

Green synthesis of silver nanoparticles from Guava (*Psidium guajava* Linn.) leaf for antibacterial, antioxidant and cytotoxic activity on HT-29 cells (Colon cancer)

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ABSTRACT

In this study, biocompatible silver nanoparticles synthesized from aqueous leaf extract of *Psidium guajava* Linn. The green synthesized AgNPs were characterized by various spectroscopic and microscopic techniques (UV Spectroscopy, FTIR, XRD and SEM). The UV-visible spectroscopic analysis showed the absorbance peak at 418nm, which confirmed the synthesis of silver nanoparticles. Identification of functional groups present in the plant extract, which involved in the reduction of silver ion to AgNPs was analyzed by Fourier transform infrared spectroscopy (FTIR). X-ray diffraction (XRD) analysis confirmed crystalline nature of the synthesized nanoparticles. The synthesized AgNPs were round and spherical shape and 20nm size were confirmed by scanning electron microscopy (SEM). Initial phytochemical analysis revealed the presence of secondary metabolites in the aqueous guava leaf extract. The antibacterial activity of guava leaf extract and AgNPs was dose-dependent manner and it was revealed synthesized silver nanoparticles showed potential antibacterial activity than guava leaf extract. The antioxidant activity of synthesized AgNPs showed highest free radical inhibition (80.2%) at 250 µg/ml as compared to guava leaf extract (62.21 %). The biosynthesized silver nanoparticles were observed to possess high cytotoxicity against colon (HT-29) cancer cells with IC₅₀- 68.26 µg/ml. In conclusion, the results obtained in the present study revealed the green synthesized silver nanoparticles from guava leaf extract exhibited considerable antioxidant, antibacterial and anticancer potential.

KEYWORDS: Silver nanoparticles; *Psidium guajava* Linn.; HT-29 cells; antioxidant; antibacterial.

Introduction

Nanotechnology is one of the active research areas in the modern material science. Nanomaterials research has received the considerable attention in worldwide due to its unique properties. A size of the nanoparticle usually ranges from one dimension less than 100nm (Lakhan et al., 2020).

Nanoparticles, nano films, nano rods and fullerenes are different variety of nanomaterials. Among these variety nanoparticles have the ability to expose zero dimension with biochemical applications. Past 10 years, the uniform size and shape dependent nanoparticles synthesized increased because which is frequently used in advanced technology (Ebrahimzadeh et al., 2020). Recently, nano-biotechnology has acquired significance in the field of biology, material science and biomedicine. An authentic and non-harmful combination of nanoparticles has a great advance in the nanoparticle's development. There are various techniques accessible in nanoparticles synthesis process such as micro wave assist process, sono-chemical, electrochemical, radiation assist and photochemical method. The critical part of these techniques was very dangerous and utilize harmful synthetic compounds which may prompt potential health effects and environmental effects. Plants sources used nanoparticles synthesizing methods have a lot of focus due to its eco-friendly and utilization of nontoxic chemicals (Bharathi & Bhuvaneshwari, 2019).

Among the various inorganic metal nanoparticles, silver (Ag) nanoparticles have received considerable attention in the field of biomedical research. AgNPs gained more attention due to its unique physical, biological and chemical properties (Roy et al., 2019). Recently synthesizing silver nanoparticles through biological method using microorganisms, plant extract and marine algae. Among these the plant mediated AgNPs has high biological properties (Shah et al., 2020). The plant associated AgNPs has excellent absorption capacity and utilizes the chemical component present in the plant materials and is not toxic to environment (Vijaya anand et al., 2019). Plants contain a wide variety of secondary metabolite including tannins, terpenoids, alkaloids, and flavonoids which have been proved to have anti-microbial, antitumor antioxidant, and other biological activities can be of great attention in therapeutic treatments (Lakshmanan et al., 2018). Plants have the capability to reduce metal ion into metal nanoparticles. Silver has been widely used as healing property and antibacterial agent for many years due to its antiseptic nature and huge therapeutic values. Furthermore, few salts and derivatives of silver economically fabricated as antimicrobial agents. At low concentration of silver-based drugs are effectively affect bacteria and viruses but not for healthy human cells (Mollick et al., 2019). Cancer kills the lives of millions of people worldwide every year. The development of anticancer drug has a great challenge in damaging cancer affected cells without unnecessary toxicity. Hence, over the past few years, the focus of researchers on the development of cancer medications from natural ingredients has increased significantly (Khandelwal et al., 2020). Malignancy is an obstruction in the mechanism of controlling of typical development, multiplication and cell death. Silver nanoparticles act as antitumor by slowing progressive growth of tumor cells (Gomathi et al., 2020).

