Original research article

Fruit waste (peel) as bio-reductant to synthesize silver nanoparticles with antimicrobial, antioxidant and cytotoxic activities

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ABSTRACT

Since last decade, biogenic synthesis of metal or metal-oxide nanoparticles is emerging as an alternative method, which is environment friendly, simple and safe to use. In this article, fruit waste (peel) extract (FWE) of three citrus fruits viz. *Citrus limon*, *Citrus sinensis*, and *Citrus limetta* were used as bio-reductant for green and sustainable synthesis of silver nanoparticles (AgNPs). As-synthesised AgNPs were characterized by using UV–vis spectroscopy, Dynamic light scattering, and High Resolution Transmission Electron Microscopy. TEM studies revealed 9–46 nm size range of synthesized AgNPs. The antimicrobial and antioxidant activities were also studied by using Agar well diffusion method and DPPH Assay, respectively. Nanoparticles showed good antimicrobial activity against both Gram positive (*S. aureus*) and Gram negative (*E. coli*) bacteria. Further, bioactivity assays revealed selective cytotoxicity (anticancer) of the nanoparticles against human lung cancer cell line A549. The nanoparticles are able to induce cancer cell specific apoptosis at G0/G1 phase of cell cycle. The results showed potential mechanism of action of nanoparticles via augmentation of antioxidant system in cancer cells. Over all, this study show multifaceted potential bioactivities of nanoparticles generated from fruit waste.

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Introduction

Nowadays green chemistry is an emphasized area of research and require some additional efforts for the implementation of sustainable methods in order to achieve the desirable products as well as to minimize and further eliminate the waste materials produced (Anastas and Warner, 1998; Raveendran et al., 2003). Nanotechnology, an emerging field of research not only concise to materials instead include biological, medical and engineering also, hence introduced nanobiotechnology, a novel multidisciplinary approach which serves as domineering technique in developing and congregation of the nanoparticles via green methods for biomedical applications (Iravani et al., 2014). Nanoparticles have

an ample of applications in various fields such as energy science, catalysis, light emitters, photo-electrochemical, single electron transistors, mechanics, optics, electronics, optoelectronics, non-linear optical devices, space industries, chemical industries, biomedical sciences, drug delivery, tissue engineering, food, health care and cosmetics (Gupta and Gupta, 2005; Iravani et al., 2014; Scaramuzza et al., 2016; Wang and Herron, 1991). Among various nanoparticles such as Ag, Au, Ce, Pd, Pt and Zn, (Ahmed et al., 2016b, 2017a) AgNPs are well known for their constructive and beneficial inhibitory biomedical properties such as antibacterial (Sharma et al., 2009), antifungal (Panáček et al., 2009), antiviral (Trefry and Wooley, 2012), larvicidal activity, anti-inflammatory (David et al., 2014), and anti-angiogenesis (Baharara et al., 2014; Veerasamy et al., 2011) against different microbes, bacteria and fungi. Amid them, AgNPs also exhibits good catalytic activity with favourable thrombolytic as well as anticoagulant applications (Azeez et al., 2017b).

Conventionally, variable methods have been developed till date such as physical methods including thermal decomposition (Esumi

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et al., 1990), evaporation-condensation or laser ablation (Scaramuzza et al., 2016) and chemical methods involving reduction of solution to form metal clusters (Goia and Matijevic, 1998), photochemical reactions in reverse micelles (Taleb et al., 1997), sonochemical (Zhu et al., 2000), electrochemical (Rodríguez-Sánchez et al., 2000), radiation assisted (Henglein, 1999, 2001) and recently via microwave assisted techniques (Chen et al., 2013). As these methods utilized precarious and environmentally lethal chemicals, required high energy, or low material conversions as well as tedious purification, hence arose different issues while going through these processes mainly regarding stabilization and aggregation of nanoparticles (Iravani et al., 2014). On the other hand, biogenic synthesis is an eco-friendly and sustainable one-pot synthesis route to synthesize the AgNPs. This method provides advancement over physical as well as chemical method as there is no need to use toxic chemicals, high energy, high temperature and pressure. Generally, plant leaf extract (Ahmed et al., 2016c), fruit peel extract (Bankar et al., 2010), microbes (Fariq et al., 2017; Hulkoti and Taranath, 2014), fungi (Anand et al., 2015) and enzymes (Liu et al., 2016) have been used as a source to synthesize nanoparticles. Furthermore, the agricultural wastes (Kumar et al., 2012), plant-derived pigments (Adelere and Lateef, 2016) and metabolites of arthropods (Lateef et al., 2016e) have also been used as a source of green synthesis of metallic nanoparticles.

