Original Article

In Silico and In Vitro Anticancer Activities of *Punica granatum* L. Peel Ethanolic Extract Against Topoisomerase II Alpha in HeLa and cMM/IPB-B4 Cell Lines

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ABSTRACT

Background: Cancer remains a leading cause of death globally, and the development of new, effective, and less toxic treatments from natural sources is a key area of research. Pomegranate peel, a rich source of bioactive compounds, has shown promise as a natural anticancer agent.

Objectives: This study examined the anti-proliferative properties of pomegranate peel ethanolic extract against cMM/IPB-B4 and HeLa cells. The study also utilized in silico approaches to explore the potential inhibitory activities of the pomegranate's major compounds, such as punicalagin, ellagic acid, and gallic acid, against topoisomerase IIα.

Methods: An ethanolic extract of pomegranate peel was prepared using the maceration method. In vitro anti-proliferative activity was assessed using a cell viability assay on HeLa and cMM/IPB-B4 cells. The IC_{50} values were determined, and the brine shrimp lethality test (BSLT) was conducted to evaluate toxicity. An in silico analysis using AutoDock Vina, PASS Online, and ADMET-AI screening was performed to study the interactions of the compounds with the topoisomerase IIα enzyme.

Results: The extract demonstrated anti-proliferative effects, with IC_{50} values of 943.43 µg/mL for HeLa cells and 1152.42 µg/mL for cMM/IPB-B4 cells. In silico docking revealed that punicalagin was the best topoisomerase IIα inhibitor (-7.24 kcal/mol). The ADMET analysis indicated that punicalagin had a moderately safety profile.

Conclusion: Pomegranate peel ethanolic extract exhibits anti-proliferative activity against both HeLa and cMM/IPB-B4 cells. The in silico findings strongly suggest that punicalagin is a key compound contributing to this effect by inhibiting topoisomerase IIα. These results support the potential of pomegranate peel extract as a novel anticancer therapy, especially against cMM/IPB-B4.

Keywords: Anticancer, cMM, HeLa cells, Pomegranate, Topoisomerase IIα

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Introduction

Cancer is a broad category of chronic, non-communicable diseases (NCDs) characterized by excessive cell division and differentiation, as well as uncontrolled cell proliferation. Even after a tumor has been entirely removed from its original location, cancer cells can spread to other parts of the body (Couffignal et al., 2000) 271 +/- 15,330 francs. An estimated 8 million fatalities occur each year, and this number is predicted to increase to 15 million by 2030, making cancer the second leading cause of death after cardiovascular disease (Fayed et al., 2020). Conventional methods, such as chemotherapy, radiation, and surgery are still considered effective, but more efficient cancer prevention treatment plans are required because of some adverse effects on healthy body cells (Newman & Cragg, 2016). A significant portion of chemotherapeutic drugs derived from natural sources are shown to be viable treatment options. If their mechanisms of action are elucidated, these findings could help reduce reliance on conventional treatments (Cragg & Pezzuto, 2016).

There are thousands of medicinal plant species in Indonesia. These biological resources have encouraged extensive exploration of secondary metabolites from medicinal plants as potential pharmaceutical agents. The National Agency of Drug and Food Control of Indonesia classifies traditional medicines into three categories based on scientific evidence: jamu (traditional herbal medicine), standardized herbal medicine, and phytopharmaca. Jamu, an indigenous Indonesian traditional medicine, is used by local communities to treat illnesses (Sholikhah, 2016). *Punica gratanum* L., commonly known by local people as "delima" (pomegranate), has been used as a traditional medicine due to its pharmacological activities, such as anticancer, anti-inflammatory, antidiabetic, antifibrotic, antimalaria, antifungal, and antibacterial activities *P. gratanum* seed oils exert anticancer activities against human lung cancer (80.3±1.2–25.4±1.2%) and colon cancer lines (83.3±1.2–28.4±1.2%) (Esther Lydia et al., 2020). In addition to the seed oils, the peel or skin exhibits cytotoxic effects against HCT116 cells, as demonstrated using a cell proliferation assay (Read et al., 2019). The HCT-116 colon cancer cell line has been reported to demonstrate dose-dependent inhibitory effects in response to plant extracts, which are attributed to their rich phenolic and flavonoid content, as well as their bioactive compounds (Hiremath et al., 2024).

The peel of *P. gratanum* has been extensively studied for its chemical constituents. Over 48 secondary metabolites have been identified in the peel, including anthocyanidins, sterols, tannins, terpenes, flavonoids, proanthocyanidins, phenolics, anthocyanins, and alkaloids (Maphetu et al., 2022). The major compounds found in the peel extract are phenolics, such as punicalin, punicalagin, ellagic acid, and gallic acid (Ge et al., 2021; Read et al., 2019). Punicalagin exhibits strong antiviral activity against Mayaro virus, with an IC_{50} of 28.2 mg/kg (Salles et al., 2021). This compound also inhibits tumor growth by eliminating tumor cells in the stomach, prostate, lung, and liver. Furthermore, punicalagin exhibits anticancer activities in various animal studies by blocking the MAPK/ERK and PI3K/AKT/mTOR signaling pathways (Hassan et al., 2025).

