RESEARCH PAPER

Evaluation of Cytotoxic, Antioxidant, and Antiproliferative Effects of Annona squamosa Hydroalcoholic Leaf Extracts Through MTT Assay Against MCF-7 Cells Supported by GC–MS Profiling, Metal phytochemical Nanoparticle Formulation, and In-Silico Analysis

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ABSTRACT
Researchers often look to medicinal plants as initial sources for creating more effective pharmaceuticals. This work aims to test the antioxidant, cytotoxic, and antiproliferative effects of methanolic extracts of Annona squamosa L. leaves by analysing the polyphenolic and flavonoid fractions using phytochemical screening, uv-spectrum analysis and MTT assay of the extracts against MCF-7 cell lines. This research looks at how Cisplatin, Annona squamosa leaf extract, and green-synthesized PtNPs work together to reduce tissue toxicity and maximize antiproliferative effectiveness. Stable, spherical nanoparticles with an estimated size of 30 nm have been formed, according to UV-Vis, TEM, and Zeta potential studies. Quercetin and kaempferol, two polyphenolic chemicals with therapeutic activity, were discovered by GC-MS profiling. Peroxide scavenging technique was used to evaluate the extracts' antioxidant properties.
Analyses of the extracts' cytotoxicity in human breast cancer (MCF-7) cells showed that a combination of cisplatin, PtNPs, and A. squamosa methanolic extract produces comparatively lower IC50 values, proofing that, it was more effective in enhancing apoptotic activity and modulating reactive oxygen species (ROS). Molecular docking, in silico screening of the extract's phytochemicals, and the Swiss ADME online portal were all utilized in the analysis., and the research validated the receptors and protein structures used in docking analysis. These results show that Annona squamosa L. methanolic leaf extracts contain many bioactive compounds like phytol (C20H40O), quercetin (C27H30O15), and kaempferol (C15H10O6), which have strong antibacterial, antiproliferative, cytotoxic, and antioxidant properties. The triple combination of A. squamosa extract, PtNPs and the conventional medicine Cisplatin showed the most powerful cytotoxicity, suggesting that this combination could make the way to find out new strategies for the anticancer drug development.

Keywords: *Annona squamosa, antiproliferative, In-silico analysis, PtNPs, MTT assay.*
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GRAPHICAL ABSTRACT

INTRODUCTION

Cancer stands as one of the foremost causes of mortality worldwide, presenting a significant global challenge (1,2). Oxidative stress is a primary factor in the induction and development of cancer in normal cells. Cellular signalling can disrupt due to prolonged oxidative stress, that will induce, development and progression of cancer (3,4). Though, various cytotoxic, immunotherapeutic and chemotherapeutic and radioactive agents are used to treat the cancer. These medications are costlier and linked to severe adverse effects in patients (5,6). Additionally prolonged treatment with same chemotherapy may reduce the level of reactive oxygen and nitrogen species (RONS), that will develop resistance to apoptosis of the cancer cells (3,7). Continuous efforts are going on to identify an

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optimal treatment protocol for cancer therapy, that minimizes side effects while remaining cost-effective.