This is due to the easy availability of plants, cost effectiveness, rapidity and proficient production. Recent literature revealed the enthusiasm of green route to synthesize AgNPs, for example, *Azadirachta indica* leaf extract (Ahmed et al., 2017b), Banana peel extract (Bankar et al., 2010), Mangosteen leaf (Veerasamy et al., 2011), *Citrus sinensis* peel (Kahrilas et al., 2014), *Mangifera indica* leaf (Philip, 2011), *Terminalia arjuna* leaf (Ahmed and Ikram, 2015), *Murraya koenigii* leaf (Philip et al., 2011), *Punica granatum* peel (Ahmad et al., 2012), *Cinnamomum zeylanicum* leaf (Gauthami et al., 2015), and so on as given in Table 1.

As per green synthetic strategy, renewable as well as non-toxic materials should be used and we here too utilized a completely environmentally benign method in order to synthesize the AgNPs from the fruit waste i.e. peel, generated from Citrus fruits namely, *Citrus limon* (lemon), *Citrus limetta* (mosambi) and *Citrus sinensis* (orange). India ranks sixth in the production of citrus fruits and so as produce their waste materials i.e. peel. Citrus fruits are widely cultivated in tropical and subtropical regions in dried and arid condition. *C. limetta* is also known as sweet lime and in India it is popular as Mosambi, having a large number of benefits regarding

health issues, like scurvy, peptic ulcer, diabetes, skin, hair, etc. Similarly, *C. sinensis* and *C. limon* have medicinal properties and hence found vast applications in various fields regarding health problems such as cancer, cormunative, stomach imbalance, diuretic and colic, bacterial infection and weak immunity besides cosmetics, skin and hair (Hussain et al., 2015; Nisha et al., 2014). Moreover, these citrus fruit waste have potent antimicrobial activity against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Shigella flexneri*, *Candida parapsilosis* and *Candida albicans* (Chabuck and Chabuck, 2014; Dubey et al., 2011; Panáček et al., 2009). Furthermore, antioxidant property of AgNPs from green methods have also been observed by many researchers and scientists mainly on leaf and flower extract (Aksoy et al., 2013; Phull et al., 2016). Cytotoxicity or cell viability of AgNPs have been observed by some researchers (Foldbjerg et al., 2011) but in this article, antioxidant and cytotoxicity was observed on blood cell as well as lung cancer cell line A549, of biologically synthesized AgNPs via a green and sustainable route from the waste materials produced, mainly for environmental concerns.

Materials and methods

Fresh lemons (*Citrus limon*), S1, oranges (*Citrus sinensis*), S2 and mosambi (*Citrus limetta*), S3 were procured from a local market in New Delhi, India. Silver nitrate was purchased from Merck Chemicals, Mumbai, India and was used as purchased. Mackonkey broth and Mackonkey Agar was purchased from HiMedia, Mumbai. Fresh blood around 5 ml have been drawn from healthy individual as per the protocol number CUPB/cc/14/IEC/4483, Government of India. For blood cells, RPMI-1640 media having 5% fetal calf serum (FCS), and RBC lysis buffer (1×) were obtained from Invitrogen. CellROX[®] Deep Red from Invitrogen. All the cell lines were acquired from National Cell Repository, NCCS, Pune, India. For lung cancer cell line, A549, DMEM media augmented with 10% fetal bovine serum (FBS) and 1 × antibiotic solution with 1% Pentsrip (50 U/ml penicillin G, 50 Ig/ml streptomycin sulfate and 1.25 Ig/ml amphotericin B) were obtained from Invitrogen. DMSO solution, Dihydroethidium (DHE) and Butylhydroxytoluene (BHT) all were purchased from Sigma-Aldrich and used without further purification. All the glassware was washed thoroughly with chromic acid followed by distilled water and then transferred to oven for being dried before the experiment.

