Ind. J. Pharm. Edu. Res., 2026; 60(4s):s1594-s1603.
https://www.ijper.org

Original Article

Cytotoxic and Apoptotic Study of Protocatechuic Acid Nanosuspensions Assessed by *in silico* Molecular Analysis and AO/EB Staining Technique

Navaneethakrishnan Subramanian[1], Vijayaraj Surendran[2,*], Rajkumar Selvam[2], Murugesan Vanangamudi[3], Thiruveni Azhagarsamy[2], Raghavendra Kumar Gunda[4,*]

[1]Department of Pharmacology, Dr. Kalam College of Pharmacy, Avanam, Thanjavur, Tamil Nadu, INDIA.
[2]Department of Pharmaceutical Chemistry and Analysis, Dr. Kalam College of Pharmacy, Avanam, Thanjavur, Tamil Nadu, INDIA.
[3]Department of Pharmaceutical Chemistry, Institute of Pharmaceutical Research, GLA University, Mathura, Uttar Pradesh, INDIA.
[4]Department of Pharmaceutics, Narasaraopeta Institute of Pharmaceutical Sciences (Autonomous), Narasaraopet, Palnadu, Andhra Pradesh, INDIA.

ABSTRACT

Background: Bone cancer, particularly osteosarcoma, is an aggressive malignancy with poor prognosis, often resistant to conventional chemotherapy. Protocatechuic Acid (PCA), a plant-derived polyphenol, holds anticancer potential but suffers from poor bioavailability. **Materials and Methods:** This study investigated the cytotoxic and apoptotic effects of PCA Nanosuspensions (PCA-NS) on MG-63 human osteosarcoma cells and evaluated its molecular interactions via docking with two target proteins, topoisomerase II alpha (1ZXN) and p53 (1TUP). **Results:** PCA-NS exhibited dose-dependent cytotoxicity with an IC_{50} of 46.10 µg/mL, and AO/EB dual staining revealed apoptosis as the predominant mode of cell death. Molecular docking confirmed stable binding interactions with both targets, supporting the anticancer potential of PCA. **Conclusion:** These results highlight PCA-NS's significance as a viable option for treating bone cancer.

Keywords: AO/EB Staining, Apoptosis, Molecular Docking, Nanosuspensions, Osteosarcoma, Protocatechuic Acid.

Correspondence:
Dr. Vijayaraj Surendran
Professor and Head, Department of Pharmaceutical Analysis, Dr. Kalam College of Pharmacy, Thanjavur, Tamil Nadu, INDIA.
Email: vijaysurender85@gmail.com
ORCID: 0000-0003-3816-4636

Dr. Raghavendra Kumar Gunda, M.Pharm., PhD., FCEM,
Associate Professor, Department of Pharmaceutics, Narasaraopeta Institute of Pharmaceutical Sciences (Autonomous), Narasaraopet, Palnadu- 522601, Andhra Pradesh, INDIA.
Email: raghav.gunda@gmail.com
ORCID: 0000-0002-4271-8614
APTI ID: AP/LM-541

Received: 13-04-2026;
Revised: 09-06-2026;
Accepted: 25-08-2026.

INTRODUCTION

Although bone cancers are relatively uncommon compared to other types of malignancies, they present considerable therapeutic challenges, because they are aggressive, have a propensity to spread to other body areas, and don't respond well to conventional treatment.[1,2] The most prevalent type of primary malignant bone tumour is osteosarcoma, primarily affecting children and adolescents.[3] The development of osteoid tissue by malignant mesenchymal cells is the histological hallmark of this disorder, which usually affects the long bones. Osteosarcoma is typically classified into two clinical stages: localized and metastatic, with the metastatic stage linked to a less favourable outcome due to the spread of the disease to other organs.[4,5]

Persistent bone pain, swelling, and limited mobility along with palpable masses frequently signal the beginning of the disease.[6,7] Usually, surgery, chemotherapy, and radiation therapy are used to treat this illness. These treatment plans are often associated with significant toxicity, suboptimal efficacy, and elevated rates of recurrence, highlighting the need for novel therapeutic strategies.[8,9]

Current osteosarcoma chemotherapy regimens (high-dose methotrexate, doxorubicin, cisplatin, and ifosfamide) face major challenges including multidrug resistance through enhanced drug efflux, DNA repair, and anti-apoptotic pathways, resulting in poor long-term survival despite multimodal treatment.[10]

DOI: 10.5530/ijper.20263446

These agents also cause severe dose-limiting toxicities such as cardiotoxicity, nephrotoxicity, and myelosuppression, compounded by poor aqueous solubility leading to suboptimal tumor exposure. Nanosuspensions overcome these drawbacks by dramatically improving solubility, bioavailability, and targeted tumor accumulation of poorly soluble drugs like protocatechuic acid while minimizing systemic toxicity.[11,12]

Over the past few years, bioactive compounds extracted from medicinal plants have become increasingly significant due to their ability to prevent and treat various types of cancer. Phytochemicals found in nature, including polyphenols, flavonoids, and terpenes, have demonstrated effectiveness in preventing cancer development by employing multiple methods, such as neutralizing oxidative stress, inducing programmed cell death, and reducing inflammation.[13] One compound of particular interest is Protocatechuic Acid (PCA), a simple phenolic compound that is widely found in fruits, vegetables, and medicinal plants. PCA has shown a range of pharmacological effects, such as antibacterial, anti-ulcer, anti-aging, and significant anticancer properties.[14]

PCA has been shown to exert anticancer effects through the induction of apoptosis, modulation of redox balance, interference with carcinogen-DNA interactions, and regulation of pro-inflammatory pathways. Despite this, its use in therapy is hindered by its poor solubility and low bioavailability.[15] Recent breakthroughs in nanotechnology, particularly in nanosuspension formulations, provide a promising approach to overcoming these challenges by increasing drug solubility, stability, and cellular absorption-ultimately enhancing pharmacological effectiveness in cancer treatment.[16]

This investigation centers on assessing the cytotoxic and apoptotic impacts of PCA Nanosuspension (PCA-NS) on MG-63 osteosarcoma cells. We assessed cellular responses using the well-established fluorescent labeling technique of Acridine Orange/Ethidium Bromide (AO/EB), which uses membrane permeability and nuclear morphology to differentiate between viable, early apoptotic, late apoptotic, or necrotic cells.[17]

To predict the binding interactions of PCA with protein targets relevant to osteosarcoma, a molecular docking study was performed. This study intends to help the advancement of bone cancer therapy by providing a thorough understanding of PCA-NS therapeutic possibilities as a novel treatment option through preliminary clinical research.

