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ORIGINAL ARTICLE

Assessment of antioxidant and cytotoxic potential of silver nanoparticles synthesized from root extract of *Reynoutria japonica* Houtt

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Abstract Free radicals, mostly consist of reactive oxygen species, are generated in human body by several exogenous and endogenous systems. Overproduction of free radicals is known to cause several degenerative disorders including cancer. The aim of this study is to synthesize silver nanoparticles (AgNPs) using root extract of *Reynoutria japonica* and to investigate its antioxidant and cytotoxic potential. AgNPs were synthesized by green approach and subsequently characterized using *UV–vis* spectroscopy, SEM, TEM, FTIR, XRD, EDS and DLS. The antioxidant activity was investigated using DPPH, FRAP, H_2O_2, and $ABTS^+$ radical scavenging assays while the cytotoxic effect was assessed using different human cancer cell lines including lung (A549), liver

Abbreviations: WHO, World Health Organization; AgNPs, Silver nanoparticles; NPs, Nanoparticles; R. japonica, Reynoutria japonica; mM, Millimolars

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(Hep-G2) and breast (MDA-MB-231) by MTS assay. Moreover, the specificity of NPs was assessed against two normal human cell lines e.g. alveolar and renal primary epithelial cells (HPAEpiC and HRPTEpiC). The *UV–vis* spectra confirmed the synthesis of AgNPs by producing a characteristic peak at 410 nm. Further analysis confirmed that AgNPs were crystalline in nature, predominantly spherical in shape, with an average width and area of 17.34 nm and 164.46 nm^2, respectively. DLS analysis revealed that NPs possess a high negative zeta potential value ($-28.5\ mV$), thus facilitating its electrostatic stabilization. AgNPs showed dose dependent antioxidant activity against DPPH, FRAP, H_2O_2 and ABTS with IC_{50} values 19.25, 22.45, 24.20 and 18.88 µg/ml, respectively. The AgNPs depicted significant cytotoxic effects against A549, Hep-G2 and MDA-MB-231 cell lines with IC_{50} values of 4.5, 5.1 and 3.46 µg/ml, respectively. Moreover, the NPs exhibited highest selectivity index (> 2.0) for A549, Hep-G2 and MDA-MB-231, confirming its specificity towards cancer cell lines. In conclusion, AgNPs prepared from root extract of *R. japonica* possess strong antioxidant and cytotoxic potential which suggests that they should be investigated further in order to develop safe and effective antioxidant and/or cytotoxic formulations.

1. Introduction

Cancer remains one of the most active area of research, being a major public health problem worldwide, with increasing mortality and cost (Ziyu et al., 2021; Hanène et al., 2020). According to the World Health Organization (WHO), approximately 21 million new cancer cases and 13 million cancer-related deaths are expected across the world by 2030 (García-Castillo et al., 2016). Breast cancer is one the most common cancer in females, accounting for about 11.6 % of all cancer-related deaths (Qiubing et al., 2022). In 2018, about 2.1 million new cases of breast cancer were reported, with an estimated 627,000 deaths (Bray et al., 2018; Fitzmaurice et al., 2017). Pakistan has the highest incident of this cancer in Asia, which is likely to increase in coming years. It has been reported that one in nine women are diagnosed with this cancer during their lifetime (Sohail et al., 2007). In Pakistan, 59 % of the patients are diagnosed at an advanced stage while 89 % at later stage due to several reasons such as socio-economic and cultural factors, fear of surgery, lack of awareness and belief in traditional treatments. Due to these reasons, the mortality rate in breast cancer patients is very high (Gulzar et al., 2019). Similarly, hepatocellular carcinoma is one of the fatal tumors in the world with the second highest mortality among all cancers (Huajun et al., 2021). It is the sixth most common cancer worldwide, with 5.7 % new cases annually with a survival rate of only 3–5 % (Parkin, 2006). According to literature review, about 82 % of cases occurred in developing countries including 55 % from China only (Raza et al., 2007). In Pakistan, its prevalence varies from 3.7 to 16 % where the most common causes are viral hepatitis C or B (68 and 22 %, respectively) (Munaf et al., 2014). Despite some global decline, lung cancer remains a major challenge for health policies, with over 470 thousand cases reported in Europe each year. This figure accounts for approximately 12 % of all tumors, 65 % of which occurred in males (Mireia et al., 2021). Similarly, in females it is the second most common cancer after breast cancer globally (Sarwar et al., 2017). According to the American cancer society, it was the leading cause of death in the United States in 2017. This cancer causes more deaths compared to breast, colon and prostate cancer combined (Saad et al., 2010).

Despite the therapeutic advances in mitigating cancer, the overall mortality rate is still very significant. Resistance to anticancer agents remains one of the hallmark of cancer therapy (Muhammad et al., 2021). The generation of free radicals in our body is a major reason for the development of cancer. It has been reported that under stress condition our body produces more reactive oxygen species than enzymatic antioxidants. These includes hydroxyl (·OH), superoxide anion ($O_2{\cdot}^-$), perhydroxyl ($HO_2{\cdot}$) and some non-free radicals i.e·H_2O_2 (Duduku et al., 2011). In the absence of effective antioxidants, they facilitate the development of many diseases such as cancer, inflammation, cardiac and a variety of neurodegenerative disorders (Nisa et al., 2013). Synthetic antioxidants (butylated hydroxytoluene and hydroxyanisole) are commonly used in medicines and food industry however, they are associated with several side effects such as carcinogenesis and liver cirrhosis (Sajjad et al., 2015). Therefore, in recent years the use of natural antioxidants have got much attention. Traditional medicines are widely used since long time to treat several health problems however; large doses are frequently required to get the therapeutic outcomes.

Nanotechnology is considered an advanced field of science that encompasses the utilization of nanoparticles (NPs) for various purposes. A variety of NPs made up of different noble heavy metals such as platinum, gold, palladium and silver (Ag) are reported in literature. However, among these AgNPs are of significant importance because of their unique structure and functions. Unlike copper and gold, silver is a naturally occurring abundant metal possessing numerous biological, physical and chemical properties including electrical conductivity, surface enhanced Raman scattering and catalytic effects. The unique biochemical properties makes it the most appropriate candidate with diverse biomedical potential i.e. as antimicrobial, antiseptic, larvicidal, significance in drug delivery, cosmetic products, optoelectronics, biomedicines and food preservation. Antimicrobials containing silver ions showed more efficacy against different microbes compared to other NPs. It has been documented that silver ions react with thiol group of proteins and leads to DNA disruption. Similarly, this metal impair DNA replication due to uncoupling of electron transport from oxidative phosphorylation; inhibits enzyme and disrupts the membrane permeability. In addition, AgNPs have shown comparatively high cytotoxic potential against a variety of cell lines as compared to other NPs (Qindeel et al., 2021; Lateef et al., 2019; Verma et al., 2019; Jabeen et al., 2021).

