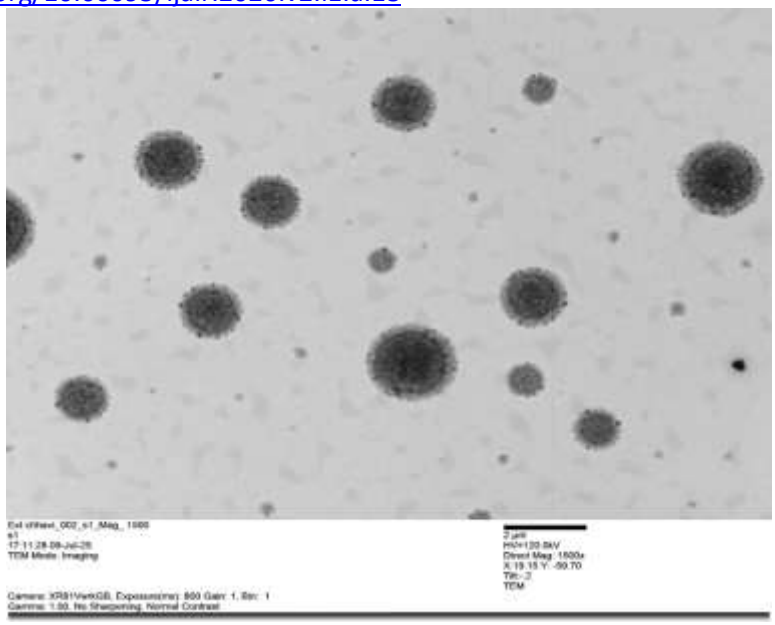

Fig. 5 Surface Morphology of nanoparticle of orange peel extract by TEM Microscopy

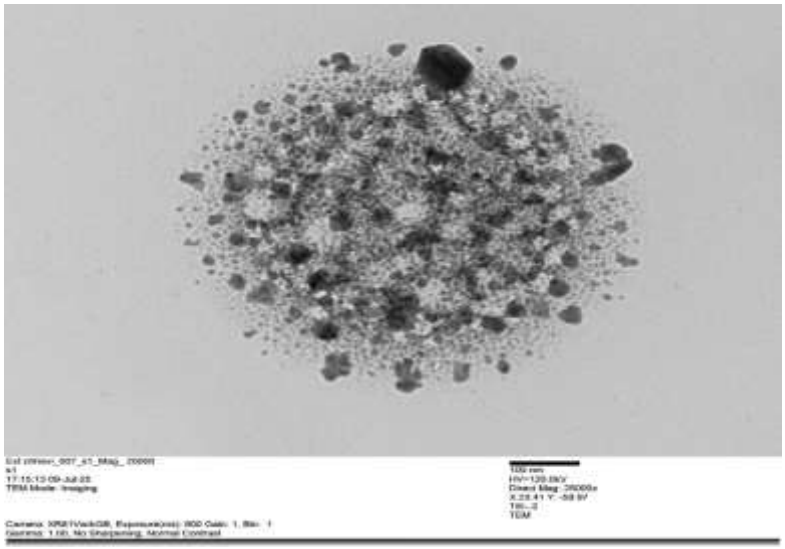

Fig.6 Surface Morphology of nanoparticle of orange peel extract by TEM Microscopy

Ref. IIT Delhi, SEM Test.

3.5. Fourier-transform infrared spectroscopy results:

The LPE showed absorption peaks at 3303.05, 2198.40, and 1987.95 cm1 (Fig. 7). While the green synthesized AgNPs showed absorption peaks at 3273.24, 2223.71, 1972.46, and 2047.29 cm1 (Fig. 8).