Numerous biological processes, such as transcription, DNA replication, and chromosome segregation, depend on the topoisomerase II alpha (topoisomerase IIα) enzyme. Its expression is highly regulated by the cell cycle, and its levels peak during the S and G2/M phases, when DNA replication occurs and chromosomes prepare to divide (Rattner et al., 1996).Topoisomerase IIα is a crucial component of the centromere, the region of a chromosome that joins sister chromatids, as demonstrated in studies of human cancer cells, such as HeLa cells. This specific association is essential for the development of the centromere and kinetochore structures that ensure proper chromosomal separation during cell division (Nitiss, 2009).

Since topoisomerase IIα is a major target for many chemotherapy medications, the association between topoisomerase IIα and HeLa cells is important in cancer research. Topoisomerase IIα is a primary target for therapeutic intervention because the high proliferation rate of cancer cells, such as HeLa cells, is associated with elevated levels of this protein. Research conducted on HeLa cells has enabled scientists to understand how anticancer drugs, such as genistein, inhibit topoisomerase IIα activity (Zhou et al., 2009). These investigations have demonstrated that such inhibition can result in apoptosis (programmed cell death) and cell cycle arrest, providing important insights into the mechanisms underlying the effects of these drugs and assisting in the development of novel cancer therapies.

In canine cancers, such as canine malignant mesothelioma (cMM), research has shown that topoisomerase IIα effects on chemotherapy similar to those observed in human cancers. cMM is a rare and aggressive tumor; however, veterinary oncologists use chemotherapy protocols that target topoisomerase IIα, as human on-

cologists do (Sato et al., 2023). Anthracyclines, such as doxorubicin, are well-known topoisomerase II inhibitors that are often used to treat various types of canine cancer, including lymphomas and sarcomas (Klimiuk et al., 2020). Research on other types of dog cancer, like canine lymphomas, has shown that the level of topoisomerase IIα expression in a tumor is associated with its response to anthracycline-based chemotherapy. Therefore, researchers may be able to use topoisomerase IIα expression in dog tumors to predict the effectiveness of a treatment, just as they study its expression in HeLa cells to learn about drug efficacy (Vail & Withrow, 2007). For the management and treatment of cMM and other proliferative canine cancers, veterinary oncology can directly benefit from the knowledge gained from research on topoisomerase IIα in human cancer cell lines.

This research investigated the anticancer activity of an ethanolic extract from the peel of *P. granatum* against HeLa cells and cMM/IPB-B4 cells using in vitro approaches. Moreover, the major phenolic compounds from *P. gratanum*, such as punicalagin, ellagic acid, and gallic acid, were analyzed for their inhibition activities against topoisomerase IIα using an in silico technique.

Materials and Methods

Extract preparation

The maceration technique was used to extract 250 g of red pomegranate fruit peel simplicia obtained from pomegranate plants distributed by CV. Herbal Anugrah Alam, Yogyakarta, in 96% ethanol at a ratio of 1:10 (250 grams of simplicia to 2.5 liters of ethanol). The extraction was conducted three times for 24 hours each, with occasional stirring. The liquid obtained was then filtered to produce the filtrate, which was stored in a sealed container. A total of 6 liters of liquid extract was concentrated using an evaporator at 80 ˚C. The concentrated pomegranate peel extract was placed in a glass container and refrigerated (Harborne, 1984).

Phytochemical analysis

The qualitative phytochemical analysis was conducted to detect flavonoids, alkaloids, saponins, tannins, quinones, steroids, and triterpenoids using Harborne's procedures (Harborne, 1984).

Antioxidant activity assay

A DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) stock solution (125 µM) was prepared by dissolving 2.5 mg of DPPH in ethanol. The sample and vitamin C were prepared by dissolving 10 mg in 1 mL of dimethyl sulfoxide/DMSO (EMSURE® Merck KGaA, Germany). The mixture was subjected to sonication until fully dissolved and subsequently vortexed. The samples were prepared at concentrations of 0, 10, 50, 100, 150, and 200 ppm. Aliquots of the samples and vitamin C (100 µL) were added to the microplate. Approximately 100 µL of DPPH solution was added to the duplicate samples. The microplate was subsequently incubated at ambient temperature in the absence of light for 30 minutes. After incubation, the absorbance was measured using an enzyme-linked immunosorbent assay (ELISA) microplate reader (MPR-H200BN Infitek, USA) at a wavelength of 517 nm. Microsoft Office Excel 2007 was used to calculate the mean and percentage standard deviations by linear regression (Salazar et al., 2011).

Toxicity assay

A container containing 250 mL of seawater was filled with 10 mg of *Artemia salina* shrimp eggs. The eggs were incubated for 48 hours using an aerator and a 25-watt light. After hatching, ten shrimp larvae were pipetted into each well of a sterile 12-well microplate. The larvae were used to test the ethyl alcohol extract of pomegranate peel at concentrations of 0, 10, 50, 100, 150, and 200 ppm, with three replicates for each test-solution concentration. The lethal concentration (LC_{50}) value was determined by recording the number of dead larvae after 24 hours a and estimating the value using Probit analysis in SPSS software, version 25.0 (SPSS, Inc., Chicago, IL, USA) based on the dose–response relationship of shrimp-larvae mortality (Ratu & Mugiyanto, 2018). All experiments were conducted at the Laboratory of Veterinary Pharmacology, IPB University, Bogor, Indonesia.