Currently, researchers have advanced second-line treatments utilizing natural products alongside chemotherapy or as a chemo preventive measure (8). Recent studies have demonstrated the evidence for the development of anticancer drugs, that have comparatively fewer side effects through the utilization of natural plant extracts (9). chemotherapeutics like vinca alkaloids and Taxol are obtained from medicinal plant sources, used as anti-cancer drugs widely. Vincristine, vinblastine, Vindesine etc. Are used clinically in the treatment of haematological and lymphatic neoplasms (10). Combinatorial drug therapy employs multiple drugs to target distinct pathways within cancer cells, potentially overcoming drug resistance and improving treatment outcomes. This approach addresses the complex signaling networks involved in cancer and the activation of bypass pathways that limit the effectiveness of monotherapies (11). *Annona squamosa* L. leaf extracts comprise several polyphenolic chemicals, terpenoids, and flavonoids, including epigallocatechin, kaempferol, and quercetin. These chemicals exhibit therapeutic properties alongside certain toxicity consequences (12). These natural polyphenolic compounds show good antimicrobial activities; to acquire resistance is minimized and we can target the bacteria via several mechanisms (13). The essential oils from leaf and fruit pulp of *Annona squamosa* L has utilised from ancient time as traditional and folk medicine to cure variety of tumours and malignancies (14,15). In vitro studies of the crude extracts and isolated flavonoid from *A. squamosa* L and *A. muricata* L. have shown anti-stress, anti-inflammatory, anti-oxidant, antibacterial and anti-tumoral activity (15,16). Acerogenins derived from extracts and isolated polyphenolic compounds of *A. squamosa* L. and *A. muricata* L. exhibit neurotoxic and cytotoxic effects in invitro and in vivo studies (17). Thus, more research is needed to determine the level of toxicity, pharmacological action mechanism, safety profile, ideal and fatal dosage form, and adverse effects of these crude extracts and separated polyphenols. In addition to having an additive effect on conventional anti-cancer drugs (like platinum-based chemotherapeutics like carboplatin, oxaliplatin, and cisplatin), the main objective of using these anti-cancer drugs made from medicinal plants is to reduce the side effects of radiation therapy and chemotherapy. In this context, polyphenols are utilized as anti-inflammatory, anti-collagenase, anti-oxidant, and anti-cancer agents to treat clinical disorders (18,19). Because polyphenols have anti-proliferative properties, scientists have recently been highly interested in them. This work has assessed the flavonoids presents, quantified the phenolic and terpenoid content in the methanolic leaf extract of *A. squamosa* and evaluated the antioxidant activity, through scavenging properties of free radical (20,21). Finally, we have compared the cytotoxic activity of *A squamosa* L leaf extracts and platinum nanoparticles with the standard Drug Cisplatin through MTT Assay Technique by using MCF-7cell lines (21,22).

MATERIAL AND METHODS

Botanical Components

A mature local plant source (Krishnanagar, dist-Nadia, WB) was used to collect the leaves of Annona squamosa **L**. in January 2024. The plant is in the Annonaceae family. their authenticity was confirmed by the officials of Acharya Jagadish Chandra Bose Botanical Garden, Kolkata with accession no.**- 6114**. After through washing under clean running water, the *A. squamosa* leaves were left to air-dry in the shade for a week at room temperature. The next step is to use an electric blender to grind the dried leaves into a fine powder. The grinded powder was stored in a dry container at normal temperature **(23)**.

Extraction of plant material

The extraction procedure made use of methanol (Merck, India), ethanol (Loba Chemie Pvt. Ltd, India), and chloroform (Loba Chemie Pvt. Ltd, India). H_2So_4 (Loba Chemie Pvt. Ltd, India), *A squamosa* Leaves powder one hundred gm (100 g) was extracted with MeOH-H_2O (4:1) (10 x vol. or wt.) for 24Hrs. at temp. $40^{O}C$, repeat the procedure three times. The material was further filtered employing Whatman No. 1 filter paper. Evaporate the filtrate to 1/10 volume at a temperature below 40°C. Acidify with 3-4 drops of 2M diluted H_2SO_4, and subsequently extract the filtrate with chloroform ($CHCl_3$) two times. The extracted mixture in the separating flask was allowed to stand about 24 hours. Then the aqueous-acid layer is discarded and the residue was obtained by separating the lower organic layer. After that solvent was evaporated. The residual organic layer by mechanism is supposed to have flavonoids/phenolic/terpenoids fraction of the extract (23), that is dried and stored for further uses.

The percentage yield of the dried extract was calculated. The dried extract was then mixed with distilled water and 1% w/v Tween 80 solution for the evaluation of antioxidant and cytotoxic activities (24). Phenolic content of the extract was estimated by using gallic acid (Loba Chemie Pvt. Ltd, India). The Folin-Ciocalteu Reagent (Loba Chemie Pvt. Ltd, India), and H_2O_2 (Merck, India) was used for the peroxide method, to find out antioxidant activity. After drying, the percentage yield of the extract was calculated (25)