Psidium guajava Linn. usually known as guava, of the family *Myrtaceae* is a native plant of tropical America, however presently developed all over the world (Perez et al., 2008). The guava leaves have a long history of medicinal uses that are still used today. Guava leaves, roots and fruits have been used for the prevention and treatment of diarrhea, gastroenteritis and dysentery. A liquid leaf extract is used to reduce blood glucose level in diabetics (Olatunde yet al., 2018). High level of antibacterial activity was detected in *P. guajava* Linn. leaves (Geetha et al., 2017). Moreover, the various bioactive compounds, such as polyphenol, flavonoids, polysaccharides, enzymes, proteins and ascorbic acid in the guava leaf was responsible for the reduction of silver ions and antioxidant activity (Wang et al., 2018). Guava leaf extract reported to have chemotherapeutic effects and suppress the proliferation of various cancer cells including colon, prostate, oral epithelium, adenocarcinoma and murine leukemia (Ryu et al., 2012).

Phytochemical screening of leaf extract of guava leaf was carried out according to the methods for identification of carbohydrates, proteins, phenols, terpenoids, alkaloids, coumarins, steroids, paleobotanics, quinones, saponins, flavonoids, and tannins using standard phytochemical method. Among all the variety of medicinal species studied, we can highlight *P. guajava Linn.* that are highly implicated in therapeutic medicine against bacterial infection, inflammation, pain and cancer, (Mahfuzul *et al.*, 2007). The aim of the present study is to synthesis a rapid and eco-friendly AgNPs using fresh leaves of *P. guajava Linn.* and its antibacterial, antioxidant and anticancer activities.

Materials and methods

Materials used:

Silver nitrate (AgNO_3), Sodium hypo chloride, 2,2- Diphenyl-1-picrylhydrazyl (DPPH), Dimethyl sulfoxide (DMSO), Luria Bertani broth and Muller Hinton Agar- HiMedia, India. Dulbecco's modified eagle medium (DMEM), Fetal Bovine Serum (FBS), Trypsin-EDTA, Antimycotic and antibiotic solution were purchased from Gibco, Thermo fishers scientific, India. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) - Sigma Aldrich.

Collection and preparation of Guava leaf extract:

Psidium guajava Linn. used for the preparation of leaf extract were collected from a local region, Chennai, Tamilnadu washed and sterilized with sodium hypo chloride. 25gm of guava leaves were accurately weighed and crushed using a blender and finely macerated. After homogenization 100 ml double deionized water was added and heated over water bath maintained at 80°C for 15 minutes; Now the leaf extract was cooled at room temperature and then filtered through Whatmann No.1 filter paper (pore size 25 μm) and used immediately for the AgNPs synthesis process. (Sun *et al.*, 2014).

Phytochemical evaluation of guava leaf extract:

Fresh extract of guava leaves were used for the phytochemical analysis, such as test for saponins, phenols, tannins, terpenoids and flavonoids for screening the bioactive chemical compounds in the guava leaf extract were carried out using the standard procedure as described by Bisht *et al.*, 2016.

Biosynthesis of silver nanoparticles:

50ml of aqueous guava leaf extract was mixed with 50ml of 1mM AgNO_3 solution. Then the reaction mixture stirred using magnetic stirrer for 24h (Ragunandhan *et al.*, 2011).

Characterization of silver nanoparticles:

The synthesized silver nanoparticles were preliminarily confirmed by UV-visible Spectroscopy (Perkin-Elmer MA, USA), range between 350-750 nm. The possible functional groups in the guava leaf extract reduced AgNPs were analyzed using Fourier Transform Infrared (FTIR Perkin-Elmer, MA, USA) spectra in the range of 4000-400 cm^{-1} . The crystalline nature of silver nanoparticles was confirmed by X-Ray Diffraction pattern (XRD-Bruker AXS, Inc., Madison, USA). The size and morphology of the nanoparticles were identified by using Scanning Electron Microscope (SEM). Energy Dispersive X-ray (EDX) used for the identification of existence elements of silver (Kumar *et al.*, 2017).

Antibacterial activity by agar well diffusion method:

Antibacterial activity of guava leaf extract and AgNPs were tested against *Escherichia coli*, *Klebsiella sp.*, *Pseudomonas sp.* Those bacterial stains were maintained in pre-cultured in nutrient broth overnight in a rotary shaker at 37°C. The cultured bacteria were spread on the Muller Hinton agar using sterile cotton swab. Wells (6mm) were created with the help of sterilized micropipette tips and different concentration (15 µl, 25 µl, 50 µl, 100 µl) of prepared AgNPs and guava leaf extract were loaded into wells on plate. The plates were then incubated for 18 h at 37°C, and zone of inhibition were recorded (Asha et al. 2017).

Determination of free radical scavenging activity by DPPH assay:

0.1 mM DPPH solution was prepared with ethanol. Different concentration of guava leaf extract, synthesized silver nanoparticles and ascorbic acid (e.g., 50, 100, 150, 200 and 250µg/mL) added into prepared DPPH solution. Ascorbic acid was considered as a standard and a blank as DPPH solution without sample was used. The mixture of solution was vortexed and then incubated at 37°C for 30 minutes. Finally, the absorbance of the solution mixture was measured spectrophotometrically at 517nm (Keshari et al. 2020).