Anti-proliferation activities of pomegranate peel extract

HeLa and cMM/IPB-B4 cells were obtained and used in the anti-proliferative activity assay, which was conducted at the Tissue Culture Laboratory, IPB University, Bogor, Indonesia. This study followed the method described by Priosoeryanto et al. (2009). Seven treatments, each with three replicates, were included in this experiment, along with a negative control, a positive control, and treatments with six different concentrations of the test substance (pomegranate peel ethanolic extract).

The negative control consisted of culture medium and a cell suspension. The positive control consisted of doxorubicin (Adooq® Bioscience, USA), Dulbecco's modified eagle medium/DMEM (Servicebio Technology, China) as the culture medium, and a cell suspension. The extract treatments consisted of a cell suspension, culture medium, and six different concentrations of the test substance. The LC_{50} value obtained from the brine shrimp lethality test (BSLT) was used to determine the extract concentration variations used in this test.

Cells were seeded in a 24-well microplate (Labselect, Beijing), with a total volume of 1 mL per well. Following the treatment protocol, 50 µL of extract at various concentrations, 50 µL of cancer cell suspension, and doxorubicin (100 µg/mL) were added to each well. After that, the microplate was incubated for three to four days at 37 ˚C with 5% CO_2.

The cancer cells were collected by removing the culture medium, trypsinizing the cells, rinsing them with EDTA-PBS (Gibco-Life Technologies, US), and agitating them uniformly to detach any cancer cells adhering to the well walls. Following their transfer to a microplate containing trypan blue dye (Elabscience®, US) the suspended cancer cells were counted using a phase-contrast microscopy (ID03 Zeiss, Germany) at 100x magnification. All cells visible in the central compartment of the counting chamber were counted (Figure 1). The Equation 1 was used to calculate the percentage inhibitory activity of the extract against the cells used in the antiproliferation assay:

1. % Growth activity = X 100%

$$\frac{\text{Average number of treatment cells}}{\text{Average number of negative control cells}} \times 100\%$$

% Inhibition activity = 100% - (% growth activity)

This experiment was carried out three times in duplicate. The experimental data were statistically analyzed using one-way analysis of variance (ANOVA), followed by Duncan's multiple comparison test, in SPSS 25.0 software (SPSS, Inc., Chicago, IL, USA). A value of $P<0.05$ was considered statistically significant.

In silico study

The PASS Online server was used to analyze the compounds' anticancer activity in this study. To identify activities linked to cancer, the SMILES representations of all compounds generated using the ChemDraw 23.1.1 application (Revvity Signals, USA) were entered into the PASS Online website. The drug similarity of the compounds was examined using Lipinski's rule by entering the SMILES representation of each compound into the SwissADME server (Widyananda et al., 2022). Furthermore, an ADMET radial plot for each compound was obtained using the ADMET-AI server to analyze the ADMET characteristics of each compound.

Autodock Vina with AutoDockTools 1.5.6 (Scripps Research Institute, USA) was used to prepare the ligands and protein for the molecular docking study. Using AutoDockTools, the topoisomerase IIα protein with PDB ID 5GWK was downloaded and prepared by adding polar hydrogens, removing its hydrogen, and adding Kollman charges. Chem3D 23.1.1 was used to optimize the ligands using the MM2 algorithm to minimize their energy. AutoDockTools was used to further prepare the optimized ligands by converting their file formats to PDBQ files (Sucahyo et al., 2023). The inhibition sites of the native ligand, located at 31.606, −23.314, and −57.776 (center_x, center_y, and center_z), served as the basis for determining the grid center. Autodock Vina used a 60×60×60 grid box with a num_nodes value of 10 and an exhaustiveness value of 16 (Trott & Olson, 2010). The optimal conformation of each compound, with the highest binding-affinity value, was selected for further analysis of its interactions with amino acid residues using BIOVIA Discovery Studio 2025 (Dassault Systèmes, USA).

Results

Extraction and phytochemical screening

Extraction of 250 g of pomegranate fruit peel yielded 75 g of concentrated ethanolic extract. As shown in Table 1, the ethanolic extract of pomegranate fruit peel contained various classes of secondary metabolites, including flavonoids, saponins, tannins, and quinones. However, other classes, like alkaloids, triterpenoids, and steroids were absent from the ethanolic extract.

Antioxidant activity

The antioxidant activity of the ethanolic extract was measured in triplicate to ensure the reliability of the data. The regression values obtained from three analyses of the ethanolic extract were consistent, with values of 0.9952, 0.9956, and 0.9928. The ethanolic extract of *P. granatum* peel exhibited antioxidant activity, with an IC_{50} of 14.2±0.91 µg/mL. This antioxidant activity was close to that of ascorbic acid, which was used as the standard (6.41±0.05 µg/mL), and the results are shown in Table 2.

Table 1. Phytochemical screening results of the ethanolic extract from pomegranate fruit peel

Class of Phytochemicals	Test/Reagent	Ethanolic Extract
Alkaloids	Dragendroff's	-
	Wagner's	-
	Mayer's	-
Flavonoids	NaOH	+
Saponins	Foam	+
Triterpenoid	H_2SO_4	-
Steroids	Salkowski	-
Tannins	KOH	+
Quinones	NH_4OH	+

Toxicity analysis

The 95% confidence limits for the Probit analysis are shown in Table 3, along with the estimated concentrations ("Estimate") needed to achieve different effect probabilities, ranging from 10% to 99%. The lethal concentration 50 (LC_{50}), or the estimated concentration required to produce a 50% effect, was 604.783 at a 50% probability, as shown in Table. The lower bound of 433.13 and the upper bound of 862.101 provide the 95% confidence limits for this LC_{50} value. This indicates that there is a 95% probability that this range contains the true LC_{50} of the substance.