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Fig.7 Lemon peel's nanoparticle

Ref. J.N.U. AIRF

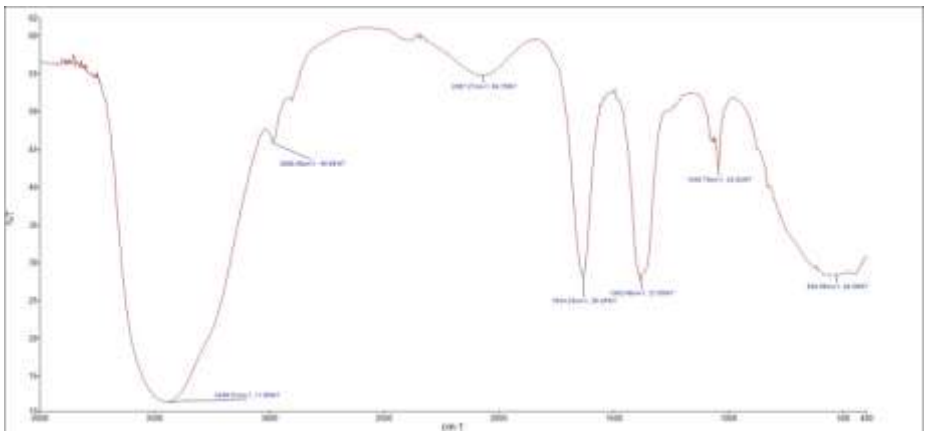

Fig.8 Orange peel's nanoparticle

Ref. J.N.U. AIRF

3.7. Cytotoxic effect of the synthesized AgNPs:

The evaluation of the cytotoxic effect of the green synthesized AgNPs were done using two types of cell lines, human breast cancer cell line (MCF-7) and human colon carcinoma cell line (HCT116). The results ravelled the concentration has a direct correlation with cell viability as shown in Fig. 8 and Tables 2&3. The 50% inhibitory concentration (IC50) of HCT-116 cell line was in 8-10 µg/ml.

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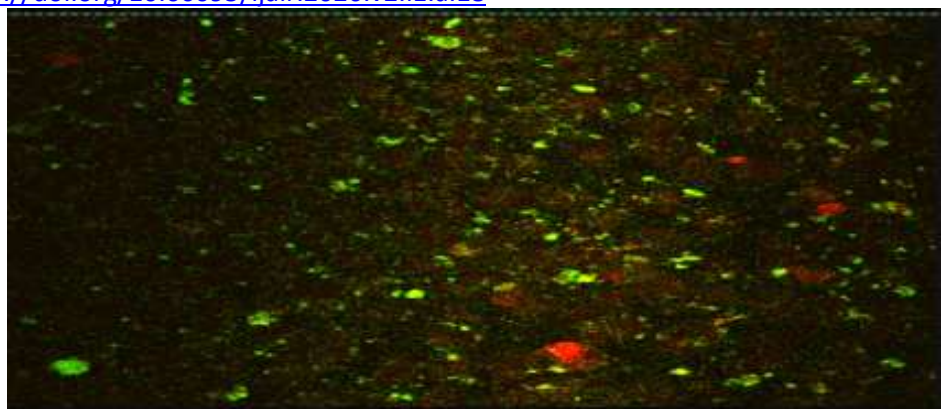