Table 1
Comparison of different source and their biosynthesized nanoparticles.

Material required	Morphology	Size	Applications	References
Leaf extract of <i>Adiantum raddianum</i>	Spherical, Truncated triangles, and Decahedron	10.9 nm	Toxicity against larvae of malaria and filariasis vector	Govindarajan et al. (2017)
<i>Lactobacillus</i> bacteria	Spherical	30–100 nm	Antioxidant activity	Dakhil (2017)
Activated sludge micromycetes, <i>Fusarium nivale</i> , <i>Fusarium oxysporum</i> , <i>Penicillium glabrum</i>	–	more than 100 nm 30–50 nm	Metal ion toxicity	Tyupa et al. (2016)
<i>Bacillus safensis</i> LAU 13	–	20–30 nm ~8.3 nm	–	Lateef et al. (2015)
Leaf and seed extracts of <i>Synsepalum dulcificum</i>	Spherical	4–26 nm	Antimicrobial, catalytic, anticoagulant and thrombolytic applications	Lateef et al. (2016a)
Pod extract of <i>Cola nitida</i>	Spherical	12–80 nm	Antioxidant activity and phytochemical contents of <i>Amaranthus caudatus</i>	Azeez et al. (2017a)
Cocoa pod husk extract	Spherical	4–32 nm	Antimicrobial, antioxidant and larvicidal activities	Lateef et al. (2016d)
Nest extract of paper wasp (<i>Polistes</i> sp.)	Hexagonal, rhomboid, 3-D triangular, rod and spherical	12.5–95.55 nm	Antimicrobial, catalytic, anticoagulating and thrombolytic applications	Lateef et al. (2016b)
Cell-free extract of <i>Bacillus safensis</i> LAU 13	Spherical	5–95 nm	Anti-candida, anti-coagulant and thrombolytic activities	Lateef et al. (2016f)

Biosynthesis of silver nanoparticles

Preparation of fruit waste (peel) extract (FWE)

First of all, fruit waste (peel) of S1, S2 and S3 were washed with distilled water for removing the dust and debris and then again thoroughly washed with double distilled water. These peels were air dried and about 10 g each S1, S2 and S3 were weighed respectively. After that they were transferred to round bottom flask containing 250 ml of double distilled water and then each were heated to boiled below 60 °C for half an hour independently. Each FWE thus obtained were filtered using Whatman No. 1 filter paper and collected in glass media bottle to store in refrigerator for further experimental usage.

Bio-reduction of AgNPs

The source of silver for all experiments was aqueous silver nitrate. 250 ml 4 mM silver nitrate aqueous solution was prepared in an Erlenmeyer flask. Then 10 ml of prepared silver nitrate was added to different concentrations of FWE in separate beakers further placed in dark chamber in order to minimize the photo-reduction of silver nitrate solution at room temperature. In lieu of varying concentration of silver nitrate, the concentration of FWEs were varied and change in colour of solution was observed from colourless to yellowish to reddish brown and further characterized by UV–vis spectroscopy, Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM) of the respective samples and finally confirmed that these waste materials generated from fruits can also be used as advantageous candidate for bio-reduction of Ag⁺ to Ag⁰.

Characterisation techniques

UV–vis spectroscopy

UV–vis spectra were collected by using the instrument Shimadzu (UV-1800, Japan) at room temperature. The appropriate aqueous fruit peel extracts were taken as blank with same cuvette throughout the experiment with 1 nm resolution from 350 to 700 nm on the basis of literature available.

Dynamic light scattering

Recently this technique is also known as quasi-elastic light scattering. It has proven to be an invaluable analytical tool for characterising the size distribution of particles suspended in the solvent. The size of synthesized AgNPs were analysed by DLS with the help of instrument DLS Spectroscatter 201.

