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Research Paper

The Potential of Ethanol Extract of Pasak Bumi Roots (*Eurycoma longifolia* Jack) as an Anti-Prostate Cancer In Vitro Against PC-3 Cells

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Abstract
The prevalence of prostate cancer cases in men is expected to continue increase. In 2040, it is estimated that there will be 2.293.818 new cases and a 1.05% increase in the death rate due to prostate cancer. *Eurycoma longifolia* Jack roots extract (ELE) has potential as an alternative treatment. This study aims to analyze ELE potential as an anti-prostate cancer agent through in silico assay and in vitro assays on the prostate cancer cell line (PC-3). ELE compounds were docked against Casp-3, Casp-8, HAX-1, p27, and PTEN. In vitro assays on PC-3 cells were used, namely cell viability (WST-8), ROS levels; cell cycle; and cell apoptosis (flow cytometry), PC-3 cell senescence (β-Galactosidase staining), Casp-3; Casp-8; HAX-1; p27; and PTEN gene expression (qRT-PCR). All proteins target were successfully docked with ELE compounds and presented binding interactions. ELE is known to reduce viability, intracellular ROS levels, live cells, necrosis, and reduce HAX-1 gene expression, and inhibit the cell cycle G0/G1 phase. ELE can also increase inhibition, senescence, late and early apoptosis, and Casp-3, Casp-8, p27, and PTEN gene expression. ELE 100 μg/mL is the most effective concentration. ELE has potential as an anti-prostate cancer agent through apoptosis, cell cycle, and antioxidant pathways.

Keywords
Anticancer, *Eurycoma longifolia* Jack, In Silico, In Vitro, PC-3

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1. INTRODUCTION

Prostate cancer known worldwide as the most frequently identified cancer among men with more than one million cases. Data shows that the prevalence of prostate cancer patients reached 358,989 or 3.8% from 1,276,106 cases caused death in men due to cancer in 2018 (Bray et al., 2018). In 2040, it is estimated that there will be 2,293,818 new cases and a 1.05% increase in the death rate due to prostate cancer (Ferlay et al., 2018). In the Asia-Pacific region, deaths due to prostate cancer in Indonesia reached 16% (Kimura and Egawa, 2018). Prostate cancer treatment strategies include surveillance in the early stages, androgen deprivation therapy, castration, chemotherapy, and other therapies. Even though there have been many developments in prostate cancer treatment strategies, there are still obstacles, namely toxicity to normal cells and non-specificity of treatment (Ghosh et al., 2021).

Typically, cancer patients show higher levels of Reactive Oxygen Species (ROS) which can elevate tumor growth, cancer cell survival, proliferation, and adaptation to low oxygen levels (Reczek and Chandel, 2017). In cancer development, senescence in cancer cells can function as a potential mechanism for cells to prevent malignant transformation (Zeng et al., 2018). Cancer cells development results in inactivation or mutation of genes that have a role in regulating the amplification of oncogenes, causing cells to lose control and causing uncontrolled cell cycle inhibition in turn disrupting cell cycle inhibition causes cell decline senescence due to active cell proliferation (Icard et al., 2019).

Cancer development is caused by abnormalities in certain genes and their related proteins, which are involved in proliferation, division, and excessive cell growth, as well as inhibition

of proteins related to apoptosis (Yunos et al., 2022). Caspase3 (Casp3) and Casp8 are genes related to the apoptosis process and play role in the onset and advancement of cancer. Decreased expression or activity of Casp3 and Casp8 has been reported in many cancer types (Pu et al., 2017). Caspase-3 is present as an inactive proenzyme in the cytosol and carries out its role by catalyzing the cleavage of the C-terminal cysteine residue, leading to the specific breakdown of the peptide bond. after the aspartic acid residue. Cancer growth is also associated with HS-1-Associated Protein X-1 (HAX1) overexpression (Trebinska-Stryjewska et al., 2019). HAX1 has been widely play a role as an anti-apoptotic factor, cell-protective properties, as well as an apoptosis-related protein (Yan et al., 2015). To prevent excessive cell growth, the body usually has the p27 protein which functions as a tumor suppressor by inhibiting the Cyclin-Dependent Kinases (CDKs) activity, namely enzymes that encourage cell division (Bencivenga et al., 2021). In addition, there is a Phosphatase and TENsin homolog deleted on chromosome 10 (PTEN) gene that makes enzymes to regulate cell division by keeping cells from growing and dividing (reproducing) too quickly or uncontrollably (Lee et al., 2018).

Natural products have many benefits because their compounds are rich resources for various types of treatment. *Eurycoma longifolia* Jack, which comes from the Simaroubaceae family, popularly used for herbal medical treatment in Asia, especially its roots (Jothi et al., 2023). In Indonesia, *E. longifolia* Jack or called Pasak Bumi known as traditional medicine for fever, malaria, energy enhancer, medicine for malaria, dysentery, fever, sexual disorders including male infertility. *E. longifolia* Jack ethanol extract (ELE) roots are known to contain flavonoids, alkaloids, tannins, saponins, phenolics, triterpenes, and quassinoids including eurycomalactone, eurycomanol, and eurycomanone (Khanam et al., 2015; Ruan et al., 2019). The biological activities reported in ELE include anticancer, antiosteoporosis, antiproliferative, antioxidant, anti-inflammatory, antimalarial, antimicrobial, aphrodisiac, antiulcer, antidiabetic, and antirheumatic (Silalahi, 2020).

Previous studies have examined *Annona muricata* (Foster et al., 2020), *Moringa oleifera*, *Phyllanthus amarus*, and *Carica papaya* (Abankwa et al., 2020) as anti-prostate cancer. Studies related to *E. longifolia* Jack as anti-prostate cancer are rarely conducted. This study aims to analyze the anticancer potential of ELE prostate against prostate cancer (PC-3) cells. PC-3 cells are recognized as a conventional cell line for prostate cancer, serving as a model for androgen-independent prostate cancer because of their elevated metastatic capability in comparison to alternative prostate cancer cell lines (Kamalidehghan et al., 2018). The focus is to analyze the effect of ELE on cytotoxicity (viability and inhibition), intracellular ROS levels, senescence, cell cycle, apoptosis, and gene expression (Casp3, Casp8, HAX-1, p27, PTEN) in PC-3 cells.

2. EXPERIMENTAL SECTION

2.1 Materials

Materials in cell line cuture were prostate cancer cells (PC-3, ATCC CRL-1435). Medium for cell culture were DMEM High Glucose (DMEM-HG) (Biowest, L0103-500) containing 20% Fetal Bovine Serum (FBS) (Biowest, S181B-500), 1% Amphotericin B (Biowest, L0009-100), 1% Nanomycopulitine Concentrtae 20x (Biowest, L-X16-100), 1% Antibiotic-Antimycotic (Biowest, L0010-100), 1% MEM Vitamins 100x (Biowest, X0556-100), 0.1% L-Glutamine 100x (Biowest, -X0550-100), and 1% MEM Non-Essential Amino Acids 100x (Biowest, X0557-100). Material employed in cell viability assay were Enhanced Cell Counting Kit 8 (WST-8/CCK8) (Elabscience E-CK-A362), DMEM-HG complete medium with 2% FBS. Meanwhile Reactive Oxygen Species Assay comprisesof ROS Fluorometric Assay Kit (Elabscience, E-BC-K138-F)and DCFH-DA reagent. Senescence assay used Senescence-Cells Histochemical Staining Kit (Sigma, CS0030) and PBS1×. Moreover, materials employed in RT-PCR method are TRI Reagent (Zymo Research, R2050-1-200), SensiFASTcDNA Synthesis Kit (Bioline, BIO-65054), and Direct-zolTMRNA Miniprep Plus (Zymo Research, R2073).