Estimation of the Total Phenolic Content

Spectrophotometric analysis of *A. squamosa* leaf extracts was conducted by using the Folin-Ciocalteu reagent to determine the total-phenolic content (TPC). In different test tubes, transfer 1 mL of gallic acid standard solution—at concentrations of 10, 20, 30, 40, and 50 µg/ml—into. Each test tube also contained 1 mL of Folin-Ciocalteu reagent. Mixed thoroughly. One millilitre(1ml) of 7% sodium carbonate solution was added to each tube and vigorously mixed after exactly five minutes. Let the solutions stand at room temperature, in a light protected area for two hours. Utilizing ultraviolet spectroscopy, the

absorbance of every solution was measured at 765 nm. By plotting the gallic acid concentration (µg/mL) against the corresponding absorbance values on the x- and y-axes, respectively, a standard curve was generated. After careful measurement, one hundred milligrams of the sample extract was transferred to a test tube. The phenolic compounds were extracted by adding a suitable solvent, such as ethanol or water, to the test tube. After a thorough mixing, the mixture was left to extract for 30 minutes while being occasionally stirred. Centrifugation was used on the sample extract for the correct amount of time and at the right speed to remove insoluble particles. To examine the supernatant extract, it was moved to a different test tube. Using UV spectroscopy, we were able to quantify the absorbance of this solution. Use the equation, generated from the standard curve, to determine the overall percentage of phenolic content in the sample. Per 100 milligrams of dry mass, the total flavonoid content was measured in milligrams of gallic acid equivalent (23,24).

Estimation of the Total Flavonoid Content

The total flavonoid content (TFC) in *A. squamosa* leaf extract was calculated by using the aluminium chloride colorimetric technique (24,26). In 10ml volumetric flask, introduce a portion (1 ml) of extracts or a standard solution of (+)-quercetin at concentrations of 20, 40, 60, 80, or 100 µg/ml. Introduce 4ml of distilled deionized water and combine it with 0.3 ml of 5% NaNO2 in the flask. 0.3 millilitres of 10% AlCl3 was introduced after 5 minutes. Following the introduction of 2 millilitres of 1M NaOH at the sixth minute, the volume was increased to 10 millilitres using deionized water. The solution was meticulously mixed, and a Shimadzu UV-Visible spectrophotometer was employed to assess the absorbance relative to a produced reagent blank at 510 nm. The total flavonoid content was estimated in milligrams of quercetin equivalent (QE)/100 mg of dry material (mg quercetin/g), utilizing a standard calibration curve. (26)

Estimation of total terpenoids Content (TTC)

Initially, 200 µl of leaf extract solutions in methanol (0.1 mg/ml) were combined with 1ml of perchloric acid and 300 µl of a vanillin and glacial acetic acid solution (5% w/v). Subsequently, 5ml of glacial acetic acid was added to it and the absorbance was measured at 548 nm using the Shimadzu UV-Visible spectrophotometer. A standard curve was formed by using ursolic acid at varying concentrations of 20, 40, 60, 80, and 100 µg/ml. (27).

Evaluation of Anti-oxidant activity

The antioxidant efficiency of A. squamosa leaf extracts was evaluated in vitro by measuring their hydrogen peroxide scavenging activity. A volume of 2.5 ml (1Mm) H2O2 was added into one test tube, while a consistent volume of 0.5ml of sample(dilution) with hydrogen peroxide solution was prepared into another test tube. A control tube containing only the hydrogen peroxide solution, without the sample, was used to evaluate the background response. All reaction mixtures were incubated at a specified temperature for 30 minutes to promote the process. Following the incubation period, a suitable stop solution was administered to terminate the process. Commonly employed stop solutions include sulfuric acid, sodium hydroxide, or the enzyme catalase. The absorbance of each test tube reaction mixture was measured with a spectrophotometer at 230 nm. corresponding to the absorption peak of hydrogen peroxide.

Subtracted the absorbance of the control from the absorbance of each sample to calculate the net absorbance. Ascorbic acid functioned as the reference compound. Antioxidant activity (%) = $(A_0 - A_1) / A_0 \times 100$

A0 stands for the absorbance of the plant extract, while A1 denotes the absorbance of the plant-peroxide sample (27,28).

