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Green Synthesis of Silver Nitrate Nanoparticles using L. of *Pyracantha crenulata* and its Anticancer Activity on Liver Cancer

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Abstract: The next-generation of anti-cancer medications have a lot of potential, thanks to the biocompatible silver nanoparticles (AgNPs). Here, AgNPs were produced utilizing *Pyracantha crenulata* leaves in a biogenic manner. Several spectroscopic methods were used to characterize and verify the produced AgNPs. The peak of the AgNP's Surface Plasmon Resonance (SPR) at 455 nm was discovered by UV spectroscopy research. Transmission electron microscope (TEM) examination was used to determine the precise size and morphology of AgNPs, which were discovered to be 60 nm in size and spherical, triangular, and hexagonal in form. X-ray Diffraction (XRD) spectrum data supported AgNPs crystallinity. The existence of elemental Ag was confirmed by (EDX) spectra, which displayed a clear, strong signal at 3.0 KeV. Particle size analysis was used to characterize the produced Ag nanoparticles. In order to characterize the silver nanoparticles, a (DLS) zetasizer particle size analysis was performed. According to a DLS investigation, the biosynthesized silver nanoparticles are 1340.8 nm in size and have a polydispersity index (PDI) of 0.6%. Silver nanoparticle stability was assessed using a zeta potentiometer. The nanoparticles generated had a zeta potential of -18.7 mV. The leaf extract's functional groups and the AgNPs that were produced, were identified using the analytical technique, FTIR. Examining AgNPs anti-cancer efficacy against liver (hep-2) cancer revealed its strong anti-cancer potential. Our findings suggest that generated AgNPs greatly inhibit the growth of liver cancer cell (hep-2) as measured by the MTT test.
Keywords: Silver nanoparticles, Pyracantha crenulata, TEM, anti-cancer activity, MTT assay

Introduction

Cancer refers to a broad variety of cancers brought on by unregulated cell proliferation. Negative consequences, such tissue breakdowns, are likely to occur if normal cells do not control fast growth. It is the second most common cause of cancer-related mortality in industrialized countries, and its prevalence is growing quickly in both developed and developing countries (Tian et al., 2020). By 2020, the World Health Organization (WHO) projects that liver cancer, the second most frequent malignancy, would be responsible for 10 million cancer-related deaths globally. Most cancers can be treated and controlled with a combination of radiation therapy, chemotherapy, hormone therapy, and surgery. Nanotechnology-based diagnostic and therapeutic approaches have recently demonstrated potential for enhancing tumor therapy (Ratan et al., 2020). Cancer nanomedicine has ushered in a new era of multidisciplinary research in biomedical engineering, pharmacology, chemistry, and biology with a focus on specific advancements in cancer detection, investigation, and management (Wang and Thanou et al., 2010). For a variety of purposes, including medical and environmental studies, nanoparticles have been developed recently (Batool et al., 2019).

Biomaterial engineering now has a broader view point thanks to nanoscience's vast variety of applications, and this method focuses on creating nanomaterials with more fascinating nanoparticles. Furthermore, it is generally accepted that using green chemistry to make eco-friendly nanoparticles is preferable. Even though substantial research on seaweed-mediated nanoparticles for a variety of therapeutic applications has been conducted due to the presence of different secondary metabolites (Sandhiya et al., 2021).