MATERIALS AND METHODS

Molecular Docking Analysis

Molecular docking research was conducted to evaluate the possible interaction of protocatechuic acid with Topoisomerase II alpha and the tumor suppressor protein p53. Chem Sketch was used to create the ligand structures, Chimera 1.12 was used to decrease energy, and the pdb format was saved. The Protein Data Bank (PDB) provided the structures of the protein receptors 1ZXN and 1TUP. The protein structure was cleared of ions, water molecules, and co-crystallized ligands. The Yasara Minimization Server was then used to reduce energy before the docking simulation. By creating protein and ligand files (in pdbqt format), configuring grid settings, and executing docking simulations via Cygwin using the Vina command-line script, AutoDock and AutoDockVina programs were utilized to predict the ideal binding conformation of ligands to target proteins.

Protein-ligand interactions were investigated by visualizing and analyzing the resultant docked complexes using BIOVIA Discovery Studio. The molecular docking study was carried out on the interphase catalytic site coordinate space (X = 40, Y = 40, and Z = 40) with 20 Å in each axis and 1 Å spacing in the Grid Box. To determine the molecular interactions between the two, the results were analyzed in the context of the maximal estimated Free Energy of Binding (FEB) for each protein-ligand combination.

Reagents and Chemicals

The study utilized various reagents and consumables from both international and local sources. Dimethyl Sulfoxide (DMSO) and the MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 5 mg/mL), along with fluorescent dyes Acridine Orange (AO) and Ethidium Bromide (EtBr) for dual staining, were procured from Sigma-Aldrich (USA). Fetal Bovine Serum (FBS), Trypsin-EDTA, and Penicillin-Streptomycin antibiotic solution were obtained from Gibco (USA). Dulbecco's Modified Eagle Medium (DMEM) and 1X Phosphate-Buffered Saline (PBS) were purchased from local vendors in India. Tissue culture plastics such as 96-well plates and wash beakers were supplied by Tarsons (India).

Instrumentation

Fluorescence-based cell imaging was carried out using the ZOE™ Fluorescent Cell Imager (Bio-Rad, USA), which facilitated real-time observation and evaluation of stained cellular samples.

Cell Culture

The National Centre for Cell Science (NCCS), located in Pune, India, provided the human osteosarcoma cell line MG-63. Cells were kept in a humidified incubator at 37°C with 5% CO_2 after being cultivated in DMEM supplemented with 10% FBS, 100 µg/mL penicillin, and 100 µg/mL streptomycin.

Methods

Formulation of Protocatechuic acid nanosuspensions

Through slight modifications, protocatechuic acid nanosuspensions have been developed using the antisolvent precipitation technique.[13] The necessary quantity of medication

was dissolved in a solvent that was soluble in water. Rapid injections of various drug concentrations produced the necessary level of supersaturation. Different solvent medication concentrations (20, 40, 60, 80, and 100 mg/mL) were employed. After that, 5 mL of the drug solution was mechanically stirred into 100 mL of water that included the stabilizer. After that, the suspension was ultrasonically agitated for 15 min in a cold environment. After that, a freeze dryer was used to lyophilize the preparations.

The effects of varying drug concentration, stabilizer concentration, stirring time, Solvent: Antisolvent (S:NS) ratio, and ultrasonication time on particle size and physical stability of nanosuspensions were investigated. The formulated nanosuspensions were characterized by SEM, zeta sizer, and particle size analysis.

In silico study

Protocatechuic Acid's (PCA) binding relationship with topoisomerase II alpha was examined using a molecular docking approach. The ATPase domain crystal structure of human topoisomerase II alpha was obtained from the Protein Data Bank (PDB ID: 1ZXN). One essential enzyme that controls DNA structure during transcription, replication, and chromosomal segregation is topoisomerase II alpha.[18] Numerous aggressive cancers, including osteosarcoma, a prevalent kind of bone cancer, have been shown to overexpress this enzyme. Apoptosis can be induced and cancer cell growth disrupted by inhibiting this enzyme.[19] With the use of Discovery Studio, the protein structure was created by eliminating water molecules and adding hydrogen atoms. Protocatechuic acid was made by optimizing its shape and minimizing energy after being obtained from the PubChem database.

After docking, the optimal binding pose was chosen based on binding affinity scores and further examined for important molecular interactions, such as hydrogen bonds, hydrophobic contacts, and π-π stacking interactions with active site residues. A grid box was centered on the ATPase domain active site, and default parameters were applied.

Cytotoxicity Assay of PCA-NS on MG-63 Cells

The cytotoxic potential of the formulated PCA-NS was evaluated *in vitro* using the MTT assay on MG-63 human osteosarcoma cells. Trypsinization was used to harvest the grown MG-63 cells, which were then collected in sterile 15 mL centrifuge tubes and plated into 96-well tissue culture plates at a seeding density of 1×10^5 cells/mL (200 µL/well) in full DMEM media that contained 10% FBS and 1% antibiotic solution. At 37°C, in a humidified atmosphere with 5% CO_2, the cells were left to adhere and multiply for 24 to 48 hr.

The wells were treated with different concentrations of PCA-NS (12.5, 25, 50, 100, and 200 µg/mL) produced in serum-free DMEM after being gently washed with sterile PBS to eliminate non-adherent cells. To guarantee repeatability, each treatment was carried out three times. Following a 24-hr exposure period, 10 µL of MTT reagent (5 mg/mL in PBS) was added to each well, and the wells were then incubated for two to 4 hr in the dark to evaluate the cell viability. Formation of purple formazan crystals, indicative of mitochondrial activity in viable cells, was confirmed by visual inspection under an inverted microscope.[20]

After incubation, the surplus MTT-containing media was carefully aspirated, and to get rid of any leftovers, wells were rinsed once with 1X PBS. Each well received 100 µL of DMSO to dissolve the formazan crystals, and the plate was gently shaken for 5 min. A microplate reader was used to measure the Optical Density (OD) at 570 nm (Thermo Fisher Scientific, USA). The following formula was used to determine the percentage of viable cells in comparison to the untreated control group.[21]

$$\text{Cell Viability}\,(\%) = (\text{Test OD} / \text{Control OD}) \times 100$$

Determination of apoptosis effect of PCA-NS using AO/EtBr Dual Staining Assay

A 96-well tissue culture plate was seeded with MG-63 cells at a density of 5×10^5 cells/mL, and the cells were then incubated for 24 hr in DMEM growth media with 10% FBS added. Following incubation, the cells were treated with serum-free DMEM based on the IC_{50} value of PCA-NS, and they were then incubated for 24 hr at 37°C in an incubator with 5% CO_2. After treatment, each well received 10 µL of a 1 mg/mL solution of Acridine Orange (AO) and Ethidium Bromide (EtBr) in a 1:1 ratio, which was well mixed. After 2 min of centrifuging the plate at 800 rpm to settle the cells, the stained cells were examined right away (within an hour).