Anti-proliferation activities

The effect of different concentrations of the extract on the colony formation of HeLa and cMM/IPB-B4 cells was investigated, and the results are shown in Figure 2. Notably, treatment with 300, 600, 900, 1200, and 1500 µg/mL of the ethanolic extract resulted in different antiproliferative activities in HeLa cells, with values of 24.31, 36.81, 48.61, 59.73, and 70.83%, respectively. A concentration-response relationship was also observed in cMM/IPB-B4 cells, with antiproliferative activities of 13.33, 25.18, 37.05, 51.85, and 65.18%, respectively. The IC_{50} values of the ethanolic extract against HeLa and cMM/IPB-B4

Table 2. Antioxidant activities of the ethanolic extract and ascorbic acid using the DPPH method

Sample	Concentration (µg/mL)	%Inhibition (n=3)		
		1	2	3
Ethanolic extract	100	84.45	84.81	84.45
	50	66.43	66.43	66.08
	25	54.77	54.77	54.42
	12.5	50.18	49.12	49.82
	6.25	45.94	45.58	44.52
Ascorbic acid	5	40.28	39.57	39.58
	2.5	21.91	21.91	21.91
	1.25	12.37	12.72	12.37
	0.625	6.71	7.42	6.71
	0.3125	1.41	2.83	1.77

Table 3. Toxicity analysis of the ethanolic extract using the BSLT with 95% confidence limits

Probability of Mortality (%)	95% Confidence Limits		
	Estimate	Lower Bound	Upper Bound
10	-312.175	-945.43	-41.755
20	2.597	-405.439	201.761
30	229.571	-40.378	401.663
40	423.511	230.496	613.53
50	604.783	433.13	862.101
60	786.054	598.752	1147.686
70	979.994	755.621	1473.557
80	1206.968	927.412	1866.73
90	1521.741	1156.619	2421.03
99	2269.299	1686.365	3752.044

cells were 943.43 and 1152.42 µg/mL, respectively. The addition of doxorubicin (100 µg/mL) to the colonies suppressed the anti-proliferative activity of the pomegranate fruit peel extract, resulting in values of 72.92% for HeLa cells and 71.11% for cMM/IPB-B4 cells.

In silico study

Only gallic and ellagic acids fulfilled Lipinski's requirements (Table 4). Because these two compounds met all of Lipinski's guidelines, they can be administered orally. Because doxorubicin and punicalagin did not comply with Lipinski's rules, they cannot be administered orally. Due to their extreme polarity, both substances have difficulty passing through the lipid bilayer membrane (Daina et al., 2017).

The pharmacological activity being investigated in this study specifically involved topoisomerase II protein inhibitors and anticancer activity. Based on the heatmap shown in Figure 3, the probability (Pa) that a compound or ligand has a particular biological activity increases with the intensity of the yellow color in the heatmap. The drug compound doxorubicin had the highest Pa value against topoisomerase II, at 0.818. Among the ligands used in this investigation, punicalagin (Pa=0.447), gallic

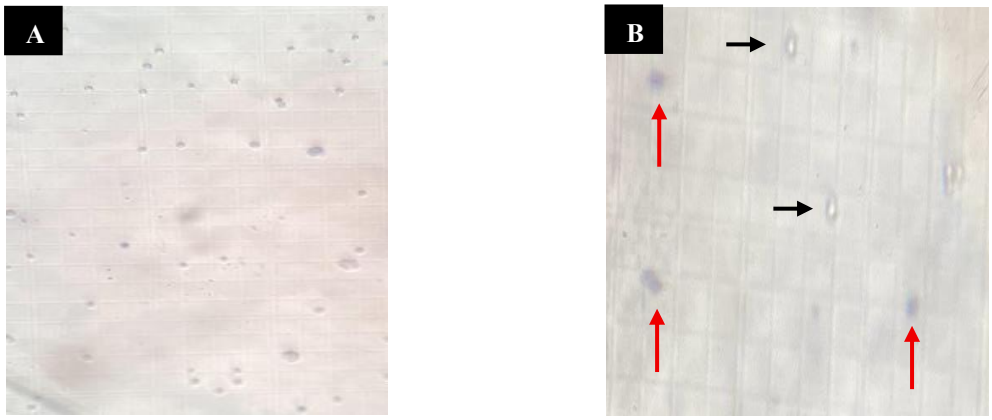


Figure 1. Cell counting and viability assessment

A) Cells in a Neubauer hemocytometer chamber, B) Trypan blue staining showing dead cells (red arrow) and live cells (black arrow)