Pyracantha crenulata, commonly known as *"Ghingharu"*, is an evergreen, spinescent undershrub or shrub that may reach a height of 5 meters and belongs to the Rosaceae family. Its leaves are narrowly oblong or oblong-lanceolate in form, 1.5 to 3 cm long, and have a crenate, sharp tip. They are densely packed at the terminals of short lateral branches. From Himachal via Bhutan, Tibet, Myanmar, and China, it may be found all over the Himalayan Mountain range. The fruit of the plant has cardiotonic properties that make it useful for treating hypertension and weakening of the myocardium. The hypotensive, coronary vasodilator, and cardio tonic properties of fruits are claimed. Burger's diseases, paroxysmal tachycardia, myocardial deterioration, and heart failure have all been treated with it. There are several phytochemicals, including tannins, saponins, cyanidin, polymeric flavone, rutine, glycosides, and cyanogenetic glycosides (Saklani et al., 2014). *Pyracantha crenulata* has showed a variety of biological qualities, including antioxidant (Guglani et al., 2022), antiproliferative (Singh and al., 2015), antiurolithogenic (Bahuguna et al., 2009), and anti-inflammatory (Otsuka et al., 1981) action. Cancer is a condition in which the body's cells proliferate unchecked. Liver cancer is the term for cancer that first appears in the liver. About 25,000 men and 11,000 women are diagnosed with liver cancer each year in the US, and the illness claims the lives of 19,000 men and 9,000 women. For several decades, the number of Americans developing liver cancer increased, but it is currently dropping. Compared to the United States, other countries of the world have a higher prevalence of liver cancer. Primary liver cancer is a condition in which the tissues of the liver develop malignant (cancer) cells. Primary liver cancer is not the same as cancer that starts in another place of the body and travels to the liver.

One of the biggest organs in the body is the liver. It occupies the top right side of the abdomen, just within the rib cage, and has two lobes. The liver's primary duties include producing bile to aid in the digestion of dietary fat, storing glycogen (sugar), which the body utilizes as fuel, and filtering toxic compounds from the blood so they may be expelled from the body through faeces and urine. The two most common kinds of adult primary liver cancer are hepatocellular carcinoma and bile duct cancer (cholangiocarcinoma). Hepatocellular carcinomas make up the majority of adult primary liver cancers. The third most common cause of cancer-related fatalities globally is this particular form of liver cancer. People of any age can develop primary liver cancer. However, paediatric therapy is not the same as adult treatment. One of the most widely utilized marketed nanomaterials for applications including antibacterial agents, medication and gene delivery systems, and biosensors is silver nanoparticles (AgNPs). Data study showed that there is no publication regarding the ecologically friendly production of AgNPs using *Pyracantha crenulata's* dissolved leaf extract. The goals of the current work were to investigate the anticancer characteristics of AgNPs synthesized from *Pyracantha crenulata* leaves extract and to design a novel, rapid, and environmentally friendly approach. Additionally, the present study's findings are consistent with those of numerous another researchers who performed on the creation of

AgNPs utilizing various extract of plants (Table-1).

Table 1 Green silver nanoparticle production from some significant medicinal herbs

S. No.	Name of the plant	Family	Plant part	Shape	Size (nm)	Ref.
1	*Prunus persica*	Rosaceae	Leaf	Spherical	40-98	(Kumar et. al., 2017)
2	*Ocimum sanctum*	Lamiaceae	Leaf	Spherical	14.6	(Jain and Mehata, 2017)
3	*Berberis vulgaris*	Berberidaceae	Leaf	Spherical	30-70	(Behravan et. al., 2019)
4	*Azadirachta indica*	Meliaceae	Leaf	Spherical	34	(Ahmed et. al., 2016)
5	*Euphorbia hirta*	Euphorbiaceae	Leaf	Spherical	40-50	(Elumalai et. al., 2010)
6	*Coriandrum sativum*	Apiaceae	Leaf	Spherical	6.45	(Khan et. al., 2018)
7	*Pyracantha crenulata*	Rosaceae	Leaf	Spherical, Hexagonal, Triangular	60	Present work

Material and Methods

Gathering and examining plant materials using resources and methods. The Herbarium Forest Research Institute confirmed the authenticity of *Pyracantha crenulata* leaves that were obtained from the Nagdev Forest area in Pauri, Uttarakhand.