Transmission electron microscopy

The morphology of the surface as well as size of the synthesized nanosilver was confirmed by TEM using the instrument 300 kV HRTEM TECHNAI G2 30S TWIN, operated at 3000 kV accelerated voltage.

Cytotoxicity analysis

The cell toxicity was evaluated using MTT assay. The human peripheral blood mononucleated cells (hPBMN) were seeded in 96-well culture plates and maintained in a RPMI-1640 medium supplemented with antibiotics and 5% FCS (Fetal Calf Serum). Cells were treated with six different concentrations (0.5, 1, 5, 10 and 50 µg/ml) for each S1, S2 and S3 with their respective extract E1, E2 and E3 (1, 0.2 and 10 µg/ml) (Supplementary Fig. 1) and non-treated cells were used as control. Cell viability was evaluated under three time periods i.e. 24 h, 48 h and 72 h at 37 °C with 5% CO₂. Then, 20 µl of MTT solution (5 mg/ml) were added to each well and incubated for 3 h in dark at 37 °C. Added 100 µl DMSO and incubated for 30 min. The resultant formazan formed by viable cells were measured using a multi-mode plate reader at a wavelength of 570 nm.

Antimicrobial assay

Prokaryotic bacteria, for example, *E. coli* and *S. aureus* were used as a source for examining antimicrobial activity using Agar well diffusion method. To subculture the bacteria, Mackonkey broth (HiMedia) as nutrient medium were prepared and incubated at 37 °C for 24 h and then it was kept for overnight. After that cultivation of bacteria was done by spreading fresh overnight cultures on Mackonkey nutrient agar plates. In each plate, double distilled water was used as control and 5 mm diameter hole was saturated with FWE and AgNPs and then again incubated at 37 °C for 24 h. Hence, on the basis of inhibition zone around the hole infused with both FWE and AgNPs antimicrobial activity were reported.

Antioxidant activity of nanoparticles

The DPPH free radical scavenging activity was evaluated using method of Marinova and Yanishlieva (1997) with few modifications. Three hundred microlitre of 200 µM DPPH (in Methanol) was added to one hundred microliter S1, S2, S3 (10 µg/ml) and their respective extract (10 µg/ml) and BHT concentration (50 mg/ml and 100 mg/ml) as standard. After mixing, the mixture was incubated at temperature 37 °C in dark environment for 30 min. Methanol (CH₃OH) was used as blank for the appropriate absorbance with wavelength 517 nm using a multi-mode plate reader. The scavenging performance of free radicals was determined with the help of a formula as follows:

$$\% \text{ Scavenging} = \frac{\text{Absorbance control} - \text{Absorbance of Test Sample}}{\text{Absorbance of Sample}}$$

Intracellular ROS production using DHE staining

In order to evaluate the intracellular Reactive Oxygen Species (ROS), the dihydroethidium (DHE) stain (non-fluorescent) was used in A549 cells, seeded in 96-well plates at $1.0\text{--}1.2 \times 10^4$ cells per well. The H₂O₂ treatment was used as positive control. The cultured cells were treated with S1, S2 and S3 (5 and 10 µg/ml) and their respective extract (10 µg/ml) for 48 h. Then, the DHE solution was added and incubated for 30 min at 37 °C in absence of light which becomes fluorescent upon superoxide oxidation. The fluorescence with an excitation and emission wavelength of 518 nm and 605 nm respectively were measured with the help of a microplate reader.

Intracellular ROS production using CellROX[®] Deep Red staining

The generation of intracellular ROS was detected by using the fluorescent probe CellROX[®] Deep Red. The cells were treated with S1, S2, S3 and their extract (10 µg/ml) for 24 h and exposed to CellROX[®] Deep Red (5 µM for 30 min) at 37 °C under 5% CO₂ atmosphere in the dark. Furthermore, the fluorescence was measured at 633 nm and 665 nm excitation and emission wavelength respectively.

Cell cycle analysis

Cells were treated with S1 and S2 (10 µg/ml) and their respective extracts (10 µg/ml) for 24 h for cell cycle analysis and uniformly fixed in ice cold 70% ethanol for staining. About 200 µl of ethanol-fixed cells processed according to Muse[™] cell cycle analysis kit. Then, the cell suspension sample transferred to a micro centrifuge tube prior to analysis on Muse[™] Cell Analyzer.

