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EFFICACY OF SALAK SEED AND POMELO PEEL EXTRACTS ON HeLa CELL APOPTOSIS AND PROLIFERATION

EFIKASI EKSTRAK BIJI SALAK DAN KULIT JERUK PAMELO TERHADAP APOPTOSIS DAN PROLIFERASI SEL HeLa

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Abstract

The development of adjuvant therapies is vital for reducing cancer recurrence and minimizing the adverse effects of conventional treatments. Salak seeds (*Salacca zalacca*) and pomelo peels (*Citrus maxima*) are rich in bioactive compounds such as terpenoids, flavonoids, lycopene, and polyphenols, making them promising candidates for adjuvant cancer therapy. This study evaluated the anticancer effects of their individual and combined extracts. Bioactive compounds in salak seed (S) and pomelo peel (J) extracts were identified using gas chromatography-mass spectrometry (GC-MS) and analyzed *in silico* to assess their binding affinities to key target proteins: COX-2 (anti-inflammatory), caspase-3 (apoptosis induction), and PI3K (cell proliferation). The extracts were tested *in vitro* on cervical cancer HeLa cells at varying treatment groups: S; J; 1S3J; 1S1J; and 3S1J. Assays evaluated anti-inflammatory activity, cytotoxicity, cell migration inhibition, and apoptosis induction. Both *in silico* and *in vitro* analyses consistently demonstrated that the extracts significantly reduced cell viability exhibited anti-inflammatory effects, inhibited cell proliferation, and enhanced apoptosis. Among the tested treatments, the 1S1J combination showed the most pronounced effects across all parameters, highlighting its synergistic interaction, and superior therapeutic potential. These findings position the 1S1J combination as an optimal candidate for adjuvant cervical cancer therapy, warranting further investigation.

Keywords*:* Anti-inflammatory; Apoptosis induction; Pomelo peel; Proliferation; Salak seeds

Abstrak

Pengembangan terapi adjuvan sangat penting untuk mengurangi kekambuhan kanker dan meminimalkan efek samping pengobatan konvensional. Biji salak (Salacca zalacca) dan kulit jeruk pamelo (Citrus maxima) kaya akan senyawa bioaktif seperti terpenoid, flavonoid, likopen, dan polifenol, yang menjadikannya kandidat potensial untuk terapi adjuvan kanker. Penelitian ini mengevaluasi efek antikanker dari ekstrak tunggal dan kombinasi biji salak serta kulit jeruk pamelo. Senyawa bioaktif dalam ekstrak biji salak (S) dan kulit jeruk pamelo (J) diidentifikasi menggunakan kromatografi gas-spektrometri massa (GC-MS) dan dianalisis secara in silico untuk menilai afinitas ikatannya terhadap protein target utama: COX-2 (antiinflamasi), caspase-3 (induksi apoptosis), dan PI3K (proliferasi sel). Ekstrak diuji secara in vitro pada sel kanker serviks HeLa dengan kelompok perlakuan S; J; 1S3J; 1S1J; dan 3S1J. Uji meliputi aktivitas antiinflamasi, sitotoksisitas, penghambatan migrasi sel, dan induksi apoptosis. Hasil in silico dan in vitro secara konsisten menunjukkan bahwa ekstrak secara signifikan mengurangi viabilitas sel, memiliki efek antiinflamasi, menghambat proliferasi sel, dan meningkatkan apoptosis. Dari semua perlakuan, kombinasi 1S1J menunjukkan efek paling signifikan pada semua parameter, menunjukkan interaksi sinergis, dan potensi terapeutiknya yang unggul. Temuan ini menjadikan kombinasi 1S1J sebagai kandidat optimal untuk terapi adjuvan kanker serviks, yang layak untuk diteliti lebih lanjut.

Kata kunci*: Antiinflamasi; Biji salak; Induksi apoptosis; Kulit jeruk pamelo; Proliferasi*

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INTRODUCTION

Cervical cancer is a serious disease requiring special attention in Indonesia. It is the second most common cancer and ranks third in cancer-related mortality in Indonesia. Currently, surgery is the primary treatment for cervical cancer, but it has limitations. Recurrence has been reported in 40% of patients following surgery. Adjuvant therapies can help reduce recurrence rates, one example being doxorubicin (Khairunnisa et al., 2022). However, high doses of doxorubicin cause systemic toxicity, while low doses risk inducing cancer cell resistance. Therefore, developing more effective adjuvant therapies is essential to minimize side effects, eliminate residual cancer cells, and reduce the risk of recurrence after primary treatment.

The exploration of natural products as adjuvant agents in cancer therapy is increasingly recognized. Natural compounds are known to contain bioactive substances with cytotoxic properties that typically exhibit fewer side effects compared to conventional pharmaceuticals (Yanto & Sulistianingsih, 2017). Salak pondoh (*Salacca zalacca* (Gaertn.) Voss) seeds and pomelo (*Citrus maxima* (Burm.) Merr.) peel show considerable potential as natural therapeutic agents against cervical cancer. Research conducted by Purwanto et al. (2015) identified that salak pondoh seeds are rich in polyphenols, alkaloids, and terpenoids, which are associated with antioxidant and cytotoxic activities. Similarly, pomelo peel contains flavonoids and lycopene, which have been shown to induce apoptosis in cancer cells (Suryanita et al., 2019). Consequently, salak pondoh seeds may function as antioxidants and anti-inflammatory agents, while pomelo peel demonstrates cytotoxic effects against cervical cancer cells. This evidence supports the potential application of these natural products as effective adjuvants in cervical cancer treatment, providing a promising alternative with reduced side effects compared to standard chemotherapy regimens.

The utilization of salak seeds and pomelo peels as research materials for sustainable practices is particularly relevant, given the significant annual increase in the production and consumption of these fruits globally (Tantrayana & Zubaidah, 2015; Aji et al., 2017). As consumption rises, organic waste, including salak seeds and pomelo peels, becomes a pressing issue, with a large portion of the pomelo fruit discarded and salak seeds often treated as waste. Their utilization supports global zero-waste initiatives aimed at reducing organic waste.