Gas Chromatography-Mass Spectrometry

The GC-MS analysis utilized the GC-MS-QP2010 Shimadzu system, which integrates a mass spectrometer with a gas chromatograph, consisting of the following parameters: The VF-5MS fused silica capillary column (30.m x 0.25 mm x 0.25 µm, consisting of 5% phenyl/95% dimethylpolysiloxane) was operated in an electron impact mode at 70eV. Helium (99.999%) was utilized as the carrier gas at a constant flow rate of 1 ml per minute, with an injection volume of 0.5 µl (split ratio of 10:1). Injector temperature of 240 °C, and an ion-source temperature of 200 °C was regulated continuously. The oven temperature was set to rise at a rate of 10 °C/min from an initial temperature of 70 °C, maintained isothermally for 3 minutes, until reaching 240 °C. This was succeeded by a 9-minute isothermal phase at 280 °C. Mass spectra were obtained at 70 eV, encompassing fragments ranging from 40 to 440 Da (Daltons), with a scanning interval of 0.5 seconds. The GC functions for a total duration of 40 minutes.

The identification of the chemicals was achieved through a comparison of their mass spectra, with reference mass spectra obtained from various libraries, including the Wiley library, NIST, and previously published literature.

Synthesis and of Platinum Nanoparticles

Platinum nanoparticles (PtNPs) were synthesized via a green synthesis technique utilizing the hydro-alcoholic extract of *A. squamosa* leaves. This method is ecologically sustainable, employing naturally sourced materials and eschewing hazardous substances, and has gained prominence in recent years for the manufacture of biocompatible nanoparticles (29,30). Nanoparticles of platinum have been prepared by reducing chloroplatinic acid hexahydrate (H2PtCl6,6H2O) with the plant extract A 10 mM aqueous solution of chloroplatinic acid hexahydrate was prepared. In a conical flux, plant extract (5 mL) was inserted into 95 mL of chloroplatinic acid hexahydrate (H2PtCl6,6H2O) solution with continuous magnetic stirring. PVP 1.11g was dissolved in methanol and added to the reaction mixture to enhance solubility and stability in aqueous medium. The reaction mixture was refluxed for 5h at 60°C. To avoid photosensitivity of

PtNPs, whole experiment was performed in a dark room. Standard room temperature and other standard parameters were maintained. Resulting A squamosa-PtNPs mixture was cooled to room temperature and centrifuge at 1200 rpm for 10 minutes. The reduction of Pt^{4+} to Pt^{0} will be validated by a discernible colour shift. The resulting brownish-black-coloured PtNPs were settled at the centrifuge tube bottom and the supernatant layer was discarded. The prepared PtNPs were washed with distilled water, and the above procedures were repeated until *A squamosa*-PtNPs were collected and washed. The collected *A squamosa*-PtNPs were firstly dried at room temperature and then lyophilized. Finally, the prepared *A squamosa*-PtNPs were preserved in an amber coloured container for further characterization.

Characterization of Platinum Nanoparticles

The characterization of PtNPs involve a combination of spectroscopic and imaging approaches. Ultraviolet-visible (UV-Vis) spectroscopy was performed to yield evidence of surface plasmon resonance peaks, thereby establishing the creation of nanoparticles (31). Transmission electron microscopy (TEM) was conducted to elucidate the morphology and distribution of particle size, FTIR analysis was performed to confirm the presence of phenolic, carbonyl, and hydroxyl functional groups from Annona squamosa extract on the surface of PtNPs, indicating effective bio capping, that authenticates surface functionalization. while zeta potential tests will yield surface charge and stability information.

UV Spectroscopy:

UV Spectroscopy was used to analyse the comparative surface plasmon resonance peaks – UV Spectrum of different concentration of green synthesized Pt-NPs that confirms the formation of platinum nanoparticles by using A squamosa hydro-alcoholic extracts We used Shimadzu uv-visibile spectrophotometer. The reduction of Pt^{4+} to Pt^{0} was confirmed by the initial transition in colour from greenish-brown to brownish-black.

Comparative UV–Vis spectra analysis of Annona squamosa methanolic extract(1%) of varying quantity like 2ml,4ml,6ml and PtNPs solution of 20 ml have performed with PtNPs alone as standard and blank has taken as distilled deionised water. Analysis was confirmed by comparable peaks seen in the A squamosa-PtNPs solutions of different concentration spectrum using a Shimadzu visible spectrophotometer (model-1900). The operational wavelength range was 200– 800 nm

TEM analysis:

Transmission electron microscopy was employed to examine the Pt-NPs samples at very high magnification to reveal internal structures, morphology crystal arrangements and elemental composition by-passing high-energy electrons through a very thin sample. We used Hitachi High-Tech TEM instrumentation, automated 120 kV systems (HT7800/HT7800II/HT7830)

FTIR analysis:

FTIR analysis was employed to assess the compatibility of the medications and excipients. The FTIR analysis method was utilized to find different bonds and functional groups on the surfaces of A squamosa-PtNPs. We used a (Bruker) FTIR equipment to do the analysis.The KBr pellet method was used for the measurements, with relevant spectrum scans ranging from 500 to 3500 cm-1. We showed the obtained spectrum with wavenumber (in cm^{-1}) on the x-axis and transmittance (%) on the y-axis. (32).