Preparation of leaf extract:

To get rid of any clinging dirt, fresh and healthy *Pyracantha crenulata* leaves were properly cleansed in double-distilled water. The leaves had dried in the shade for 15 days when they had attained their constant weight. Following the crushing of these dried leaves with a mortar and pestle, 10 g of Pyracantha crenulata was added to 500 ml of double-distilled water in a 500 ml Erlenmeyer conical flask and heated at 69 °C for 21 minutes. The extract was then filtered through Whatman filter paper no. 1 in a separate conical flask after being allowed to cool to room temperature.

Synthesis of silver nanoparticles:

A 1:11 ratio of 3 mM aqueous $AgNO_3$ solution and *Pyracantha crenulata* leaf extract was utilized in a 2 L Erlenmeyer flask. It was kept for 70 hours in a dark place. The hue of the solution shifted to a deep red when AgNPs were produced. After centrifuging the mixture for 20 minutes at 7500 rpm to remove any remaining impurities, it was washed in distilled water and acetone. The resulting material was dried at 50 °C for 24 hours in an oven before being broken up in a mortar and pestle to create fine powdered greyish black AgNPs for the purpose of characterizing the AgNPs and their anti-cancer activities.

Characterization

The formation of silver nanoparticles was initially seen using the Elite twin beam UV-visible spectrophotometer on a regular basis. Then, using the X'PERT-PRO Diffractometer, PAN analytical; CuK radiation, max = 1.54; and reporting their spectra in the range of 2, from 0º to 75º, silver nanoparticles were subjected to X-ray diffraction (XRD) analysis. FTIR (Spectrophotometer Perkin Elmer Model RZX) analysis at wavelengths between 4000-400 cm^{-1} was used to pinpoint the bioactive plant extract components in charge of capping and stabilizing the nanoparticles. The average size of silver nanoparticles might be determined using Debye Scherrer's equation. In the equation, the average crystallite size $D = K\lambda/\beta\cos(\theta)$, where 'K' is the Scherrer's

constant, 'λ' is the wavelength, 'β' is full width at half maximum (FWHM), and 'θ' is Bragg's diffraction angle.

Result and Discussion

AgNPs were produced in the current work utilizing an aqueous leaf extract of *Pyracantha crenulate*

UV-Visible Analysis

The bioactive components of the leaf extract were essential to produce AgNPs. The change of the solution from colourless to dark red may be due to the production of AgNPs. The solution's broad absorption peak at maximum 455 nm, with a projected energy band gap of between 2.72 eV, as shown in Fig. 1, served as evidence that AgNPs were present in the mixture.

XRD Analysis

The average crystallite size of the AgNPs was calculated using Debye-Scherrer's equation (Das et al., 2010; Azizi et al., 2013; Karthik et al., 2014). The crystalline nature of the silver nanoparticles was shown by the XRD spectrum. Sharp peaks at 2θ = 38.125°, 38.223°, 44.28°, 44.40°, 64.476° and 64.656º, were observed in the XRD spectra of the synthesized nanoparticles, which are indexed to the (111), (200), (220), (311), (222), (400), (331), (420), and (422) lattice planes of cubic phase in (**Table 2), (Fig. 2).**

EDX Analysis

EDX was employed to ascertain the NP's composition. A distinct signal in Fig. 4 at about 3 keV indicates the presence of elemental silver, and there is also a weak peak for carbon at 0.25 keV, which may have originated from biomolecules bound to the surface of AgNPs. The extra signals noticed in the spectra might be a result of the bioactive compounds present in plant extract

TEM Analysis

Using TEM analysis, the surface morphology of Ag nanostructures was studied (Fig. 3). These results confirmed the AgNP's spherical, hexagonal, and triangular in shape and average size of less than 60 nm.

DLS Analysis

The generated *Pyracantha crenulata* silver nitrate nanoparticles were characterized by particle size analysis using a DLS zetasizer. According to DLS research, the biosynthesized AgNPs in Fig. 5 had a size of 1340.8 nm and a polydispersity index (PDI) of 0.6%. It was determined Whether AgNPs were stable using a zeta potentiometer. The manufactured AgNPs zeta Potentiometer was -18.7 mV in Figure 6.

