Antioxidant and Radical Scavenging Activity of Silver Nanoparticles Synthesized by *Punica granatum* Fruit Peel Extract

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Abstract

Synthesis of silver nanoparticles by biological methods has been suggested as possible eco-friendly alternative to chemical and physical methods. In this present study, silver nanoparticles (AgNPs) were synthesized by the aqueous extract of *Punica granatum* fruit peel extract which was used as reducing and capping silver nanoparticles. The antioxidant activity of the synthesized silver nanoparticles were evaluated in the present study using various *in vitro* methods such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) ($ABTS^+$) assay, Hydroxyl radical scavenging (HRS) assay and Nitric oxide scavenging (NOS) assay. The results indicated that the synthesized AgNPs had dose dependent antioxidant activity as compared to ascorbic acid the standard reference used. The antioxidant activity of these nanoparticles may be attributed to the polyphenolic compounds of plant extracts which are responsible for the silver nanoparticle formation and hence these silver nanoparticles are found to have potential application to reduce oxidative stress with health benefits.

Keywords: *Punica granatum* fruit peel, Antioxidant activity, DPPH assay, $ABTS^+$ assay, HRS assay.

1. Introduction

Aerobic organisms need oxygen for their survival but it may become toxic if supplied at superior concentrations level. Dioxygen in its ground state is relatively unreactive; its incomplete reduction gives rise to reactive oxygen species (ROS) such as singlet oxygen, super oxide anion radical, hydroxyl radical, hydrogen peroxide etc. These ROS are formed as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis. However, during the times of environmental stress (e.g., UV or heat exposure), ROS levels can increase dramatically [1]. This may result in significant damage to cell structures. Cumulatively, this is known as oxidative stress. ROS are also generated by exogenous sources such as ionizing radiation. A free radical can be defined as any molecular species capable of independent existence that contains an unpaired electron in an atomic orbital. Many radicals are unstable and highly reactive. They can either donate an electron to or accept an electron from other molecules, therefore behaving as oxidants or reductants [2]. The most important oxygen-containing free radicals in many disease states are hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, nitric oxide radical and peroxynitrite radical. These are highly reactive species, capable of damaging biologically relevant molecules such as DNA, proteins, carbohydrates and lipids [3]. Thus, free radicals lead to cell damage and homeostatic disruption. Targets of free radicals include all kinds of molecules in the body. Among them, lipids, nucleic acids and proteins are the major targets.

In plants and animals these free radicals are deactivated by antioxidants. These antioxidants act as an inhibitor of the process of oxidation, even at relatively small concentration and thus have diverse physiological role in the body. The body is constantly exposed to the negative and lethal effects of oxidants during normal physiological processes [4]. Generally, up to 5% of inhaled oxygen may be converted to reactive oxygen species (ROS) which may cause oxidative stress. It is raised as a result of an imbalance between free radical production and antioxidant defenses. Oxidative stress is now thought to make a significant contribution to all inflammatory diseases and many others [5], [6]. The protective mechanism is capable of intercepting or stopping the chain reactions which are typical of free radicals, converting them into less harmful molecules. As a result, antioxidants efficiently repair oxidative damage in cells [7]. For more than fifty years, synthetic antioxidants have been used as food additives to prevent peroxidation of fats and oils. Butylated-hydroxytoluene (BHT), butylated-hydroxyanisole (BHA), tertbutyl hydroquinone (TBHQ) are effective and common antioxidants preventing oxidation and off-flavor development in fats and oils. However, those chemicals are now doubted for their safety and they are associated with health risks in their use in food products [8].

Therefore, the attention is now increasingly paid to the development and utilization of more effective, natural and non-toxic antioxidants from natural sources such as plants. Medicinal plants used in the traditional medicine are well-known significant sources of natural antioxidants. Medicinal plants-derived natural antioxidants, which are in the form of raw extracts and chemical constituents, are very efficient to block the process of oxidation by neutralizing free radicals [9]. The antioxidant property of many plant materials are due to the presence of active phytochemicals which include the vitamins, poly phenols, flavonoids, terpenoids, carotenoids, cumarins, saponin, plant sterols etc [10]. Poly phenols and flavonoids in plant crude extract can act as reducing agents for the reduction of Ag^{+} ions. As these polyphenols and flavonoids are used as antimicrobial and antioxidant agents by the plants to protect themselves from various pathological conditions, the silver nanoparticles synthesized from them also have antioxidant activity [11].