Results and discussion

Visual observation

On adding varying concentration of FWE to 10 ml of 4 mM silver nitrate solution, there is a colour change in the solution from colourless to yellowish and then to reddish brown after 30 min of

Fig. 1. Visual representation of biosynthesis of AgNPs with varying concentration of FWE.

addition, indicating the deepening of colour with increase in time (Fig. 1). This colour change inferred the formation of AgNPs, due to the excitation of surface plasmon vibrations. When the electrons of conduction band oscillate or vibrate collectively in resonance, then there exhibit a unique optical property known as Surface Plasmon Resonance (SPR) which in turn responsible for the absorption ranging between 400 and 500 nm in UV–vis spectra for the synthesized silver nanoparticles. Out of the citrus FWE engaged in the experiment, *C. limon* comparatively took more time in the formation of AgNPs. This method of using fruit waste as bio-reductant is time saving with complete reduction of silver as reported in published literature (Lateef et al., 2016a; Kaviya et al., 2011; Prathna et al., 2011).

UV–vis spectral studies

The colour change inferred the formation of AgNPs and it was further confirmed by the UV–vis spectrophotometer. In order to determine the minimum time required for colour change, the experiment was performed at varying time interval and simultaneously their UV–vis spectral data were recorded. It was thus found that overall time required in synthesizing AgNPs were about 30 min. for S2 and S3; and 1 h for S1 (Fig. 2). The concentration of aqueous solution of each of FWE was 1 ml, 3 ml, 5 ml and 7 ml keeping silver nitrate concentration constant. It was observed that the absorption wavelength of AgNPs for S1 were 448 nm, 440–450 nm for S3 and 442–460 nm for S2. Lateef et al. (2016c) showed

Fig. 2. UV–vis spectra of (a) S1, (b) S2 and (c) S3 at varying concentration of 1 ml, 3 ml, 5 ml, 7 ml in 4mM silver nitrate solution.

431.5 nm wavelength for *Cola nitida* synthesized AgNPs and hence in good agreement with our results. A red shift in S2 has been observed on increasing the time interval of each concentration. This red shift was due to the holes injected to the clusters according to Henglein (1993) theory which deals with the actions of the plasmon shifts observed for bare clusters. Literature confirms many bio-organic compounds found in these citrus FWE such as *n*-octanal, perilla aldehyde, 2,6-dimethyl-2,6-octadiene-1,8-dial, 4-isopropenyl-1-methyl-1,2-cyclohexanedial, limonene, β -linalool, α -terpineol, geraniol, 1-octanol, β -linalool, 1-nonanol, 2-(4-methylenecyclohexyl)-2-propen-1-ol, perilla alcohol and 8-hydroxylinalool (Kahrilas et al., 2014). The concentration of each extract was optimized to 1 ml, and was used for further evaluation of AgNPs.

Transmission electron microscopy (TEM)

The shape and size of the nanoparticles were further evaluated and confirmed by using HRTEM analysis. The high resolution HRTEM studies revealed that the synthesized AgNPs of citrus FWE were spherical in shape and polydispersed and irregular. The measured size of these nanoparticles was in the range of 9–46 nm and the electron diffraction pattern has also been analysed as shown in Fig. 3. This data has been found in good agreement when

compared with the reports available (Durand and Storz, 2017; Philip, 2011). For instance, Kaviya et al. (2011) showed 35 ± 2 nm size range at room temperature and Lateef et al. (2016c) revealed size range of 12–80 nm of biologically synthesized AgNPs.

Dynamic light scattering (DLS)

The average size of AgNPs analyzed by DLS spectrometer was found to be 37.1 nm, 86.4 nm and 98.1 nm synthesised by S1, S2 and S3, respectively. The measured size of nanoparticles using DLS technique was large as compared to the TEM measurements. This is due to the fact that the water molecules present in the solution sample leads to increase the size of nanoparticles and this technique measured the hydrodynamic radius of the particle examined. The DLS histograms of the synthesized AgNPs from all the three FWE solution have been depicted in Fig. 4. Anandalakshmi et al. (2016) revealed similar results of particle size distribution in the range of 10–150 nm (average size 73.14 nm) from the leaf extract of *Pedaliu murex*.