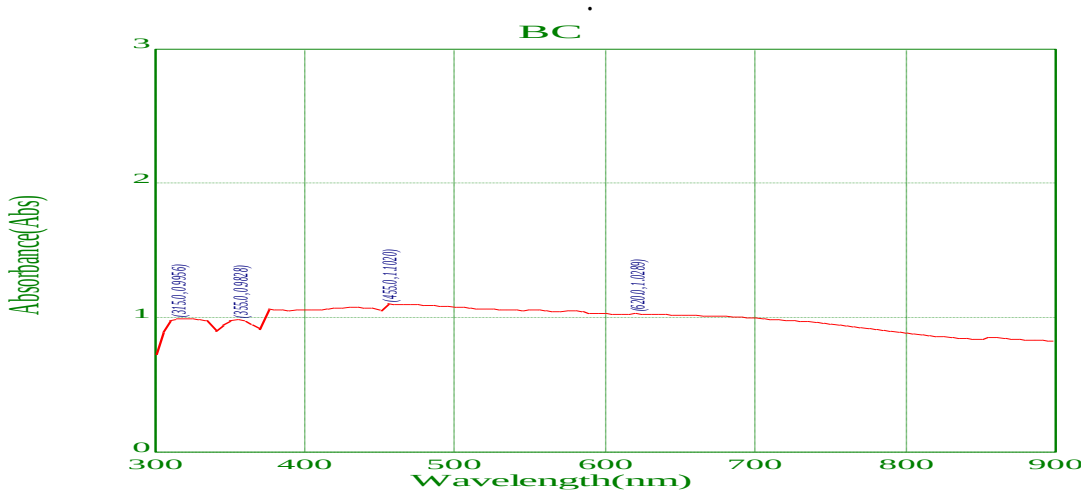

Figure 1 UV-Visible spectrum of AgNPs of *Pyracantha crenulata* leaves part

Table 2. Peak list for average size calculation of AgNPs, Where 'θ' is the Bragg's diffraction angle, (h,k,l) are miller indices and d-spacing is interplanar distance.

2θ (in ^{0})	h,k,l	d-spacing	Rel. Int. (%)
38.125	111	2.35857	100.00
38.223	200	2.35857	50.00
44.28	220	2.04396	25.04
44.40	311	2.04396	12.52
64.476	222	1.44403	30.86
64.656	400	1.44403	15.43