Fruits and vegetables wastes and by-products, which are formed in great amounts during industrial processing, represent a serious problem as they exert an influence on environment and need to be managed and utilized. On the other hand, these waste materials are very rich in bioactive components, which are considered to have a beneficial effect on health. For the last decade, efforts have been made to improve methods and ways of re-using fruits and vegetables wastes. The important purpose is the valorization of the antioxidants and other biocomponents in by-products from fruit and vegetable industries [12]. The peels and pomace are a source of sugars, minerals and organic acids, dietary fibers and phenolics which have a wide range of actions which includes antioxidants, antimutagenic, cardio preventive, antibacterial and antiviral activities. Use of waste as a source of polyphenols and antioxidants may have considerable economic benefit to food processors [13].

The *Punica granatum* (Pomegranate) is one of the oldest edible fruits and is widely grown in many tropical and subtropical countries [14]. Most pomegranate fruit parts are known to possess substantial antioxidant activity. The flower, seed oil, seed extract and peel extract of pomegranate have a potent antioxidant activity [15]. There are reports for the use of water decoction of pomegranate peel powder as a multifunctional vaginal suppository and for the prevention and cure of venereal disease [16]. Previous researches reported that *P. granatum* peel possess antioxidant activity besides their antibacterial activity. The antioxidant potential of the *P. granatum* peel is mainly depends upon the amount of phenolic compounds present in the peel [17].

The phytochemical screening of *P. granatum* fruit peel aqueous extract showed the presence of polyphenols and flavonoids which may be responsible for the reduction of silver nitrate to silver nanoparticles. So the antioxidant potential of silver nanoparticles synthesized using the aqueous extract of *P. granatum* fruit peels were evaluated in the present study using various *in vitro* methods such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) ($ABTS^{+}$) assay, Hydroxyl radical scavenging (HRS) assay and Nitric oxide scavenging (NOS) assay

2. Materials and Methods

2.1 Synthesis of Silver Nanoparticles

20g of pomegranate fruit peel was weighted and added in 100 ml of distilled water in 250 ml Erlenmeyer flask and boiled for 10 minutes. With the help of Whatmann filter paper (NO.3), the boiled materials were filtered to get aqueous fruit peel extract which was used as such for metal nanoparticles synthesis. 1mM aqueous solution of silver nitrate was prepared for 100 ml. To this 5 ml of filtrate was added and kept for incubation with intermittent shaking. The formation of dark brown colour was observed after 6 hr incubation at room temperature and λ max was taken using UV-Visible spectroscopy from 200-800 nm. It was characterized by Fourier Transform Infrared Spectrometer (FTIR) analysis, X-ray Diffraction (XRD), Scanning Electron Microscopy (SEM), Energy-dispersive X-ray spectroscopy (EDX), Transmission Electron Microscopy (TEM). After characterization, these nanoparticles were used for the detection of antioxidant activity.

2.2 Antioxidant Activity

2.2.1 DPPH Radical Scavenging Activity

4ml of different concentrations (20, 40, 60, 80 and 100µg/ml) of silver nanoparticles were individually mixed with 1.0 ml of methonolic DPPH to a final concentration of 0.2 mM of DPPH. The mixture was shaken well and left to stand for 30 min. DPPH radical is scavenged by antioxidants through the donation of a proton which the results in the formation of reduced DPPH. The colour change from purple to yellow after reduction can be quantified at 517 nm in UV-Visible Spectrophotometer against methanol as a blank. DPPH methanol reagent without sample was used as control and Vitamin C as standard. The percentage of inhibition was calculated by using the following formula [11].

% of inhibition= $[(Absorbance_{control} - Absorbance_{test}) / Absorbance_{control}] \times 100$

2.2.2 $ABTS^+$ Assay

$ABTS^+$ decolourisation assay involves the generation of the $ABTS^+$ chromophore by the oxidation of ABTS with potassium persulphate. The ABTS radical cation was prepared by mixing equal quantities of 7 mM ABTS solution and 2.45 mM of potassium per sulfate solution and incubating the mixture at room temperature in dark for 16 h. ABTS working solution was obtained by diluting it with methanol to give an absorbance of 0.85 ± 0.20 at 734 nm. 1.0 ml of different concentration (20, 40, 60, 80 and 100 µg/ ml) of silver nanoparticles and fraction of diluted methanol was added to 3.0 ml of ABTS working solution. The reaction mixture was incubated at room temperature for 5 min and then the absorbance was measured at 734 nm using UV-Vis Spectrophotometer against ABTS without sample as blank sample. Vitamin C was used as a positive control. [18], [19]. The inhibition was calculated according to the equation,