A fluorescent imaging system (ZOE™, Bio-Rad, USA) was used to analyze at least 100 cells per well in order to determine necrosis and apoptosis based on fluorescence staining patterns. Green was the color of viable cells, green with condensed or shattered nuclei was the color of early apoptotic cells, and orange to red was the color of late apoptotic or necrotic cells.[22,23]

RESULTS

Docking analysis

Promising interactions between Protocatechuic Acid (PCA) and topoisomerase II alpha (PDB ID: 1ZXN) are shown by the docking investigation, which supports PCA's potential use as an anticancer drug to treat bone cancer. PCA showed a strong affinity for the ATPase domain of topoisomerase II alpha in the current investigation, indicating that it may be able to disrupt the enzyme's activity. A modest but perhaps physiologically significant interaction was indicated by the calculated binding energy of -6 kcal/mol.

The protein ligand complex illustration demonstrates that PCA is well suited to the binding pocket of Topo IIα and participates in a number of stabilizing interactions Figure 1. Notably, PCA forms six conventional hydrogen bonds, which are essential for binding stability and specificity: ASN A:91, SER A:149, ASN A:150, SER A:148, LEU A:168, and GLY A:164. These residues are situated close to the DNA cleavage domain, indicating that PCA may hinder the DNA religation step, a characteristic mechanism of Topo IIα inhibition Van der Waals interactions with residues like ALA A:167, GLY A:166, and TYR A:165 provide further stability in addition to hydrogen bonding, enhancing PCA's fit within the active site. These interactions imply that PCA occupies a region of the enzyme essential to its function.

The potential of Protocatechuic Acid (PCA) as a small-molecule modulator of p53 activity in the context of bone cancer is demonstrated by the molecular docking study of PCA with the tumor suppressor protein p53 (PDB ID: 1TUP), which shows a strong interaction profile. p53 is a master regulator of DNA repair, apoptosis, and cell cycle arrest. One important tactic in cancer treatment is its functional restoration or activation, especially in osteosarcoma, where p53 mutations or misregulation are prevalent (Figure 2).

DT E:1011, DT E:1012, DA F:1112, and DG E:1013 are nucleotide bases that show that PCA intercalates or aligns within the DNA-binding groove of the p53-DNA complex. Conventional hydrogen bonds were observed between PCA and ARG B:248 - a well-known "hotspot" residue for mutations in various cancers, essential for DNA contact and structural stabilization. Carbon hydrogen bond interaction is seen with DG F:1113, suggesting a non-covalent stabilizing force. APi-alkyl interaction is also detected, possibly adding hydrophobic stabilization near DA F:1111, improving PCA's anchoring within the binding site.

Two probable routes of action are suggested by this network of interactions, which shows that PCA may be able to disrupt or imitate DNA interactions: a. Stabilization of the DNA-p53 complex, which may increase the transcriptional activity of wild-type p53 and improve its binding affinity to target DNA. b. In mutant p53 variations, allosteric or competitive inhibition may partially restore DNA-binding function or prevent carcinogenic activity.

Crucially, the relationship with ARG248 is very important. This residue's participation indicates that PCA may interact substantially even in p53-mutant situations, since it is commonly mutated in bone malignancies like osteosarcoma. PCA showed a binding affinity of -5.9 kcal/mol for p53 (1TUP), which is like that found for 1ZXN. Another suitable range for a flexible ligand docking to a complex site, such as the DNA-binding interface of p53, is the RMSD value of 4.142 Å. Notably, PCA interacts with many DNA bases (DT E:1011, DG E:1013, DA F:1112) and important residues like ARG248, a residue that is often altered in bone cancer, indicating that it may imitate or maintain DNA connections with p53. The data was presented in Table 1.

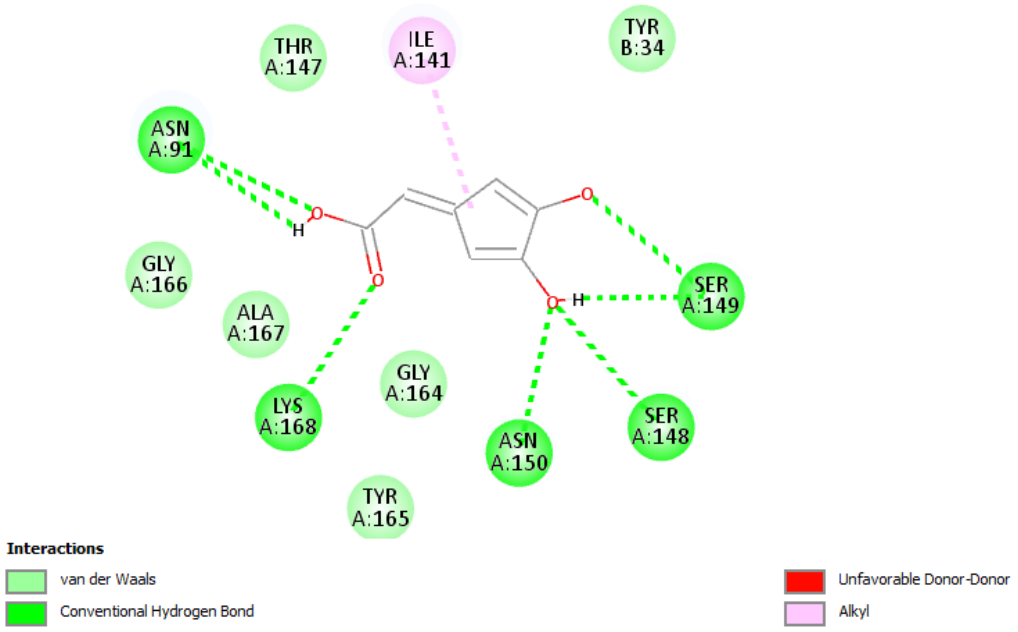

Figure 1: Interacting residues between docked Protein (code: 1ZXN) and Protocatechuic acid.

Table 1: Binding affinity of PCA with proteins.