Determination of invitro cytotoxic activity by MTT assay:

The present work was carried out in human colon cancer cells (HT-29). The cell lines were purchased from National Centre for Cell Science (NCCS), Pune, India. They were grown in DMEM (Dulbecco's Modified Eagle's Medium) medium with 10% Fetal Bovine Serum, 1% antibiotic and antimetabolic solution and maintained in 5% CO₂ incubator at 37°C. The HT-29 cells (1×10⁴ cells/well) were seeded into 96 well plates and treated with AgNPs in various concentration (15, 25, 50, 75 and 100 µg/mL) for 24 h with serum free media. The spent medium was discarded from cells at the end of the treatment period. 0.5mg/mL MTT prepared in 1 X PBS was added and incubated at 37°C for 4 h using CO₂ incubator. The medium containing MTT was aspirated from the cells after incubation period and washed using 200µL of PBS. The developed crystals were dissolved using 100µL of DMSO and the formazan dye turns to purple blue color. The absorbance was measured at 570nm using microplate reader (Khorrami et al. 2019).

Result

Phytochemical analysis:

The guava leaf extract has been analyzed for their phytochemical constituents. The test results confirmed that the leaves extract contains secondary metabolites like carbohydrates, alkaloids, flavonoids, phenolic compounds, saponins, sugars, etc. The results were qualitatively expressed by positive sign and show the observed color changes in various phytochemical tests (Table 1).

Table 1. Phytochemical analysis

S. No	Phytochemical components	Guava leaf
1	Carbohydrates	-
2	Tannins	+

3	Saponins	+
4	Alkaloids	-
5	Flavonoids	-
6	Quinones	-
7	Glycosides	+
8	Terpenoids	+
9	Phenols	+
10	Steroids	+
11	Anthraquinones	+

Characterization of synthesized AgNPs

UV-visible spectroscopy analysis:

The formation of silver nanoparticles from guava leaf extract observed using UV-visible spectroscopy. When the leaf extract was mixed with silver nitrite solution, slowly it started to change colour from greenish brown to dark brown color due to the reduction of Ag^+ to Ag^0 (Fig 1). The surface plasmon resonance peak was raised after 24 h of incubation. A broad peak was observed at 418nm which further confirmed the synthesis of AgNPs as shown in (Fig 2).

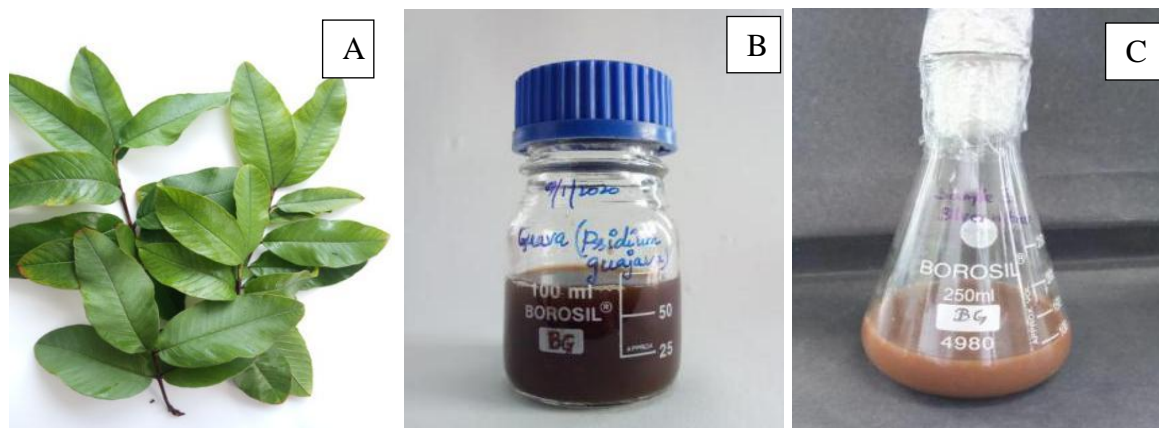


Figure 1 - A) *Pidium guajava* leaf B) leaf extract of *Pisidium guajava* Linn. and C) synthesized silver nanoparticles after 24 h of incubation

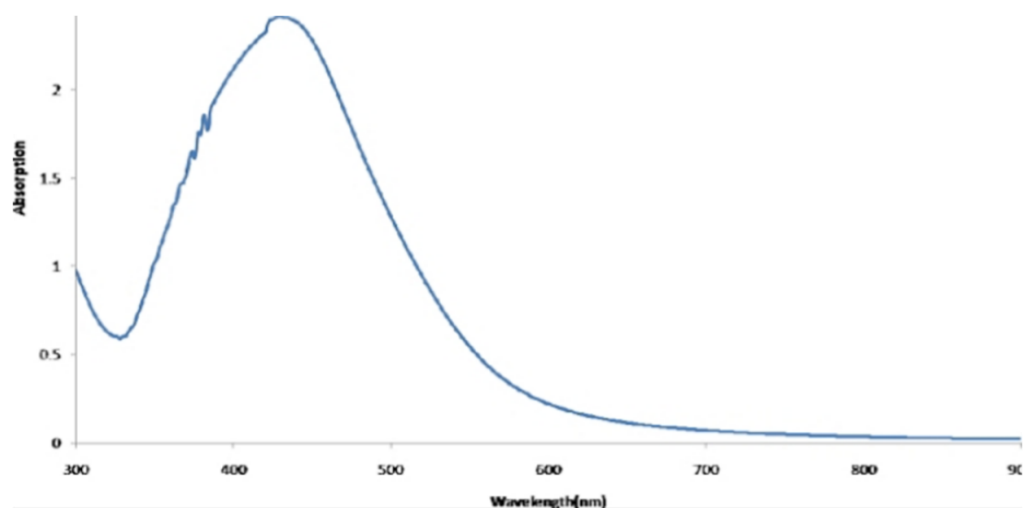


Figure 2- UV – VIS spectra of Biosynthesis of silver Nanoparticles

Fourier transform infra-red analysis:

FTIR analysis was used to identify the functional groups associated with AgNPs, biosynthesized from *P. guajava Linn.* leaf extract, which is responsible for capping, reduction and efficient stabilization of the AgNPs. (Fig 3) shows some pronounced absorbance bands observed at 3739 cm^{-1} [N-H stretching (amine group)], 2918 cm^{-1} [C-H (sym/Asym) aliphatic], 2360 cm^{-1} [aromatic rings], 2029 cm^{-1} [aliphatic nitro compounds NO_2 symmetric], 1975 cm^{-1} [Ether linkage], 1053 cm^{-1} [C-C (aromatic benzene)]. There is a significant change in the vibrational spectra of stabilized nanoparticles when compared with increase in the concentration of leaf extract of *P. guajava Linn.* The absorption peak at 3739 cm^{-1} gets narrower and broader due to proteins, which are formed between the amine groups.

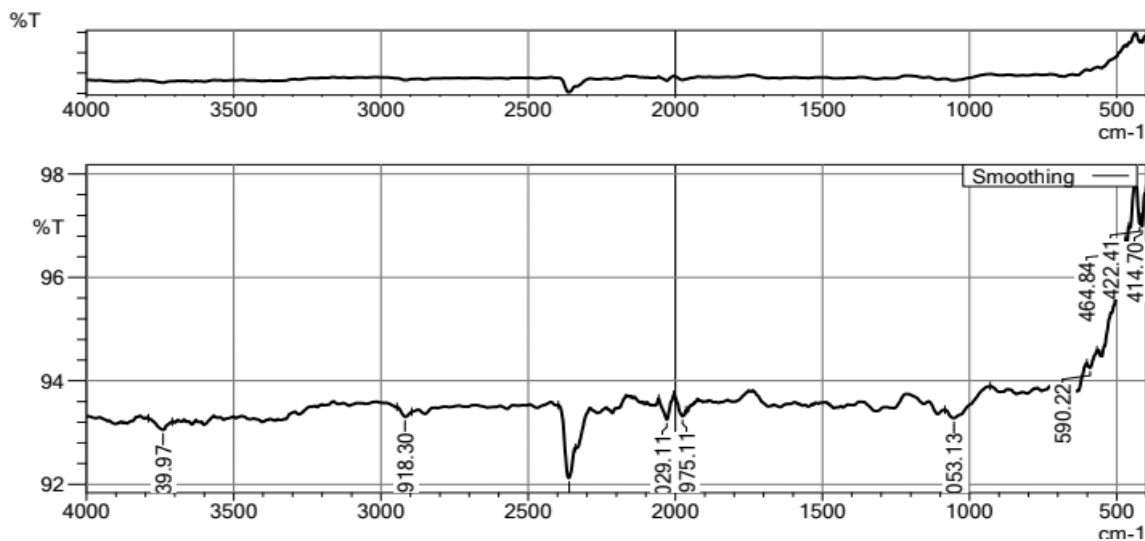


Figure 3. Fourier transform infrared spectrum of silver nanoparticles

X-ray diffraction analysis:

Analysis of AgNPs using X-ray diffraction confirmed the crystalline nature of particles (Fig 4). The XRD spectrum shows four main predominant Bragg diffraction peaks which are positioned at 2θ values of 38.0° , 44.1° , 64.4° , and 77.4° which corresponds to 111, 200, 220, and 311 facets of the face-centered cubic structure of AgNPs. These diffraction peaks are consistent with the standard Ag crystals, indicating that the synthesized AgNPs are of crystalline in nature. The unassigned peaks (*) observed in the XRD spectrum indicates the crystallization of plant phytochemicals phase presence in guava leaf extract occurred on the surface of the AgNPs.