These NPs are prepared using different techniques including chemical, physical and biological. The chemical method is less time consuming and require least labour however; it utilizes various hazardous chemicals for reduction and stabilization of synthesized NPs (Ponsanti et al., 2020). In contrast, green synthesis is an eco-friendly technique, using biological materials such as plants, algae, bacteria, and fungi as reducing and stabilizing agents. Several microalgal species have been used for the synthesis of iron, silver, palladium and gold NPs due to their high rate of intracellular metal accumulation. Extracellular synthesis of NPs is highly advantageous due to their simplicity in the purification process. Several biological applications of microorganism mediated NPs have been reported in literature (Adelere and Lateef, 2021). Similarly, enzyme-mediated synthesis of NPs is one of the recent advancements in the field of nanotechnology. Several enzymes including sulfite reductase, cellulose, laccase, ligninase and nitrate reductase have been used for the synthesis of metal NPs. These enzymes acts a natural reducing and capping agents in the formation of NPs. Enzyme based NPs have been reported for different biological

activities (Adelere and Lateef, 2016). According to literature review, plant mediated synthesis of nanoparticles has been shown to produce particles with comparable shape and size to those produced through physical and chemical procedures. In addition, plants contain several phytochemicals such as phenols and flavonoids that play a vital role in reducing Ag^+ (Lim et al., 2020). Several plants including *Acacia nilotica*, *Conocarpus Lancifolius*, *Datura inoxia Mill*, *Persea Americana*, *Hyptis suaveolens, Annona muricata, Opuntia ficus-indica* and *Sambucus wightiana* have been reported to fabricate AgNPs with improved biological activities (Saratale et al., 2019; Oves et al., 2022; Bagewadi et al., 2019; Adebayo et al., 2019; Lateef et al., 2020; Badmus et al., 2020; Adebayo et al., 2019; Fazli et al., 2021). Beside plants and microorganisms, animal waste materials have been used for the synthesis of AgNPs. These NPs have shown considerable potentials as antioxidant, anticoagulant and thrombolytic agents (Akintayo et al., 2020). Considering the importance of using plant extracts for green synthesis of NPs, the present study is therefore designed to prepare AgNPs, using the crude extract of *Reynoutria japonica*.

Reynoutria japonica Houtt. (*Polygonaceae*) known as Japanese knotweed is a herbaceous perennial plant, widely distributed in Asia, Japan, China and Korea (Anna et al., 2019). The first mention of *R. japonica* for the treatment of abdominal masses and gallstones appeared in a Chinese medicine monograph, Mingyi Bielu, which was written during China's Han dynasty (B.C. 202–A.D. 200) (T'ao, 1986). Its clinical applications were recorded in Bencao Gangmu, another Chinese herbology book that was first published in Korea in 1613. Literature review showed that this plant has been used for the treatment of various disorders including hepatitis, cancer, skin burns and inflammation (Vastano et al., 2000). In addition, it has shown antimicrobial and antioxidant activities (Chan et al., 2008; Feng et al., 2006; Kim et al., 2008). Phytochemical analysis of the plant showed the presence of physiologically active substances, such as Physcion, polydatin, Quercetin, resveratrol, citreorosein, (+)-Catechin and emodin (Chang et al., 2012; Yi et al., 2007). Resveratrol from this plant has been reported to prevent lung cancer in mice. It has also shown anticancer activity in mice bearing neuroblastoma, melanoma and ovarian cancer (Kimura et al., 2001; Guo et al., 2010). Because of its diverse biological activities, biosynthesis of silver nanoparticles from *R. japonica* extract will exhibit greater therapeutic benefits.

2. Material and methods

2.1. Chemicals

Chemicals and reagents used in present experiment include: silver nitrate (99 %), FRAP (99 %), DPPH (99 %), H_2O_2, potassium persulfate and ABTS, 1 % penicillin–streptomycin solution, Dulbecco's Modified Eagle's Medium supplemented with 10 % fetal bovine serum (FBS), Methanol (99 %), NaOH (98 %), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and acetate buffer. All the reagents and chemicals were purchased from Sigma-Aldrich, Germany.

2.2. Plant material

The plant was purchased from Kyung-Dong Oriental Market, Seoul, Korea and was authenticated by Dr. Mi-Jeong Ahn, Professor of Pharmacy, at College of Pharmacy, Gyeongsang National University, Korea (Specimen no. GSC-104).

2.2.1. Preparation of extract

The coarse powder (100 g) was soaked in deionized water (1 L) and subsequently heated at 45 to 55 °C for about 40 min. Following cooling, the extract was filtered through a nylon cloth and the filtrate was centrifuged (7000 rpm) for 10 min to remove any dissolved impurities. The supernatant was collected and stored at 4 °C until further use.

2.3. Nanoparticles synthesis and purification

Fixed volume of plant extract (5 ml *v*/*v*) was added to different ratios (3, 5, 10, 15, 20, 50 and100 ml *v*/*v*) of silver nitrate aqueous solution (1 mM) followed by stirring (300 rpm; 25 °C) for about 60 min. The change in color from light green to yellowish-brown indicates the formation of AgNPs, which was further confirmed by UV visible spectroscopy (350 and 800 nm). For optimum synthesis of NPs, the mixture was incubated at 37 °C for 24 hrs. The resulting NPs were subjected to centrifugation at 10,000 rpm for about 5 min. The obtained pellets were washed, three time with distilled water for removal of any free ions and unreacted moieties. Finally, the pure colloidal NPs were freeze dried and stored at 25 °C for further characterization and biological activities (Ilahi et al., 2021).

2.4. Physical characterization

The following techniques were used for the analysis of AgNPs.

2.4.1. UV–vis spectrophotometry

Initially, the synthesis of AgNPs was confirmed by UV–*vis* spectrophotometry (Perkin-Elmer, Lambda 35, Germany). The reduction of silver ions (Ag^+) in the reaction mixture was periodically monitored by taking samples at different time interval and were scanned in 350–800 nm range.

2.4.2. SEM and TEM analysis

The surface morphologies and crystalline nature of AgNPs were confirmed using SEM (S-2500, Japan) and TEM techniques, respectively. For TEM analysis, the colloidal particles were dispersed in distilled water and a drop of this dispersion was put on a staining mat. A "carbon coated Copper-grid" was inserted into the drop (coated side upward), and after 10 min. it was removed and dried in air and finally screened using Hitachi-7650 (Japan) transmission electron microscope.

2.4.3. FTIR analysis

The possible role of different functional groups involved in the green synthesis and stabilization of AgNPs were analyzed using FTIR (Version 10.5.1, Perkin-Elmer). The spectra was recorded in 400–4000 cm^{-1} range. Vibrations at different modes were identified and assigned to determine functional groups present in the extract and NPs. Finally, the spectra's were compared.

2.4.4. XRD analysis

The crystalline nature of NPs was investigated using XRD (JDX-3532 JEOL, Japan) operated at a specific voltage and current (40 kV and 30 mA, respectively) with Cu Kα radiation in θ-2θ configurations. The size of crystal was calculated from the width of XRD peaks using Debye-Scherrer's equation.

$$D(nm) = \left(\frac{0.9\lambda}{\beta\cos\theta}\right)$$

Where D: mean crystal size; λ: X-ray wavelength; β: full-width at half maximum (FWHM); θ: diffraction angle.

2.4.5. EDX analysis

The elemental composition such as the presence of silver, carbon, oxygen and other such elements in the synthesized NPs was investigated using EDX (NOVA-450 instrument). EDX analysis is used to confirm the purity of NPs.