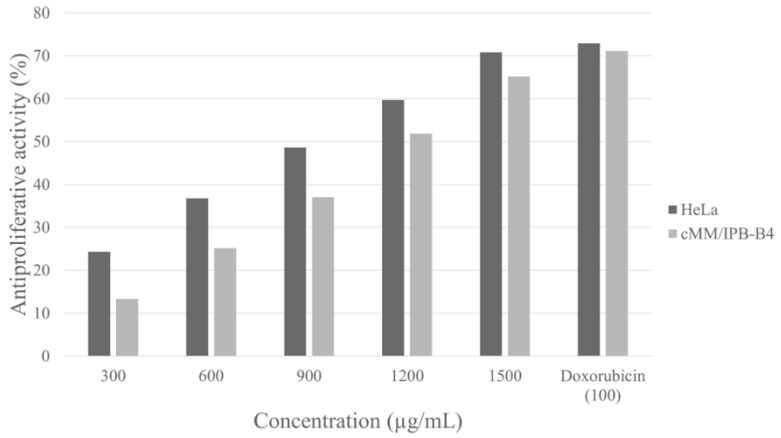


Figure 2. Antiproliferative activity of the ethanolic extract from pomegranate fruit peel

acid (Pa=0.323), and ellagic acid (Pa=0.225) were expected to exhibit the strongest inhibitory activity against topoisomerase II.

Because the root mean square deviation (RMSD) value from the redocking results was 0.87 Å, Figure 4 demonstrates that the conformational changes were minimal. Consequently, the docking method used in this study was validated (Odhar et al., 2023).

According to Table 5, ellagic acid interacted with doxorubicin through the same amino acid residue, Arg487. Ellagic acid also exhibited the same interactions as the drug at DT9 and DG13 in the ligand–DNA interaction at the topoisomerase IIα receptor. Gallic acid exhibited interactions similar to those of the drug at Arg487, DA12, and DT9. The punicalagin compound had only one interaction in common with doxorubicin, namely, at the DG13 region of the DNA. Despite having only one similar interaction, punicalagin formed six hydrogen bonds, which provided the greatest complex stability compared with gallic acid and ellagic acid.

The ADMET analysis presented in Figure 5 that profile of doxorubicin is not optimal. The ADMET profile of doxorubicin is not optimal. Doxorubicin can easily cross the blood-brain barrier (BBB). Additionally, its interaction with hERG ion channels and low hERG Safe value indicate a significant risk of cardiovascular damage. This compound is thought to have low bioavailability due to its limited solubility. However, the low toxicity profile of doxorubicin makes it an extremely effective pharmaceutical agent.

The ADMET profile of ellagic acid is moderate. Its moderate ability to cross the blood–brain barrier suggests that it may be able to do so. Its suitably high hERG Safe value indicates that this compound has a favorable

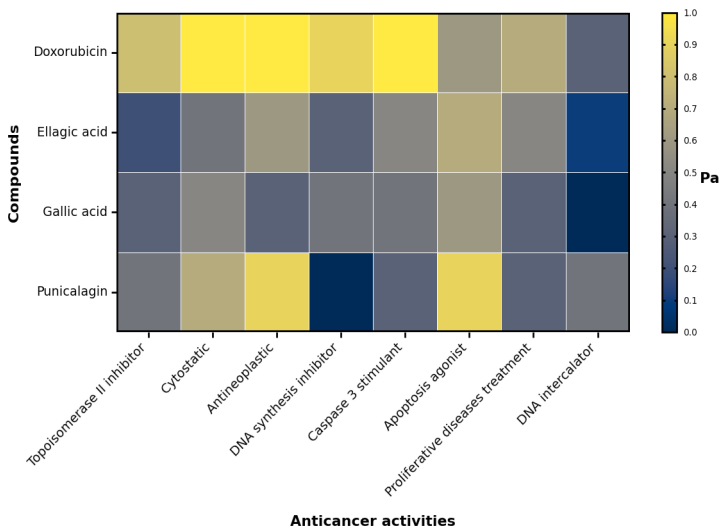


Figure 3. Screening of cancer-related bioactivities

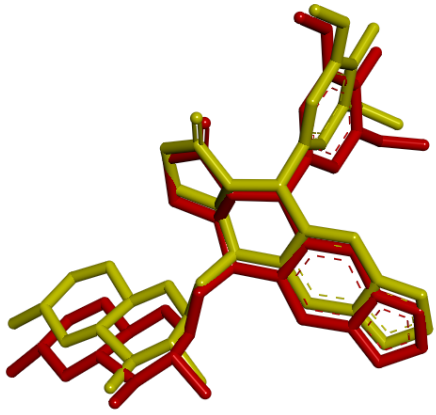


Figure 4. Conformation of the native ligand before (yellow) and after (red) the re-docking process.

safety profile with respect to hERG ion channels, thereby lowering the risk of heart attack. Because of its relatively high solubility, ellagic acid has intermediate bioavailability. Its high non-toxic score indicates that ellagic acid has a low level of toxicity and a favorable safety profile.

Of the four substances tested, gallic acid had the most favorable ADMET profile. Its effective penetration capability indicates a strong potential to cross the blood–brain barrier. Given its high hERG safe rating, which suggests a low risk of cardiac damage, this compound demonstrates notable safety with regard to hERG ion channels. The high solubility of gallic acid contributes to its exceptional bioavailability. Its high non-toxic rating suggests that it has a very favorable safety profile and low toxicity.