Antimicrobial assay

Silver have been used in varying field of sciences and technology from ancient times and extend prestige from last ten

Fig. 3. TEM images of green mediated synthesized AgNPs from (a) S1, (b) S2 and (c) S3.

Fig. 4. DLS histograms of AgNPs of solution (a) *C. limon* (S1), (b) *C. sinensis* (S2) and (c) *C. limetta* (S3).

years due to its inherent antimicrobial property (Ahmed et al., 2016a). Generally, the inherent antimicrobial efficacy of green synthesized AgNPs were found to be dependent on the shapes and size of the nanoparticles and being confirmed by studying the zonal inhibition of bacterial growth by differentially shaped nanoparticles (Morones et al., 2005). As we have synthesized the AgNPs via green route so their antimicrobial assay was immediately done against both Gram positive, *S. aureus* as well as Gram negative, *E. coli* bacteria (Fig. 5 and Table 2) so as to show their efficacy by inhibition zone. In this study, each FWE and control independently, didn't possess any antimicrobial activity against these bacteria (Nisha et al., 2014). *C. sinensis* showed highest zone of inhibition of 28 mm against *S. aureus* whereas *C. limetta* showed 20 mm against *E. coli*. Lesser antibacterial activity of AgNPs is observed by *C. limon* for 20 μl/ml. The study presented similar results as revealed in the literature available (Lateef et al., 2016c). The mechanism proposed for this can be described as: the extremely large surface area of AgNPs provide a better contact with the cell wall of the bacteria. Thus, the nanoparticle interacts with the DNA and S-containing proteins of the bacterial cell wall and finally penetrates into the cell creating a reservoir of low molecular weight region at the centre and attacks the mitochondria, cell division lead to death of the cell. Releasing of the Ag⁺ ions in the bacterial cells enhanced the bactericidal activity (Ahmed et al., 2016a; Feng et al., 2000).

Green nanoparticles with anti-cancer properties

Cell viability was evaluated by doing MTT [3-(4, 5-dimethylthiazolyl)-2, 5-diphenyl-tetrazolium bromide] assay which involves

the reduction of MTT by mitochondrial dehydrogenase to purple formazan in living cells. This reflects the functioning of mitochondria of viable cells, allowing the measurement of cell viability and cytotoxicity.

Reaction involved:

In this study, effect of silver nanoparticles were evaluated on the growth of these cells by MTT assay using A549 cancer cell lines at different concentrations (0.5, 1, 5, 10 and 50 μg/ml) and at three different time periods (24, 48 and 72 h).

Our data revealed that all the samples showed very high anti proliferative activity at 50 μg/ml at all the three time points (Fig. 6A–C) while at 0.5 μg/ml approximately 20–30% inhibition at 72 h which was proven non-toxic at lower time points. Similarly, concentrations of 5 and 10 μg/ml also showed significant reduction in cell numbers at all the three time points. There were 50% of inhibition at concentrations 5 μg/ml and 10 μg/ml after 24 h of treatment with silver nanoparticle S1, whereas maximum inhibition (80%) was observed at highest concentration of 50 μg/ml as shown in Fig. 6A. Growth inhibition

Fig. 5. Visual representation of antimicrobial activity shown by AgNPs against *E. coli* and *S. aureus*.

Table 2

Antimicrobial zone of inhibition (mm) obtained by Agar well diffusion method.