2.4.6. Dynamic light scattering (DLS)

The average particle size, polydispersibility index (PDI) and zeta potential of AgNPs were analysed using a Zetasizer (Malvern, UK). The sample was prepared by dispersing the NPs in distilled water filtered through 0.45 μm filter paper. Particle size and PDI were measured at room temperature with a scattering angle of 90. For zeta potential measurement, the sample was placed in a disposable zeta cell at room temperature and was measured by PALS technology. The analysis was performed in triplicate.

2.5. Stability and optimization of AgNPs synthesis

The effect of substrate concentration, pH and temperature on the biosynthesis of AgNPs were optimized. To optimize substrate concentration, fixed volume of crude extract was added to different ratios of silver nitrate solution (1:3, 1:5, 1:9, and 1:13 *v*/*v*) and maximum absorbance was determined using UV–Vis spectroscopy. Temperature optimization was conducted by mixing crude extract with silver nitrate at varying temperature (25, 40, 60 and 80 °C) and the corresponding spectra's were obtained. The pH was optimized by dispersing AgNPs in phosphate buffer at different pH (4,6,8 and 12). Moreover, the short-term stability was assessed after 30 min, 48 and 96 hr following synthesis of AgNPs and the SPR peaks were anayased for maximum absorbance.

2.6. Antioxidant assay

2.6.1. DPPH assay

The antioxidant potential of AgNPs from *R. japonica* extract was evaluated using a reported method (Sagar and Sing, 2011). Different concentrations (62.5, 125, 250, 500 and 1000 μg/ml) of AgNPs and ascorbic acid were prepared in methanol. Similarly, DPPH solution (0.3 mM) was also prepared in the same solvent. Thereafter, from each working solution 100 μl was mixed with 1 ml of the reagent solution in separate test tubes and vortexed thoroughly. The reaction mixture was incubated in dark place for 15 min. For control, 2 ml of methanol was added instead of AgNPs and simultaneously run with the test samples. Finally, the maximum absorbance was measured at 517 nm and the percentage radical scavenging activity of AgNPs and extract were calculated using the formula.

$$\%Radicalscavengingpotential = \frac{C_{Abs} - S_{Abs}}{C_{Abs}} \times 100$$

Where S_{Abs} and C_{Abs} represents the absorbance of standard/test samples and control respectively.

2.6.2. FRAP assay

The assay was conducted following the method of Iris and Strain (Iris and Strain, 1999). This assay measures the antioxidant potential in test samples via reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}); the later forms a complex with 2,4,6-tripyndyl-s-triazine (TPTZ), that can be quantified at 593 nm using colorimetric techniques. In brief, acetate buffer (300 mM; pH 3.5) was freshly prepared. Then a mixture of 10 mM TPTZ (0.25 ml) in 40 mM HCL, 20 mM $FeCl_3$ (0.25 ml) and acetate buffer in the ratio of (1:1:10 *v*/*v*) was prepared for FRAP reagent. The assay was conducted using a 96-well plate. The FRAP reagent (170 μl) and sample solutions (20 μl) was added to each well, mixed in dark at ambient temperature and allowed to incubate for 30 min. Finally, the maximum absorbance was measured at 593 nm using a microplate reader.

2.6.3. Hydrogen peroxide scavenging assay

Different concentrations of tested samples and H_2O_2 (2 mM) was mixed (1:1 v/v) and incubated for 20 min at ambient temperature. Following this, the sample solutions and H_2O_2 was mixed with methanol (10 μl) and FOX reagent (0.9 ml). This mixture was thoroughly mixed using a vertex mixer and subsequently incubated at 25 °C. The maximum absorbance of the formed complex was measured at 560 nm. The percent H_2O_2 scavenging activity was then determined and compared to sodium pyruvate (standard) (Chandan et al., 2014).

2.6.4. ABTS scavenging assay

The $ABTS^{\cdot+}$ radicals was produced by the reaction between potassium persulfate (2.5 mM) and ABTS (7 mM) in 1:1 (*v*/*v*) ratio and subsequently stored away from light at 25 °C for 10–15 hr before use. The reagent solution was diluted with methyl alcohol until an absorbance of 0.700 was achieved at 734 nm. Silver NPs and/or extract (5 μl) was mixed with ABTS solution (4 ml) and after 30 min, the absorbance was measured. All the anti-oxidant assays were performed in triplicate (Chandan et al., 2014). Finally, the percent radical scavenging activity was determined.

2.7. Cytotoxicity assay

The MTS assay was used to investigate the cytotoxic potential of *R. japonica* mediated AgNPs against various cell lines including hepatocellular carcinoma (Hep-G2), lung adenocarcinoma (A549) and breast adenocarcinoma cells (MDA-MB-231) as well as two normal cell lines i.e. renal and alveolar primary epithelial cells (HPAEpiC and HRPTEpiC) (Fazli et al., 2022). In this assay the soluble MTT salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide is reduced to an insoluble colored formazan product which is subsequently measured using a spectrophometer. The level of MTT salt indicates the normal function of mitochondrial dehydrogenase enzymes which in turn represents the number of viable cells.

Briefly, the cells treated with AgNPs and plant extract were incubated (37 °C; 48 h; 5 % CO_2). The MTS reagent (25 μl) in phosphate buffer solution was added to each well and incubated for 30 min at 37 °C The insoluble formazan crystals were collected and solubilized in DMSO (100 μl) and the absorbance was measured at 490 nm using a microplate reader.

The cells treated with sample were compared with controls. The MTT salt was reduced to colored formazan by cellular enzymes in viable cells only. Therefore, the amount of formed formazan dye directly correlates with the number of viable cells in culture media which is measured as function of total absorbance. The % cell viability was calculated using the following equation.

$$\%Viable\ cells = \left(\frac{abs_{sample} - abs_{blank}}{abs_{control} - abs_{blank}}\right) \times 100$$

To find out the specificity of the NPs for cancer cell lines, their selectivity index was determined. It was calculated by dividing the IC_{50} values of the normal cells with the IC_{50} values for cancer cell lines. A value of two or more indicated high specificity.

2.8. Statistical analysis

The analytical determinations were performed in triplicate. One-way ANOVA (GraphPad Software Inc., San Diego, California, USA) was used for data analysis. The data were presented as mean ± SEM (n = 5).

3. Results and discussion

3.1. Synthesis of AgNPs

The addition of 1 mM silver nitrate solution changed the color of the extract from light green to yellowish-brown. The rapid synthesis of NPs shows the presence of high levels of phenolics and flavonoids which are predominately involved in the reduction of Ag to AgNPs. This method is comparatively safe, rapid and do not involve the use of hazardous chemical substances.

3.2. Characterization of AgNPs

3.2.1. Physical characterization

Initially, AgNPs were characterized with color change resulted from the reduction of Ag^+ by the green extract. As the reduction of Ag^+ increased, an intense color was produced that become stable upon the completion of reduction process. In present study, the color of NPs solution changed from light green to yellowish-brown. This color change was observed within 30 min to 1 hr. No such change in color was observed up to 24 hrs following incubation. The short duration for completion of biochemical reduction indicates the presence of high levels of phenolics and flavonoids that could be involved in the reduction of Ag to AgNPs (Yugal et al., 2017).

3.2.2. UV–vis spectrophotometry

This reduction was further investigated using UV–vis spectroscopy which is a valuable technique used to detect the characteristics surface plasmon resonance (SPR) pattern of the metallic NPs. Fig. 1A depicts the UV–visible spectrum for AgNPs at 410 nm, a characteristic band for Ag. The absence of any other peaks at this range confirms the synthesis of AgNPs. The observed value are well within the range reported for AgNPs (Yugal et al., 2017).