Fig.10: Images of cancer cell line Hep-G2 growth with crude orange extract

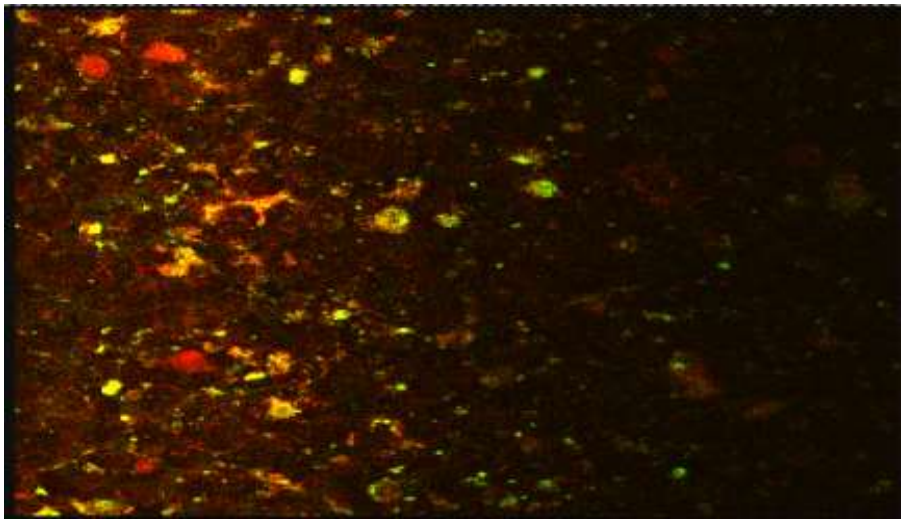

Fig.11: Images of cancer cell line of Hep-G2 with silver nanoparticle

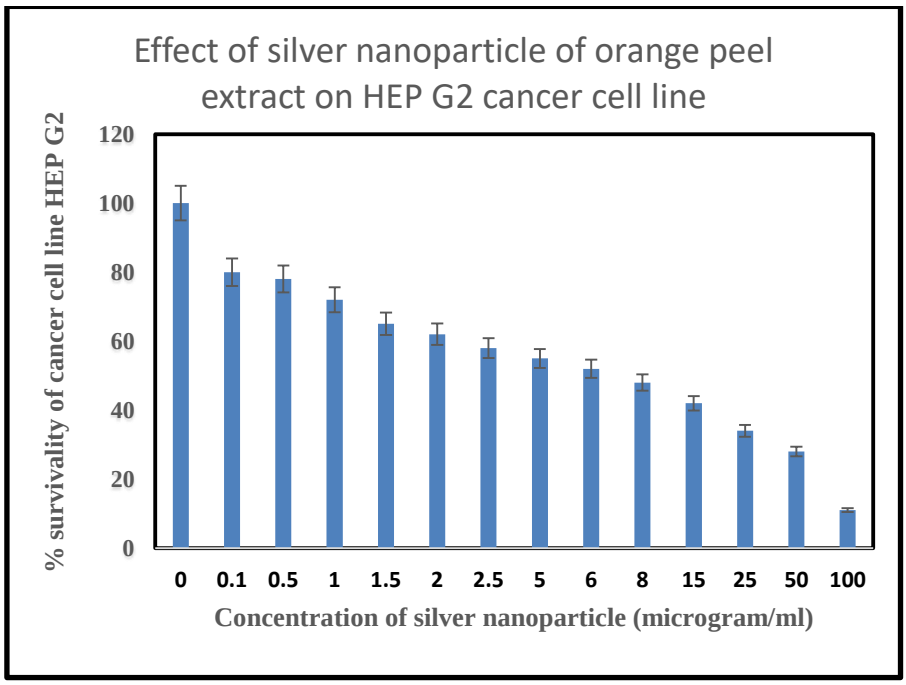

Fig.12. *In Vitro* study of silver nanoparticle of orange extract against the growth of HEP-G2 cancer cell lines

4. Discussion:

The present study demonstrated the green biosynthesis of AgNPs using extract of Citrus limon peels, that were treated with 1 mM AgNO3.The colourless AgNO3 solution started turning brown after 15 min; this change could possibly be due to the reduction of Ag ions to silver nanoparticles [13]. For characterization, UV–visible spectrophotometry revealed the maximum peak of the AgNPs at 437 nm; the peak was due to the excitation of the surface plasmon resonance. During the DLS evaluation, the semi-broad peak of the green synthesized AgNPs from LPE (59.74 r. nm) indicated variation in size or an aggregated structure of metal nanoparticles. The Pdi of 0.463 indicated mono-dispersed nanoparticles [14] TEM images confirmed the existing of spherical and rod-like shaped nanoparticles. The results also showed an aggregated structure of the silver nanoparticles; the aggregation could be due to the layer (capping agents) covering the NPs, which causes the NPs to be attached to each other resulting in decreased space between the NPs. These results corroborated with those obtained from UV–visible spectrophotometry and DLS analysis. From the EDX analysis, the absorption peak was observed at 3 KeV indicating the presence of a silver element [15]. The presence of copper and carbon could be due to the grid's composition. Other signals were also observed indicating the presence of oxygen in the range of 0.0 – 1.5 KeV, chlorine was observed near 3 KeV, and chromium was seen in the range of 0.0 – 6.0 KeV; thus, these could also be components of the peel extract. In FTIR, LPE revealed several peaks at 3303.05, 2198.40, and 1987.95 cm1. Peak 3303.05 cm-1may correspond to alcohols, phenolics, mono-substituted alkynes, aliphatic primary amines, sodium salt, amino acid, or SiOH alcohol. Peak 2198.40 cm-1 may correspond to NH3 or alkynes. Peak 1987.95 cm-1 may correspond to cumulated alkenes, indicating a typical aromatic benzenoid compound. The green synthesized AgNPs showed absorption peaks at 3273.24, 2223.71, 1972.46, and 2047.29 cm1. The peak 3273.24 cm1 may represent alcohols, phenolics, monosubstituted alkynes, aliphatic primary amines, sodium salt, amino acid, or SiOH alcohol. The peak 2223.71 cm1 could correspond to alkynes or ammonium. Peak 1972.46 cm-1maycorrespond to cumulated alkenes, indicating a typical aromatic benzenoid compound. Peak 2047.29 cm-1could correspond to NH3 [16]. From the comparison between the spectra of the LPE and the green synthesized AgNPs, the shifts in the positions of the peaks indicated the presence of the functional groups that reduced the silver ions to silver nanoparticles. Regarding the