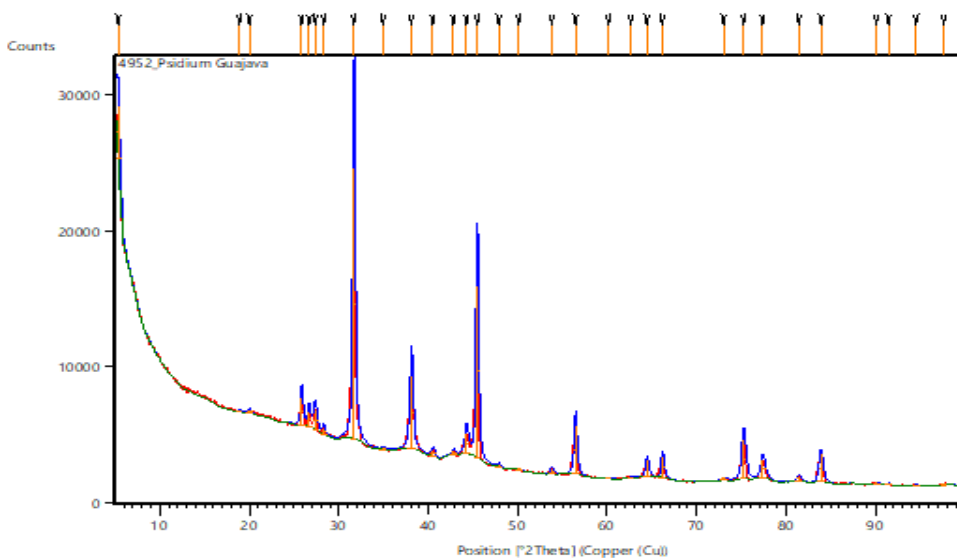


Figure 4- X-ray diffraction spectrum of silver nanoparticles

Energy dispersive X-ray spectrometer analysis:

Energy dispersive X-ray (EDX) spectrometer set up the presence of natural identification of the silver and homogenous assignment of silver nanoparticle. Assessment of AgNPs by Energy Dispersive X-ray (EDX) spectrometer showed the existence of elemental indication of the Ag and homogenous distribution of synthesized AgNPs. The sharpe sign pinnacle of a capably settled the decrease of AgNO_3 to AgNPs. The upstanding pivot communicates the quantity of X-ray tallies while the equal hub shows energy in KeV. Detection lines for the main releasing energy for Ag were clarified and these communicate with peaks in the spectrum, thus giving affirmation that Ag has been properly recognized and present in the solution (Fig 5).

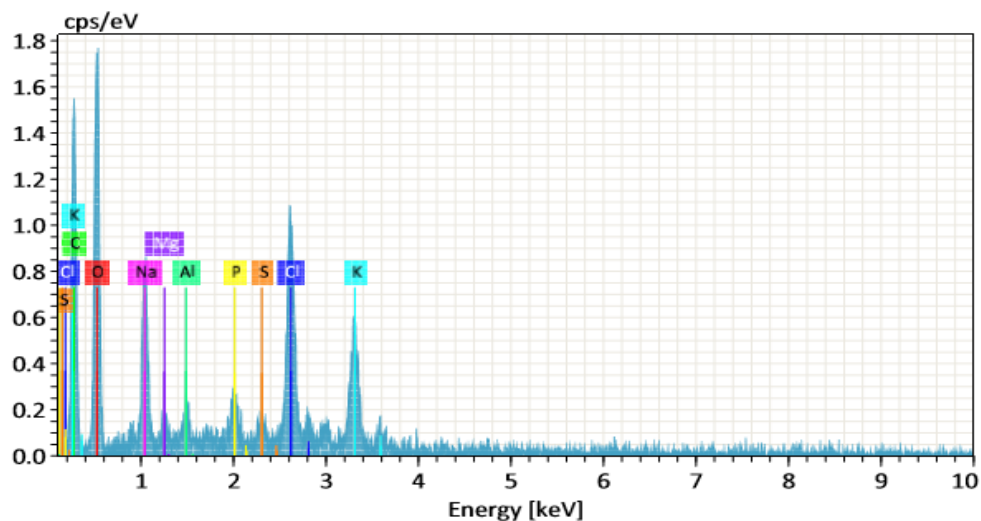


Figure 5- Energy dispersive X-ray spectrum of silver nanoparticles

Scanning electron microscopy (SEM) analysis:

The Scanning electron microscopy (SEM) has been used to identify the size, shape and morphology of silver nanoparticles. Size and shape of the silver nanoparticles depend on the precursor, reducing agents used to synthesize nanoparticles. The size of silver nanoparticles were found at 20 nm and spherical and some particles were round in shape (Fig 6).

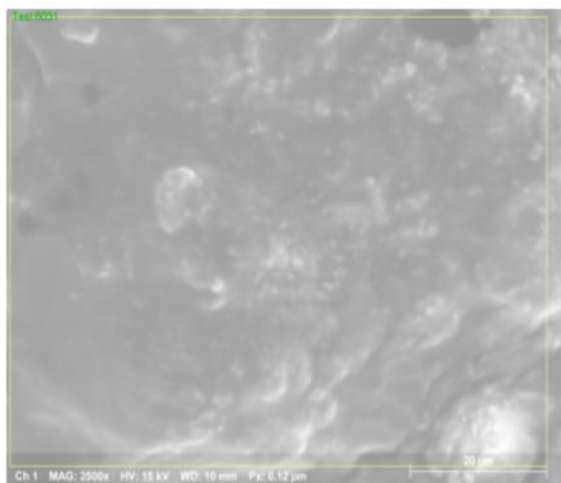


Figure 6- Scanning electron microscope image of silver nanoparticles

Antibacterial activity by agar well diffusion assay:

The agar well diffusion method was employed for screening the antibacterial activity of green synthesized silver nanoparticles and aqueous extract of *P. guajava* Linn against Gram negative bacteria. The aqueous leaf extract and green synthesized AgNPs showed antibacterial activity in all concentrations (15, 25, 50 and 100 µL) against test pathogens such as *E. coli*, *Pseudomonas sp.* and *Klebsiella sp.* and the results are shown in table 2. The highest zone of inhibition was found against *Pseudomonas sp.* and minimum level antibacterial activity were observed in *E. coli* for aqueous extract of *P. guajava* Linn. The synthesized AgNPs showed highest zone of inhibition was found in *Klebsiella sp.* and lowest inhibition was found in *E. coli* (Fig 7). The present study clearly indicates the antibacterial activity was dose-dependent manner and it was

revealed synthesized silver nanoparticles showed potential antibacterial activity than aqueous extract of *P. guajava* Linn.

Table 2: Antibacterial activity of silver nanoparticles

Bacterial species	Zone of inhibition							
	15 μ l		25 μ l		50 μ l		100 μ l	
	Leaf extract	AgNPs	Leaf extract	AgNPs	Leaf extract	AgNPs	Leaf extract	AgNPs
<i>E. coli</i>	4 mm	4 mm	5 mm	7 mm	7 mm	6 mm	8 mm	10 mm
<i>Klebsiella sp.</i>	5 mm	5 mm	6 mm	6 mm	7 mm	21 mm	10 mm	25 mm
<i>Pseudomonas sp.</i>	5 mm	5 mm	7 mm	6 mm	8 mm	9 mm	14 mm	10 mm

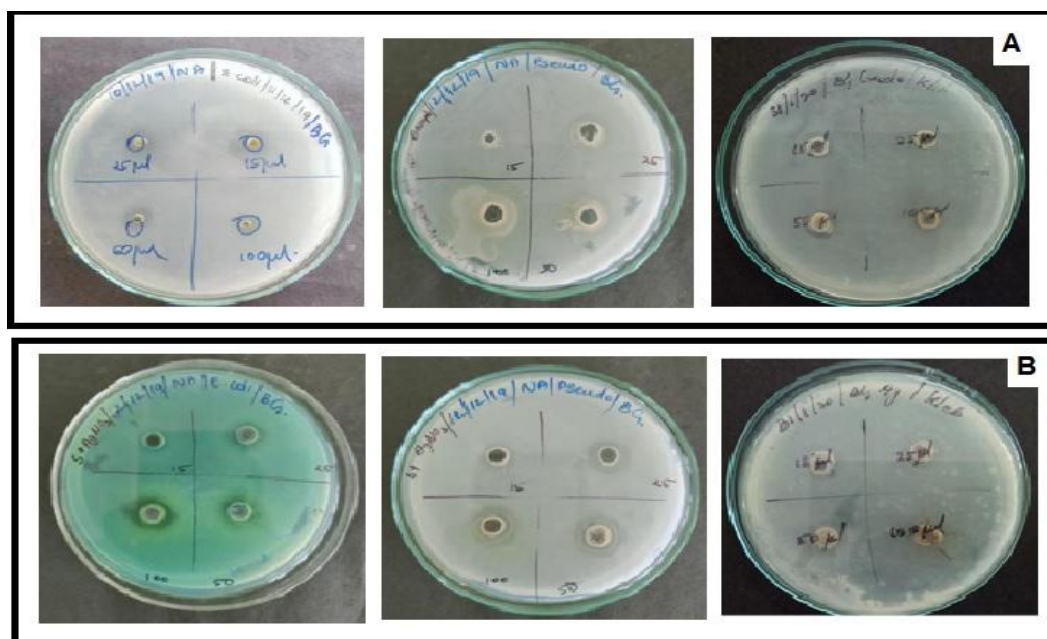


Figure 7- A) Silver nanoparticles treated bacterial pathogen and B) Guava leaf extract treated bacterial pathogens.

Antioxidant activity by DPPH assay:

The antioxidant properties of guava leaf extract and synthesized AgNPs were examined by a DPPH radical scavenging assay. In this study the antioxidant activity was found in dose-dependent way. The synthesized AgNPs displayed the highest free radical inhibition (80.2%) at 250 μ g/ml. Likewise, the guava leaf extract showed the highest scavenging effect (62.21 %) respectively (Fig 8). The DPPH scavenging activity of synthesized nanoparticles exhibited significant inhibition when compared with standard ascorbic acid. Hence the biosynthesized silver nanoparticles showed higher antioxidant activity than guava leaf extract.