3.2.3. SEM and TEM analysis

SEM and TEM analysis were conducted to investigate the surface and inner morphological features of AgNPs. SEM results

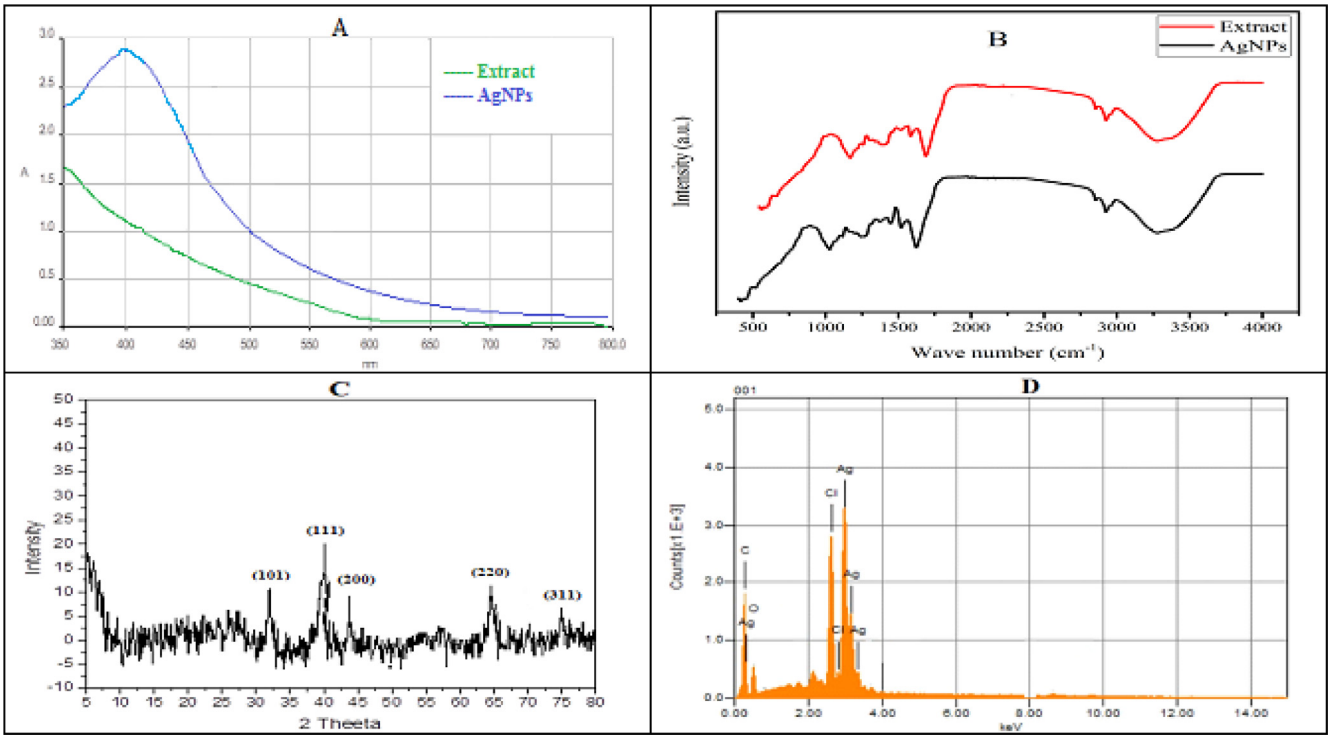

Fig. 1 (A) UV–visible spectra (B) FT-IR spectra (C) XRD pattern and (D) EDX spectra of AgNPs.

confirmed the formation of heterogeneously dispersed AgNPs (Fig. 2). TEM analysis revealed different shapes of AgNPs such as cubic, hexagonal, trigonal and polygonal with an average area of 164.46 nm^2 and width of 17.34 nm (Fig. 3). The formation of poly-dispersed particles may be due to the early and later stages of nucleation.

3.2.4. FTIR analysis

FTIR spectra was used to identify different functional groups involved in the synthesis of AgNPs (Fig. 1B). The spectra of the extract and AgNPs showed different stretching vibration pattern of the functional groups. The broader band at 3315 cm^{-1} are due to the stretching vibration of OH group while the bands at 2933, 1618, 1376 and 1022 cm^{-1} are assigned to C—H, C═O, N—O and C—N groups, respectively. According to literature review, functional groups responsible for the reduction and stabilization of synthesized AgNPs includes but not limited to phenolic, alcoholic and carboxylic acid.

3.2.5. XRD analysis

The XRD analysis showed diffraction peaks at 2θ values of 32.5, 38.2, 44.5, 64.6, and 77.3°which were assigned to the planes (111), (200), (220), and (311) facet of Ag crystal, respectively. The diffractogram (Fig. 1C) was compared to JCPDS card: silver file number 04–0783. The average size of AgNPs was calculated using Scherrer's equation and was found to be 20.5 nm.

3.2.6. EDX analysis

The EDX analysis was conducted to confirm the presence of elemental silver. Fig. 1D carries a prominent Ag-peak that shows the presence of silver in the sample. The spectrum also showed peaks for other elements such as O and C that may be due to capped biomolecules on the surface of silver NPs. The spectrum did not showed any other peak, which confirm the high purity of synthesized NPs.

3.2.7. Dynamic light scattering

The size and zeta potential of the AgNPs was determined using Zetasizer. By studying the DLS, the average size of AgNPs reveals a narrow size distribution (approx. 35–100 nm) (Fig. 4). These results are in concurrence with those of the TEM images. Similarly, the analysis showed the presence of AgNPs with low PdI (0.185). Moreover, the apparent zeta potential recorded was − 28.5 mV. The negative zeta potential of AgNPs may be due to the capping of particles by the –OH groups of different biomolecules. According to literature review, particles with zeta potential above + 20 mV or below − 20 mV are considered stable. The obtained value of zeta potential showed that AgNPs remained highly stable and dispersed even after 96 hrs of its synthesis. This may also be the reason for producing particles with a very narrow size distribution index.

3.3. Optimization of AgNPs

Fixed volume of crude extract was mixed with different ratios of silver nitrate solution (1:3, 1:5, 1:9, and 1:13 v/v). Optimum synthesis occurred at 1:5 v/v (Fig. 5A). The synthesis of AgNPs was also investigated at different pH (Fig. 5B). The synthesized NPs showed maximum absorption at acidic pH while at basic pH the SPR peaks gradually diminished. The stability of AgNPs was also studied at different temperature ranging from 25, 40, 60 and 80 °C Maximum absorbance was observed at 80 °C (Fig. 5C). Similarly, the short-term stability was assessed after 30 min, 48 and 96 hrs by storing the AgNPs in a dark place. An increase in maximum absorbance was observed with the passage of time, which was also noticed with naked eye as darkening of the solution. Maximum absorbance was observed after 96 hrs of synthesis (Fig. 5D).