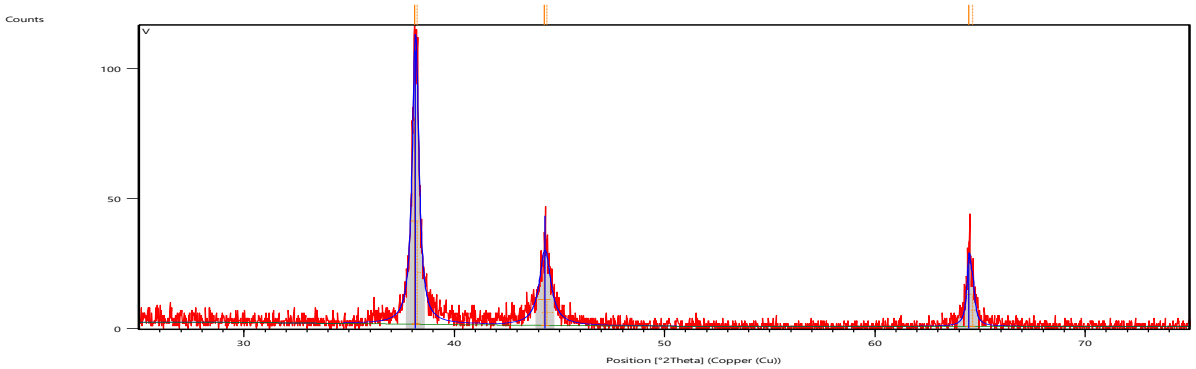

Fig. 2: XRD spectra of synthesized AgNP's of *Pyracantha crenulata* leaves part

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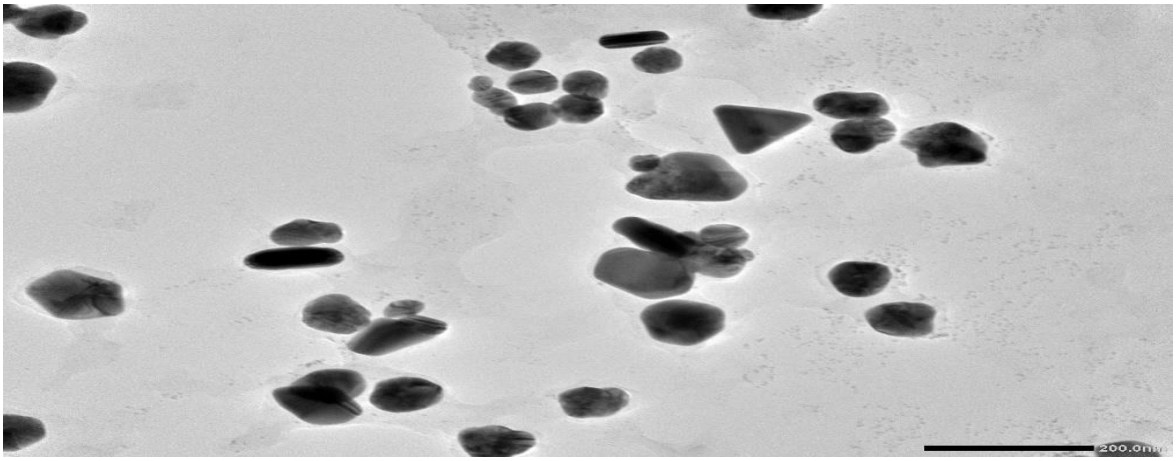

Figure 3. TEM image of synthesized AgNPs of *Pyracantha crenulata* leaves part

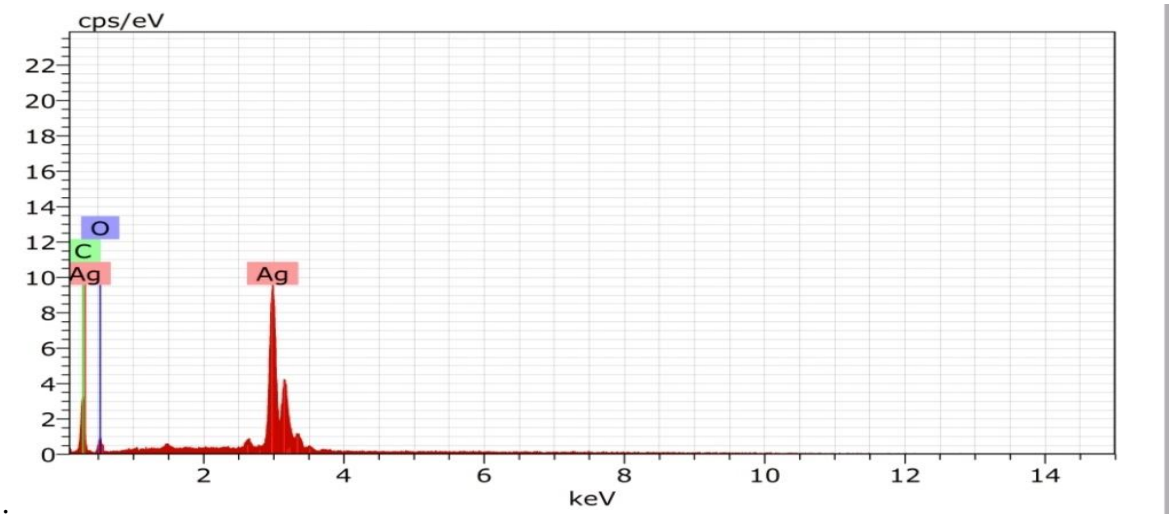

Figure 4. EDX pattern of synthesized AgNP's of *Pyracantha crenulata* leaves part.