The objective of this study is to evaluate the effectiveness of combined salak seed and pomelo peel extracts compared to individual extracts as potential cervical cancer therapies. It investigates their anti-inflammatory activity, cytotoxicity, apoptosis induction, and antiproliferative effects on HeLa cells. The novelty of this research lies in its exploration of the synergistic effects of these extracts, which have not been previously studied. By profiling their phytochemical compositions and analyzing their impact on cell migration, proliferation, apoptosis, and apoptosis-related protein expression using *in silico* and *in vitro* methods, this study provides valuable insights into their anticancer potential. The findings offer promising avenues for developing more effective and eco-friendly cervical cancer treatments.

MATERIALS AND METHODS

Location and Time

The study was conducted from April 1 to July 31, 2024, at the Biochemistry Laboratory of the Faculty of Biology, Universitas Gadjah Mada (UGM) for the extraction of pomelo peel and salak seeds. *In vitro* assays (MTT assay, anti-inflammatory tests, apoptosis tests, and migration proliferation tests) were performed at the Parasitology Laboratory of the Faculty of Medicine, Public Health, and Nursing. Gas chromatography-mass spectrometry (GC-MS) analysis was conducted at the Integrated Research and Testing Laboratory (LPPT) of UGM.

Equipment

The equipment used included a maceration chamber, rotary evaporator, mill, maceration vessel, a complete set of glassware, oven, magnetic stirrer, pH meter, centrifuge, mortar, pestle, dropper pipette, GC-MS instrument, 5% CO_2 incubator, ImageJ software, microplate reader, BD FACSCalibur™ flow cytometer (California, USA), 96-well plates, micropipettes (10; 100; and 1,000 µL), blue tips, red tips, yellow tips, UV/Vis spectrophotometer, and light microscope.

Materials

The materials used included salak seeds and pomelo peel, 70% ethanol, 96% ethanol, albumin, sodium diclofenac, phosphate-buffered saline (PBS), HeLa cells, Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), streptomycin, dimethyl sulfoxide (DMSO), MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), 10% SDS, FITC Annexin V Apoptosis Detection Kit with PI (BioLegend, USA), 4% paraformaldehyde, Tris-buffered saline, concentrated HCl, magnesium ribbon (Mg), and Mayer's hematoxylin.

Collection, Plant Determination, Sample Preparation of Salak Pondoh Seeds and Pomelo Peel

The salak pondoh seed samples were obtained from small businesses (UMKM) in Sleman Regency, Special Region of Yogyakarta, while the pomelo peel samples were collected from UMKM in Sukoharjo Regency, Central Java Province. The seeds and peels were selected from ripe fruits, with dark brown salak pondoh seeds and greenish-yellow pomelo peels. Plant species determination was conducted by a botanist at the Plant Systematics Laboratory, Faculty of Biology, Universitas Gadjah Mada (UGM), to confirm the species used in the research. Sample preparation began with the weighing and thorough washing of salak pondoh seeds and pomelo peels, followed by cutting them into smaller pieces. The samples were then dried and ground into a fine powder.

The Extraction of Salak Pondoh Seed

The extraction of salak pondoh seeds was performed using the maceration method according to Nurihardiyanti et al. (2015). A total of 300 g of seed powder was soaked in 96% ethanol. The mixture was periodically stirred over two days, followed by remaceration with 96% ethanol. After combining the resulting macerates, the solvent was evaporated to obtain a concentrated extract, and the yield was calculated.

The Extraction of Pomelo Peel

The extraction of pomelo peel was carried out using the maceration method according to Wenas et al. (2021). A total of 250 g of pomelo peel powder was macerated with 70% ethanol. The sample was periodically stirred over two days, followed by remaceration with 70% ethanol. The ethanol extract was then evaporated using a rotary evaporator to obtain a concentrated extract, and the yield was calculated.

Preparation of Combined Extracts of Salak Pondoh Seeds and Pomelo Peel

The combination extract ratios of salak pondoh seeds (S) and pomelo peel (J) were defined as S (salak pondoh seed extract only), J (pomelo peel extract only), 1S3J (1 portion of salak pondoh extract and 3 portions of pomelo peel), 1S1J (1 portion of salak pondoh extract and 1 portion of pomelo peel), and 3S1J (3 portions of salak pondoh extract and 1 portion of pomelo peel). All extracts were prepared at a concentration of 1,000 ppm using DMSO as the solvent, according to the respective combination ratios. The mixtures were then stirred and homogenized using a centrifuge. Each ratio was prepared in triplicate for both salak pondoh seed extract and pomelo peel extract. The stock solution was further diluted to final concentrations of 1,000; 500; 250; 125; and 62.5 ppm. Each concentration was tested with three replications. The method for preparing the combined extracts was based on the protocol established by Noviardi et al. (2019).

Analysis of Phytochemical Profiles and *In Silico* Testing

The *in silico* analysis aimed to evaluate the interaction potential between ligand compounds and target proteins. Ligand structures, derived from GC-MS data, were retrieved from the PubChem Compound database (https://pubchem.ncbi.nlm.nih.gov/) and converted from MOL SDF format to PDBQT files using PyRx to define atomic coordinates. Target protein structures were obtained from the Protein Data Bank (https://www.rcsb.org/). Both ligands and proteins were prepared by adding hydrogen atoms, analyzing charge distribution, and identifying binding sites using AutoDock Tools version 4.2.6. Molecular docking results were visualized and assessed using PyMOL version 3.1, while detailed analyses of interactions, including hydrogen bonds and hydrophobic contacts, were

performed with BIOVIA Discovery Studio 2021 Client. This workflow provided a comprehensive evaluation of binding affinities and interaction dynamics between the ligands and their target proteins (Muhammad & Fatima, 2015).