3.4. Antioxidant activity

Antioxidants play a significant rule in the prevention of various human diseases. Herbal remedies with antioxidant potential may serve as hydrogen donors, free radical scavengers and single oxygen quenchers, thereby protecting the body from a variety of degenerative diseases including cancer. These damaging reactive species are generated during cellular metabolism that are responsible for initiating a state of oxidative stress. Subsequently, it leads to the pathogenesis of several diseases by damaging DNA, lipids and proteins. Polyphenols are the most abundant compounds present in plants with high potential for antioxidant activity. The most common phenolic compounds are flavonoids, tannins, phenolic acids and lignins. These compounds possess significant redox properties; allow them to act as potential reducing agents (Ankita et al., 2015). According to literature review, plant mediated AgNPs have shown significant antioxidant activities against DPPH, FRAP, H_2O_2 and ABTS scavenging assays (Adebayo et al., 2019); Badmus et al., 2020).

3.4.1. DPPH assay

In present study, root extract of *R. japonica* was investigated for their antioxidant potential using different *in vitro* models. The IC_{50} values and % free radical scavenging activity of the extract and NPs are summarized in Table 1.. Silver NPs caused 10.45, 21.58, 30.65, 51.75 and 67.50 % inhibition at 62.5, 125, 250, 500 and 1000 µg/ml concentrations, respectively. Crude extract showed much lower activity at the same concentrations as evident from the respective IC_{50} values. The IC_{50} values for crude extract, NPs and standard were 27.80, 19.25 and 16.98 µg/ml, respectively. It has been documented that DPPH free radical scavenging effect occur due to the neutralization of these radicals by different phytochemicals mainly by the transfer of hydrogen and/or an electron. The results showed that silver NPs exhibited significant antioxidant activity compared to crude extract in a dose dependent manner therefore, it may be considered as an effective and safe alternative herbal therapy for the mitigation of a variety of degenerative disorders. Adebayo *et al.* (Adebayo *et al*, 2109) reported DPPH free radical scavenging activities of *Persea americana* fruit feel extract mediated AgNPs as 57.82–63.25 %. The activity may be assigned to the presence of biomolecules adhere at the surface of AgNPs.

3.4.2. FRAP assay

The results of this assay are summarized in Table 2. Crude extract, silver NPs and standard revealed 42, 56 and 78 % inhibition at 1000 µg/ml, respectively. The respective IC_{50} values

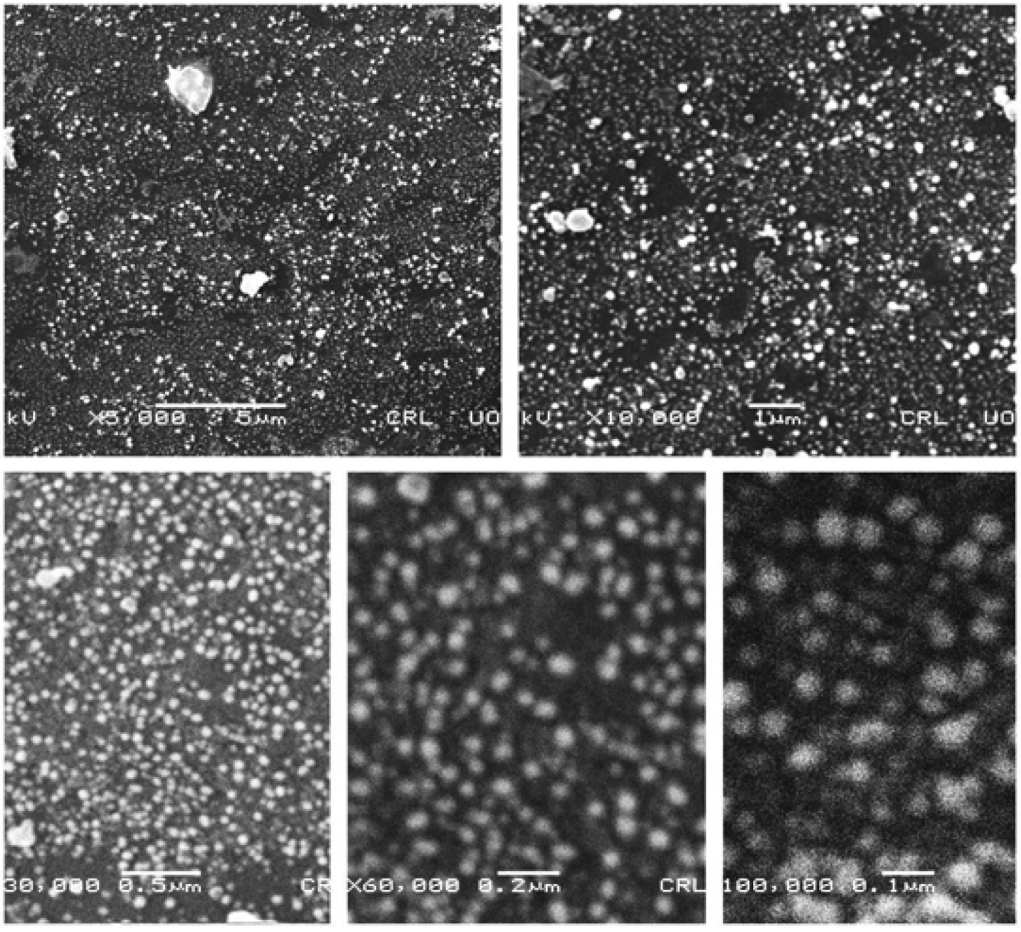

Fig. 2 SEM images of silver nanoparticles at various resolutions.

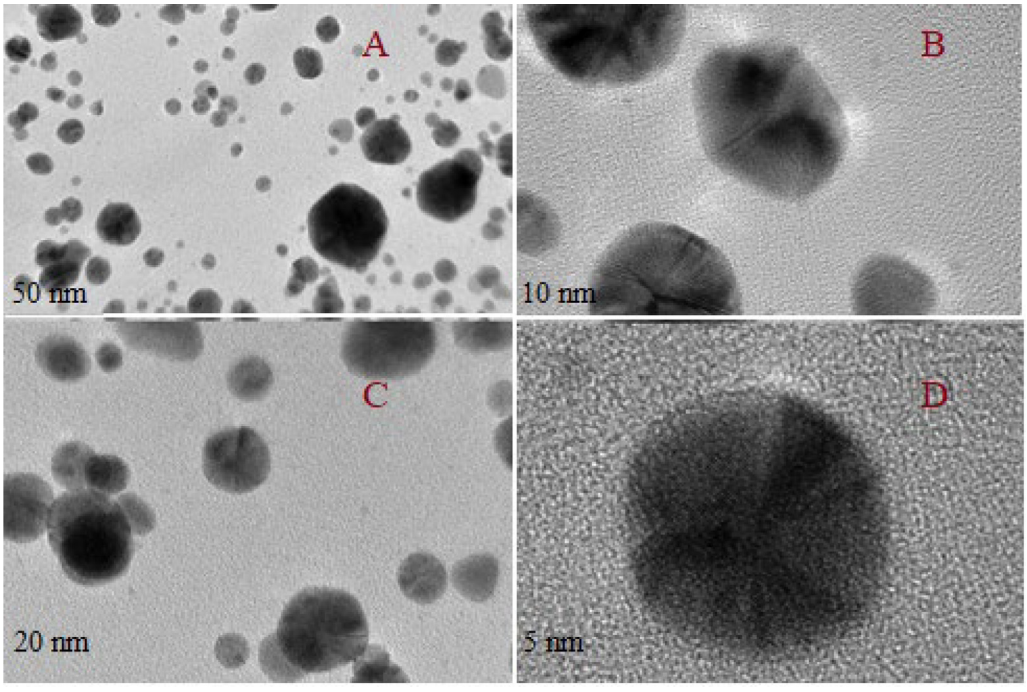

Fig. 3 TEM images of silver nanoparticles (A: 50 nm; B: 10 nm; C: 20 nm; D: 5 nm).