Sl. No.	Compound name	Protein Code	Binding affinity	RMSD
1.	Protocatechuic acid	1ZXN	-6	4.275
2.		1TUP	-5.9	4.142

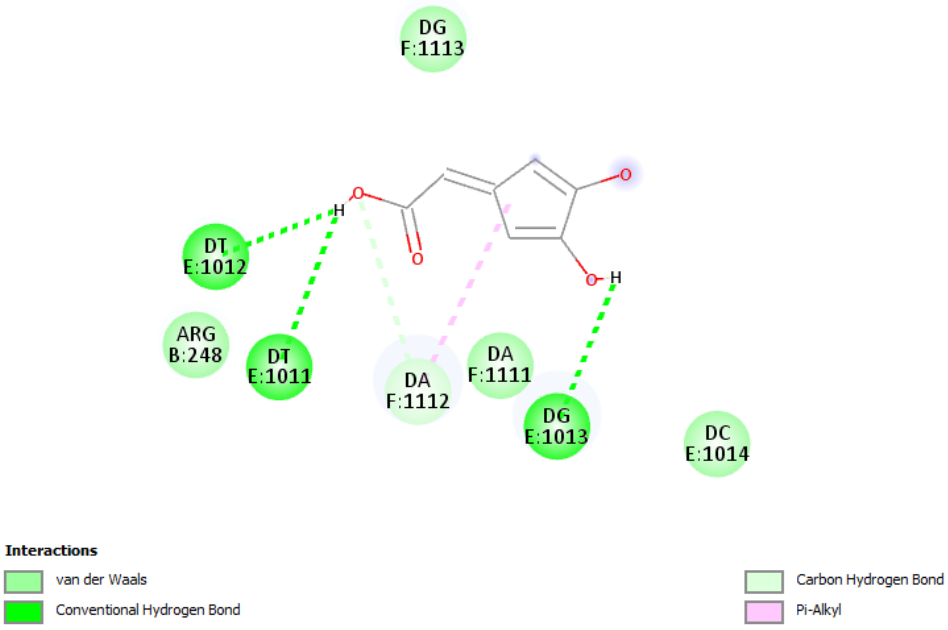

Figure 2: Interaction type and interacting residues between docked Protein (code: 1TUP) and Protocatechuic acid.

PCA's good complementarity and binding stability within the active site are further highlighted by the numerous hydrogen bonds and favorable non-covalent interactions that were observed. Its polyphenolic structure, which contains available hydroxyl and carboxyl groups, facilitates these interactions, making it a promising scaffold for additional optimization.

Assessment of Cytotoxic Effects of PCA-NS Using MTT Assay

The MTT assay was used to assess PCA-NS's cytotoxic capability on MG-63 osteosarcoma cells. It was discovered that the decrease in cell viability depended on concentration. The viability of cells gradually decreased as the PCA-NS concentration rose from 12.5 to 200 µg/mL. As PCA-NS concentration increased, the intensity of the dark-blue color generated in the assay, which is correlated with the number of metabolically active cells lower, suggesting decreased cell viability, as seen in Figure 3a.

At 12.5 µg/mL, the cell viability was 77.72%; at 200 µg/mL, it was 33.49%. The untreated control group exhibited 100% viability, with no observed cytotoxicity. The IC_{50} value of PCA-NS, with a regression coefficient (R^2) of 0.932, which indicates a strong match to the dose-response model, the concentration at which 50% reduction of cell viability occurred was found to be 46.10 µg/mL (Table 2).

In comparison to the untreated control, treatment with PCA-NS at a dose of 200 µg/mL dramatically decreased the vitality of MG-63 osteosarcoma cells, suggesting a strong cytotoxic effect. The mean ± SEM of three separate experiments conducted in triplicate is shown by the data. Compared to the untreated control, $p<0.001$.

A progressive reduction in formazan crystal formation was observed with increasing concentrations of PCA-NS, indicating a dose-dependent decrease in cell viability compared to the control.

Determination of apoptosis effect of PCA-NS using AO/EtBr Dual Staining Assay

Analysis of cell populations treated with PCA-NS showed distinct stages of cell death. Around 4.5% of the cells were found to be dead, with a small portion (2%) retaining their viability. Cells exhibiting pro-apoptotic characteristics, indicative of the initial stages of apoptosis, comprised 9% of the total cell count. A significant increase in late apoptotic cells was observed, representing 76.5% of all cell death, suggesting that the main mechanism of PCA-NS-induced cell death is apoptosis. Furthermore, necrotic cells

made up approximately 7.5% of the observed cell population, as depicted in Figure 3b.

These findings indicate that PCA-NS effectively triggers apoptosis rather than necrosis, as evidenced by the dominance of late apoptotic cells and the same was presented as Figures 4a and 4b.

Apoptotic markers were distinctly visible at the IC_{50} concentration, which supported apoptosis initiation, in contrast to control cells that showed typical, undisturbed cellular architecture. These apoptotic indicators were clearly evident at the IC_{50} concentration, confirming apoptosis induction, whereas control cells exhibited normal, intact morphology.

Cells in control group A & B possess intact nuclei, displaying consistent green fluorescence indicative of cell viability. Cells exposed to PCA-NS at its IC_{50} concentration of 46.10 µg/mL showed distinct morphological alterations: dead cells emitted a red fluorescence, pro-apoptotic cells appeared yellowish-green, and apoptotic cells exhibited yellowish-orange fluorescence with prominent membrane-bound apoptotic bodies.

DISCUSSION

A naturally occurring polyphenol called protocatechuic acid has shown a variety of pharmacological actions, including anti-inflammatory, cancer-preventive, and antioxidant qualities. Anticancer activity of PCA was studied by docking analysis with proteins 1ZXN and 1TUP. The present study explored the anticancer potential of Protocatechuic Acid (PCA), focusing on its interaction with molecular targets and its biological effects on osteosarcoma cells. Molecular docking studies revealed that PCA exhibits significant binding affinity with topoisomerase IIα and p53 proteins, two critical targets in cancer therapy. Specifically, PCA demonstrated a binding energy of -6 kcal/mol with topoisomerase IIα, interacting with key active site residues such as Arg112, Asn 120, and Glu 87. These interactions suggest that PCA may act as a competitive inhibitor, disrupting ATPase activity and thus interfering with DNA replication and repair mechanisms-essential processes in rapidly proliferating cancer cells. Stabilization through π-π stacking and hydrogen bonding further underscores PCA's potential as a topoisomerase IIα inhibitor with a safer toxicity profile compared to traditional agents like doxorubicin or etoposide.