Anti-Inflammatory Test

The anti-inflammatory effect was confirmed using the albumin denaturation method. Albumin was mixed with PBS and the sample extract at various concentrations. Diclofenac sodium was used as the standard solution. Absorbance readings were measured at a wavelength of 280 nm using a UV/Vis spectrophotometer. The obtained data were calculated using the protein denaturation inhibition percentage formula as an indicator of efficacy (Madhuranga & Samarakoon, 2023).

Preparation and Culture of HeLa Cells

HeLa cell stocks were placed in a dish containing DMEM medium supplemented with 10% fetal bovine serum (FBS) and streptomycin. The cell culture was incubated at 37 °C with 5% CO_2 humidity. Once the cells entered the logarithmic phase, they were harvested and transferred to a 96-well plate at a density of 2×10^3 cells per well (Bai et al., 2021). The cells were divided into seven treatment groups, including a negative control group treated with 0.1% DMSO medium, a positive control group treated with doxorubicin, and five treatment groups (S; J; 1S3J; 1S1J; and 3S1J) treated with sample extracts prepared from salak pondoh seeds and pomelo peel in different combination ratios as previously described. Each combination ratio was prepared in triplicate. The stock solutions were then serially diluted to final concentrations of 1,000; 500; 250; 125; and 62.5 ppm. Each treatment group, including each concentration within each combination ratio as well as the positive and negative controls, was tested in three replications. All samples were incubated for 48 hours, during which their effects on the cells were assessed.

Cell Viability Test

Cytotoxicity was assessed using the MTT assay according to a modified protocol by Tunjung et al. (2021). The cytotoxicity assay was performed at a cell density of 2×10^3 cells per well. Following the removal of the culture medium and DMSO solution, the cells were washed with phosphate-buffered saline (PBS) to remove residual compounds. Next, 10 µL of 5 mg/mL MTT solution was added to each well and incubated for 2–4 hours. The reaction was terminated by adding 10% sodium dodecyl sulfate (SDS; Merck), followed by overnight incubation at room temperature. Absorbance was measured at 595 nm using an ELISA plate reader (Bio-Rad 680XR), and the data were converted into percent viability to calculate the IC_{50} value.

Apoptosis Test

The cytotoxicity assay was performed by treating HeLa cells with five experimental extracts (S; J; 1S3J; 1S1J; and 3S1J) or doxorubicin (as the control group) for 48 hours. After treatment, the cells were collected and washed three times with phosphate-buffered saline (PBS) to eliminate any remaining substances. The cells were then resuspended in 200 µL of staining buffer and incubated with 5 µL of propidium iodide (PI, 50 µg/mL) and 10 µL of annexin V-FITC (20 µg/mL) for cell staining. Apoptosis was evaluated using the FITC Annexin V Apoptosis Detection Kit with PI, following the manufacturer's guidelines. The stained cells were analyzed using a BD FACSCalibur™ flow cytometer to quantify the percentage of apoptotic cells as an indicator of effectiveness (Bai et al., 2021)

HeLa Cell Proliferation Inhibition Test

HeLa cells (2×10^5 cells/well) were plated in a 24-well plate and cultured for 24 hours to achieve more than 90% confluence. A vertical scratch was made using a 200 µL pipette tip, and the wells were washed three times with PBS to remove debris. Serum-free medium was added, along with five experimental extracts (S; J; 1S3J; 1S1J; and 3S1J) or control (doxorubicin). The plates were incubated at 37 °C for 24 hours, with cell migration assessed at 0; 12; 24; and 36 hours. Scratch areas were quantified using ImageJ software to calculate the percentage of wound closure (Bai et al., 2021).

Data Analysis Method

Qualitative data analysis was performed descriptively. Quantitative data analysis was conducted using ANOVA and Principal Component Analysis (PCA) with Minitab software, applying multivariate data analysis techniques.

RESULTS

Extraction of Salak Pondoh Seeds and Pomelo Peel

The powder from the salak seeds that underwent maceration and evaporation yielded 5.15 g of concentrated extract, resulting in a yield of 1.1%. Meanwhile, the pomelo peel powder, after maceration and evaporation, produced 15.68 g of concentrated extract, corresponding to a yield of 3.2%.

Phytochemical Compounds in Salak Pondoh Seed and Pomelo Peel Extracts

Table 1 shows that the pomelo peel extract contains flavonoid compounds, indicated by a color change to dark red. In contrast, the salak pondoh seed extract does not contain flavonoid compounds, as no color change was observed.

Table 1. Detection of flavonoids in the salak pondoh seed and pomelo peel extracts

Sample	Color	Flavonoid
Salak pondoh seed	Yellowish clear	-
Pomelo peel	Dark red	+

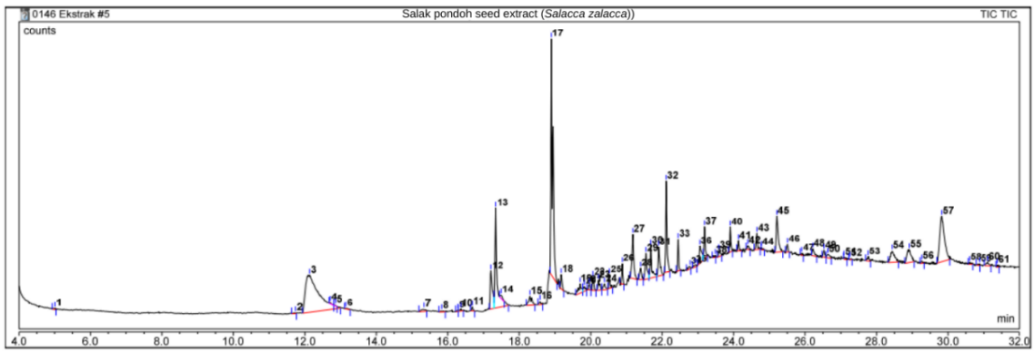

Figure 1. Total ion chromatogram (TIC) of salak pondoh seed extract

Figure 1 illustrates the total ion chromatogram of the salak pondoh seed extract. A total of 61 peaks were detected, representing 15 distinct compounds within the extract. This complex chromatographic profile not only underscores the chemical diversity of the seed extract but also suggests that these compounds may contribute synergistically to its biological activities.