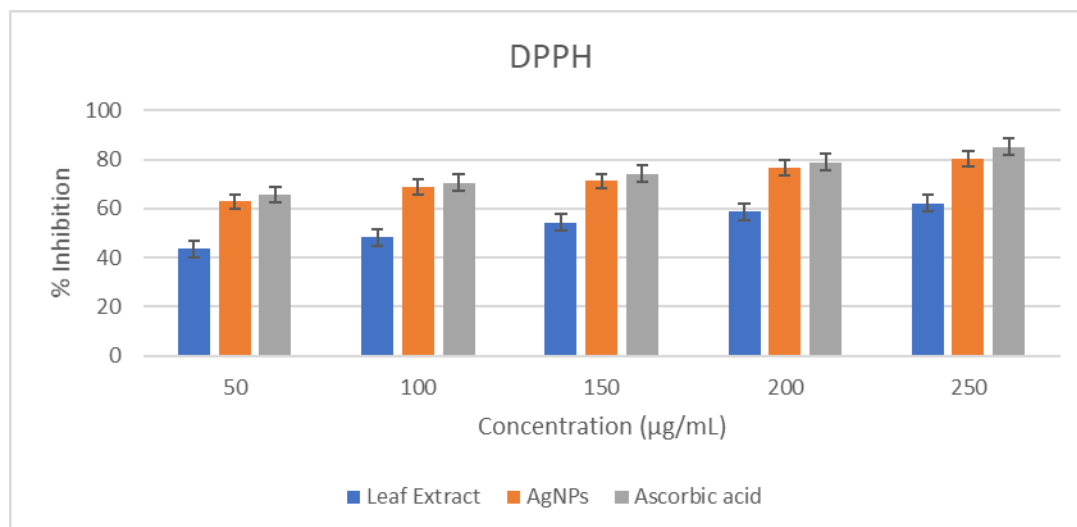


Figure 8: DPPH radical scavenging activity

Invitro cytotoxic activity by MTT assay:

Invitro cytotoxic activity of guava leaf mediated silver nanoparticles was analyzed by MTT assay against HT-29 colon cancer cells. The cells were treated with different concentration of AgNPs, the untreated cells considered as control. The cell viability was assessed following the treatment of cells with different concentrations of AgNPs (15, 25, 50, 100 and 500 µg/mL). The results of the MTT assay revealed that the cell viability decreased with increase in the concentration of the synthesised AgNPs Fig C. The IC₅₀ value was found around 68.26 µg/ml. The synthesized silver nanoparticles treated colon cancer cells changed its morphology and the cell count was reduced when compared to control well (Fig 9). It proved the AgNPs from guava leaf extract has a tendency to kill cancer cells and it acted as anti-cancer activity for colon cancer.

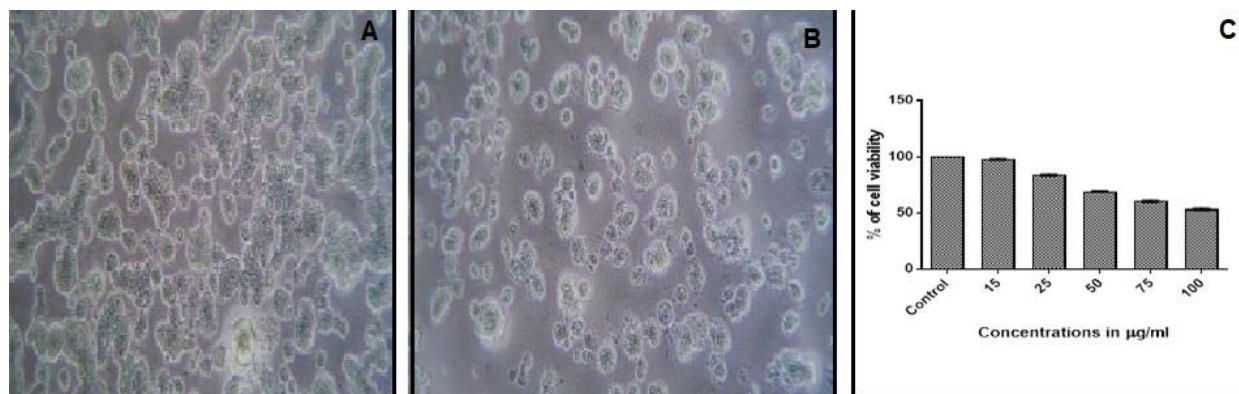


Figure 9: A) HT-29 control cells, B) AgNPs treated cells and C) Cytotoxicity of AgNPs

Discussion

Green synthesis of silver nanoparticles was done by guava leaf extract. Guava leaf extract has important biological components like Tannins, Saponins, Glycosides, Phenols, Anthraquinones detected by phytochemical analysis and alcoholic, protein and nitrogen groups were found by FTIR analysis acts as potential reducing and capping agent to synthesize AgNPs (Nasar et al., 2019; Roy et al., 2019). The synthesized silver nanoparticles were confirmed by visual observation of color changes in solution. The dark brown colored colloidal solution indicating the

silver ions reduction reaction due to the response of surface plasmon resonance (Pirtarighat et al., 2019). UV spectroscopy analysis used to assess the color change of reaction mixture. The increasing color intensity of colloidal solution increases adsorption rate due to the bulk amount of silver ions reduced and more nanoparticles integrated (kaur et al., 2019). Our report ultimately similar to previous studies. (Parvataneni et al., 2020).