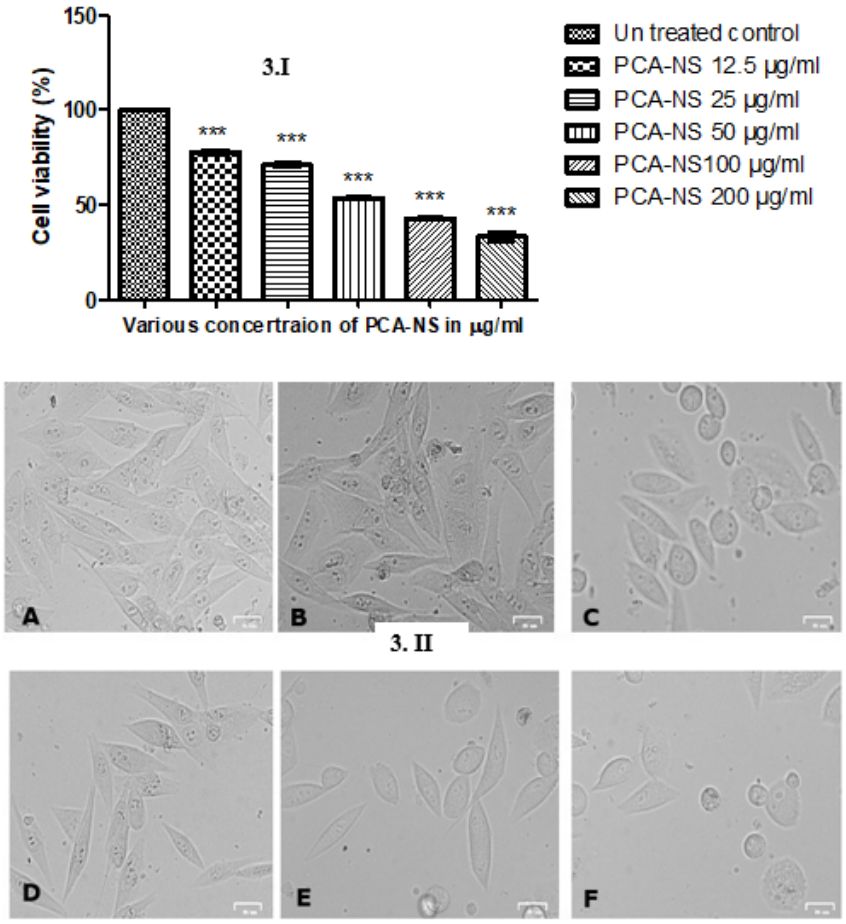

Figure 3: a: Cytotoxicity assay of PCA-NS on MG-63 cells; b: Effect of PCA-NS on cell viability assessed by formazan crystal formation following MTT treatment. The influence of increasing concentrations of PCA-NS on MG-63 cell viability is demonstrated through the intensity of formazan crystal formation: (a) untreated control, (b) 12.5 µg/mL, (c) 25 µg/mL, (d) 50 µg/mL, (e) 100 µg/mL, and (f) 200 µg/mL.

Table 2: IC_{50} Value and Regression Analysis Parameters for PCA-NS in the MTT Cell Viability Assay.

Parameter	Value
Log IC_{50}	1.664
IC_{50} (µg/mL)	46.10
95% CI (IC_{50})	42.73 to 49.75
Hill Slope	-2.319
95% CI (Hill Slope)	-2.745 to -1.894
Standard Error (Log IC_{50})	0.01613
R^2 (Goodness of Fit)	0.932
Degrees of Freedom	28
Sum of Squares	1869
Standard Deviation (Sy.x)	8.170
Number of X values	30
Number of Y values analysed	30

PCA demonstrated encouraging binding to the tumor suppressor protein p53 (PDB ID: 1TUP), an essential regulator of the cell cycle and apoptosis, in addition to its interaction with topoisomerase IIα.According to molecular docking, PCA forms hydrogen bonds with the mutation hotspot residue ARG248 and other nucleotide bases by aligning itself within the p53-DNA complex's DNA-binding groove. This implies that PCA may either stabilize the p53-DNA complex or mimic DNA interactions to restore p53 function, which is often lost or diminished in osteosarcoma due to mutations. The comparable binding affinity for p53 (-5.9 kcal/mol) and a low RMSD value support PCA's suitability for targeting this complex binding interface. Earlier research had also revealed that for phytochemicals, moderate binding scores are often acceptable due to their high molecular flexibility, polyphenolic nature of the chemical and multiple weak but cooperative interactions. In the present study, Protocatechuic acid formed five hydrogen bonds and six Vander waals interaction residues with the protein 1ZXN; formed three hydrogen bonds, five Vander waals interaction residues with the protein 1TUP. Hydrogen bonding account for specificity and vander waals interaction account for stability and flexibility and ensure that PCA accommodate hydrophobic cavity of protein effectively.

The cytotoxic potential of PCA-loaded Nanosuspensions (PCA-NS) was further validated *in vitro* using MG-63 osteosarcoma cells. With an IC_{50} value of 46.10 µg/mL, the MTT experiment clearly showed a dose-dependent decrease in cell viability. This indicates a strong anti-proliferative effect, corroborated by the reduction in formazan crystal formation at higher concentrations. However, since MTT assays cannot differentiate between apoptosis and necrosis, additional assays were necessary to confirm the mode of cell death.

Dual fluorescent staining with acridine orange and ethidium bromide provided insights into the mechanism of cytotoxicity.29 PCA-NS treatment predominantly induced late-stage apoptosis, accounting for 76.5% of total cell death, alongside a smaller proportion of necrotic and early apoptotic cells. These findings were supported by distinct morphological changes, including membrane blebbing, nuclear condensation, and apoptotic body formation. In fluorescent microscopy analysis, MG-63 cells exposed to PCA-NS at a concentration of 46.10 µg/mL exhibited distinct morphological features indicative of both apoptosis and necrosis. The normal cells remained intact with green-fluorescing nuclei. Dead cells were visible due to the red fluorescence caused by EB penetration. Early apoptotic cells appeared with a granular yellow-green colour, whereas late apoptotic cells showed intense orange fluorescence concentrated in their condensed and fragmented nuclei. Cells with necrosis were distinguished by irregular, orange-red staining around the nucleus, and there was no indication of chromatin coiling.