2.2 Methods

2.2.1 *Eurycoma longifolia* Jack Extraction

Eurycoma. longifolia Jack root part was used in the extraction. ELE is produced by PT Industri Jamu Borobudur (Batch 053PU 01.1). Ethanol 70% was used as an extraction solvent. Extraction is carried out based on Good Manufacturing Practice (GMP) standards by the Indonesian Food and Drug Supervisory Agency (BPOM). After the extraction process, ELE is stored at 25 ± 2°C (Widowati et al., 2024).

2.2.2 PC-3 Cell Culture

PC-3 (ATCC ®CRL-1435™) from Aretha Medika Utama Laboratory, Bandung, Indonesia. PC-3 cells were cultured using DMEM High Glucose (DMEM-HG) (Biowest, L0103-500) containing 20% Fetal Bovine Serum (FBS) (Biowest, S181B-500), 1% Amphotericin B (Biowest, L0009-100), 1% Nanomycopulitine Concentrate 20× (Biowest, L-X16-100), 1% Antibiotic-Antimycotic (Biowest, L0010-100), 1% MEM Vitamins 100× (Biowest, X0556-100), 0.1% L-Glutamine 100x (Biowest, X0550-100), and 1% MEM Non-Essential Amino Acids 100x (Biowest, X0557-100). PC-3 cells were cultured in an incubator 5% CO_2 at 37°C (Sun et al., 2019; Widowati et al., 2023).

2.2.3 Biology Activity Prediction

Eurycoma longifolia Jack compounds underwent additional analysis to determine their potential as antineoplastic agents. WAY2 DRUG PASS Druzhilovskiy et al. (2017), was used to enter the isomeric SMILES of each molecule. The findings were displayed as Pa (probability of activity) values. In this investigation, a cut-off value of 0.7 of Pa was applied.

2.2.4 ADMET Compounds Prediction

Predictions of Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) were carried out on chemicals associated with *E. longifolia*, such Eurycolactone E and Eurycolactone F. Protox 3.0 was used for ADMET prediction (Widowati et al., 2019). The Lipinski's rule predicts how closely a chemical would resemble recognized medications that exhibit successful or unsuccessful metabolism. According to Lipinski's criterion, the compound needs to possess a minimum of two of the following attributes: 1) Molecular weight <500 Daltons; 2) high lipophilicity (shown by a LogP <5); 3) less than five donors of hydrogen bonds; and 4) fewer than ten acceptors of hydrogen bonds; LD50 and toxicity class (25). Class I with LD50 ≤ 5 is the most toxic; Class II with 5 <LD50 ≤ 50 is the least toxic; Class III with 50 <LD50 ≤ 300 is the most toxic; Class IV with 300 <LD50 ≤ 2000 is the most hazardous; Class V with 2000 <LD50 ≤ 5000 is potentially hazardous if consumed; and Class VI with LD50 <5000 is the least toxic (Priyandoko et al., 2019).

2.2.5 Molecular Docking

E. longifolia Jack's potential as a preventative measure against prostate cancer was illustrated using molecular docking (Rawla, 2019). The target proteins used included Casp-3, Casp-8, HAX-1, PTEN, and P-27 respectively, the proteins were obtained from the RCSB protein data bank, and Autodock 4.2 was used to prepare them by releasing water and native ligands (Ramadhani et al., 2024). AutoDock Vina was used for molecular docking. To verify binding locations, natural ligands were re-docked onto protein targets (Tong et al., 2015). The root mean square deviation (RMSD) cutoff value, as determined by PyMOL, was 2 Å. The protein-compound interaction's binding affinity value is the outcome of molecular docking. In Discovery Studio 2021, interactions between 3D and 2D were visualized (Ye et al., 2022).

2.2.6 Cell Viability Assay

Cell viability assay performed using the Enhanced Cell Counting Kit 8 (WST-8/CCK8) (Elabscience E-CK-A362) followed the manufacturer's protocol. Briefly, cells were seeded in $5x10^3$ in 96 well plates with 200 μL DMEM-HG complete medium with 2% FBS and incubated in an incubator (5% CO_2, 37°C) for 24 hours. The medium was replaced then the cells were treated with a test group consisting of negative control (NC) (not treated), DMSO (treated with 10% DMSO), and ELE (6.25; 12.5; 25; 50; 100) μg/mL (given ELE treatment with different concentrations) in 3 replications. Cells were incubated again for 24 hours. WST-8 reagent 20 μL was added to wells. And then, cells were then incubated (37°C, 5% CO_2) for 3 hours. Results were measured using a spectrophotometer at OD 490 nm (Widowati et al., 2023; Liu et al., 2022). From the cytotoxicity test, IC_{50} is produced, then three ELE concentrations are selected for further testing.

2.2.7 Intracellular ROS Levels Assay

ROS levels in PC-3 cells were measured with ROS Fluorometric Assay Kit (Elabscience, E-BC-K138-F) followed the protocol from manufacturer. Cells were plated in 6-well plates with 2 mL media then incubated for 24 hours. 200 μL samples were added to the well. The ELE concentration variation used in the test were obtained from the cell viability assay (ELE 25 μg/mL; 50 μg/mL; 100 μg/mL) IC_{50} values. Briefly, cells that had been treated in 6 well plates and incubated for 24 hours (37°C, 5% CO_2), cells were rinsed using PBS 1x. 1 mL of DCFH-DA was added to each well for incubated 60 minutes (37°C, 5% CO_2). Then the cells were deteached using trypsin and intracellular ROS levels were detected using flow cytometry MACSQuant Analyzer 10 (Miltenyi Biotec) with FITC fluorescence detection (Widowati et al., 2021; Cheng et al., 2023).

2.2.8 Senescence Assay

The senescence test on PC-3 cells was performed based on Priyandoko et al. (2019) with modifications. Senescence assay was conducted with Senescence Cells Histochemical Staining Kit (Sigma-Aldrich, CS0030-1KT) followed the protocol from manufacturer. The treated cells then incubated for 24 hours. The cell medium was discarded, then the cells were washed with PBS 2×. Cells were then fixed using 1× fixation buffer for 7 minutes. Cells were washed again with PBS 1×. The staining mixture was then added to wells and the cells were incubated at 37°C for overnight. The cell staining results were observed using an inverted microscope and the senescence cells number was counted manually.

2.2.9 Cell Cycle Assay

PC-3 cells were seeded (5x105) in 6 well plates then added DMEM-HG complete medium containing 2% FBS. Briefly, to the prepared samples, absolute ethanol was added which had been stored at -20°C (overnight). Cell cycle assay on PC-3 cells using the Cell Cycle Assay Kit (Re Fluorescence) (Elabscience, E-CK-A351) based to the manufacturer's protocol. Cell cycles were measured using MACSQuant Analyzer 10 (Miltenyi Biotec) (Zhong et al., 2023).

2.2.10 Apoptosis Assay

Live cells, necrosis, early and late apoptosis were analyzed using flowcytometry. Cells that had been plated in 6 well plates and treated were washed with FACS buffer 1 mL. Cell pellets were added with 500 μL of 1× Annexin binding buffer then stained with Annexin V-FITC/PI Apoptosis Kit (Elabscience, E-CK-A211). Then incubation for 30 minutes at 4°C (dark room), cells were analyzed using MACSQuant Analyzer 10 (Miltenyi Biotec) (Widowati et al., 2019, 2023).