The XRD analysis used to determine the crystalline nature of silver nanoparticles. The crystalline size of the synthesized AgNPs was calculated by the Debye-Scherrer equation, $D = K\lambda/\beta \cos \theta$. Where D = average crystallite size, K - shape factor (0.9), λ - wavelength of Cu K α radiation (1.541 Å), β - FWHM of reflection (in radians) located at 2θ and θ - angle of reflection (in degrees) was used to relate the crystallite size to the line broadening. The average crystallite size of prepared AgNPs was found to be about 25.8 nm (Saxena et al., 2012). Babak sadhegi et al., 2015 reported that green synthesized silver nanoparticles using seed aqueous extract of *pistacia atlantica* showed similar results. The average size, distribution and surface morphology of synthesized silver nanoparticles have been analyzed using SEM coupled with EDX respectively. Integrated silver nanoparticles have been found to be crystalline aggregated and spherical in size and different size ranges. Such differentiations in size and shape are common when using various biological compounds using synthesis process (Kedi et al., 2018). Previously similar results have been found reported for silver nanoparticles from plant extract as reducing agents (logeshwari et al., 2015; Awwad et al., 2009).

The antibacterial mechanism of silver nanoparticles which releases silver ion into the bacterial cells and inactivated the bacterial enzymes. It inhibits DNA replication, cause cytoplasm damage and reduced adenosine triphosphate level and destroy the bacterial cells (Hajmohamadi et al., 2019). Because nanoparticles are smaller in size and have a larger surface area gives more effective in antibacterial activity (Bilal et al., 2017). The smaller nanoparticles have a tendency to penetrate into the bacterial cell membrane for the destruction of DNA and cause cell death (Bhakya et al., 2015). Table-2 represented the biosynthesized silver nanoparticles showed potential antibacterial activity than guava leaf extract. Radhika Parvataneni, 2018 reported that aqueous leaf extract of *Scoparia dulcis* L. synthesized AgNPs showed maximum antibacterial activity against *E. coli* (13 mm) and *Pseudomonas sp.* (11 mm) at 1mg/mL of concentration. *Ephedra procera* C. A. Mey. mediated silver nanoparticles showed good antibacterial activity against *E. coli* and *Klebsiella sp.* (Muhammad et al., 2019). There are many studies and reports that plant mediated silver nanoparticles has strong antimicrobial activity. These AgNPs were effective in killing a wide variety of bacterial pathogens involved in various infectious diseases (Guerra et al., 2020).

The increased formation of reactive oxygen species (ROS) in the living cells as a results of oxidation process, and it is extremely unhealthy due to vigorous oxidation-reduction potential. Under normal biological condition, the generation of ROS and antioxidant capacity of cells ought to be balance, whereas beneath imbalance condition, the cells start the oxidation process to develop oxidative stress. (Roy et al., 2019) Free radicals can cause cellular damages in the human body, which is generally neutralized by antioxidants substances in the plant materials. Most of the antioxidants are known to slow down the progression of chronic diseases (Ansar et al., 2020). The antioxidant capacity of guava leaf extract and synthesized silver nanoparticles were found that that AgNPs have more radical scavenging activity than leaf extract. The antioxidant capacity was observed due to the presence of phytochemicals like phenolic functional groups in the plant extract as capping agents of the nanoparticles (Kumar et al., 2016).

In the current research, the impact of guava leaf extract synthesized silver nanoparticles on the cytotoxicity of HT-29 cells was analyzed. Colon cancer is the third most dangerous cancer in

world wide. The silver nanoparticles were able to inhibit the HT-29 cells in a dose dependent manner (Muthu Irulappan *et al.*, 2010). Silver nanoparticles have pro-apoptotic and antioxidant potential which acted as therapeutic agent for cancer cells. When silver ions enter into the cancer affected cells which blocks the DNA repair enzymes it can suppress DNA multiplication (Biresaw *et al.*, 2020). This might be attributed to two effects, intracellular Reactive Oxygen Species (ROS) generation by AgNPs and its activity on the key cellular components, leading to cell death (Samari, F *et al.*, 2019). Factors such as particles size and surface charges have greater dissemination and high penetration in the tumor cells. Cancerous cells having a different pore size compared to other cells, the size-controlled silver nanoparticles can be exhibited effectively in the therapy of cancer (Amendola *et al.*, 2010). Previous study reported that a *Zingiber officinale* synthesized AgNPs inhibits the HT-29 cells at 150.8 µg/ml (Venkatadri *et al.*, 2020). In our study reported that the cytotoxic effect of guava leaf synthesized silver nanoparticles that can halt the cell proliferation at lower concentration in colon cancer.

Conclusion

In conclusion, the silver nanoparticles developed using *P. guajava Linn* leaf aqueous extract by rapid, non-toxic and environment friendly green approach method. The synthesized silver nanoparticles were characterized by UV-spectroscopy, SEM, XRD and FTIR. The biosynthesized AgNPs exhibited DPPH scavenging property and excellent antibacterial activity against *E. coli*, *Pseudomonas sp.* and *Klebsiella sp.* and, the excellent anticancer activity against HT-29 colon cancer cells. Our finding confirmed that the green synthesized silver nanoparticles showed potent pharmacological activity.

Conflict of interest

The authors have no conflicts of interest regarding this investigation.

Acknowledgments

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