PCA's efficacy is further enhanced by its formulation into nanosuspensions, which likely improve cellular uptake, solubility, and bioavailability which are critical limitations of many phytochemicals. The literature supports this advantage, noting that nanoparticles facilitate targeted delivery and enhance intracellular drug accumulation, thus boosting therapeutic efficacy. Additionally, the polyphenolic structure of PCA has anti-inflammatory and antioxidant properties that may help lower oxidative stress in tumor microenvironments and provide a more comprehensive approach to cancer treatment.

The mechanisms behind Protocatechuic acid's ability to prevent cancer involve blocking carcinogen activation, improving detoxification processes, reducing inflammatory compounds, slowing tumor cell growth, and triggering cell death through apoptosis.[24-27]

Studies have shown that PCA triggers programmed cell death in several types of cancer, including liver cancer, as indicated by research.[28] The cytotoxic effects of PCA-loaded Nanosuspensions (PCA-NS) on MG-63 human osteosarcoma cells were examined in this study. The quantity of alive, metabolically active cells is directly correlated with the intensity of the blue-purple hue seen in the MTT experiment. As the PCA-NS concentration increased from 12.5 µg/mL to 200 µg/mL, a decrease in cell survival was observed, suggesting a dose-dependent cytotoxic effect on cells. Based on the IC_{50} value, the 50% inhibitory concentration was found to be 46.10 µg/mL.

At this concentration, cell death rates were substantially higher than in the control group, where virtually no cell damage was observed. PCA-NS demonstrated a cytotoxic effect of 33.4% at its maximum tested dose of 200 µg/mL. This implies the effective inhibition of osteosarcoma cell growth, positioning PCA-NS as a potential anticancer compound. It's crucial to acknowledge that the MTT assay is successful in assessing cell viability but lacks the ability to distinguish between various forms of cell death, including apoptosis and necrosis.[29-32]

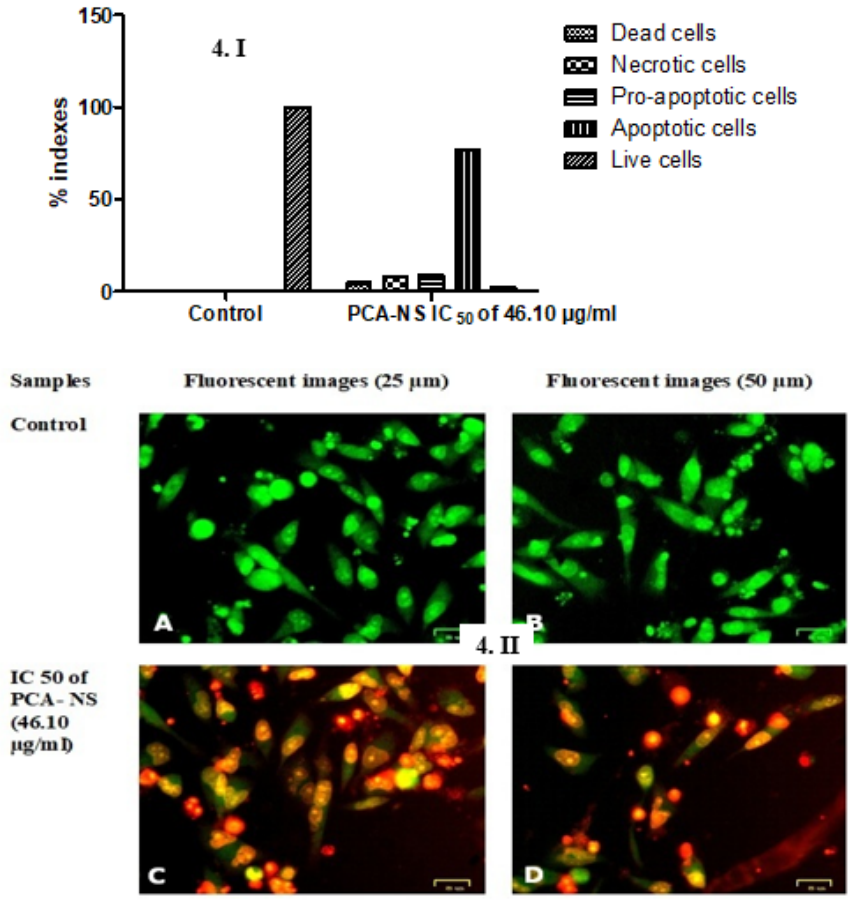

Figure 4: a: Cellular morphology following treatment with PCA-NS at its IC_{50} concentration, in comparison to untreated controls, as observed through dual staining with Acridine Orange and Ethidium Bromide; b: Effect of PCA-NS on AO/EB fluorescent staining for detecting morphological changes associated with apoptosis.

Nanoparticle delivery systems such as PCA-NS further enhance this apoptotic effect by improving bioavailability and targeted delivery.[33,34] Literatures discussed the advantages of nanoparticle-mediated drug delivery in increasing intracellular uptake and promoting apoptosis in cancer cells.[35] This justifies the high percentage of late apoptotic cells seen in the present analysis.

The late apoptotic cell prevalence indicates that PCA-NS activates both intrinsic and extrinsic apoptotic pathways. Fulda and Debatin found in 2006 that anticancer agents trigger both apoptotic pathways, resulting in late-stage apoptosis in approximately a majority of treated cells, thus supporting the current research results.[36]

The efficacy of PCA-NS as an anticancer agent in osteosarcoma MG-63 cells is supported by our findings. The combination of MTT cytotoxicity assays and AO/EB dual staining indicates that PCA-NS causes cell death mainly through the process of apoptosis. However, limitations include evaluation in a single cell line and lack of*in vivo* validation. Due to limited project time duration, formulation scalability, long-term stability, and comprehensive ADME profiling could not be assessed.*In silico* binding predictions require experimental validation. Our future research will incorporate multiple cell lines, animal models, and extended stability studies to overcome these time-constrained limitations.

CONCLUSION

This investigation underscores the significant anticancer properties of a Protocatechuic Acid-loaded Nanosuspension (PCA-NS) in inhibiting the growth of human osteosarcoma (MG-63) cells, indicating its potential in controlling cancer cell proliferation. Due to its natural source and low toxicity, PCA presents potential as a safe, multifunctional therapeutic substance for bone cancer treatment. Molecular docking studies of Protocatechuic Acid (PCA) with the cancer-associated proteins topoisomerase II alpha (1ZXN) and the p53 DNA-binding domain (1TUP) highlight significant binding interactions, suggesting PCA's potential utility as a therapeutic agent in bone cancer treatment. These results validate the function of PCA-NS in triggering programmed cell death, which is crucial for the creation of successful cancer treatments. PCA's capacity to trigger apoptosis while causing minimal harm to healthy cells highlights its potential as a therapeutic agent. Furthermore, the

nano formulation of PCA improved its bioavailability and cellular absorption, which contributed to the observed cytotoxic effects. PCA-NS is a promising candidate for use in anticancer treatment, particularly in the case of osteosarcoma treatment. Further validation of the clinical usefulness and mode of action of this compound will require additional research involving molecular pathway analysis, *in vivo* testing, and pharmacokinetic studies.