Table 2. Phytochemical compounds in salak pondoh seed extract

Peak	Retention time	%Peak area	Compound name
1	5.01	0.07	(2-phenyl-1,3-dioxolan-4-yl)methyl (E)-octadec-9-enoate
31	21.90	2.18	Ethyl iso-allocholate
3	12.13	20.17	Lactose
6	13.15	0.11	2-myristynoyl pantetheine
11	16.69	0.19	Methyl 4-[2-[[2-[[2-[(2-pentylcyclopropyl)methyl]cyclopropyl]methyl]cyclopropyl]methyl]cyclopropyl]butanoate
12	17.21	3.36	N-Hexadecanoic acid
19	19.70	1.05	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester
13	17.34	8.14	Hexadecanoic acid, ethyl ester
30	21.68	2.18	[(Z)-7-methyltetradec-8-enyl] acetate
17	18.90	17.05	Ethyl octadeca-9,12-dienoate
18	19.17	0.77	Eicosanoic acid
20	19.82	0.38	3-methoxy-4-hydroxy-cinnamic acid
57	29.82	8.83	Sitosterol
27	21.18	3.27	2-monoolein
32	22.11	4.53	Bis(6-methylheptyl) benzene-1,2-dicarboxylate

Based on the results presented in Table 2, the peak area with the highest percentage is lactose (20.07%), followed by ethyl octadeca-9,12-dienoate (17.05%), and hexadecanoic acid, ethyl ester (8.14%). Additionally, compounds such as (2-phenyl-1,3-dioxolan-4-yl)methyl (E)-octadec-9-enoate, sitosterol, and 3-methoxy-4-hydroxy-cinnamic acid were also identified, which possess antioxidant and anti-inflammatory properties.

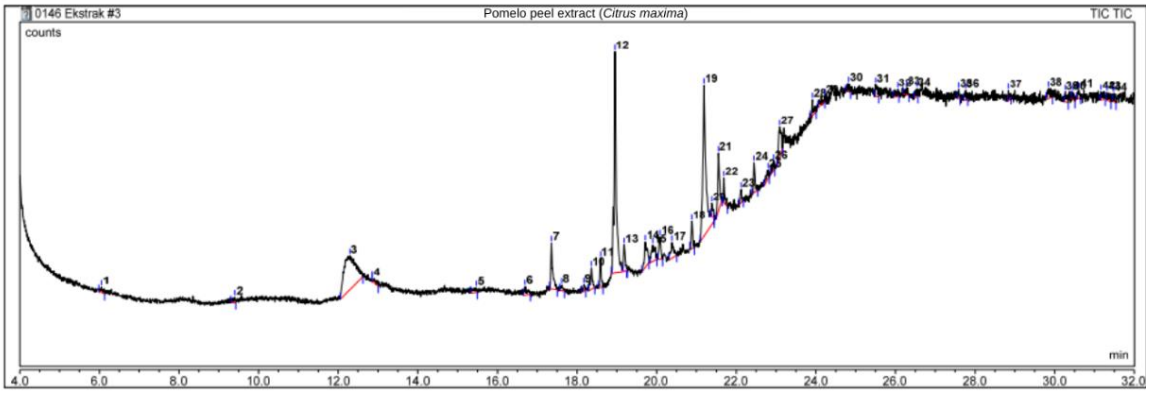

Figure 2. Total ion chromatogram (TIC) of pomelo peel extract

Figure 2 presents the total ion chromatogram obtained from the pomelo peel extract. A total of 44 distinct peaks were observed, corresponding to 15 unique compounds present in the sample. This complex chromatographic profile highlights the intricate chemical composition of the extract and suggests potential synergistic interactions among the identified compounds.

Table 3. Phytochemical compounds in pomelo peel extract

Peak	Retention time	%Peak area	Compound name
1	6.05	0.49	Methyl 8-[2-(2-hexylcyclopropyl)cyclopropyl]octanoate
2	9.40	0.51	2-myristynoyl pantetheine
3	12.29	15.84	Melezitose
27	23.09	3.27	Ethyl iso-allocholate
7	17.36	3.55	Hexadecanoic acid, ethyl ester
10	18.36	1.68	Methyl octadec-11-enoate
11	18.59	1.64	Methyl 4-[2-[[2-[[2-[(2-pentylcyclopropyl)methyl]cyclopropyl]methyl]cyclopropyl]methyl]cyclopropyl]butanoate
12	18.96	18.89	Ethyl (E)-octadec-9-enoate
14	19.72	2.75	(2-hexadecanoyloxy-3-methylbutyl) hexadecanoate
15	19.90	2.23	Propanoic acid, 2-methyl-, (dodecahydro-6a-hydroxy-9a-methyl-3-methylene-2,9-dioxoazuleno[4,5-b]furan-6-yl)methyl ester, [3aS-(3a.alpha.,6.beta.,6a.alpha.,9a.beta.,9b.alpha.)]-
18	20.89	1.49	[(Z)-7-methyltetradec-8-enyl] acetate
19	21.19	17.82	2-monoolein
21	21.55	4.17	Glycidyl oleate
34	26.55	0.49	[5,6-dihydroxy-7-(hydroxymethyl)-3,11,11,14-tetramethyl-15-oxo-4-tetracyclo[7.5.1.01,5.010,12]pentadeca-2,7-dienyl] hexadecanoate
38	29.85	1.19	[(10E)-4,9,13-triacetyloxy-3,6,6,10,14-pentamethyl-2-oxo-16-oxatetracyclo[10.3.1.01,12.05,7]hexadec-10-en-8-yl] pyridine-3-carboxylate

According to Table 3, the most dominant compound in the pomelo peel extract is ethyl (E)-octadic-9-enoate (18.89%), followed by 2-monoolein (17.82%), and melezitose (15.84%). Additionally, the pomelo peel extract contains propanoic acid, 2-methyl-, (dodecahydro-6a-hydroxy-9a-methyl-3-methylene-2,9-dioxoazuleno[4,5-b]furan-6-yl)methyl ester, [3aS-(3a.alpha.,6.beta.,6a.alpha.,9a.beta.,9b.alpha.)]-, and ethyl iso-allocate, which have potential as anticancer agents.