The ADMET profile of punicalagin is varied. Its moderate penetration of the blood–brain barrier indicates limited permeability across the BBB. Its suitably high hERG safe value indicates that this chemical has an excellent safety profile with respect to hERG ion channels. Despite its modest solubility, punicalagin is thought to have limited bioavailability. Its moderate to high non-toxic score indicates that punicalagin has moderate toxicity and a somewhat favorable safety profile.

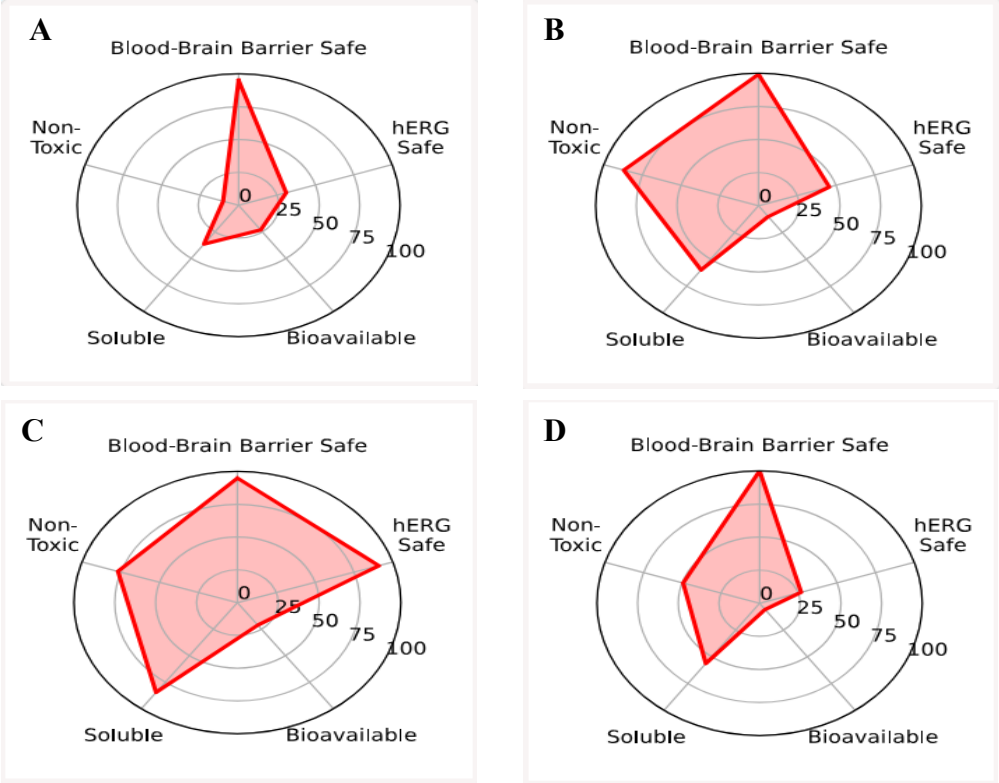


Figure 5. ADMET characteristics of doxorubicin (A), ellagic acid (B), gallic acid (C), and punicalagin (D)

Table 4. Oral drug screening using Lipinski's rules

Compounds	MW	HBA	HBD	TPSA	LogP	Lipinski
Doxorubicin	543.53	12	6	206.07	0	×
Ellagic acid	302.19	8	4	141.34	1.31	✓
Gallic acid	170.12	4	4	97.99	0.5	✓
Punicalagin	1084.72	30	17	518.76	1.96	×

Abbreviations: MW: Molecular weight; HBA: Hydrogen bound acceptor; HBD: Hydrogen bound donor; TPSA: Topological polar surface area.

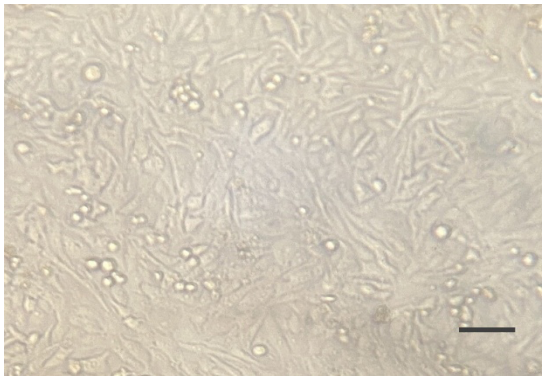


Figure 6. Representative morphology of HeLa cells, which appeared strongly adherent, polygonal, and widely spread after 72 hours of incubation (phase-contrast microscopy)

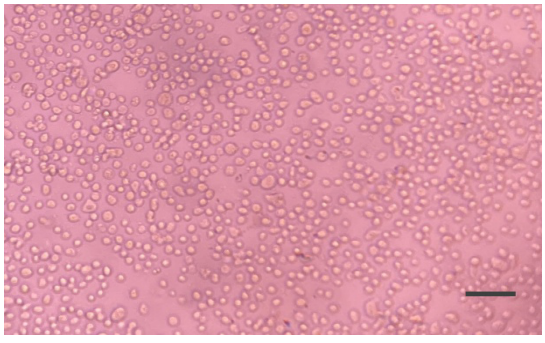


Figure 7. Representative morphology of cMM/IPB-B4 cells, which appeared floating, round, and variable in size after 72 hours of incubation (phase-contrast microscopy)

Table 5. Binding energy values and amino acids and DNA regions interacting with the ligands