ACKNOWLEDGEMENT

The authors acknowledge the management of Dr. Kalam College of Pharmacy, Thanjavur, Tamil Nadu for providing financial support and necessary facilities to carry out the work.

ABBREVIATIONS

AO/EB: Acridine Orange/Ethidium Bromide; **CDK:** Cyclin-Dependent Kinase; **DMEM:** Dulbecco's Modified Eagle Medium; **DMSO:** Dimethyl Sulfoxide; **IC_{50}:** Half Maximal Inhibitory Concentration; **µg/mL:** Micrograms per milliliter.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING SOURCE

The research work received seed amount from the Institutional research fund of Dr. Kalam College of Pharmacy, Thanjavur, Tamil Nadu.

SUMMARY

The study investigates the anticancer potential of protocatechuic acid nanosuspensions (PCA-NS) against MG-63 human osteosarcoma cells, combining *in-vitro* cytotoxicity tests with *in-silico* molecular docking analysis. Treatment options for osteosarcoma, an aggressive bone cancer, are limited. Despite its well-established anticancer properties, Protocatechuic Acid (PCA) has low bioavailability and solubility. The goal of nanosuspension formulation is to overcome these constraints. PCA-NS was created by antisolvent precipitation and its stability was assessed. PCA docked with topoisomerase II alpha (1ZXN) and p53 DNA-binding domain (1TUP) to predict molecular interactions. MTT assay conducted on MG-63 cells to determine IC_{50}.AO/EB dual staining to differentiate between viable, apoptotic, and necrotic cells. Docking showed strong binding to both topoisomerase IIα (-6 kcal/mol) and p53 (-5.9 kcal/mol) with stabilizing hydrogen bonds. MTT assay revealed a dose-dependent cytotoxic effect with IC_{50} = 46.10 µg/mL. AO/EB staining indicated apoptosis as the primary mode of cell death, with 76.5% late apoptotic cells. PCA-NS exhibits significant cytotoxic and pro-apoptotic effects on osteosarcoma cells, likely due to enhanced bioavailability and effective molecular interactions with cancer-related proteins. The formulation appears promising as a possible treatment strategy for bone cancer, indicating the need for additional *in-vivo* and clinical research.

REFERENCES

1. Wang CC, Schulz MD. Ewing's sarcoma: A study of fifty cases treated at the Massachusetts General Hospital, 1930-1952 inclusive. N Engl J Med. 1953;248(14):571-6. doi: 10.1056/NEJM195304022481401.
2. Zöllner SK, Amatruda JF, Bauer S, Collaud S, De Álava E, DuBois SG, *et al.* Ewing sarcoma-diagnosis, treatment, clinical challenges and future perspectives. J Clin Med. 2021;10(8):1685. doi: 10.3390/jcm10081685, PMID 33919988.
3. Arndt CA, Rose PS, Folpe AL, Laack NN. Common musculoskeletal tumors of childhood and adolescence. Mayo Clin Proc. 2012;87(5):475-87. doi: 10.1016/j.mayocp.2012.01.015, PMID 22560526.
4. Bielack S, Jürgens H, Jundt G, Kevric M, Kühne T, Reichardt P, *et al.* Osteosarcoma: the COSS experience. Pediatr Adolesc Osteosarcoma Jaffe N. 2009;152:289-308. doi: 10.1007/978-1-4419-0284-9_15, PMID 20213397, Bruland, O. S, Bielack, S, Eds, Cancer Treatment and Research; Springer US: Boston, MA, 2009; 152:289-308.
5. Sheng G, Gao Y, Yang Y, Wu H. Osteosarcoma and metastasis. Front Oncol. 2021;11:780264. doi: 10.3389/fonc.2021.780264, PMID 34956899.
6. Senthelal S, Li J, Ardeshirzadeh S, *et al.* Arthritis. StatPearls Publishing; 2025.
7. Pickering ME, Delay M, Morel V. Chronic pain and bone-related pathologies: A narrative review. J Pain Res. 2024;17:2937-47. doi: 10.2147/JPR.S469229, PMID 39253740.
8. Yu J, Mu Q, Fung M, Xu X, Zhu L, Ho RJ. Challenges and opportunities in metastatic breast cancer treatments: nano-drug combinations delivered preferentially to metastatic cells may enhance therapeutic response. Pharmacol Ther. 2022;236:108108. doi: 10.1016/j.pharmthera.2022.108108, PMID 34999182.
9. Abbasi E, Aval SF, Akbarzadeh A, Milani M, Nasrabadi HT, Joo SW, *et al.* Dendrimers: synthesis, applications, and properties. Nanoscale Res Lett. 2014;9(1):247. doi: 10.1186/1556-276X-9-247, PMID 24994950.
10. Garcia-Ortega DY, Cabrera-Nieto SA, Caro-Sánchez HS, Cruz-Ramos M. An overview of resistance to chemotherapy in osteosarcoma and future perspectives. Cancer Drug Resist. 2022;5(3):762-93. doi: 10.20517/cdr.2022.18, PMID 36176756.
11. Brar GS, Schmidt AA, Willams LR, Wakefield MR, Fang Y. Osteosarcoma: current insights and advances. Explor Target Antitumor Ther. 2025;6:1002324. doi: 10.37349/etat.2025.1002324, PMID 40547807.
12. Kortam S, Lu Z, Zreiqat H. Recent advances in drug delivery systems for osteosarcoma therapy and bone regeneration. Commun Mater. 2024;5(1):168. doi: 10.1038/s43246-024-00612-2.
13. Mir SA, Dar A, Hamid L, Nisar N, Malik JA, Ali T, *et al.* Flavonoids as promising molecules in the cancer therapy: an insight. Curr Res Pharmacol Drug Discov. 2024;6:100167. doi: 10.1016/j.crphar.2023.100167, PMID 38144883.
14. Kakkar S, Bais S. A review on protocatechuic acid and its pharmacological potential. ISRN Pharmacol. 2014; 2014:952943. doi: 10.1155/2014/952943, PMID 25006494.
15. Cadena-Iñiguez J, Santiago-Osorio E, Sánchez-Flores N, Salazar-Aguilar S, Soto-Hernández RM, Riviello-Flores ML, *et al.* The cancer-protective potential of protocatechuicacid:Anarrativereview.Molecules.2024;29(7):1439.doi:10.3390/molecules29071439, PMID 38611719.
16. Liu Y, Liang Y, Yuhong J, Xin P, Han JL, Du Y, *et al.* Advances in nanotechnology for enhancing the solubility and bioavailability of poorly soluble drugs. Drug Des Dev Ther. 2024;18:1469-95. doi: 10.2147/DDDT.S447496, PMID 38707615.
17. Liu K, Liu PC, Liu R, Wu X. Dual AO/EB staining to detect apoptosis in osteosarcoma cells compared with flow cytometry. Med Sci Monit Basic Res. 2015;21:15-20. doi: 10.12659/MSMBR.893327, PMID 25664686.
18. Nitiss JL. DNA topoisomerase II and its growing repertoire of biological functions. Nat Rev Cancer. 2009;9(5):327-37. doi: 10.1038/nrc2608, PMID 19377505.
19. Broadhead ML, Clark JC, Myers DE, Dass CR, Choong PF. The molecular pathogenesis of osteosarcoma: a review. Sarcoma. 2011; 2011:959248. doi: 10.1155/2011/959248, PMID 21559216.
20. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65(1-2):55-63. doi: 10.1016/0022-1759(83)90303-4, PMID 6606682.
21. Alley MC. Proc Am Assoc Cancer Res, Washington, DC. 1986;27:389.
22. Jangid AK, Pooja D, Jain P, Rompicharla SV, Ramesan S, Kulhari H. A nanoscale, biocompatible and amphiphilic prodrug of cabazitaxel with improved anticancer efficacy against 3D spheroids of prostate cancer cells. Mater Adv. 2020;1(4):738-48. doi: 10.1039/D0MA00189A.
23. Abotaleb M, Samuel SM, Varghese E, Varghese S, Kubatka P, Liskova A, *et al.* Flavonoids in cancer and apoptosis. Cancers. 2018;11(1):28. doi: 10.3390/cancers11010028, PMID 30597838.
24. Woo CS, Lau JS, El-Nezami H. Herbal medicine. Adv Bot Res. 2012;62:365-84. doi: 10.1016/B978-0-12-394591-4.00009-X.
25. Ramakrishna W, Kumari A, Rahman N, Mandave P. Anticancer activities of plant secondary metabolites: rice callus suspension culture as a new paradigm. Rice Sci. 2021;28(1):13-30. doi: 10.1016/j.rsci.2020.11.004.