In Silico Testing

Table 4 provides compelling evidence of the bioactivity of the compounds present in the salak pondoh seed extract. Notably, 3-methoxy-4-hydroxy-cinnamic acid exhibits a binding affinity to

COX-2 that is nearly equivalent to its native ligand (–9.4 versus –9.5 kcal/mol), suggesting a strong potential for anti-inflammatory activity. In addition, the interactions observed, such as hydrogen bonding and hydrophobic contacts with key residues including SER 146, GLU 140, and LEU 238, support the specificity of this compound toward the COX-2 active site. Furthermore, the results from docking studies on caspase-3 indicate that both sitosterol and (2-phenyl-1,3-dioxolan-4-yl)methyl (E)-octadec-9-enoate have binding affinities that are competitive relative to their native peptide inhibitors (-7.2 and -6.4 kcal/mol vs. -6.5 kcal/mol, respectively). This suggests that these compounds may effectively engage with the caspase-3 enzyme, thereby potentiating apoptotic pathways. Additional docking analyses involving the PI3K target (5ITD) reveal moderate binding affinities for both sitosterol and (2-phenyl-1,3-dioxolan-4-yl)methyl (E)-octadec-9-enoate. While these affinities are slightly lower than those observed with the COX-2 and caspase-3 targets, they nonetheless indicate a potential contributory role in signaling pathways that may lead to the modulation of cell survival and apoptotic processes.

Table 4. Results of molecular docking for salak pondoh seed extract

Compound (ligand)	Group of compound	Target protein	Native ligand	Binding Affinity (kcal/mol)		Amino-acid residues
				Native ligand	Ligand	
3-methoxy-4-hydroxy-cinnamic acid	Fatty acid	5KIR (COX-2)	COH	-9,5	-9,4	SER 146, GLU 140, LEU 238, ASN 144, ARG 242
		5KIR (COX-2)	COH	-9,5	-8,4	ARG 61, ARG 44, THR 62, LYS 546, ASP 125, HIS 122, PRO 542, ALA 543
Sitosterol	Phytosterol	3GJQ (Caspase-3)	Peptide Inhibitor	-6,5	-7,2	LYS 137, GLY 125, GLU 124, ARG 164, PRO 201, TYR 197
		5ITD (PI3K)	6CY	-7,3	-6,7	SER 774, MET 772, SER 919, ILE 932, THR 856, MET 922, GLN 859, TRP 780
(2-phenyl-1,3-dioxolan-4-yl)methyl (E)-octadec-9-enoate	Fatty acid	5KIR (COX-2)	COH	-9,5	-8,1	SER 353, LEU 531, TRY 355, LEU 359, VAL 349
		3GJQ (Caspase-3)	Peptide Inhibitor	-6,5	-6,4	VAL 266, PRO 201, TYR 197
		5ITD (PI3K)	6CY	-7,3	-6,7	ILE 800, TRP 780, MET 772

Table 5. Results of molecular docking for pomelo peel extract

Compound (ligand)	Group of compound	Target protein	Native ligand	Binding affinity (kcal/mol)		Amino-acid residues
				Native ligand	Ligand	
Ethyl iso-allocholate	Steroid	5KIR (COX-2)	COH	-9,5	-7,0	PRO542, HIS 122, SER 126, TYR 373, GLN 372, GLN 370, PHE 367, ARG 61
		3GJQ (Caspase-3)	Peptide inhibitor	-6,5	-8,6	VAL 266, MET 268, LYS 137, TYR 195, LEU 136, THR 140, TYR 197, GLY 125, PRO 201, GLU 124, ARG 164
		5ITD (PI3K)	6CY	-7,3	-7,0	ASP 933, ILE 932, THR 856, MET 922, TRP 780, MET 772, ILE 800
Propanoic acid, 2-methyl-, (dodecahydro-6a-hydroxy-9a-methyl-3-methylene-2,9-dioxoazuleno[4,5-b]furan-	Fatty acid	5KIR (COX-2)	COH	-9,5	-7,2	SER 143, LEU 224, LEU 145, SER 146, ASN 144, GLU 236, LEU 238, THR 237
		3GJQ (Caspase-3)	Peptide inhibitor	-6,5	-8,1	VAL 266, TYR 197, GLY 125, PRO 201, GLU 124, ARG 164, CYS 264

Compound (ligand)	Group of compound	Target protein	Native ligand	Binding affinity (kcal/mol)		Amino-acid residues
				Native ligand	Ligand	
6-yl)methyl ester, [3aS-(3a.alpha.,6.beta.,6a.alpha., 9a.beta.,9b.alpha.)]-		5ITD (PI3K)	6CY	-7,3	-7,8	MET 772, LYS 802, ASP 933,PHE 934, ASP 810, LEU 807, ILE 932, TYR 836, ILE 800, ILE 932
Hesperidin	Flavonoid	3GJQ (Caspase-3)	Peptide inhibitor	-6,5	-9,4	Pro201, Arg164, Val266, Arg164, Tyr197, Glu124

Based on Table 5, the extract of pomelo peel is capable of inducing apoptosis and exhibiting antiproliferative effects. The ability to induce apoptosis in caspase-3 is demonstrated by the compounds ethyl iso-allocholate and hesperidin, which show a better binding affinity compared to their native ligands. Additionally, the compound propanoic acid, 2-methyl-, (dodecahydro-6a-hydroxy-9a-methyl-3-methylene-2,9-dioxoazuleno[4,5-b]furan-6-yl)methyl ester, [3aS-(3a.alpha.,6.beta.,6a.alpha.,9a.beta.,9b.alpha.)]- can induce apoptosis and has antiproliferative effects on PI3K.