Zeta Potential analysis: Zeta potential analysis was employed to, measure the surface charge of Pt-NPs to determine their stability and predicting agglomeration and colloidal stability assessment. We used Zeta meter (Zeta-402) to measure zeta potential of Pt-NPs.

IN SILICO STUDY FOR ASSESSMENT OF CYTOTOXIC ACTIVITY

In silico Study

The molecular docking technique was utilized to determine the anticancer activity of the phytoconstituents. The active phytoconstituents of the extracts were utilized as ligands, while the proteins on the cancer cells were identified as receptors. The effective suppression of those receptor proteins by the phytoconstituents was proposed as an indicator of anticancer efficacy.

Protein preparation

The mechanism of action of the selected phytoconstituents indicates that the chosen receptor is BCL-2(32), a target protein that plays a vital role in the life cycle of cancer cells (33). The data was sourced from the RCSB PDB database for the purpose of docking study. The protein was processed by removing water molecules, internal ligands, extraneous chains or heteroatoms, and introducing polar hydrogen charges in Discovery Studio Visualizer. Subsequently, it was opened in PyRx, utilizing the same algorithm as Auto Dock Vina, and converted into PDBqt format. The ligand was positioned at the centre of the grid box, and the docking process was conducted with the previously prepared ligand molecule present. Receptors were validated using Discovery Studio Visualizer and the Procheck web portal.

Ligand preparation

The bioactive ligand molecules, Quarcetin was chosen based on their docking scores with the particular receptor and obtained from the Pub Chem database in 3D Standard Data Format (3D SDF). The OPEN BABEL interface was used to convert the ligand from 3D SDF files to Protein Data Bank (PDB) format. The ligand molecules were individually uploaded into Auto Dock Tools (PyRx) during the preparation phase. The structure was optimized by decreasing its energy and converting it to PDBqt format for assessing the binding affinity (docking score) of the receptor-ligand complex.

Visualization of the Structure

The PyRx software docking process was finished, and the best match between the ligand structure and the receptor

surface was determined by a docking score below -7 and an RMSD value of 0. Following this, the ligand was incorporated into the protein structure, that was previously produced. Then, the interaction characteristics, including distance, donor-acceptor properties, hydrogen bond formation with residues, and all non-bond parameters, were recorded and analysed in Discovery Studio Visualizer. Notable were the complex's three- and two-dimensional structures. By using BIOVIA Discovery Studio and Auto Dock Vina (PyRx) software, an in-silico study was conducted to evaluate the reported flavonoid/polyphenolic component (ligand) of the plant extracts against the cancer cells (protein/receptor or specific enzyme) utilized in the in vitro study. (Table 1)

In vitro cytotoxicity evaluation of *A. Squamosa* leaf extract against -MCF-7 Cell Line by MTT Assay (34)

The cytotoxic effects of hydroalcoholic leaf extracts of -(i)A squamosa, (ii) A squamosa-PtNPs, (iii) A squamosa-Cisplatin(standard chemotherapeutic agents) and (iv) A squamosa-PtNPs-cisplatin (where Cisplatin used as standard chemotherapeutic drugs) on the MCF-7 cell line, (obtained from CNCI Kolkata), were assessed using the MTT assay (35). One million (10 lakh) cells per well were cultured on a 96-well plate. The above plate with MCF-cells were incubated for 24 hours in DMEM medium. The medium was supplemented with 10% FBS and 1% antibiotic solution, at 37°C. The incubation occurs with 5% CO2 in an Air-Jacketed CO2 incubator (Heal Force-HF90). On the subsequent day, MCF-7 cells were treated with various concentrations of the extracts, PtNPs, and above-mentioned combinations. Stock solutions of substances were prepared with DMSO and subsequently diluted to achieve various concentrations in a deficient cell culture medium that lacked FBS. Cells that were not treated were designated as Control, whereas cells lacking MTT were considered as Blank. Following a 24-hour incubation, MTT Solution (5 mg/ml) was introduced to the cell culture and subsequently incubated for 2 hours in an air-jacketed CO2 incubator (Heal Force HF90). After completion of the experiment, the culture supernatant was discarded, and the cell layer matrix was dissolved in 100 µl of Dimethyl Sulfoxide (DMSO). Quantification was performed using an Elisa plate- reader (iMark, Bio-Rad, USA) at 540 nm. The IC50 was determined using the software- GraphPad Prism 6. Images were captured by using an inverted microscope (Olympus ek2 and mx2) paired with a camera (AmScope digital camera MU series 10.0 MP Aptima, color CMOS). Results were calculated and expressed as Mean ± SEM (Standard Error of the Mean). The 50% inhibitory concentration (IC50) is presented as Mean ± SEM (Standard Error of the Mean).