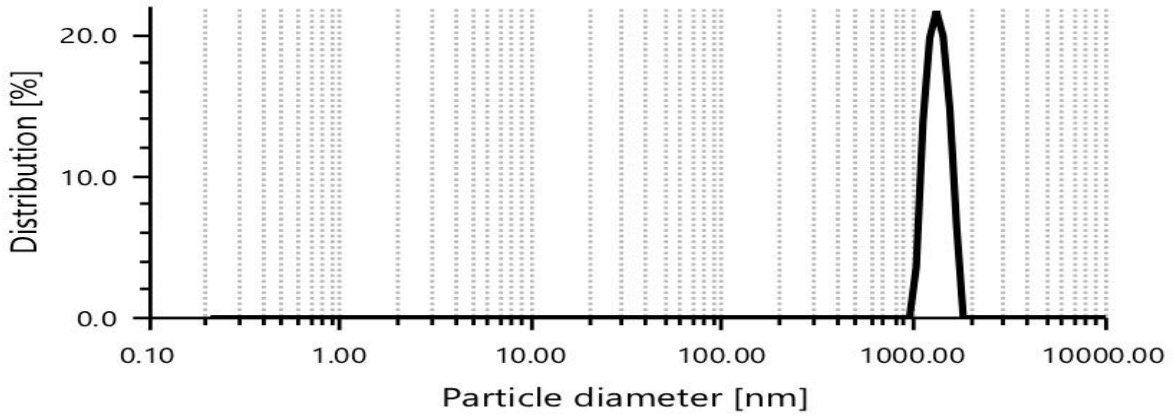

Figure 5. Particle size distribution for synthesized AgNP's of *Pyracantha crenulata* leaves part

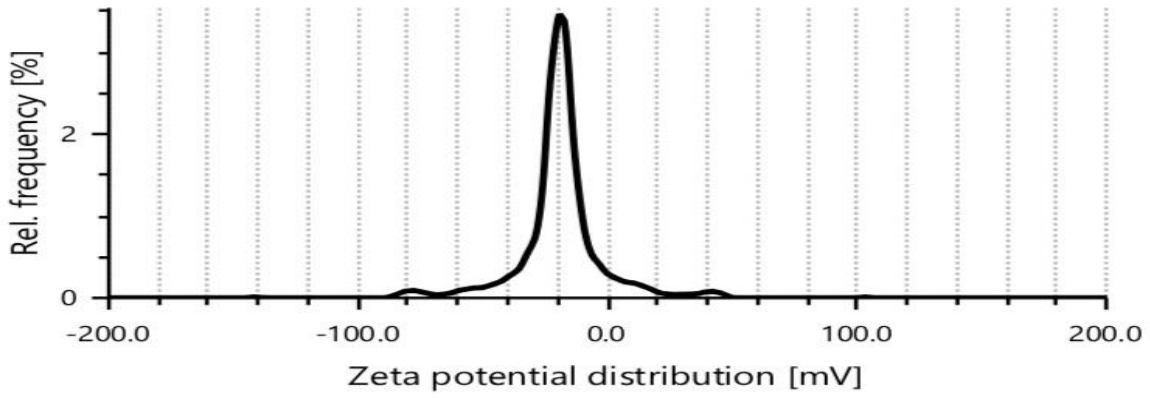

Figure 6. Zeta Potential distribution for synthesized AgNPs of *Pyracantha crenulata* leaves.