antimicrobial activity of the prepared AgNPs, the results showed varying degrees of antibacterial activity against human pathogenic bacteria. It is elucidated that these nanoparticles have significant antimicrobial behaviour against the tested Gram-negative (E. coli, Salmonella typhimurium and P. aeruginosa) and Gram-positive (S. aureus) bacteria. Based on the inhibitory and bactericidal behaviour of the nanoparticles, it was revealed that these nanoparticles are able to inhibit the growth of microbial strains when used in very low concentrations. The antibacterial effect for these particles might be due to the ability of the AgNPs to enhance the permeability of the cell membrane, formation of free radicals, and interaction with thiol groups, prevent DNA replication, affect cellular signalling, and prevent biofilm formation [17]. Four types of mechanisms have been proposed to interpreting the mechanism of antimicrobial activity of AgNPs, these were; (1)Interaction of AgNPs with cell membranes, alterations in the membrane permeability, and perturbation of respiratory chain enzymes; (2)Gradual diffusion of nanoparticles into the cells, which could both adversely affect the activity of cellular enzymes and restrict the transcription process by conjugation of silver particles to DNA; (3) Leakage of subcellular components as a result of nanoparticles interaction with the plasma membrane leading to cell death and (4) Generation of free radicals when the cell membrane is affected by silver ions [18,19]. For the cytotoxicity evaluation, different concentrations of the AgNPs were used. By increasing the concentration of the AgNPs, the cell viability decreased, resulting in IC50 of 23.5 ± 0.97 mL/100 mL for MCF-7 and IC50 of 37.48 ± 5.93 mL/100 mL for HCT-116. The possible cytotoxic effect might be attributed to the ability of AgNPs to stimulate reactive oxygen species generation in the cellular components, resulting in cell death [20]. It has been postulated that AgNPs interact with mitochondria and disrupt the cellular electron transfer chain function leading to an increase in the ROS level [21, 22]. Consequently, the oxidative stress generated by ROS could be considered as a main toxicity mechanism of AgNPs against cells. The elevated anticancer activity of the AgNPs could be attributed to a synergy between AgNPs and the covering polyphenols. It is proposed that the superior cytotoxicity of AgNPs against cancerous cells occurs owing to the highest uptake of nanoparticles by these cells rather than healthy cells, given that cancerous cells have an abnormal metabolism and high proliferation rate, which in turn makes them more vulnerable [23]. The simultaneous effect of AgNPs and polyphenols not only increases the ROS generation but also inhibits the transcription process. It is noteworthy that antioxidants such as polyphenols show cytotoxicity only against nonhealthy cells [24]. This report is in good agreement with the data in the literature, which

report the concentration-dependent toxicity of nanoparticles, particularly at lower levels [25, 26 27]. It seems that the prominent cell death mechanism is conjugation of nanoparticles with cells and change in the permeability of plasma membrane, which leads to free-radical and ROS generation. This assumption is further augmented by the emergence of pigments (such as beta carotene) in the tested bacteria as a defence mechanism against the oxidative stress.

Silver nanoparticles (AgNPs) are of great interest due to their unique and controllable characteristics. A significant improvement in the cytotoxicity characteristics of the green synthesized Ag nanoparticles against a cancerous cell line. These findings imply that the synthesized nanoparticles using green nanotechnology could be an ideal strategy to combat cancer and infectious diseases. The synthesized AgNPs proved to possess improved anticancer, antimicrobial activity in comparison with the extract. The method of AgNPs synthesis introduced in this study, therefore, holds great potential as a simple, low-cost, and environmentally-friendly approach for producing value-added products from waste material. The synthesized AgNPs exhibited selective cytotoxicity toward the cancerous cell line when compared to their effect on the normal cell line tested. These findings are very promising in utilizing the biological effects of the AgNPs synthesized using walnut green husk extract.