Percentage of Inhibition = $A_0 – A_1/A_0$ x 100

Where,

A_0 = Absorbance of control

A_1 = Absorbance of Sample

2.2.3 Hydroxyl radical scavenging activity

The ability of silver nanoparticles to scavenge hydroxyl radicals was determined according to the method of Elizabeth and Rao [20]. The hydroxyl radical attacks deoxyribose, which results in thiobarbituric acid reacting substance (TBARS) formation. The reaction mixture was prepared with deoxyribose (2.8 mM), ferric chloride (0.1 mM), ethylene diamine tetra acetic acid (0.1 mM), hydrogen peroxide (1 mM), ascorbic acid (0.1 mM), potassium hydrogen phosphate/potassium hydroxide buffer (20 mM, pH 7.4) and various concentrations (50,100,150,200 and 250 µl/ml) of the silver nanoparticles. The reaction mixture was incubated for 1 h at 37°C. Deoxyribose degradation was measured as TBARS and percentage inhibition was calculated. Deoxyribose degradation was measured as Thiobarbituric acid reactive substances (TBARS) by adding 0.5 ml of (TBA) and 0.5 ml of (HCl) Hydrochloric acid, boiled in a water bath for 20 min, cooled and measured the absorbance at 532 nm (Madhe, 2014). The scavenging activity of hydroxyl ion by silver nanoparticles and the standard (vitamin C) was calculated using the formula,

Scavenging activity (% inhibition) = $(A_0-A_1)/A_0 \times 100$

Where,

A_o = Absorbance of control

A_1 = Absorbance of sample

2.2.4 Nitric oxide scavenging activity

The reaction mixture (3 ml) containing 2 ml of sodium nitro prusside, 0.5 ml of phosphate buffered saline and 0.5 ml of different concentration (50,100,150,200 and 250 µl/ml) of silver nanoparticles was incubated at 25°C for 30 min. Control without test compound was kept in an identical manner. After incubation, 0.5 ml of Griess reagent was added. The absorbance of the chromophore formed was read at 540 nm and the percentage inhibition was calculated by the following formula [21], [22].

Scavenging activity (% inhibition) = $(A_0 - A_1) / A_0 \times 100$

Where,

A_0 = Absorbance of control

A_1 = Absorbance of sample.

3. Results and Discussion

3.1 Synthesis of Silver Nanoparticles

The characterization of synthesized silver nanoparticles by the aqueous extract of *P. granatum* peels were found to be 17.2nm in diameter and spherical in shape.

3.2 Antioxidant activity

3.2.1 DPPH radical scavenging activity

The silver nanoparticles synthesized using the aqueous extract of *P. granatum* peels showed maximum DPPH scavenging activity of 60.25% whereas, ascorbic acid (standard) exhibited 63.15% at concentration of 100 µg/ml (Table.1). The present study revealed that the silver nanoparticles synthesized using the aqueous extract of *P. granatum* peels showed increasing DPPH radical scavenging activity with increasing concentration. The antioxidant potential of silver nanoparticles could be attributed by functional groups adhere to nanoparticles from fruit peel extract.

Table 1: DPPH radical scavenging activity

Concentration (µg/ml)	Ascorbic acid Inhibition (%)	PgFP AgNPs Inhibition (%)
20	19.11 ± 0.55	12.33 ± 0.25
40	35.22 ± 0.47	25.70 ± 0.55
60	48.97± 0.81	40.05 ± 0.36
80	57.15 ± 0.74	53.77 ± 0.70
100	63.15 ± 0.33	60.25 ± 0.25

Values are mean ± S.D of three replicates

3.2.2 $ABTS^+$ Assay

ABTS, a protonated radical, has characteristic absorbance maxima at 734 nm which decreases with the scavenging of the proton radicals [23]. $ABTS^+$ is generated by oxidation of such ABTS with potassium persulfate and is reduced in the presence of such hydrogen - donating antioxidants. The influences of both the concentration and duration of reaction on the inhibition of the radical cation were taken into account when determining antioxidant activity. The percentage scavenging of the silver nanoparticles synthesized using the aqueous extract of *P. granatum* peels and the standard ascorbic acid were showed in Table 2.