2.2.11 Quantification of Casp3, Casp8, HAX1, p27, and PTEN Genes Expression

The expression of the Casp3, Casp8, HAX1, p27, and PTEN genes was tested using the qRT-PCR method (Widowati et al.,

2024). Direct-zol RNA Miniprep Plus Kit (Zymo, R2073) was used for RNA isolation with procedures followed the protocol from manufacturer. The purity and concentration of RNA can be seen in Table 1. Then c-DNA synthesis was performed with SensiFASTcDNA synthesis kit (Bioline, BIO-65054) with procedures followed the protocol from manufacturer. The cDNA synthesis steps include priming for 5 min at 25°C, reverse transcription for 20 min at 46°C, RT inactivation for 1 min at 95°C, and optimization step at 4°C. Next, SYBR NO-ROX kit (Bioline, BIO-98005) was used for Casp3, Casp8, HAX1, p27, and PTEN gene expression analysis. GAPDH is used as a housekeeping gene. Primary sequences of genes can be seen in Table 2.

2.2.12 Statistical Analysis

The data was analyzed with SPSS 26.0 software with results presented as mean ± SD. The One-way ANOVA with the Tukey HSD test (P<0.05) was used for normal and homogeneous data. Dunnett's T3 test (P<0.05) was used for non-homogeneous data. The Kruskal-Wallis and Mann-Whitney U Test (P<0.05) was used for non-normal data (Gondokesumo et al., 2025).

3. RESULTS AND DISCUSSION

3.1 Compound in *Eurycoma longifolia* Jack

Phytochemicals of *E. longifolia* were obtained from KNApSAcK Family database. The KNApSAcK Family database showed four compounds that contained in *E. longifolia* Jack. The compounds are Picrasidine L, Picrasidine O, Eurycolactone E, and Eurycolactone F (Table 3).

3.2 Biology Activity Prediction

Total 4 compounds were tested, only two compounds showed potential bioactivity as antineoplastic (Table 4). The Pa <0.7 value of both compounds indicated high potential as antineoplastic. Further tests were conducted on both compounds.

Total 2 compound from *E. longifolia* Jack (Eurycolactone E and Eurycolactone F) were analyzed for bioactivity potential as a antineoplastic agent. The both compounds were showed antineoplastic potential with Pa value higher than 0.7. The Pa and Pi values varied from 0 to 1 and were independent of each other. A compound's potential is indicated by a Pa value greater than 0.7. The derived prediction function's accuracy is shown by the Pa value, where a larger Pa value indicates a higher level of accuracy.

3.3 ADMET Compounds Prediction

ADMET characteristics contribute to the discovery of new drugs (Table 5). Both compounds Eurycolactone F and Eurycolactone E demonstrate compliance with five of Lipinski's rule, which is often used to predict the oral bioavailability of drug-like molecules. Lipinski's rule was satisfied by both Eurycolactone E and Eurycolactone F. According to Zeiz et al. (2024), compounds that satisfy Lipinski's criteria correspond to the drug and are likely to exhibit superior membrane permeability and oral bioavailability.

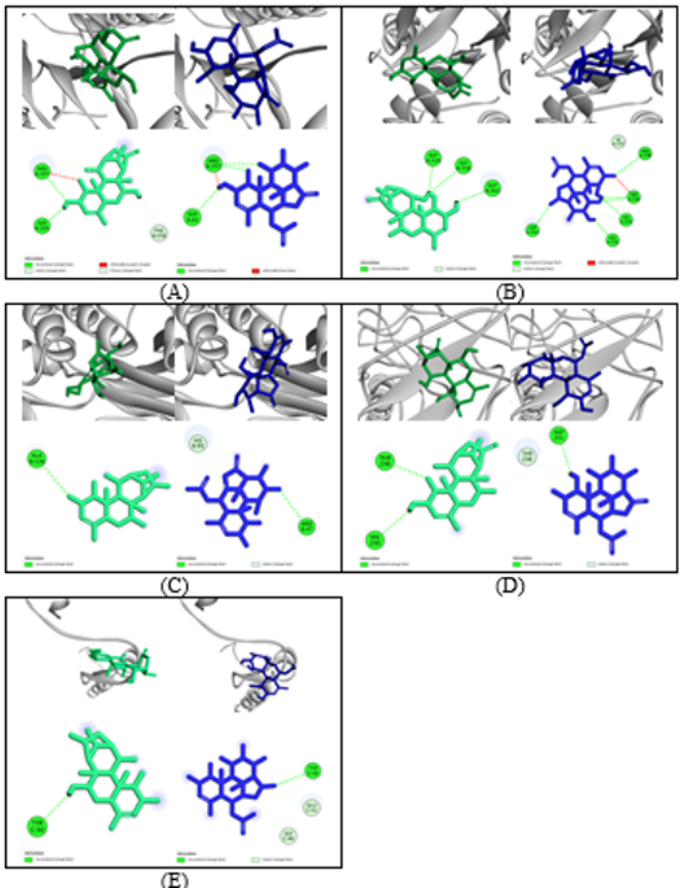


Figure 1. 3D and 2D Visualization of Molecular Docking Results. Green Compound Represented Eurycolactone E and Blue Compound Represented Eurycolactone F against (A) CASP-3, (B) CASP-8, (C) PTEN, (D) HAX-1, (E) P27

Paired with all target proteins. The molecular docking results visualized in 3D showed that the test compounds successfully bind to the active sites of the proteins (Figure 1). The 2D visualization shows the protein-ligand interaction, green circles indicate hydrogen bonds and pink or purple circles indicate hydrophobic interactions (Figure 1). These compounds have different affinity values (Table 6). The smaller the affinity value indicates the highest binding and the higher the affinity value indicates the lowest binding. Binding affinity values of Eurycolactone E were between -7.4 and -3,9 kcal/mol compared to Eurycolactone F were between -6,9 and -3,5 kcal/mol. Both compounds were exhibited lowest binding affinity values against CASP-3 and highest binding affinity values against PTEN.

3.4 Molecular Docking

Eurycolactone E and Eurycolactone F were analyzed by molecular docking against CASP-3, CASP-8, PTEN, HAX-1, and P-27 proteins. Each protein was re-docked and showed RMSD <2 Å(data not shown) to validate the active site of the protein.

Table 1. RNA Purity and Concentration

Sample	Concentration (ng/ μL)	Purity (λ260/ λ280 nm)
Untreated PC-3 cells (I)	12.24	1.2956
PC-3 + DMSO 1% (II)	12.72	1.2938
PC-3 + ELE 25 μg/mL (III)	10.56	1.3243
PC-3 + ELE 50 μg/mL (IV)	11.52	1.3068
PC-3 + ELE 100 μg/mL (V)	13.52	1.2859

Table 2. Primer Sequences of Genes

Gene	Primer sequences (5'-3')	Product size (bp)	Annealing (°C)	Cycle	Reference
Casp3 (Human)	F: AGAACTGGACTGTGGCATTG R: GCTTGTCGGCATACTGTTTCAG	191	58	40	NM_054 350958.1
Casp8 (Human)	F: AGGCCAGATCTTCACTGTCC R: GGTCACTTGAACCTTGGGAA	114	58	40	NM_001 228.5
HAX1 (Human)	F: GAGGAGGGATACGTTTCCACGA R: GTCTCTGACTCAGGACCTGGAA	133	63	40	NM_006 118.4
p27 (Human)	F: GCAGATCAAGGCCAACTATGAC R: ATAATGTTGCAGCCCAGCAG	237	63	40	NM_001 261400.3
PTEN (Human)	F: ATTGCAGAGTTGCACAATATCC R: CATATCATTACACCAGTTCGTCC	163	55	40	NM_000 314.8
GAPDH	F: GAAGGTGAAGGTCGGAGTC R: GAAGATGGTGATGGGATTTC	172	58	40	NM_001 289745.3

Both compounds were successfully paired with all target proteins. The molecular docking results visualized in 3D showed that the test compounds successfully bind to the active sites of the proteins (Figure 1). The 2D visualization shows the protein-ligand interaction, green circles indicate hydrogen bonds and pink or purple circles indicate hydrophobic interactions (Figure 1). These compounds have different affinity values (Table 6). The smaller the affinity value indicates the highest binding and the higher the affinity value indicates the lowest binding. Binding affinity values of Eurycolactone E were between -7.4 and -3,9 kcal/mol compared to Eurycolactone F were between -6,9 and -3,5 kcal/mol. Both compounds were exhibited lowest binding affinity values against CASP-3 and highest binding affinity values against PTEN.