Invitro Anticancer Activity

Absorbance values that are lower than those of the control cells indicate that there is less cell growth. A higher absorption rate, on the other hand, indicates more cell development. It has been demonstrated that liver Hep-2 cell lines include in-vitro viable cells. The proportion of viable cells was determined using the Trypan blue dye exclusion technique. The SRB and MTT tests were used to assess the cytotoxicity activity.

The liver (hep-2) cell line displayed 80.02% cell viability as assessed by the Trypan blue dye exclusion procedure, according to the data listed in Table 3 and Figure 7. It was discovered that when treated with the dye, viable cells had a pink color whereas non-viable cells displayed a blue color.

When the nanoparticle's cytotoxicity was tested on the liver (hep-2) cell line, 50% cytotoxicity was found at a concentration of 10 g/ml for each of the cancer cell lines investigated. Cyclophosphamide monohydrate, the standard positive medication, demonstrated 50% cytotoxicity against the liver (hep-2) cell line at a concentration of 5 g/ml. Accordingly, the IC50 value of *Pyracantha crenulata's* silver nanoparticles was shown to be 10 g/ml against cancer cell lines, whereas the IC50 value of cyclophosphamide monohydrate was discovered to be 5 g/ml against the liver (hep-2) cell line. There were no cytotoxic values for DMSO. Table 4 presents the outcomes.

Before the anticancer experiment, both cancer cell lines had elevated cell counts (cell concentration). The liver (hep-2) cell line was tested with the nanoparticles (20 g/ml) using the Sulphorodamine B assay and the Microculture Tetrazolium (MTT) assay, respectively. Silver nanoparticles from *Pyracantha crenulata* were shown to be effective against the test cell line. It was found that the results of the tests, Sulphorodamine B and MTT assay, were connected.

According to results of a sulphorodamine B assay, silver nanoparticles from *Pyracantha crenulata* (10 g/ml) suppress cancer cells in the liver (hep-2) cancer cell line by 78.45%. Cyclophosphamide monohydrate (5 g), a positive control, inhibited cancer cells in liver (hep-2) cancer cell lines by 85.0%. The outcomes are displayed in Table 5.

According to MTT assay results, the silver nanoparticles of *Pyracantha crenulata* (10 g/ml) induce a 75.62 decrease in cancer cells in the liver (hep-2) cancer cell line. The MTT experiment revealed that the positive control, cyclophosphamide monohydrate (5 mg), inhibited liver cancer cells (hep-2) by 82.23%. Table 6 presents the outcomes.

Table 3. Percent cell viability and characterization of cell lines via Trypan blue assay (before anticancer assay)

Cell lines	Percent viability	Live cell count	Total cell count	pH
Liver (Hep-2)	80.02	$1.23x10^6$	$2.18x10^6$	7.4

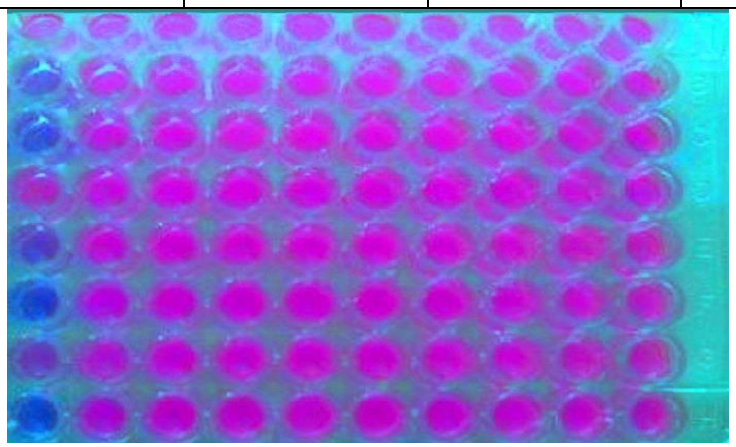

Figure 7. Viability of cells in cancer cell lines in titer plate as determined by Trypan blue assay (Pink colored wells shows viability of the cells. Blue colored wells shows non-viable cells)