Table 2: 2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) ($ABTS^+$) assay

Concentration (µg)	Ascorbic acid Inhibition (%)	PgFP AgNPs Inhibition (%)
20	22.45 ± 0.92	14.67 ± 0.34
40	33.22 ± 0.44	28.27 ± 0.47
60	46.88 ± 0.31	39.25 ± 0.83
80	58.78 ± 0.55	50.35 ± 0.65
100	68.89 ± 0.34	61.48 ± 0.15

Values are mean ± S.D of three replicates

The results showed that, there was a concentration dependent increase in the $ABTS^+$ radical scavenging activity of the silver nanoparticles obtained from the aqueous extract of *P. granatum* peels. They showed the maximum inhibition of 61.48% at a concentration of 100 µg/ml which was lesser than the standard ascorbic acid with 68.89%.

3.2.3 Hydroxyl Radical Scavenging Assay

Hydroxyl radicals are highly reactive and consequently short-lived. The hydroxyl radical can damage virtually all types of macromolecules: carbohydrates, nucleic acids, lipids and amino acids. Hydroxyl radical scavenging assay is used to find the scavenging activity of free hydroxyl radicals (which damage the body cells) like hydrogen peroxide in the presence of different concentrations of sample. The percentage of hydroxyl radical scavenging activity of the silver nanoparticles synthesized using the aqueous extract of *P. granatum* peels were exposed in Table 3.

Table 3: Hydroxyl radical scavenging assay

Concentration (µg)	Ascorbic acid Inhibition (%)	PgFP AgNPs Inhibition (%)
50	23.37 ± 0.22	19.61 ± 0.20
100	36.12 ± 0.83	31.25 ± 0.42
150	50.74 ± 0.62	40.50 ± 0.78
200	58.99 ± 0.41	52.68 ± 0.22
250	70.10 ± 0.94	65.32 ± 0.52

Values are mean ± S.D of three replicates

The results showed a dose response increase in the capacity to quench hydroxyl radicals for all the concentrations studied. The silver nanoparticles synthesized using the aqueous extract of *P. granatum* peels showed maximum hydroxyl radical scavenging activity of 65.32%, whereas standard ascorbic acid exhibited 70.10% at 250 µg/ml concentration. The obtained results described that the silver nanoparticles synthesized using the aqueous extract of *P. granatum* peels showed reasonable hydroxyl radical scavenging activity like that of standard. The radical scavenging capacity of the sample might be attributed to phenolic compounds in the peels.

3.2.4 Nitric oxide scavenging activity

The nitric oxide radical scavenging activity was determined by different concentration of silver nanoparticles using the nitrating agent of sodium nitroprusside and N-(1- Naphthyl) ethylenediamine (Griess reagent). The nitric oxide scavenging activity of the nanoparticles was detected by its ability to inhibit the formation of nitrite through direct competition with oxygen and oxides of nitrogen in reaction mixture. The percentages of nitric oxide radical scavenging activity of the silver nanoparticles synthesized using the aqueous extract of *P. granatum* peels are presented in Table 4.

Table 4: Nitric oxide scavenging activity

Concentration (µg)	Ascorbic acid Inhibition (%)	PgP AgNPs Inhibition (%)
50	17.55 ± 0.34	10.25 ± 0.60
100	29.74 ± 0.28	25.66 ± 0.53
150	45.96 ± 0.20	38.15 ± 0.40
200	60.15 ± 0.51	53.56 ± 0.60
250	72.11 ± 0.45	65.23 ± 0.94

Values are mean ± S.D of three replicates

The present study showed that the silver nanoparticles synthesized using the aqueous extract of *P. granatum* peels had nitric oxide scavenging activity. An increase in concentration of nanoparticles, increases the nitric oxide radical scavenging activity. They showed maximum nitric oxide scavenging activity of 65.23% at 250 µg/ml of nanoparticles concentration. The scavenging activity of the nanoparticles was lesser as compared to standard ascorbic acid which was 72.11% at 250 µg/ml.

4. Conclusion

The present study indicated that the silver nanoparticles synthesized using the aqueous extract of *P. granatum* peels showed reasonable antioxidant activity by inhibiting and reducing DPPH, ABTS, hydroxyl radicals and Nitric oxide. The antioxidant activity of these nanoparticles may be attributed to the polyphenolic compounds of plant extracts which are responsible for the silver nanoparticle formation and hence these nanoparticles are found to have potential application to reduce oxidative stress with health benefits.

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