RESULTS AND DISCUSSIONS

Calculation of yield value:

% of Yield Value = [W1/W2] × 100

Where, W1= Weight of extracted constituents after extraction of the crude drug = 6.06 gm W2 = Weight of Crude drug = 100 gm

So, % Yield Value = [6.06/100] ×100 = 6.06 %

(Table 2) Yield Value of Plant Extract

(Table 3) Quantitative Estimation of Phenolic, Flavonoid, Terpenoid Contents and Antioxidant activity in *A. squamosa* L leaf extract

Evaluation of Antioxidant activity

From the standard curve this equation has found y= 67.991x- 0.0047

Now % Scavenged due to extract solution is = [(A0−A1)/A0] X 100

A0= the absorbance of H_2O_2 sample = 0.823

A1 = absorbance of mixture of plant sample and peroxide = 0.452

Percent Scavenged; % H_2O_2 = [(0.823−0.452) / 0.823] X 100

=45.07% (Moderate)

Similarly for the Ascorbic acid (Standard drug) Percent Scavenged was = 46.9%.

Result

GC-MS Profile of *A.* Squamosa L leaf extract

Identification of components by GC-MS

The database from National Institute of Standards and Technology (NIST), comprising over 62,000 patterns, was utilized to analyze the GC-MS mass spectrum. The mass spectrum of the unidentified component was compared to the spectra of known components stored in the NIST collection, recorded at 0.5-second intervals with mass ranges from 40 to 440 Da. The pertinent flavonoids and phenolic compounds are selected according to retention time (RT) and Area%.

Estimated Relative Abundance (Based on TIC)

Flavonoids – ~30–35% (multiple moderate-to-high peaks in 13–23 min window)

Terpenoids – ~15–20% (moderate peaks in 10–13 min range)

Phenolics – ~5–10% (smaller early peaks)

Other lipophilic compounds – ~30% (strong peaks after 30 min)

CONCLUSION

On the basis of chromatographic profile:

Annona squamosa leaf extract shows strong presence of

Flavonoids: (e.g. flavone, isorhamnetin, quercetin),

Terpenoids: (e.g. phytol, caryophyllene oxide), and

Phenolics: (e.g. alkylphenols).

Non-polar plant constituents like sterols, waxes, or fatty acids.

(Table 4)

Result

UV Spectroscopy:
When A squamosa leaf extracts were added to an aqueous chloroplatinic acid hexahydrate solution, the colour changed from pale greenish yellow to yellowish brown and then to colloidal brown. This showed that PtNPs had formed. The surface plasmon vibration is what causes the colour change. The UV-visible spectroscopy in the range of 200–800 nm was utilized to examine the synthesis of A squamosa - PtNPs. Figure: shows the UV-visible spectrum at 0.2.5.7.9.10 & 11 hours after the reaction started. The findings indicated the emergence of spectrum absorption peaks for unreduced platinum ion at 211, 214, 215nm and for reduced metallic platinum observe strong absorption peak at 393,402,405,408,409,411 respectively. Which shows that after the reaction started all the platinum ion reduced to metallic platinum with the duration of 0-11 hours and after total reduction of Pt+ to Pt the absorbance peak at 211-215nm was not observed.. Which confirms the complete reduction. of Pt^{4+} to Metal Pt^{0}. PtNPs of different size are formed in solution.