Compounds	Binding Energy (kcal/mol)	Amino Acids and DNA
Native ligand	-11.30	Gly462, Asp463, Arg487, Met762, Pro803, DC8, DG13
Doxorubicin	-9.66	Gly462, Asp463, Leu486, Arg487, Gly488, Gly760, DC8, DT9, DA12, DG13
Ellagic acid	-6.36	Arg487, DT9, DG13
Gallic acid	-3.87	Arg487, DRG13, DA12, DT9
Punicalagin	-7.24	Met762, Lys798, Ser800, Pro803, DG13, DC11, DA12

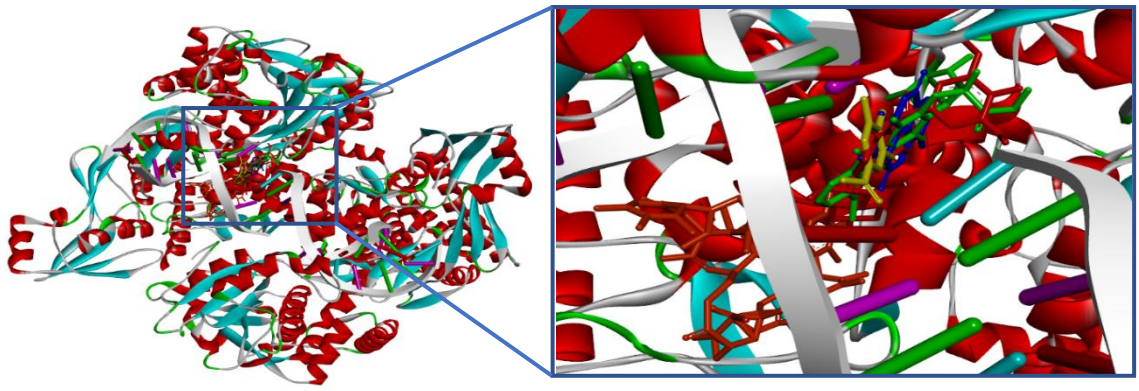


Figure 8. Binding positions of the native ligand (red), doxorubicin (green), ellagic acid (blue), gallic acid (yellow), and punicalagin (orange) on the topoisomerase IIα receptor

Discussion

The current study demonstrated that the concentrated ethanolic extract of pomegranate fruit peel contains different phytochemical classes, such as flavonoids, saponins, tannins, and quinones. Magangana et al. also found all secondary metabolite classes detected in this study (Magangana et al., 2020). However, some classes were not detected in the ethanolic extract, possibly because environmental factors can directly alter the constituents of pomegranates grown in different countries (Qaderi et al., 2023). The ethanolic extract of *P. gratanum* peel demonstrated potent antioxidant activity, with an IC_{50} of 14.2±0.91 µg/mL, which may be attributed to its high concentrations of phenolic compounds and flavonoids (Saparbekova et al., 2023). The hydroxyl groups in those classes may contribute to the low IC_{50} because they can inhibit DPPH free radicals by transferring hydrogen atoms to them (Santos-Sánchez et al., 2019).

The pomegranate peel extract's LC_{50} value, as determined by the BSLT, was 604.783 ppm (µg/mL). This value, along with the 95% confidence limits of 433.130 to 862.101, indicates that the extract has an intermediate level of toxicity. A substance with an LC_{50} of less than 1000 µg/mL is generally considered toxic (Konan et al., 2022). Different toxicity levels have been reported in other studies of pomegranate peel extracts, depending on the specific pomegranate variety and extraction solvent used. For example, a study on white pomegranate fruit extract found that it had an LC_{50} of 248.6 µg/mL, indicating that it was toxic and possibly anti-cancer (Firnanda et al., 2021). An even lower LC_{50} of 114.090 µg/mL was found in another study on black pomegranate peel extract, indicating that it has strong acute toxicity (Achmad et al., 2019). These results are consistent with the concept that a material may exhibit cytotoxic activity and antitumor potential if its LC_{50} value in the BSLT is less than 1000 µg/mL. Thus, the LC_{50} value of 604.783 µg/mL for this particular pomegranate peel extract indicates that it contains bioactive compounds that may be useful for further investigation as anticancer agents.

Furthermore, this study investigated the anti-proliferative activity of the ethanolic extract against HeLa and cMM/IPB-B4 cells. A concentration-response relationship was observed, where increasing concentrations of the extract resulted in increased antiproliferative activity. The extract showed antiproliferative activities against HeLa cells ranging from 24.31% to 70.83% at concentrations of 300 to 1500 µg/mL, respectively. In cMM/IPB-B4 cells, the activities ranged from 13.33% to 65.18% over the same concentration range. The IC_{50} values of the ethanolic extract were 943.43 µg/mL for HeLa cells and 1152.42 µg/mL for cMM/IPB-B4 cells. Doxorubicin, the positive control at 100 µg/mL, exhibited anti-proliferative activities of 72.92% for HeLa cells and 71.11% for cMM/IPB-B4 cells.