Sample		Zone of Inhibition (mm)	
		<i>E. coli</i>	<i>S. aureus</i>
1.	Control	NZ	NZ
2.	<i>Citrus sinensis</i>	17	28
3.	<i>Citrus limetta</i>	20	18
4.	<i>Citrus limon</i>	7	8

NZ* = No Zone of inhibition.

by silver nanoparticles S1 and S2 in 48 h were 20% and 30% respectively whereas S3 showed 50% of inhibition at 5 $\mu\text{g/ml}$ as depicted in Fig. 6B. The S2 sample Exhibit 80% of inhibition at 10 $\mu\text{g/ml}$, whereas S1, S2 and S3 shows 90% growth inhibition at 50 $\mu\text{g/ml}$. After 72 h of treatment, S1, S2 and S3 (5 $\mu\text{g/ml}$) shows 60%, 46% and 41% respectively, but at 10 $\mu\text{g/ml}$ there were about 61%, 75%, and 52% of inhibition exerted by S1, S2, and S3, respectively as shown in Fig. 6C. The anti-proliferative potential of the pure extracts E1, E2 and E3 is shown in Supp. Fig. 1.

These results indicated that nanoparticles at lower concentration were also able to inhibit cancer cell growth in a time dependent fashion. Similar experiment was repeated for hPBMN cells as normal cell control. The results showed that there is a little or no toxicity of the nanoparticles at different concentrations and even at higher time points no toxicity were observed as depicted in Fig. 6D–F.

AgNPs as potent antioxidant agents

Several natural extract based anticancer potential was due to the presence of strong antioxidants in these extracts. Thus we decided to test the antioxidant potential of the extract coated silver nanoparticles using cell based as well as *in vitro* assays.

The *in vitro* free radical scavenging ability of nanoparticles was determined using DPPH free radical scavenging assay. In this study, DPPH tests demonstrated these nanoparticles as good free radical scavengers (Fig. 7). The 50 and 100 $\mu\text{g/ml}$ concentration of BHT showed scavenging activity about 78.18% and 94.77% respectively when used as reference compound. On the other hand, S1, S2 and S3 showed 87.43%, 67.50%, and 95.13% scavenging activity,

respectively. So, these biologically synthesized nanoparticles exhibit potential to combat the free radicals generated as a result of oxidative stress. In this context, we further monitor the cellular antioxidant activity by inducing oxidative stress using H_2O_2 and pesticides. Antioxidant activity of AgNPs were similar to those found by Lateef et al. (2016c).

The intracellular ROS scavenging was measured by CellROX[®] Deep Red and DHE assay via inducing oxidative stress using H_2O_2 and pesticides. The cells were treated with respective nanoparticles as well as H_2O_2 pre-treatment (one hour) followed by nanoparticles (24 h). Next day, the treated cells were exposed to DHE in absence of light to evaluate intracellular ROS in cell in response to the AgNPs. It was found that the non-fluorescent DHE easily permeates cell membranes that get oxidized by O_2^- and converted to ethidium bromide (fluorescent) intercalates into nuclear DNA. The H_2O_2 treatment results in significant increase in the fluorescence intensity indicating higher intracellular ROS production (Fig. 8A). In contrast, both the concentration of S1, S2 and S3 AgNPs (0.5 and 10 $\mu\text{g/ml}$) demonstrated less intensity as compared to control. Interestingly, the fluorescence intensity decreases in cells treated with H_2O_2 followed with nanoparticles indicating that the AgNPs have potential to quench the induced reactive oxygen species. Similarly, the free radical scavenging activity was evaluated in response to oxidative stress induced using pesticide exposure and H_2O_2 . It was found that the free radicals produced as a result of pesticides, were scavenged by the AgNPs treatment, that enter into the food chain as reported by many researchers (Gonzalez-Rodriguez et al., 2011; Popp et al., 2013). The intracellular ROS generation using fluorescent probe CellROX[®] Deep Red revealed similar results as obtained using DHE. The nanoparticles treatment decreased the oxidative stress induced due to H_2O_2 as well as pesticide treatment (Fig. 8B).