The active site is accurate with smaller error, as indicated by the docking validation results of RMSD <2 Å. Lower affinity indicates a stronger binding of the medication to the target values. As a result, the binding affinity values and distance have a comparable meaning (i.e., the stronger the relationship between the two entities, the shorter the distance). Hydrogen bonds play an essential role in determining the binding affinity between proteins and ligands, making them essential to many biological processes. Water molecules, with their unique properties, perform multiple functions within protein-ligand complexes. One of their key roles is mediating hydrogen bonds between interaction partners, since water can function as both a hydrogen bond donor and acceptor, thus facilitating and stabilizing interactions.

One of cancer's defining characteristics is impaired apoptosis. A family of cysteine proteases called calpain family interacts with caspase-3 and -8, important regulators of the apoptotic response, during the tumorigenic process (Pu et al., 2017). *E. longifolia* Jack extracts have the ability to activate caspase-8, an initiator caspase that plays a role in the extrinsic pathway

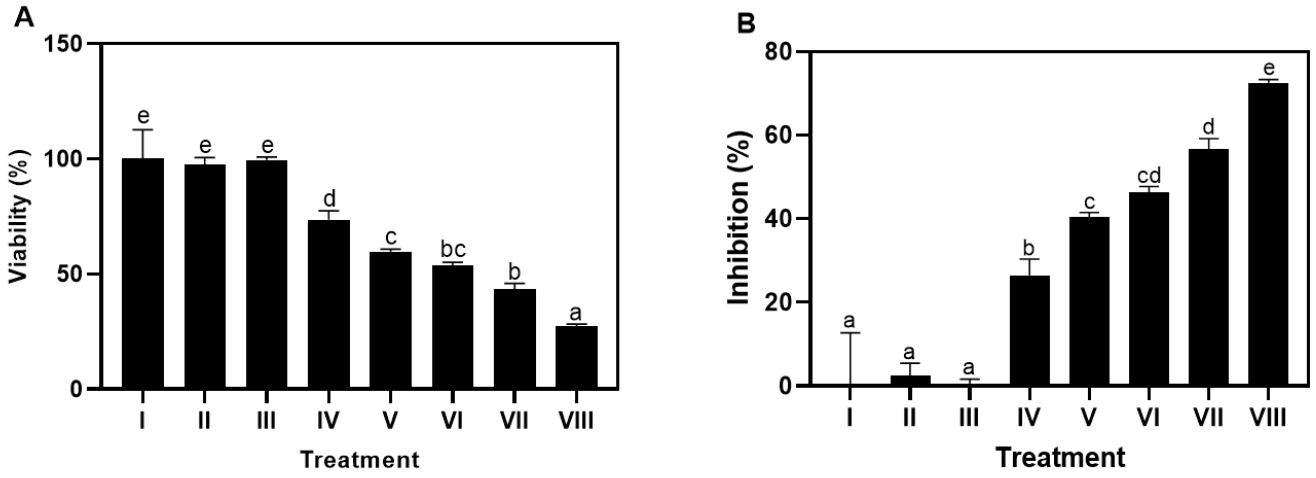


Figure 2. Effects of ELE on PC-3 cell viability and inhibition. (A) Viability and (B) inhibition. * I (NC), II (DMSO 1%), III (ELE 3.13 μg/mL), IV (ELE 6.25 μg/mL), V (ELE 12.5 μg/mL), VI (ELE 25 μg/mL), VII (ELE 50 μg/ mL), VIII (ELE 100 μg/mL). * Data are presented as mean ± SD. Different superscript marks indicate significant differences ($p<0.05$) Dunnett's T3 post hoc test.

Table 3. Data of Compounds in *E. longifolia* Jack

Compounds	PubChem CID	Molecular Formula	Molecular Weight (g/mol)
Picrasidine L	5324104	$C_{15}H_{10}N_2O_2$	250.25
Picrasidine O	5320558	$C_{16}H_{12}N_2O_3$	280.28
Eurycolactone E	636592	$C_{19}H_{26}O_6$	350.4
Eurycolactone F	10938426	$C_{21}H_{28}O_8$	408.4

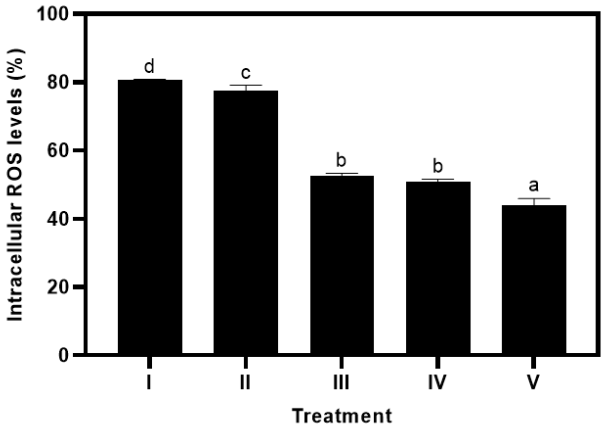


Figure 3. Effect of ELE on Intracellular ROS levels of PC-3 Cells. *I (NC), II (DMSO), III (ELE 25 μg/mL), IV (ELE 50 μg/mL), V (ELE 100 μg/mL). *Data are Presented as Mean ± SD. Different Superscript Signs Indicate Significant Differences ($p<0.05$) Tukey's HSD.

of apoptosis. The activation of caspase-8 leads to a cascade that activates downstream executioner caspases like caspase-3, facilitating cell death. Once activated by upstream signals such as those from caspase-8, caspase-3 executes the final steps of apoptosis. It is involved in cleaving multiple cellular substrates, resulting in the distinct morphological changes associated with programmed cell death. The presence of *E. longifolia* Jack extracts has been correlated with increased levels of active caspase-3 in treated cancer cells, indicating its role in mediating apoptosis (Jothi et al., 2023).

PTEN (Phosphatase and Tensin Homolog deleted on Chromosome 10) is a critical tumor suppressor enzyme that is crucial in regulating cellular processes, including growth, survival, and metabolism. As a dual-specificity phosphatase, PTEN primarily dephosphorylates phosphatidylinositol-3,4,5-trisphosphate (PIP3), converting it back to phosphatidylinositol-4,5-bis-phosphate (PIP2). This action inhibits the PI3K/Akt signaling pathway, which is frequently activated in various cancers, leading to enhanced cell proliferation and survival. The loss or mutation of the PTEN gene is one of the most common alterations found in human tumors, including glioblastoma, breast cancer, and prostate cancer (Chen et al., 2023). The study found that Eurycoma longifolia root extract can modulate the PTEN/PI3K/Akt pathway. Specifically, the extract appears to enhance PTEN expression and activity, which can counteract the aberrant signaling caused by its loss. This modulation is