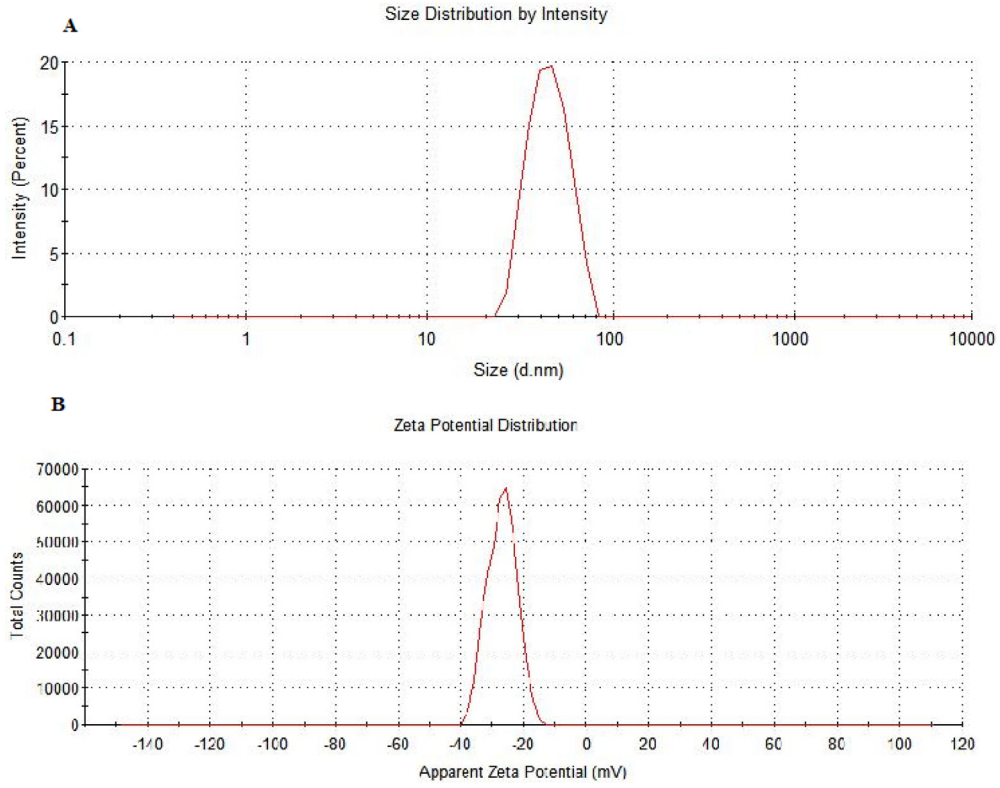

Fig. 4 DLS spectra of (A) hydrodynamic size distribution and (B) Zeta potential (mV) of synthesized silver nanoparticles.

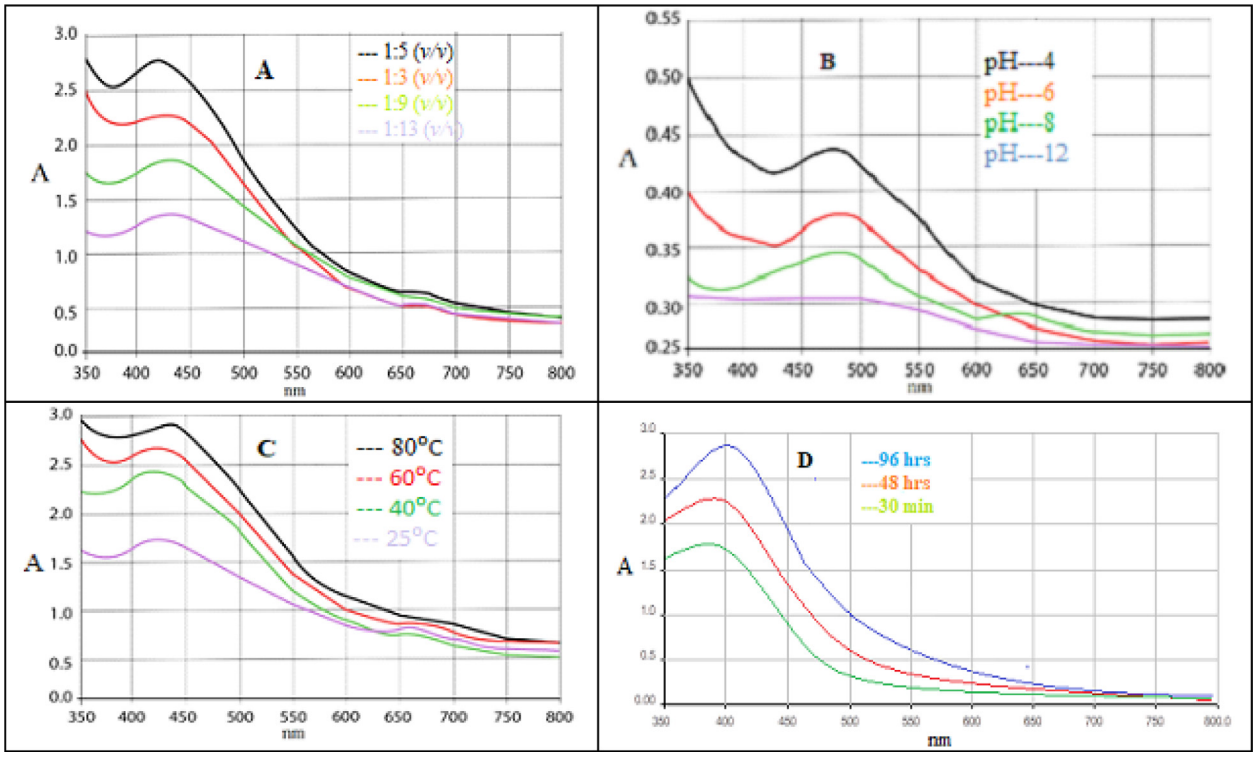

Fig. 5 (A) UV–Vis absorption spectra of silver nanoparticles at different concentration of silver salt; (B) pH; (C) temperature and (D) at different time interval.

Table 1 DPPH free radical scavenging activity of extract and silver nanoparticles.

Concentration (µg/ ml)	DPPH Scavenging activity (%)		
	Extract	AgNPs	Ascorbic acid
62.5	–	10.45 ± 0.32	18.75 ±
125	9.25 ± 0.85	21.58 ± 0.98	28.80 ± 1.25
250	24.76 ± 1.55	39.65 ± 2.34	46.75 ± 2.80
500	36.42 ± 2.11	51.75 ± 3.10	61.88 ± 4.45
1000	48.90 ± 4.45	67.50 ± 5.59	82.65 ± 6.70
IC_{50}	27.80	19.25	16.98

Table 2 FRAP scavenging activity of extract and silver nanoparticles.