Anti-inflammation Test

Based on Figure 3, the highest percentage of inflammation inhibition is demonstrated by the positive control (doxorubicin) at 61.57%, followed by the 1S1J combination extract (49.04%) and the single salak pondoh seed extract (47.63%). The lowest percentage is observed in the single pomelo peel extract (24.56%).

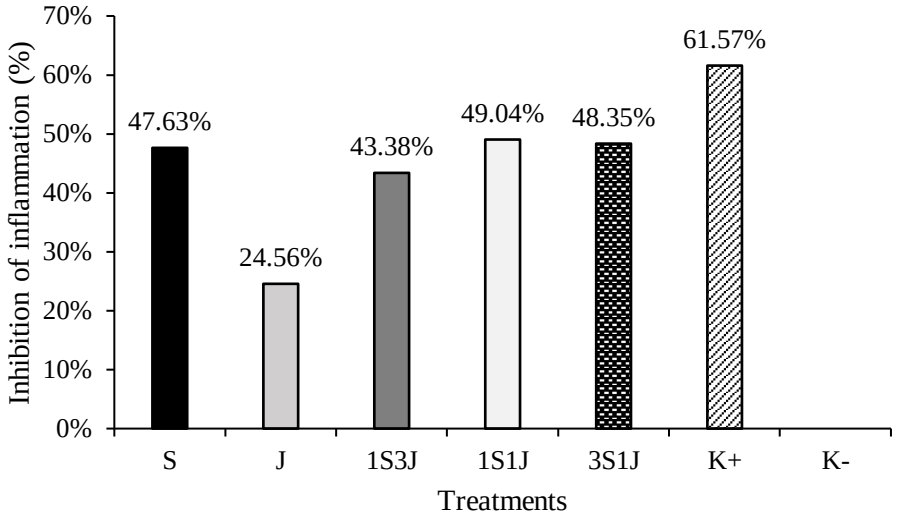

Figure 3. Percentage of inflammation inhibition of salak pondoh seed and pomelo peel extracts. Note: S= salak pondoh seed extract, J= pomelo peel extract, 1S3J= 1:3 combination, 1S1J= 1:1 combination, 3S1J= 3:1 combination, K+= positive control, K-= negative control

HeLa Cell Viability Test

Figure 4 shows that the IC_{50} value for the positive control (doxorubicin) is 6.063 µg/mL, while the IC_{50} value for the negative control (medium only) is 1505.65 µg/mL. The IC_{50} values for the extracts are as follows: 762.47 µg/mL for S; 1015.42 µg/mL for J; 864.59 µg/mL for 1S1J; and 922.68 µg/mL for 1S3J. Based on these IC_{50} values, the treatments S; 1S3J; and 1S1J are classified as having moderate cytotoxicity.

Apoptosis Test

Figure 5 illustrates that each treatment successfully induced apoptosis. The treatment with pomelo peel extract exhibited the highest percentage of cell death due to apoptosis and necrosis, at 31.5% and 54.9%, respectively. Additionally, the combination extracts demonstrated a decrease in the percentage of necrosis while still inducing apoptosis.

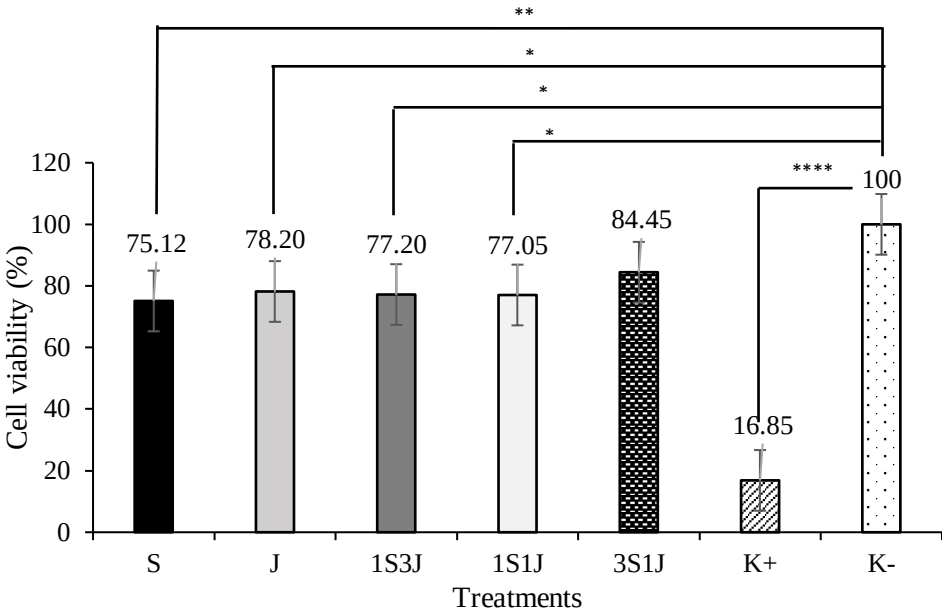

Figure 4. HeLa cell viability percentage. Note: S= salak pondoh seed extract, J= pomelo peel extract, 1S3J= 1:3 combination, 1S1J= 1:1 combination, 3S1J= 3:1 combination, K+= positive control, K-= negative control