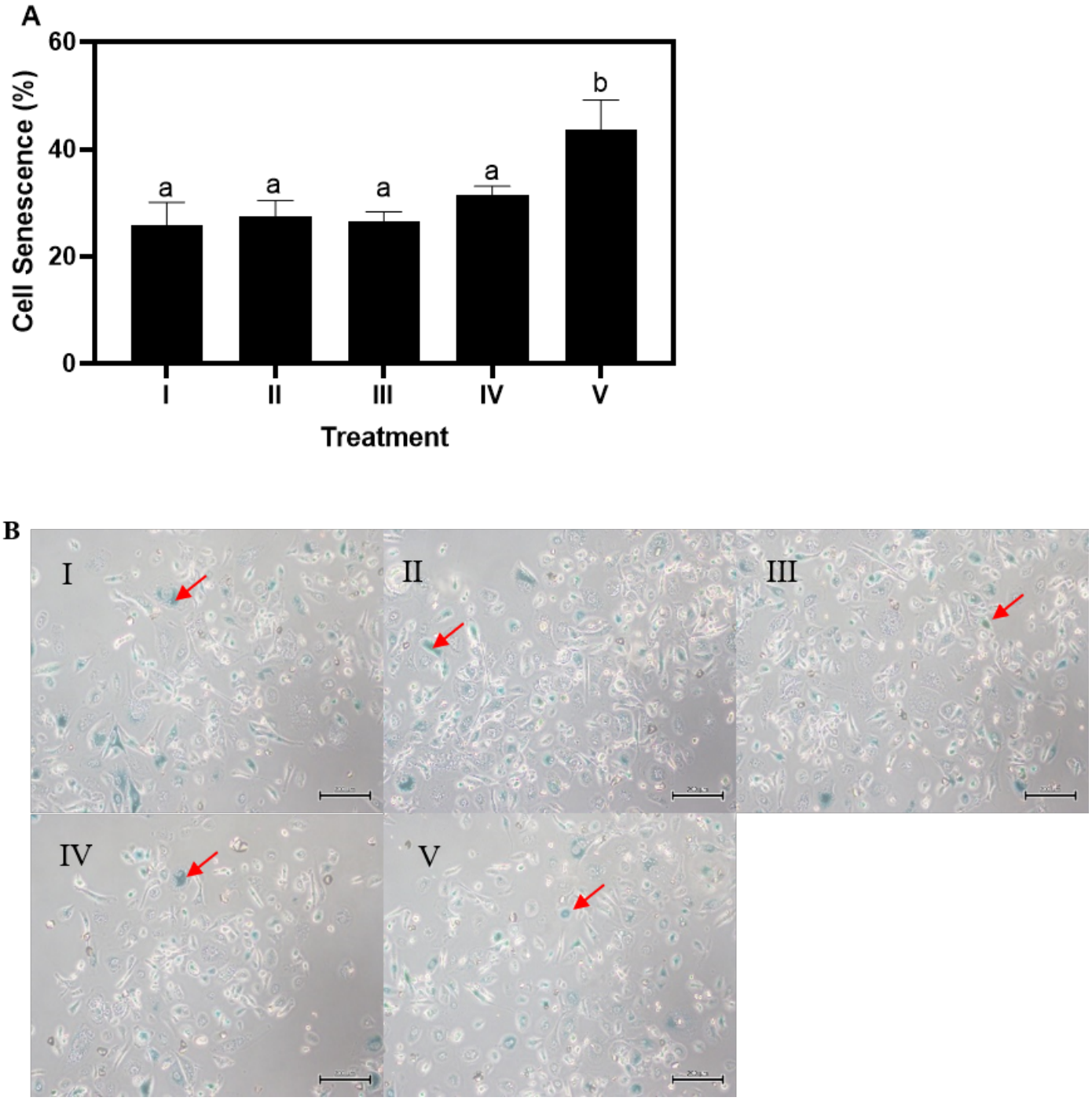
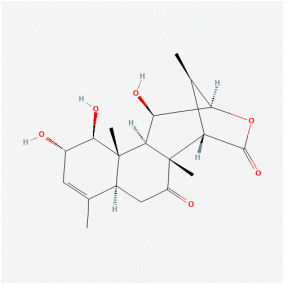
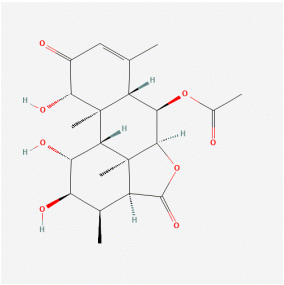


Figure 4. (A) Effect of Various Concentration ELE on PC-3 Cell Senescence, (B) Senescence Morphology of PC-3 (10x Magnification. PC-3 Cell Senescence (red arrow)). *I (NC), II (DMSO), III (ELE 25 μg/mL), IV (ELE 50 μg/mL), V (ELE 100 μg/mL). *Data are presented as mean ± SD. Different superscript signs indicate significant differences ($p<0.05$) Mann Whitney Post-HocTest.

significant because restoring PTEN function can enhance the sensitivity of prostate cancer cells to apoptosis.

HAX1 is essential in regulating apoptosis and cell survival across different types of cancer. Overexpression of HAX1 has been linked to the development of chemoresistance, particularly in breast cancer cells, where its knockdown can resensitize drug-resistant cells to chemotherapeutic agents like cisplatin and doxorubicin. In prostate cancer, HAX1 inhibits apoptosis

Table 4. Prediction of Compound in *E. longifolia* Jack as Antineoplastic Agent

Antineoplastic Bioactivity	Chemical Structure	Pa	Pi
Eurycolactone E		0.943	0.004
Eurycolactone F		0.984	0.004

by suppressing the activation of caspase-9, and its downregulation enhances the sensitivity of these cells to apoptosis-inducing stimuli (Yan et al., 2015). The p27 tumor suppressor gene (p27 gene) undergoes hypermethylation in prostate cancer. It has been shown that hypermethylation of its promoter activates the p27 gene in prostate cancer. In many cancers, including breast, prostate, and colorectal cancers, p27 expression is often found to be decreased or mislocalized, which contributes to uncontrolled cell growth and tumor progression. The reduction in p27 levels is frequently due to enhanced proteolytic degradation via the ubiquitin-proteasome pathway, which is linked to poor prognosis and aggressive tumor behavior.

3.5 Cell Viability

In this study, the ability of ELE to reduce the viability of PC-3 prostate cancer cells was analyzed using the WST-8 cytotoxicity test. The results showed that ELE 100 μg/mL could significantly reduce viability (42.51%) and increase inhibition (57.49%) of PC-3 cells compared to NC (100.00%) (0.00%) (Figure 2). Inhibition of PC-3 cell growth was demonstrated by an IC_{50} value of 45.150 μg/mL.

Prostate cancer is the second malignant cancer that often occurs in men, following lung cancer, throughout the world and prostate cancer is diagnosed in almost 60% of men over the age of 65 (Rawla, 2019). As stated by the World Health Organization (WHO), 85% of the global population depend on herbal medicine for their treatment and health. One herbal plant that is widely available in Southeast Asia and Indo-China and is popular is *E. longifolia* Jack and in particular the root part of this plant is believed to have a higher therapeutic effect than the other parts (Jothi et al., 2023).

The inhibition of PC-3 cell growth was demonstrated by an IC_{50} value of 45.150 μg/mL. Another studies have reported the biological activity of ELE as an anticancer. This is influenced by the quassinoid content in ELE. In the study from Tong et al. (2015) reported that the cytotoxic activity of 40% quassinoid in ELE affected the viability of cancer cells including RWPE-1 cells (IC_{50} = 59.26 μg/mL), WRl 68 cells (IC_{50}= 27.69 μg/mL), PC-3 cells (IC_{50} 87.94 μg/mL), LNCaP cells (IC_{50} 5.97 μg/mL). Quassinoids, especially eurycomanone in ELE, are known to inhibit colon cancer viability by influencing autophagy which is characterized by the formation of reen fluorescent protein (GFP)–fused LC3 (GFP-LC3) puncta and regulation of LC3-II as well as activating Mechanistic Target of Rapamycin (mTOR) signaling (Ye et al., 2022). Apart from that, other studies reported that eurycomanone was effective in cancer cell viability in several cell lines tested, namely MCF-7 (IC_{50} 44.1 μM) and MCG-803 (IC_{50} 14.1 μM). This is caused by the presence of olefinic bonds in the eurycomanone compound, namely in the C-13 and C21 chains, which may have an anticancer role (Ryu et al., 2017; Li et al., 2019).