Concentration (µg/ ml)	FRAP Scavenging activity (%)		
	Extract	AgNPs	Ascorbic acid
62.5	3.20 ± 0.25	9.80 ± 0.75	21.25 ± 1.12
125	7.65 ± 0.70	18.78 ± 0.56	34.67 ± 1.89
250	18.90 ± 1.25	32.56 ± 3.45	46.28 ± 3.36
500	29.75 ± 1.87	43.92 ± 5.10	59.73 ± 5.90
1000	42.56 ± 3.11	56.36 ± 7.80	78.80 ± 7.78
IC_{50}	29.45	22.45	17.46

Table 3 Hydrogen per oxide scavenging activity of extract, nanoparticles and standard at different concentrations.

Concentration (µg/ ml)	H_2O_2 Scavenging activity (%)		
	Extract	AgNPs	Sodium pyruvate
62.5	3.55 ± 0.30	8.45 ± 0.55	15.78 ± 0.80
125	7.12 ± 0.25	11.90 ± 0.55	22.56 ± 1.25
250	11.45 ± 0.65	21.56 ± 2.66	38.29 ± 2.56
500	19.80 ± 1.0	34.56 ± 4.45	55.39 ± 5.89
1000	34.35 ± 3.78	50.0 ± 6.90	77.80 ± 6.88
IC_{50}	33.66	24.20	18.80

were 29.45, 22.45 and 17.46 µg/ml, respectively. The results showed that there is a concentration dependent radical scavenging activity, the maximum activity being observed at 1000 µg/ml. This assay is based on the ability of antioxidants to facilitate the reduction of Fe^{3+} to Fe^{2+} ions in the presence of TPTZ, producing Fe^{2+}-TPTZ complex (intense blue) that show maximum absorption at 593 nm. The decrease in maximum absorption has direct correlation with the antioxidant contents (Zahra et al., 2015). It has been reported that *R. japonica* contain phenolic compounds and has been widely used in traditional medicines for the treatment of various ailments including cancer, inflammation and a number of pathological conditions. Results of the study suggests that AgNPs have strong antioxidant activity due to the presence of bioactive compounds most probably phenolics on their surface. Therefore, we suggests that suitable formulation of AgNPs might be used in the treatment of mentioned human diseases.

3.4.3. Hydrogen per oxide scavenging assay

Results of this assay are presented in Table 3. AgNPs showed considerable hydrogen peroxide scavenging activity compared to sodium pyruvate. Crude extract, AgNPs and standard revealed 34, 50 and 77 % inhibition at the highest concentration (1000 µg/ml) with IC_{50} values 33.66, 24.20 and 18.80 µg/ml, respectively. Hydrogen peroxide is a weak oxidizing agent, responsible for inactivation of several enzymes via oxidation of essential thiol (-SH) groups. It can rapidly cross the cell membrane and has the ability to react Cu^{2+} and Fe^{2+} ions to form $^{-}$OH radicals that in turn initiate cell damage *in vivo*. Scavenging this radical remained an important antioxidant activity for the protection of living cell and mitigating diseases initiated by free radicals (Giovanni et al., 2022).

3.4.4. ABTS scavenging assay

Table 4 represents the ABTS radical scavenging effects of the test samples. The % inhibition values observed at 1000 µg/ml for crude extract, AgNPs and standard were 61, 74 and 80 %, respectively. The corresponding IC_{50} values were 25.45, 18.88 and 15.34 µg/ ml, respectively. All the test samples exhibited dose dependent activity. ABTS is a stable radical cation capable of donating hydrogen ions. It has been reported that phenolics have more ability to quench ABTS free radicals (Nataraj et al., 2013; Oana et al., 2020). The present results revealed that beside silver NPs, crude extract demonstrated strong antioxidant activity compared to standard. Since, the extract has the potential to scavenge free radicals, and thereby

Table 4 ABTS radical Scavenging activity of extract, nanoparticles and standard at different concentrations.

Concentration (µg/ ml)	ABTS radical Scavenging activity (%)		
	Extract	AgNPs	Ascorbic acid
62.5	7.35 ± 0.25	19.60 ± 0.80	24. 55 ± 1.10
125	19.56 ± 1.0	25.58 ± 2.34	29.25 ± 1.80
250	28.32 ± 1.88	41.60 ± 3.30	45.50 ± 3.30
500	42.67 ± 3.80	58.10 ± 5.38	63.78 ± 7.75
1000	61.70 ± 6.35	74.48 ± 7.12	80.58 ± 8.80
IC_{50}	25.45	18.88	15.34

prevent lipid oxidation it could serve as potential nutraceutical. Similarly, silver NPs showed improved antioxidant effects compared to crude extract therefore; it could be used to prepare suitable formulation for the safe and effective treatment of several diseases. Previous studies have reported the antioxidant potential of plant mediated AgNPs using Annona muricata, *Persea americana* and *Opuntia ficus-indica* extracts as good scavengers of free radicals. These NPs have comparatively many advantages over synthetic antioxidants including improved bioavailability, stability and targeted delivery (Badmus et al., 2020; Adebayo et al., 2019).

3.5. Cytotoxicity assay

In this study, the cytotoxic potential of *R. japonica* root extract and its NPs were evaluated against different human cancer cell lines including A549, MDA-MB-231 and Hep-G2 using MTS assay. Furthermore, the safety profile of the test samples were investigated against two normal human cell lines e.g. HPAEpiC and HRPTEpiC. Silver NPs have encouraging applications in mitigating wound healing, breast, skin, lung and liver cancers (Oana et al., 2020). To validate and support the development of anticancer formulations, the synthesized silver NPs were investigated for their cytotoxic potential against the mentioned cell lines. In present study, the % cell viability treated with different concentrations of extract and NPs were determined (Figure S1-S3) and the corresponding IC_{50} values were calculated (Fig. 6).

Silver NPs demonstrated significant cytotoxic activity against A549, Hep-G2 and MDA-MB-231 cell lines with IC_{50} values 4.50, 5.15 and 3.46 µg/ml, respectively, compared to standards cyclophosphamide (IC_{50} values 0.98, 0.89 and 0.97 µg/ml, respectively) and doxorubicin (IC_{50} values 1.86, 1.38 and 0.95, µg/ml, respectively). On the other hand, crude methanol extract showed relatively week cytotoxic activity against the mentioned cell lines with IC_{50} values 6, 7.52 and 6.45 µg/ml, respectively. In order to assess the safety profile of the test samples, they were tested against two normal human cell lines. The percent cell viability values are depicted in figures S4 & S5. The corresponding images for different cell lines are depicted in figures S6-S9. The IC_{50} values of the extract and NPs were 18.45 and 12.22 µg/ml, respectively against HPAEpiC and 15.24 and 10.58 µg/ml, respectively against HRPTEpiC. The results showed that IC_{50} values remained high against the normal human cell lines that preliminary confirms its safety against normal cell. In order to further elaborate the selectivity of the test samples, the respective selectivity index values were determined (Figs. 7 & 8).