The cytotoxic and oxidative effects of silver nano-particles synthesised from orange peel extracts were examined in human cancer cell lines. The study demonstrated that the nano-formulation had anticancer activity [28]. Khateef and colleagues examined the cytotoxicity of silver nanoparticles at various concentrations. It was noticed that the inhibition of cell growth was enhanced with increasing concentrations of the nanoparticle

Further research should be performed in in vivo lung cancer models to support our findings and to explain the mechanism of action at the molecular level.

Silver nanoparticles at an average size of 10 ± 5 nm and concentrations of 2.5 and 0.25 ppm slightly increased the production of IL-12 in Normal Human Epidermal Keratinocytes (NHEK).

Silver NPs encapsulated with chitosan Nano formulations inhibited MCF-7 breast cancer cell lines in a dose-dependent manner [29]. Silver NPs synthesized from Panax ginseng Meyer and Origanum vulgare, inhibited the viability, migration and phosphorylation of A549, MCF-7, and HepG2 cell lines.

NPs synthesized from fruit extract of Cleome viscose induced cytotoxicity on lung A549 and PA1 ovarian cancer cell lines in a dose-dependent manner [30]. Silver nanoparticles embedded into a specific polysaccharide.

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Conclusion

Silver nanoparticles (AgNPs) are of great interest due to their unique and controllable characteristics. A significant improvement in the cytotoxicity characteristics of the green synthesized Ag nanoparticles against a cancerous cell line. These findings imply that the synthesized nanoparticles using green nanotechnology could be an ideal strategy to combat cancer and infectious diseases. The synthesized AgNPs proved to possess improved anticancer, antimicrobial activity in comparison with the extract. The method of AgNPs synthesis introduced in this study, therefore, holds great potential as a simple, low-cost, and environmentally-friendly approach for producing value-added products from waste material. The synthesized AgNPs exhibited selective cytotoxicity toward the cancerous cell line when compared to their effect on the normal cell line tested. These findings are very promising in utilizing the biological effects of the AgNPs synthesized using walnut green husk extract. The effect of silver nanoparticles on gene expression in the human lung epithelial cell line was analysed. The study revealed that exposure to silver nanoparticles influenced the cell cycle and directed to an arrest in the G2/M phase [31]. It has recently been reported that silver nanoparticles induced autophagy in cancer cells through activating the Ptdins3K signalling pathway. Moreover, wortmannin, an inhibitor of autophagy, significantly enhanced the antitumour effect of silver nanoparticles in a melanoma cell model [32] and green synthesised silver nanoparticles showed a dose-dependent response based on the human lung cancer study [33].

Notably, for anticancer treatment, activating apoptotic pathways that do not cause inflammation is preferred. We believe that these results will pave the way for the use of $AgNO_3$ in in vitro studies of other types of cancer.

Ethical Approval and Consent to participate:

- **Human Ethics:** we have not performed any In Vivo study using animal model. This is reviewing article, therefore, eethical approval is not applicable.
- **Consent for publication:** The review article is original and we both authors are mutually agree to submit this review article in this journal
- **Availability of supporting data:** The article includes the data collected from referred journal and authenticate site

-- **Funding:** *The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.*

- **Authors' contributions***: All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Ms. Princy Rajput, Mrs. Meena (Research Scholar), UG student (Arin Jain & Varsha Goyal). Dr. Priyanka Singh* (as supervisor) has guided her for designing of experiment, compilation of data and drafting of manuscripts*. All authors have read and approved the final manuscript.*

-**Acknowledgements:** Authors are thankful to NIMS University Rajasthan for providing lab facility for completion of this research work. We are also tankful for CRF Institute of IIT Delhi and AIRF centre of JNU for giving facility for SEM, TEM analysis and FTIR characterization

- **Declaration statement:** The authors have no relevant financial or non-financial interests to disclose. We agree that all data, materials as well as software application has supported their published claims and complied with field standards.

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