26. George BP, Chandran R, Abrahamse H. Role of phytochemicals in cancer chemoprevention: insights. Antioxidants (Basel). 2021;10(9):1455. doi: 10.3390/antiox10091455, PMID 34573087.
27. De Flora S, Ferguson LR. Overview of mechanisms of cancer chemopreventive agents. Mutat Res. 2005;591(1-2):8-15. doi: 10.1016/j.mrfmmm.2005.02.029, PMID 16107270.
28. Punvittayagul C, Luangsuphabool T, Wongpoomchai R. Protocatechuic acid as a potent anticarcinogenic compound in purple rice bran against diethylnitrosamine-initiated rat hepatocarcinogenesis. Sci Rep. 2022;12(1):10548. doi: 10.1038/s41598-022-14888-2, PMID 35732709.
29. Kari S, Subramanian K, Altomonte IA, Murugesan A, Yli-Harja O, Kandhavelu M. Programmed cell death detection methods: A systematic review and a categorical comparison. Apoptosis. 2022;27(7-8):482-508. doi: 10.1007/s10495-022-01735-y, PMID 35713779.
30. Kasibhatla S, Amarante-Mendes GP, Finucane D, Brunner T, Bossy-Wetzel E, Green DR. Acridine orange/ethidium bromide (AO/EB) staining to detect apoptosis. Cold Spring Harb Protoc. 2006; 3:pdb.prot4493.
31. Darzynkiewicz Z, Bruno S, Del Bino G, Gorczyca W, Hotz MA, Lassota P, *et al.* Features of apoptotic cells measured by flow cytometry. Cytometry. 1992;13(8):795-808. doi: 10.1002/cyto.990130802, PMID 1333943.
32. Baskić D, Popović S, Ristić P, Arsenijević NN. Analysis of Cycloheximide-Induced Apoptosis in Human leukocytes: fluorescence Microscopy Using annexin V/propidium iodide versus acridin Orange/ethidium bromide. Cell Biol Int. 2006;30(11):924-32. doi: 10.1016/j.cellbi.2006.06.016, PMID 16895761.
33. Pooja YS, Rajana N, Yadav R, Naraharisetti LT, Godugu C, Mehra NK. Design, development, and evaluation of CDK-4/6 inhibitor loaded 4-carboxy phenyl boronic acid conjugated pH-sensitive chitosan lecithin nanoparticles in the management of breast cancer. Int J Biol Macromol. 2024;258(1):128821. doi: 10.1016/j.ijbiomac.2023.128821, PMID 38110163.
34. Yoon HY, Koo H, Kim K, Kwon IC. Molecular imaging based on metabolic glycoengineering and bioorthogonal click chemistry. Biomaterials. 2017;132:28-36. doi: 10.1016/j.biomaterials.2017.04.003, PMID 28399460.
35. Deivayanai VC, Thamarai P, Karishma S, Saravanan A, Yaashikaa PR, Vickram AS, *et al.* A comprehensive review on advances in nanoparticle-mediated cancer therapeutics: current research and future perspectives. Cancer PathogTher, 2024: S2949713224000880.
36. Fulda S, Debatin KM. Extrinsic versus intrinsic Apoptosis Pathways in Anticancer Chemotherapy. Oncogene. 2006;25(34):4798-811. doi: 10.1038/sj.onc.1209608, PMID 16892092.

Cite this article: Subramanian N, Surendran V, Selvam R, Vanangamudi M, Azhagarsamy T, Gunda RK. Cytotoxic and Apoptotic Study of Protocatechuic Acid Nanosuspensions Assessed by *in silico* Molecular Analysis and AO/EB Staining Technique. Indian J of Pharmaceutical Education and Research. 2026;60(4s):s1594-s1603.