3.6 ROS Level

ROS level has been performed at Figure 3. The results showed that ELE can reduce intracellular ROS levels compared NC (80.53%), with the most effective ELE concentration being

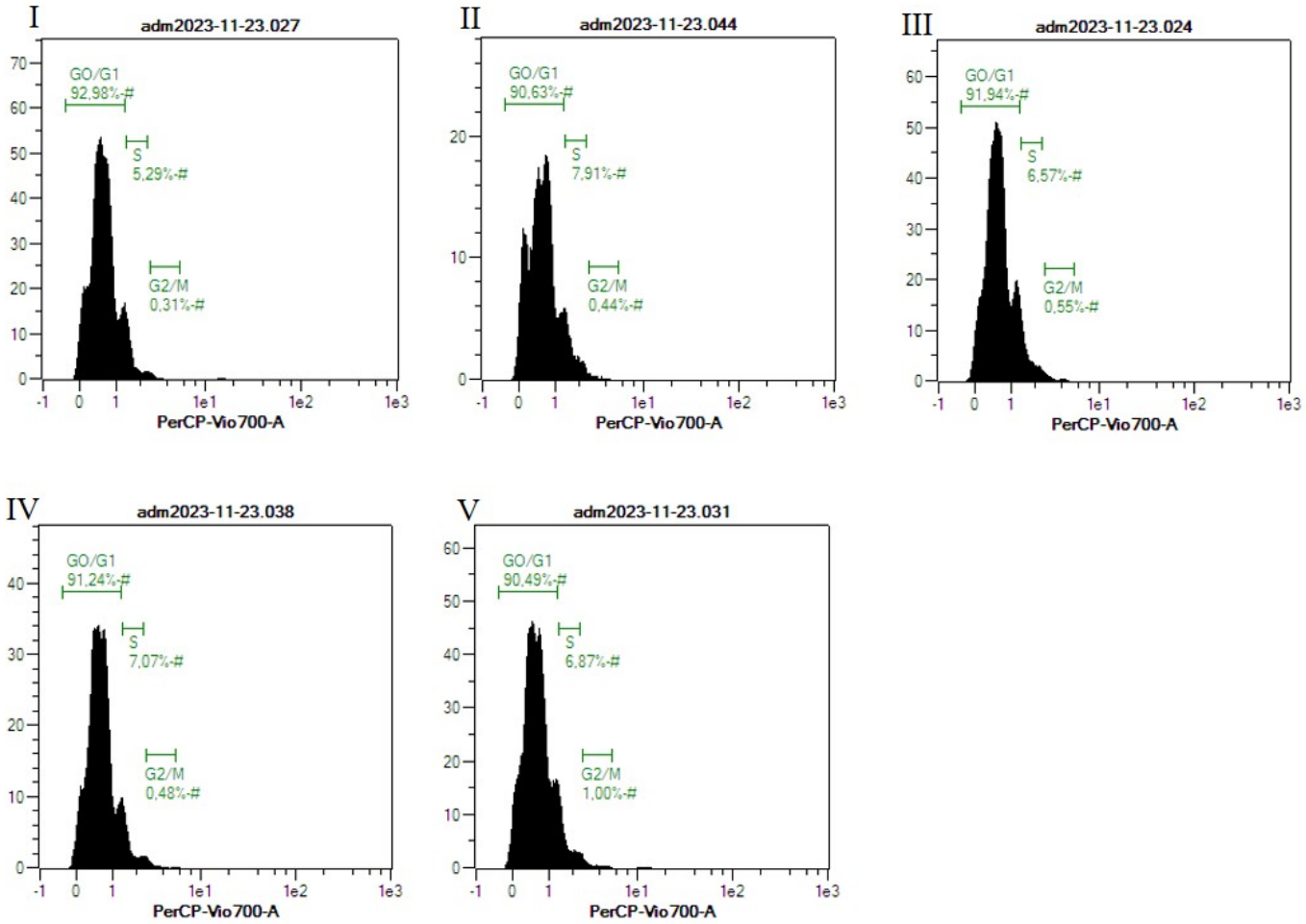


Figure 5. Dot Blot Representative of the Effect Various Concentrations ELE on PC-3 Cell Cycle *I (NC), II (DMSO), III (ELE 25 μg/mL), IV (ELE 50 μg/mL), V (ELE 100 μg/mL)

ELE 100 μg/mL (44.01%). These results are caused by the compounds contained in ELE acting as antioxidants.

Cancer cells development is characterized by an imbalance in redox homeostasis caused by an increase in intercellular ROS. This imbalance affects cell proliferation, differentiation and migration, causing cancer cells to actively proliferate and increasing the malignancy of the cancer cells (Daviu et al., 2024). The results of antioxidant activity tests using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method show that the results of ELE extraction using various methods have strong and moderate antioxidant abilities (Anwar et al., 2022). Antioxidant activity test conducted by Mohamad and Ismail (2022) also showed the same results with the DPPH and Ferric Reducing Antioxidant Power Assay (FRAP) methods. These antioxidant compounds play a role in overcoming the imbalance in redox homeostasis that occurs.

3.7 Senescence Level

The results showed that ELE could increase cell senescence with the most significant concentration of ELE causing an increase in PC-3 cell senescence being ELE 100 μg/mL (43.72%) compared NC (25.93%) (Figure 4A). The morphology of PC-3 senescence cells can be seen in Figure 4B which is marked with a red arrow. Apart from an imbalance in redox homeostasis, cancer cell development is marked by unchecked growth and proliferation. This is due to uncontrolled cell control of various cell cycles (Otto and Sicinski, 2017). In the process, there is a decrease in cell senescence due to an increase in the G0/G1 phase of the cell cycle (Ligasová et al., 2023).

3.8 Cell Cycle

ELE 100 μg/mL (90.34%) also can inhibit PC-3 cell proliferation by decreasing the G0/G1 phase compared NC (92.63%) (Table 7), the dot blot can be seen in Figure 5. Research by Tong et al. (2015) reported that standard quassinoids that contain 40% of the total quassinoids contained in ELE against

Table 5. ADMET Characteristics of Compounds in *E. longifolia* Jack (Protox 3.0 prediction

	Eurycolactone E	Eurycolactone F
LD50 (mg/kg)	2589mg/kg	25mg/kg
Toxicity class	5	2
Average similarity	70.35%	75.44%
Prediction accuracy	69.26%	69.26%
Molecular weight	350.41	408.44
Num. of H-bond acceptors	6	8
Num. of H-bond donors	3	3
Num. of atoms	25	29
Num. of bonds	28	32
Num. of rotable bonds	0	2
Octanol/water partition coefficient (logP)	0.44	-0.02

Table 6. Binding Affinity and Interaction of Amino Acid Residue

Protein	Eurycolactone E Binding Affinity (kcal/mol)	Hydrogen Bond	Eurycolactone F Binding Affinity (kcal/mol)	Hydrogen Bond
CASP-3	-7.4	ARG207, SER205, PHE256	-6.8	ARG207, SER65
CASP-8	-6.8	GLY318, ASP319, ASP363	-6.9	SER256, ARG258, ASP363, LYS253, HIS255, ILE257
PTEN	-3.9	ALA126	-3.5	ARG47, HIS93
HAX-1	-5.1	THR238, VAL230	-5	ASP231, THR238
P-27	-4	TYR50	-3.7	TYR50, GLY48, GLU51

the prostate cancer cell line LNCaP can inhibit the growth of LNCaP cancer cells by inhibiting the G0/G1 phase depending on the dose and time of treatment through downregulating the expression of contributing proteins. against G1 to S phase, Cyclin D1, Cyclin-dependent kinase 2 (CDK2), CDK4, as well as upregulating the cyclin inhibitory protein, p21WAF1/Cip1. Previous research also reported that quassinoids have an effect on preventing purine synthesis thereby inhibiting Deoxyribonucleic (DNA)/Ribonucleic acid (RNA) synthesis which possibly contributes to the G1/S phase (Xie et al., 2021). In addition, a study conducted by Al-Salahi et al. (2014) also revealed that various fractions of ELE methanol extract with various concentrations and different time intervals in vitro and in vivo had an effect on the termination of the G1 and S phase cell cycle. Based on this, it is known that the compound content in ELE, especially quassinoids, plays an active role in senescence and cell cycle (Al-Salahi et al., 2014).