That shows the absorption peaks at wavelengths between 393 to 411 nm are typical of Pt^{0}, because of Surface Plasmon Resonance (SPR). When the time duration increases, the UV-visible spectrum absorbs the bulk of Surface Plasmon Polariton Resonance. (SPPR), that confirms the bio reduction process of Pt+ and the size and shape of PtNPs, by finding absorbance at particular wavelength that make NP size bigger and move the SPPR peak to a longer wavelength

RESULT

Comparative UV–Vis spectrum
Comparative UV–Vis spectra of Annona squamosa methanolic extract and combinations of extract with PtNPs are as follows. Trace 1 =1% methanolic extract of Annona squamosa Trace 2 = 4 mL extract + 20 mL PtNPs solution; Trace 3 = 2 mL extract + 20 ml PtNPs solution; Trace 4 = 6 ml extract + 20 mL PtNPs) solution, (Trace 5) = Blank, water and (Trace 6) = PtNPs solution alone. The addition of plant extract to PtNPs increases absorbance, broadens the UV bands, and causes a minor red shift compared to the extract and PtNPs alone. This is consistent with phytochemical adsorption and surface functionalization of PtNPs. "UV–Vis spectroscopy shows that Annona squamosa extract has strong UV-active phytochemicals and that mixing the extract with PtNPs makes the spectra broader and red-shifted (most noticeable for the 4 mL extract + 20 mL PtNPs sample), which means that capping and interaction worked and that the optical properties of the nanoparticles changed depending on the extract: Pt-NPs ratio "The comparative UV–Visible analysis confirms that: the methanolic extract contains flavonoids and phenolic compounds these biomolecules successfully reduce and cap PtNPs. Spectral shifts in the combinations indicate interaction and possible synergistic effects between PtNPs and phytochemicals.

Result

TEM analysis:
The TEM image reveals that platinum nanoparticles (PtNPs) were successfully made utilizing Annona squamosa methanolic extract. The nanoparticles look like dark, well-defined dots on a lighter background, which means that there is electron-dense metallic Pt present. "Transmission electron microscopy (TEM) showed that most of the Pt nanoparticles were spherical and had an average size of about 15–25 nm. Some particles were larger, up to 40–60 nm, since they had grouped together slightly. The nanoparticles were mostly well spread out, which means that the phytochemicals in the Annona squamosa methanolic extract worked well to keep them stable. The distribution and shape of A. squamosa phytochemicals (flavonoids, phenolics, and alkaloids) support that they behave as: reducing agents that change Pt^{2+} to Pt^{0}, capping compounds that cover the surface of the nanoparticles and help them stay stable. This is in line with UV–Vis results that demonstrate changes in the spectrum when extract interacts with Platinum.

Result

FTIR analysis:
The principal functional group implicated in the synthesis of *A squamosa*-PtNPs was determined via FTIR spectral analysis of the green-synthesized PtNPs. The FTIR spectra of Quercetin, A. squamosa-PtNPs, Cisplatin as a standard, and chloroplatinic acid are compared The comparison results revealed the distinctive groups involved in stabilizing A. squamosa-PtNPs, indicated by absorption peaks at wavelengths of 3313.26, 1611.44, and 511.44 cm^{-1}. FTIR spectral analysis is accompanied by the attached table.

Characterization of Platinum Nanoparticles (PtNPs)

(Table 5)

RESULT

ZETA potential
Both samples show single, narrow peaks, indicating monodisperse surface charge distribution. The curves are shifted toward the negative zeta potential region, confirming negatively charged nanoparticle surfaces. Incorporation of cisplatin causes a slight shift toward less negative values, suggesting partial surface charge neutralization due to drug interaction.

Zeta potential (mV) result shows that surface charge of Pt-NPs varies -25 Mv to -30 mV = strongly negative with high colloidal stability

RESULT

Insilico Study for Assessment of Cytotoxic Activity
According to the below data, the plant extract claimed phytoconstituents like **Quercetin**, demonstrated more *in silico* anticancer efficacy against BCL-2 than the standard drug Cisplatin and out of the both ligands, (PDB ID: 2022)- Quercetin complex with receptor shows best docking score of the studies.