Pomegranate peel ethanolic extract has been shown to be cytotoxic to HeLa cells. One study found that, after 24 hours of incubation, the half-maximal inhibitory concentration, or IC_{50}, was 450 µg/mL (Nurfazriah et al., 2024). According to another study, HeLa cell proliferation was significantly inhibited at a concentration of 50 µg/mL (Leesombun et al., 2022). The extract promotes programmed cell death, or apoptosis, in cancer cells, as observed in Figures 6 and 7 after the incubation periods. This finding was demonstrated by observations of DNA fragmentation, a sign of apoptosis. One of the main causes of the ex-tract's anti-cancer effects is its effect on apoptosis (Abdul Gany et al., 2010). The differences in values between this study and other investigations may be attributed to differences in the constituents of the ethanolic extracts. Similar anticancer activity was also observed in A431 cells and was primarily mediated by

reactive oxygen species (ROS), which triggered apoptosis and G1 phase arrest, thereby inhibiting cell proliferation (Dinarvand et al., 2024).

Research on cMM/IPB-B4 is limited, and there is a recognized lack of established cell lines and experimental models, which makes it difficult to con-duct in-depth studies on novel treatments, including natural compounds. However, this study was able to evaluate the antiproliferative activity of the extract against cMM/IPB-B4 cells, yielding an IC_{50} of 1152.42 µg/mL. The ethanolic extract of pomegranate peel exhibited moderate effectiveness against cMM, an uncommon and aggressive cancer with a poor prognosis (Moberg et al., 2022).

The in silico study was conducted on major compounds in pomegranate peel, including punicalagin, ellagic acid, and gallic acid to determine whether these compounds exhibit anticancer activity against topoisomerase IIα. Drug-likeness screening based on Lipinski's rules showed that punicalagin is not a good candidate for use as an oral drug because of the numerous hydrophilic groups in the compound, which make it less able to penetrate lipid membrane layers (Santos et al., 2025). In the bioactivity screening using PASS Online, punicaligin exhibited anticancer activity, as indicated by high Pa values for cytostatic, antineoplastic, and apoptosis-agonist activities. Investigations into the mechanisms underlying these anticipated activities have revealed that punicalagin functions as a cytostatic agent by inducing cell cycle arrest and inhibiting survival pathways, such as PI3K/AKT/mTOR and MAPK/ERK, in a variety of cancer cell lines (Hassan et al., 2025). Its ability to trigger the caspase cascade—specifically, by upregulating caspase-3, -8, and -9—and to alter the balance of Bcl-2 family proteins by increasing proapoptotic Bax and decreasing antiapoptotic Bcl-2, thereby inducing programmed cell death, further supports its function as an apoptosis agonist. In addition to these direct effects, studies have shown that punicalagin has antimetastatic and anti-angiogenic properties by inhibiting matrix metalloproteinases and VEGF, respectively. These findings demonstrate that it has a multifaceted strategy for preventing tumor growth and spread (Zhang et al., 2020).

The well-known chemotherapeutic drug doxorubicin mainly functions as a topoisomerase IIα inhibitor. It works by intercalating into DNA, thereby trapping the topoisomerase IIα enzyme after it has cleaved the DNA double strand (Nitiss, 2009). Figure 8 shows you that all ligands used in this study occupy the same active site as doxorubicin. Therefore, all ligands are predicted to have the same topoisomerase IIα inhibition mechanism as the drug. However, punicalagin, with a binding affinity of −7.24 kcal/mol, had a relatively closer binding affinity to doxorubicin than the other ligands. Punicalagin interacted with DG13, DC11, and DA12 on the DNA strand of topoisomerase IIα (Table 5). This strong correlation between punicalagin's favorable binding and inhibitory potential in silico and the anti-proliferative and apoptosis-inducing effects observed in the in vitro experiments strongly suggests that punicalagin is a primary active compound responsible for the anti-cancer properties of pomegranate peel extract.

Punicalagin has a suitably high hERG safe value, indicating a low risk of cardiotoxicity and an excellent safety profile with regard to hERG ion channels. Despite its moderate solubility, punicalagin is thought to have limited bioavailability (Cerdá et al., 2003). According to its moderate to high non-toxic score, punicalagin has moderately low toxicity and a somewhat favorable safety profile. Despite certain restrictions in its pharmacokinetic characteristics, this balanced profile emphasizes its potential therapeutic use.

Conclusion

The in vitro and in silico studies demonstrated a strong correlation between the antiproliferative activity of pomegranate peel extract and Topoisomerase IIα inhibition. The induction of apoptosis by the ethanolic extract of pomegranate peel, a known mechanism of topoisomerase IIα inhibitors, is consistent with the in vitro results, which demonstrated that the extract effectively inhibited the growth of both HeLa and cMM/IPB-B4 cells. Punicalagin was identified as a major contributor by the in silico analysis, providing a molecular basis for this activity. Punicalagin was found to have a high likelihood of inhibiting topoisomerase IIα and a favorable binding affinity. The molecular docking study also demonstrated that punicalagin and doxorubicin, a well-known inhibitor of topoisomerase IIα, share an active site on the enzyme, indicating a similar mode of action. These data support the idea that punicalagin's capacity to inhibit topoisomerase IIα is at least partially responsible for the pomegranate peel extract's anti-proliferative effects on HeLa and cMM/IPB-B4 cells. However, further studies are needed to purify the biologically active molecules and evaluate their effects in animal models through in vivo experiments to further investigate their anticancer activity.

Ethical Considerations

Compliance with ethical guidelines

The were no ethical considerations to be considered in this research.

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

All authors declared no conflict of interest.

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