3.9 Apoptosis Level

The results show that ELE significantly influenced PC-3 cell apoptosis. The most effective ELE treatment can reduce live cells, necrosis, and increase early and late apoptosis of PC-3 cells compared to the I group (live cells 93.41%) (necrosis 0.91%) (early apoptosis 0.00%) (late apoptosis 0.14%) is ELE 100 μg/mL (live cells 45.19%) (necrosis 0.42%) (early apoptosis 38.02%) (late apoptosis 16.40%) (Figure 6A), dot blot can be seen in Figure 6B.

In the progression of cancer cells, apoptosis is necessary. Apoptosis is a form of cell death that is preprogrammed to eliminate unnecessary or potentially dangerous cells. Most of the time there is a decrease in apoptosis in patients suffering from cancer, so therapeutic agents that can re-induce apoptosis mechanisms and prevent cancer cell proliferation are urgently needed (Li et al., 2023). The results indicate that ELE significantly affected PC-3 cell apoptosis. Previous research reported that the Eurycomanone and Eurycomalactone content in ELE for HeLa and HT29 cell lines in in silico tests had the potential to induce apoptotic cell death by inhibiting Tumor Necrosis Factor Alpha (TNF-α) and Dihydrofolate reductase (DHFR) (Yunos et al., 2023). Apart from that, the 9-methoxycanthine-6-one content in ELE against cell lines A2780, SKOV-3, MCF-7, HT29, A375, and HeLa is also known to induce apoptosis using the Hoechst 33342 assay method by influencing the expression of apoptosis-related proteins, namely pyruvate, galectin 3 (LGAL3), pyruvate kinase (PKM), peroxiredocin 3 (PRDX3), annexin A2 (ANXA2), glyceraldehyde-3 phosphate dehydrogenase (GAPDH), and nuclear heterogeneous nucleoprotein A1 (HNTNP1A1) (Yunos et al., 2022).

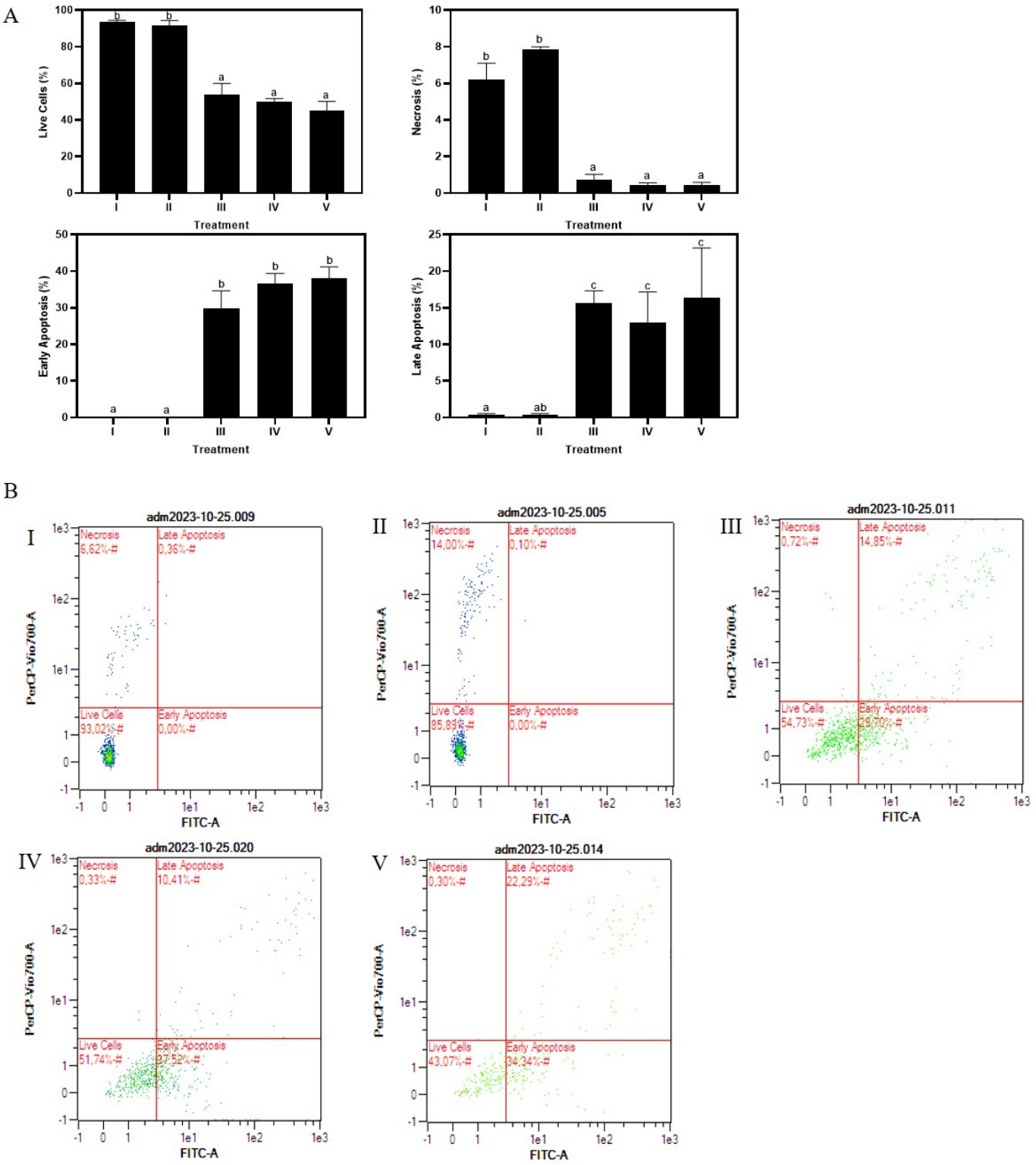


Figure 6. (A) Effect and (B) Representative Dot Blot of Different Concentrations of ELE Toward Apoptosis (Live Cells, Necrosis, Early and Late Apoptosis) on PC-3 Cells. *I (NC), II (DMSO), III (ELE 25 μg/mL), IV (ELE 50 μg/mL), V (ELE 100 μg/mL). *Data are Presented as Mean ± SD. Different Superscript Signs Indicate Significant Differences ($p<0.05$) Mann Whitney Post-Hoc Test