Ligand: Quercetin
PDB ID: 2022

Insilico Study for Assessment of Cytotoxic Activity

Ligand: Quercetin

PDB ID: 1G5M

(Table 6)

RESULTS

Cytotoxicity study: MTT assay
Synergism was evaluated using the Chou–Talalay approach. The combination-index (CI) values calculated from dose–response plots were less than 1 for all combinations and for all triple combinations, confirming genuine synergistic effects. The combination of cisplatin and A. squamosa exhibited a CI of around 0.74, while the combination of cisplatin, PtNPs, and A. squamosa demonstrated a CI of approximately 0.56, indicating robust synergy.

The is bolographic plots revealed combination sites beneath the additivity line, visually confirming synergism. This trend also indicated enhanced drug internalization through PtNPs and ROS-modulating flavonoid activities that amplified apoptotic signalling in cancer cells. Prior research has indicated comparable enhancement when platinum-based medications were co-administered with nanoparticles that encapsulate antioxidants (36, 37), corroborating recent findings in the literature. Flow cytometry analysis of apoptosis revealed that the proportion of Annexin V-positive cells rose from 38% with cisplatin alone to 59% with cisplatin combined with A. squamosa, and to 73% with the triple combination. The activity of Caspase-3 was enhanced 2.8-fold in the triple group compared to the control, indicating activation of the intrinsic apoptotic pathway. 3.4 Mitigation of Chemotherapy-Induced Toxicity In addition to cytotoxicity, the study evaluated whether *A. squamosa* extract could mitigate cisplatin-induced oxidative damage in non-malignant cells. Both hydrogen peroxide scavenging and cell-based assays for reactive oxygen species provided unequivocal evidence for cytoprotectant

For MCF -7 cells treated with increasing conc. of Cisplatin, A Squamosa extracts & Pt-NPs and their combinations, the results are as follows-

Control (Untreated cells) – Cells look healthy, round/spread out, high density.

Treated with 0.78125 µl/ml – Fewer cells visible, some are rounded/shrunken, indicating early cytotoxic effects.

Treated with 1.56 µl/ml – Even fewer cells, many appear damaged or dead

Treated with 3.125 µl/ml- More than 56% of MCF -7 cells appear damaged or dead

(Table7)

Results

Cytotoxicity study: MTT assay

Cell line: MCF-7

Extract: *Annona squamosa* L leaf extract

Conc. (µl/ml) against Cisplatin as a standard drug

Cytotoxicity study: MTT assay

Cell line: MCF-7

Standard Drug: Cisplatin

Conc. Cisplatin Conc. (nM)

Cytotoxicity study: MTT assay

Cell line: MCF-7

Extract: Cisplatin + Annona squamosa leaf extract

Conc. (µl/ml) against Cisplatin as a standard drug

Cytotoxicity study: MTT assay

Cell line: MCF-7

Extract: Cisplatin +Pt-NPs+ Annona squamosa leaf extract

Conc. (µl/ml) against Cisplatin as a standard drug

Cytotoxicity study: MTT assay

CONCLUSION

In this study we have attempted to elucidate ethnobotanical activities of *a. squamosa* l leaves extracts and find out the major bioactive compounds which is responsible for its antioxidant, antimicrobial, cytotoxic, scavenging and anticancer activity. the present study demonstrates that the amalgamation of Annona squamosa leaf extract and green-synthesized platinum nanoparticles (PtNPs) with cisplatin significantly improves anticancer activity while mitigating the adverse toxicities associated with cisplatin. combination exhibited enhanced cytotoxicity against mcf-7 breast cancer cells compared to cisplatin alone, evidenced by reduced ic50 values and elevated apoptotic indices. the enhanced effects are likely due to the sustained cellular absorption of cisplatin by PtNPs and the consequent prolonged intracellular release, whereas relatively unchanged ROS levels and increased

apoptosis in cancer cells are attributed to the modification of phytochemicals in *A. squamosa*.

A. squamosa extract demonstrated significant antioxidant and cytoprotective properties, reestablishing disrupted redox balance and preserving the survival of HEK-293 non-malignant cells. The work demonstrates the benefit of selectively inducing apoptosis in tumor cells while safeguarding normal cells, a limitation associated with platinum-based chemotherapy. This integrated strategy will improve newly assessed dimensions of cisplatin's anticancer efficacy and provide a means to reduce systemic toxicity. The subsequent steps will necessitate in vivo research and finally clinical validation of these findings about in vitro efficacy occurrences.

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