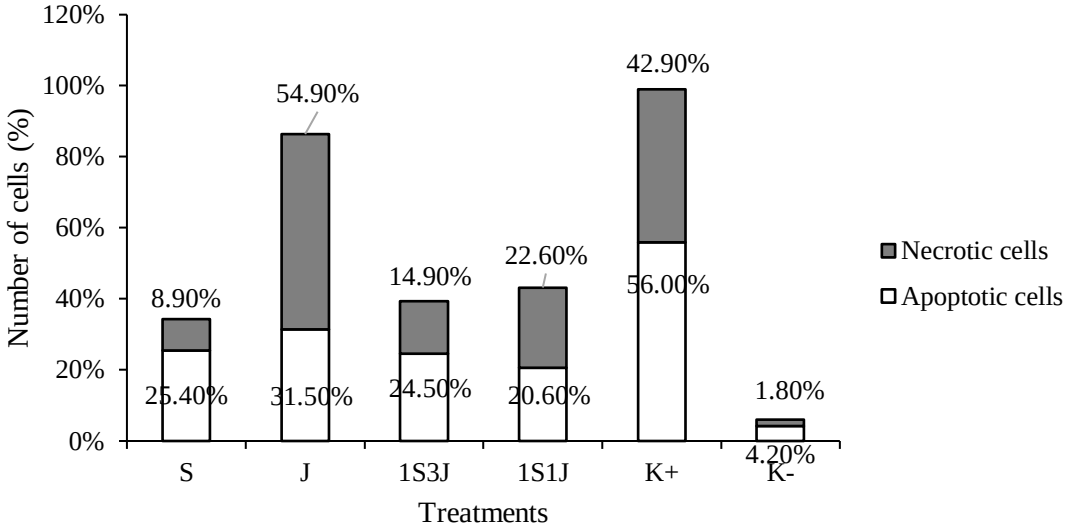

Figure 5. Percentage of HeLa cells undergoing apoptosis and necrosis**.** Note: S= salak pondoh seed extract, J= pomelo peel extract, 1S3J= 1:3 combination ,1S1J= 1:1 combination, K+= positive control, K-= negative control

HeLa Cell Proliferation Inhibition Test

According to Figure 6, each treatment group was able to inhibit wound closure. Overall, the combination of salak pondoh seed extract and pomelo peel extract was more effective in hindering wound closure compared to the positive control (doxorubicin), although the difference was not statistically significant. This wound closure capability reflects the rate of cell proliferation; thus, a slower wound closure indicates that the treatment effectively inhibits cancer cell proliferation. The 1S1J treatment represents the optimal combination and shows potential as an antiproliferative agent for HeLa cells.

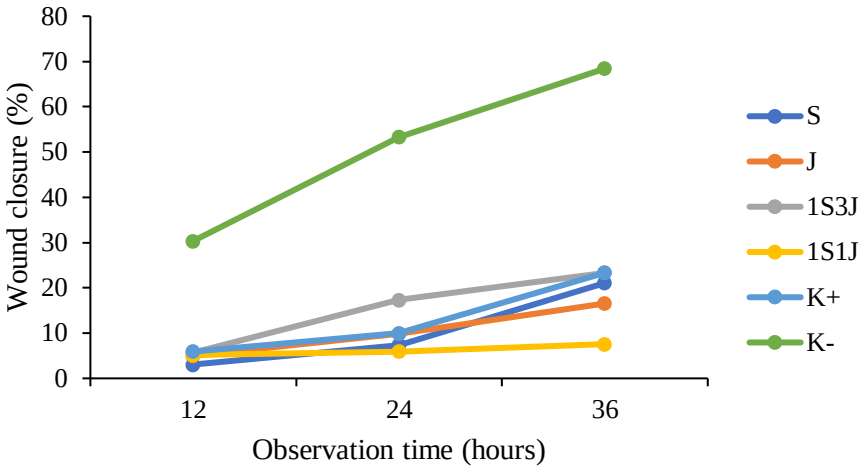

Figure 6. Percentage of wound closure in HeLa cell culture medium**.** Note: S= salak pondoh seed extract, J= pomelo peel extract, 1S3J= 1:3 combination, 1S1J= 1:1 combination, K+= positive control, K-= negative control

Principal Component Analysis (PCA)

Based on Figure 7, it is shown that the PCA results indicate that the 1S1J treatment is the most effective. Showing the closest alignment with the evaluated indicators, especially in the anti-inflammatory, cytotoxicity, and migration assays.

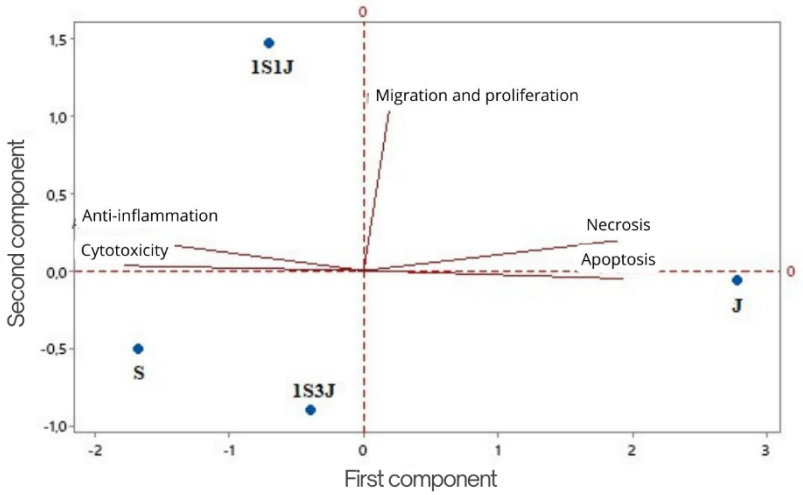

Figure 7. The principal component (PC) in PCA analysis

DISCUSSION

Phytochemical Composition of Salak Pondoh Seed Extract and Pomelo Peel

In this study, it was found that pomelo peel extract contains flavonoids, whereas no flavonoids were detected in salak pondoh seed extract (Table 1). Flavonoids are known to have anticancer properties, primarily due to their strong antioxidant activity, which safeguards cellular components from oxidative damage induced by free radicals key contributors to cancer initiation and progression. Furthermore, flavonoids enhance immune system responses and mitigate the risks of inflammation, stroke, cardiovascular diseases, and cancer through their molecular mechanisms (Anh et al., 2021; Su et al., 2024). This finding aligns with the study conducted by Suryanita et al. (2019), which reported that pomelo peel extract contains several anticancer compounds, including flavonoids and lycopene. This is also supported by Priani and Fakih (2021), who reported that pomelo peel contains the flavonoid hesperidin. Hesperidin is a bioactive compound believed to have potential as an anticancer agent, as it can induce cell death through apoptosis and autophagy in cancer cells.