Table 4. Cytotoxicity assay of nanoparticles against Hepatic (Hep-2) cancer cell line (IC50 values determination)

Samples	**IC_{50} values (µg/ml)**
	Cancer cell lines used
	Liver (Hep-2)
Cyclophosphamide monohydrate	**5.0**
Silver nanoparticles of *Pyracantha crenulata*	**10.0**
DMSO	**0.0**

Table 5. Sulphorodamine assay in hepatic (hep-2) cell line

	Percent inhibition of cancer cells	
Sulphorodamine B assay In hepatic (hep-2) cell line	**Silver nanoparticles of *Pyracantha crenulata* (10µg/ml)**	**Cyclophosphamide monohydrate (5 µg)**
	78.45	**85.0**

Table 6. MTT assay in hepatic (hep-2) cell line

	Percent inhibition of cancer cells	
MTT assay In hepatic (hep-2) cell line	**Silver nanoparticles of *Pyracantha crenulata* (10µg/ml)**	**Cyclophosphamide monohydrate (5 µg)**
	75.62	**82.23**

In vivo Anticancer Activity

This study examined the in vivo anticancer activity of the liver (hep-2) cancer cell line using the nanoparticles *Pyracantha crenulata* (10 g/ml). In addition to evaluating tumor weight, viable and non-viable cells, RBC and WBC counts, and haemoglobin content, anticancer tests were also carried out. The findings of the current study imply that nanoparticles were successful in reducing the

number of cancer cells in Swiss albino mice's livers. Cyclophosphamide monohydrate (10 g/ml), used as a positive control, was compared to the study results. The hepatic cells of tumor development were further homogenized in the animal models (positive control, negative control, and treatment groups). Hepatic (hep-2) cancer cell line negative control groups in the first set of negative controls were found to have tumor weights of 1.56 g. In the second and third groups, the tumor weights were respectively 0.52 g and 0.56 g in **(Table 7).**

Table 7. Parameters determined in different sets of animal models for evaluation of anticancer activity

Groups	Parameters evaluated					
	Tumor weight (g)	**Viable cell count (cellsx10^6/ml)**	**Non-viable cell count (cellsx10^6/ml)**	**RBC (cellsx10^6/µl)**	**WBC (cellsx10^6/µl)**	**Hemoglobin (g/dl)**
1st Group- Negative Control	1.56±0.82	4.23±0.45	0.32±0.75	7.23±0.92	7.56±0.92	12.23±0.62
2nd Group- Positive Control	0.52±0.53	2.56±0.48	0.67±0.53	4.54±0.75	5.57±0.64	8.37±0.48
3rd Group- Test -A	0.56±0.18	2.23±0.18	2.05±0.25	6.23±0.34	6.23±0.43	13.23±0.15

* ±SD; p<0.5 (level of significance) ***Note:**
1st Group- Negative Control - Mice administered with Hepatic (Hep-2) cancer cell lines only;
2nd Group- Positive Control- Mice administered with Hep-2 cancer cell lines injected with Cyclophosphamide monohydrate (5 µg/ml);
3rd Group- Test-A- Mice administered with Hepatic (Hep-2) cancer cell lines injected with silver nanoparticles of *Pyracantha crenulata* (10 µg/ml).

Conclusion

Silver nanoparticle production may be accomplished quickly, safely, and profitably using *Pyracantha crenulata* leaf extract. We produce crystallized, spherically, hexagonally, and triangulated silver nanoparticles with an average size of 60 nm. According to reports, bioactive plant extract components are what reduce, cap, and stabilize the silver nanoparticles. Because the green route to synthesis doesn't require any potentially harmful substances, it offers a viable substitute for tried-and-true chemical and physical procedures.

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