Crude extract and silver NPs exhibited high selectivity index values for A549, Hep-G2 and MDA-MB-231. The SI values of crude extract for the mentioned cell lines were 3.07, 2.16 and 2.19, respectively when investigated against HPAEpiC. The corresponding SI values of silver NPs for the same cell lines were 2.17, 2.45 and 3.08, respectively. SI values of two or more indicated high specificity. Thus, the results suggest that *R. japonica* extract and its NPs exhibit strong antiproliferative effects without affecting the normal cells. The possible mechanism(s) for the said activity many include but not limited to ROS generation and activation of several cellular metabolic pathways e.g. tumor necrosis factor-alpha (TNF-α) and nuclear factor kB (NF- kB) which ultimately triggers

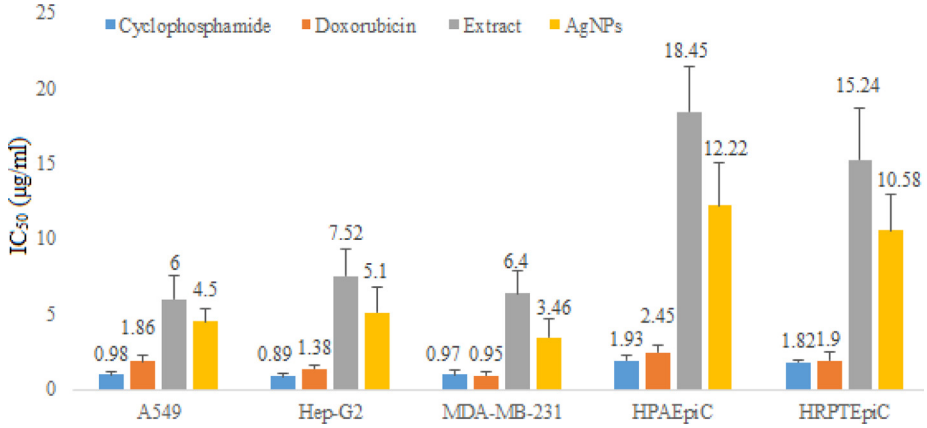

Fig. 6 IC_{50} values of extract and silver nanoparticles against different cell lines using cyclophosphamide and doxorubicin as positive and DMSO as negative control.

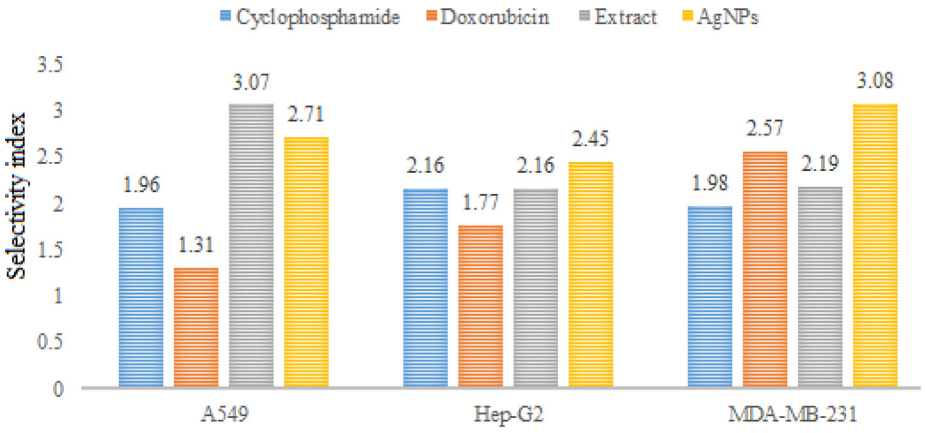

Fig. 7 Selectivity indices of standards, extract and silver nanoparticles against HPAEpiC.

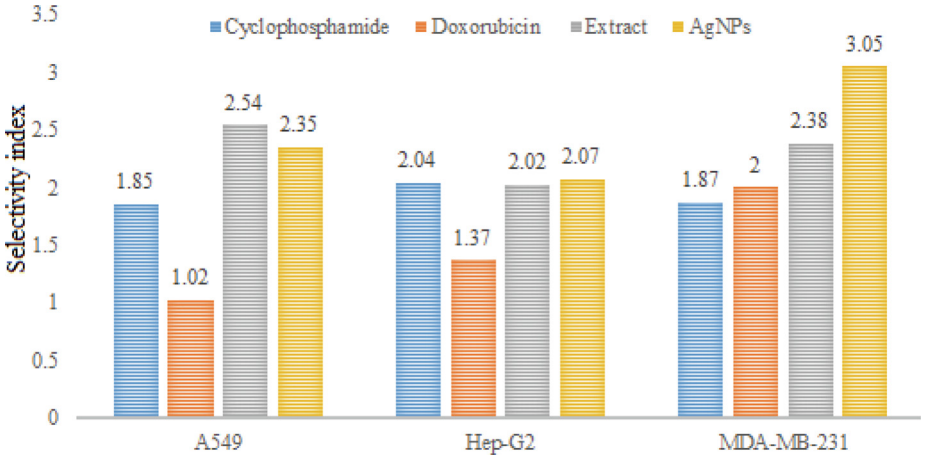

Fig. 8 Selectivity indices of standards, extract and silver nanoparticles against HRPTEpiC.

apoptosis and cell death. Plant mediated AgNPs have shown significant cytotoxic activity against different cell lines including Hep-G2 (liver cancer), PC3 (human prostate cancer), HeLa (cervical cancer), A549 (lung cancer), BxPC-3 (pancreas cancer) and MCF-7 (human breast cancer). AgNPs synthesized from mint extract have shown promising activity against HCT116 colon cancer cell lines; the author attributed the cytotoxic activity to the delayed cell division in G1 phase. In another study, AgNPs from leaf extract of *Piper nigrum* showed significant activity against MCF-7 and Hella cancer cell lines. Significant disruption in plasma membrane integrity and inhibition in cell growth was found in the studied cells (Jabeen et al., 2021). Similarly, the cytotoxicity of AgNPs can be attributed to; formation of stable S-Ag bond with thiol group of specific enzymes in cell membrane and its deactivation; denaturation of DNA by breaking H-bonds between nitrogen base of DNA; production of free radicals and ATP leakage (Patil and Kumbhar 2017; Shahzadi et al., 2022; Ragupathi et al., 2016). In last few years, a number of potent cytotoxic drugs have been developed however, the emergence of resistance and side effects associated with these drugs demand for search of safe and effective cytotoxic agents. Present study strongly revealed the cytotoxic potential of synthesized AgNPs. This activity may be due to the synergistic effect of nano sized silver and bioactive molecules attached on their surface. However, we suggests further studies to find out the exact mechanism for cytotoxic activity associated with these nanoparticles.

4. Conclusion

Herein, a simple and ecofriendly method was adopted for the synthesis of silver NPs using *R. japonica* root extract, which produced spherical shaped NPs without using any harmful reducing or capping agents. The role of phytochemicals in the synthesis of AgNPs was confirmed by FTIR studies. The AgNPs exhibited strong antioxidant potential through DPPH, FRAP, H_2O_2 and ABTS radical scavenging assays. This activity is believed to be due to the adsorption of phenolics and flavonoids on the surface of AgNPs. Moreover, AgNPs showed significant cytotoxic effects against the selected cancer cell lines. This activity is believed to be due to the small size and improved bioavailability of AgNPs as compared to crude extract. In addition, the NPs remained highly selective for the mentioned cell lines as evident from its selectivity index values. Current study provides an insight into the usage of *R. japonica* root extract as a potential natural source of antioxidant and could have great importance as therapeutic agent in ameliorating or preventing oxidative stress related diseases such as cancer. However, further studies are needed to confirm the role of *R. japonica* mediated AgNPs in mitigating the mentioned type of cancers and to validated its proper mechanism.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.arabjc.2022.104327.

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