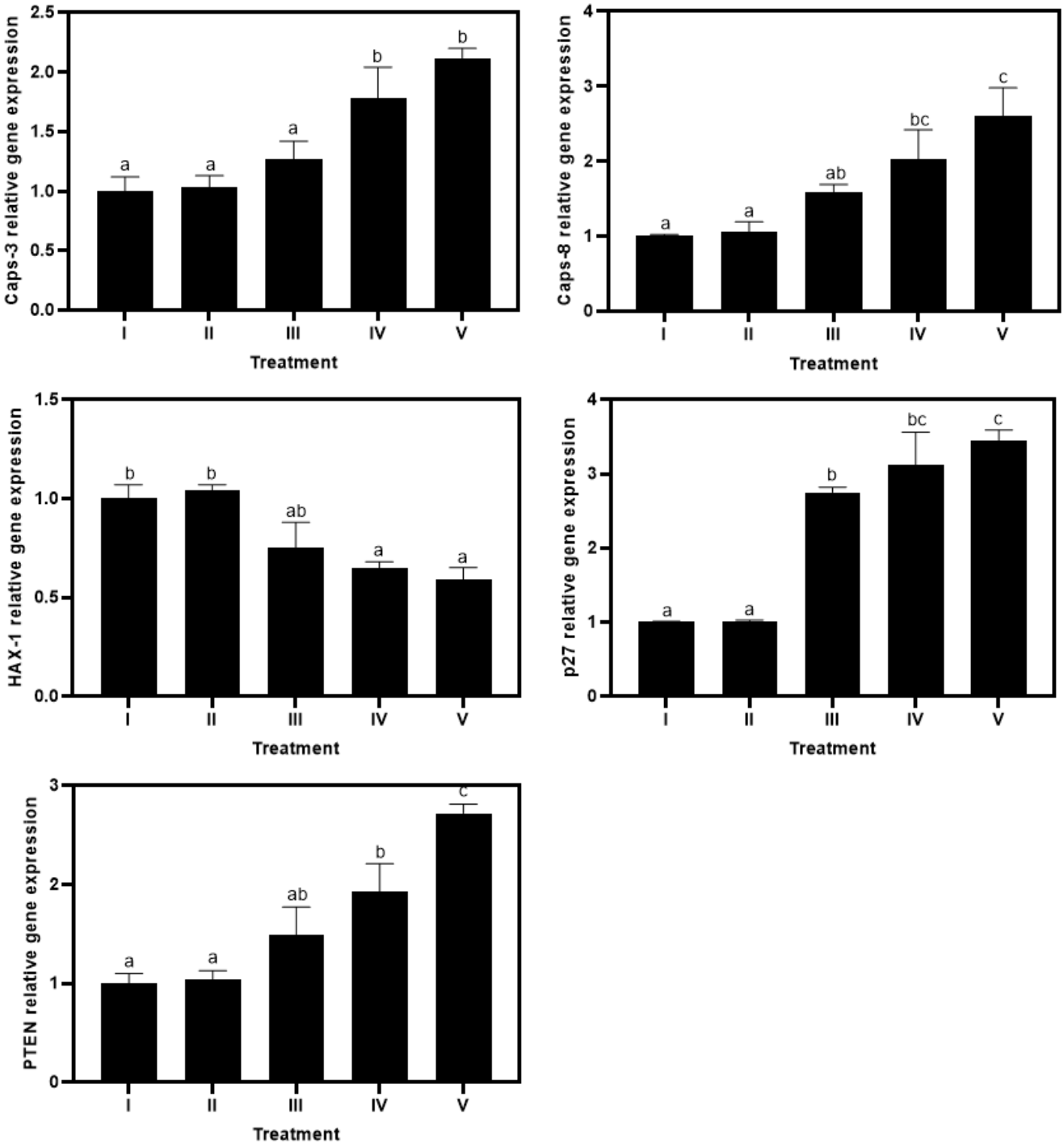


Figure 7. Effect of Different Concentrations of ELE on the Relative Gene Expression of Casp3, Casp8, HAX1, p27, and PTEN. *I (NC), II (DMSO), III (ELE 25 μg/mL), IV (ELE 50 μg/mL), V (ELE 100 μg/mL). *Data are presented as mean ± SD. Different superscript signs indicate significant differences ($p<0.05$) Mann Whitney Post-Hoc Test

3.10 Gene Expression of Casp3, Casp8, HAX1, p27, and PTEN

The results of the gene expression test in the study showed that ELE can increase the expression of the Casp3 and Casp8 genes and reduce the expression of the HAX-1 gene with the most effective ELE concentration being ELE 100 μg/mL compared to group I (Figure 7). Based on this, the research results are

Table 7. Effect of Various Concentration ELE on the Cell Cycle (G0/G1, S, and G2/M Phases) of PC-3 Cells

Sample	Cell cycle		
	G0/G1 phase	S phase	G2/M phase
Untreated PC-3 cells (I)	92.63 ± 1.30	5.91 ± 1.12	0.40 ± 0.19
PC-3 + DMSO 1% (II)	92.23 ± 2.65	6.35 ± 2.04	0.46 ± 0.25
PC-3 + ELE 25 μg/mL (III)	91.64 ± 0.63	6.55 ± 0.79	0.52 ± 0.17
PC-3 + ELE 50 μg/mL (IV)	90.96 ± 1.11	7.20 ± 1.03	0.65 ± 0.16
PC-3 + ELE 100 μg/mL (V)	90.34 ± 0.55	7.37 ± 0.52	0.75 ± 0.29

from previous research and ELE is known to have potential as an anticancer agent which plays a role in increasing apoptosis through increasing Casp-3 and -8 gene expression and decreasing HAX1 gene expression. ELE can also increase the expression of the p27 and PTEN genes.

There are changes in apoptosis, proliferation, and growth in cancer cells, this is due to gene malfunction. Malfunctioning of genes affects their translation proteins associated with uncontrolled cell proliferation, division, and growth and/or suppression of apoptosis-related proteins (Tamizhazhagan et al., 2017). Caspases constitute a group of cysteine proteases responsible for triggering apoptosis, of which Casp2, Casp8, Casp9, and Caps10 act as initiator caspases which then activate executioner Caspases (Casp3, Casp6, and Casp7) (Rodríguez-Berriguete et al., 2015). Activation of Casp3 is an important marker in the apoptosis signaling pathway (Srivastava and Saxena, 2023). Conversely, inhibition of cell apoptosis is due to inactivation of Casp9 which is caused by increased expression of the HAX1 gene. This was proven in research by Yan et al. (2015) which showed that prostate cancer cell lines, one of which was PC-3, demonstrated a significant increase in HAX1 mRNA and protein levels compared to primary prostate epithelial cells. The gene expression test results in the study showed that ELE 100 μg/mL can increase the expression of the Casp3 and Casp8 genes and reduce the expression of the HAX-1 gene (Figure 7). Based on this, the research results are from previous research and ELE is known to have potential as an anticancer agent which plays a role in increasing apoptosis through increasing Casp-3 and -8 gene expression and decreasing HAX1 gene expression. Apart from that, the research results also show that ELE can enhance the expression of the p27 and PTEN genes. This is by previous research which states that in the adult prostate gland, the p27 gene is expressed and its downregulation occurs in most prostate cancers, where the p27 gene functions to control cell proliferation by stopping the cell cycle. On the other hand, PTEN gene expression plays a critical role as a tumor suppressor gene, regulating cell growth, survival, and metabolism in prostate cancer by controlling the phosphatidylinositol 3-kinase (PI3K)-protein kinase B (AKT) signaling pathway Chen et al. (2023).

Based on the findings of conducted research, it is known that ELE has the potential to serve as an anti-prostate cancer agent, with the most effective ELE treatment being ELE at 100 μg/mL. due to the various compounds contained in ELE, especially quassinoids. ELE is known to reduce viability and increase PC cell inhibition with an IC_{50} of 45.150 μg/mL, can reduce intracellular ROS levels, reduce live cells and necrosis, increase senescence, increase late and early apoptosis, inhibit cell proliferation by inhibiting the G0/G1 phase. ELE can also increase the expression of the Casp3, Casp8, p27, and PTEN genes and reduce the expression of the HAX1 gene

4. CONCLUSIONS

The results of the research show that *E. longifolia* Jack root extract is toxic, thereby reducing the viability of prostate cancer cells (PC-3) and is known to have anticancer potential by increasing cell senescence, late and early apoptosis and gene expression Casp3, Casp8, p27, and PTEN. Apart from that, ELE is also known to reduce intracellular ROS levels, live cells and necrosis, reduce HAX1 gene expression and inhibit the G0/G1 phase in cell cycle tests on PC-3 cells.

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