AgNPs actively scavenge induced ROS in lung cancer cells

The cell toxicity of AgNPs and extract on cancer cells as well as on the oxidative stress induced by H_2O_2 has been further studied. The cells were pre-treated with H_2O_2 followed by incubation with AgNPs. The results showed that H_2O_2 is able to increase ROS levels

Fig. 6. Percent Cell viability upon treatment of S1, S2 and S3 green AgNPs on lung cancer cell lines A549 for (A) 24 h, (B) 48 h and (C) 72 h time points and on hPBMN for (D) 24 h, (E) 48 h and (F) 72 h time points.

by more than 2 folds, but in presence of AgNPs, the phenomenon is reversed and ROS levels remain closer to the control cell (Fig. 8A). Interestingly, all samples S1, S2 and S3 showed potent and active quenching of H_2O_2 induced ROS to compare its response with biologically synthesized AgNPs. The results revealed that H_2O_2 cause severe toxicity about 90.4% inhibition in cell viability, but only extract samples protect the cells from stress induced by H_2O_2 whereas the cell death observed with nanoparticle treatment in combination with H_2O_2 treatment. So, this suggests that extract quench the free radicals produced due to H_2O_2 and promote the cell survival (Supp. Fig. 2). Literature available also suggested higher reactive oxygen species in cancerous cell (Durand and Storz, 2017; Zhou et al., 2014).

Fig. 7. Antioxidant activity of S1, S2, S3 nanoparticles using DPPH Assay.

Fig. 8. (A) Intracellular ROS generation evaluation using DHE assay; (B) Intracellular ROS generation induced using H_2O_2 and Pesticide analysis using fluorescent probe CellROX[®] Deep Red.

Fig. 9. (A) Effect of AgNPs and their respective extract on cell cycle; (B) DNA content profile of control; (C) S1; (D) E1 extract; (E) S2 sample; (F) E2 treated samples.

Green nanoparticles induced cell death by causing cell cycle arrest at G1 phase

Since cancer cell specific cytotoxicity by the nanoparticles was evaluated, thereafter the effect of AgNPs on cell cycle were observed. In this study, cells were treated with AgNPs followed by cell cycle analysis using MUSE cell analyzer (Merck-Millipore). Our results displayed peaks at G0/G1 (33.3%), G2/M (16%), S (22.9%) phases for control sample. In contrast, the S1 nanoparticle treatment results increase in 10.21% G0/G1 peak values and decrease of 26.25% G2/M whereas extract decreased S and G2/M phase about 34.5 and 75% respectively, (Fig. 9) with respect to control sample. On the other hand, the S2 and E2 treatment resulted increase of 31.83% and 63.96% of G0/G1 phase respectively, whereas S and G2/M phase decrease about 28.38% and 33.13% (S2 treatment), 80.35% and 87.5% respectively (E2 treatment). The results of cell cycle analysis uncovered that A549 cells are arrested at the G0/G1 checkpoint with AgNPs treatment as compared to untreated cells. The prime reason for the cell cycle arrest was DNA damage and chromosomal aberrations as reported by AshaRani et al. (2009).

Conclusion

In this study, peels of three citrus fruits namely *C. limon*, *C. limetta* and *C. sinensis* have been used as waste material to successfully synthesize the AgNPs and accomplished under room temperature. We have demonstrated the cost effective, time saving, renewable, green and sustainable route for AgNPs to be formed. The FWE itself acts as bio-reductant, stabilising as well as capping agent. The confirmation for the formation of nanoparticles were observed by colour change and UV-vis spectra obtained and further the size and shape determination has been done by DLS and HR-TEM. The size of the synthesized spherical AgNPs were found to be in the range of 9 to 46 nm. These

nanoparticles showed respectable antimicrobial activity against *E. coli* and *S. aureus* (20 μ l/ml). Moreover, DPPH assay revealed potent antioxidant activity of these AgNPs. Besides, cause apoptosis by cell cycle arrest at G1 phase and hence these biogenically AgNPs are found to be quite safe for normal human cells when tested *in vitro*. Overall, the study showed that the nanoparticles which we synthesized from the waste material (peel) of the citrus fruits not only exhibit the antimicrobial activity but also possess beneficial antioxidant and anticancer activity as well. This may open a new avenue of anticancer therapies which requires further exploration.

Author's contributions

Annu and SA have biologically synthesised and characterized AgNPs. SS and SI have designed all the biological experiments. GK and PS have performed various biological assays in primary cells as well as cancer cell lines.

Conflict of interests

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.jab.2018.02.002>.

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