In this study, GC-MS analysis of salak pondoh seed extract revealed the presence of (2-phenyl-1,3-dioxolan-4-yl)methyl (E)-octadec-9-enoate, sitosterol, and 3-methoxy-4-hydroxy-cinnamic acid (Table 2), all of which have antioxidant and anti-inflammatory properties. In contrast, pomelo peel

extract contains propanoic acid, 2-methyl-, (dodecahydro-6a-hydroxy-9a-methyl-3-methylene-2,9-dioxoazuleno[4,5-b]furan-6-yl)methyl ester, [3aS-(3a.alpha.,6.beta.,6a.alpha.,9a.beta.,9b.alpha.)]-, and ethyl iso-allocholate. Thakur and Ahirwar (2019) reported that these compounds have anticancer potential by inducing apoptosis.

In Silico Testing with Molecular Docking

This study highlights the bioactivity of compounds in salak pondoh seed extract, focusing on their anti-inflammatory and pro-apoptotic effects. Specifically, 3-methoxy-4-hydroxy-cinnamic acid exhibits anti-inflammatory properties, while sitosterol and (2-phenyl-1,3-dioxolan-4-yl)methyl (E)-octadec-9-enoate demonstrate apoptotic potential. *In silico* docking studies revealed weak interactions between 3-methoxy-4-hydroxy-cinnamic acid and the COX-2 protein (5KIR), with a binding affinity of -9.4 kcal/mol, involving five amino acid residues. Despite this low binding affinity, the compound may exert its anti-inflammatory effects through alternative pathways. COX-2, a key enzyme in prostaglandin synthesis, is instrumental in mediating inflammation, pain, and fever.

The anti-inflammatory mechanism of 3-methoxy-4-hydroxy-cinnamic acid appears multifaceted, including interactions with COX-2 that reduce the activity of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6). Additionally, it is associated with decreased levels of the inflammatory enzyme myeloperoxidase (MPO), as reported by Rezaei et al. (2024). Similarly, sitosterol and (2-phenyl-1,3-dioxolan-4-yl)methyl (E)-octadec-9-enoate also exhibit low binding affinities toward COX-2, suggesting their anti-inflammatory effects may operate through alternative or complementary pathways rather than direct COX-2 inhibition. Binding affinities below -8 kcal/mol are typically indicative of strong interactions; thus, the weaker affinities observed here underscore the likelihood of indirect mechanisms of action.

In addition to their anti-inflammatory potential, sitosterol and (2-phenyl-1,3-dioxolan-4-yl)methyl (E)-octadec-9-enoate exhibit apoptotic activity, potentially via the caspase-3 pathway. Endrini et al. (2014) demonstrated that sitosterol derived from *Strobilanthes crispus* induces apoptosis in HepG2 and Caco-2 cell lines. Similarly, Ahmad et al. (2018) reported that 9-octadecenoic acid methyl ester, a structurally related compound found in *Callistemon lanceolatus*, induces apoptosis in HepG2 cells. Furthermore, Pagliari et al. (2023) highlighted the anti-inflammatory activity of cinnamic acid derived from *Cinnamomum verum* against Caco-2 cells, providing additional context for the therapeutic potential of these bioactive compounds.

The extract from pomelo peel exhibits apoptosis-inducing activity through the caspase-3 pathway and antiproliferative effects, supported by the strong interactions of its bioactive compounds. *In silico* docking studies reveal low binding affinities for ethyl iso-allocholate and propanoic acid, 2-methyl-, (dodecahydro-6a-hydroxy-9a-methyl-3-methylene-2,9-dioxoazuleno[4,5-b]furan-6-yl)methyl ester, [3aS-(3a.alpha.,6.beta.,6a.alpha.,9a.beta.,9b.alpha.)]- with 5ITD protein, and hesperidin with the 3GJQ protein. These low binding affinity values indicate strong interactions with these target proteins, which are pivotal in regulating apoptotic and proliferative pathways.

The 5ITD protein is primarily associated with cell survival regulation, while 3GJQ plays a critical role in apoptosis modulation. Effective targeting of these proteins by the identified compounds enhances their potential to induce programmed cell death and suppress cancer cell proliferation. These findings align with previous studies, including Thakur and Ahirwar (2019), who demonstrated that ethyl iso-allocholate from *Trigonella foenum-graecum* induces apoptosis in A549 lung cancer cells.

Furthermore, hesperidin, a compound found in tea and *Citrus maxima*, has been shown to exert apoptosis-inducing effects on HepG2 and Bel7402 cells via the PI3K/AKT/mTOR pathway (Wen et al., 2022). Additionally, Dian et al. (2012) reported that cinnamic acid from incense at a dosage of 100 mg/kg exhibited significant anti-inflammatory activity in Wistar rats. These results collectively highlight the therapeutic potential of pomelo peel extract, emphasizing its role in modulating apoptosis and inhibiting cancer cell proliferation, likely through interactions with key proteins in these pathways.

Anti-Inflammatory Activity and Viability Testing

The anti-inflammatory activity of the single extract from salak pondoh seeds exhibited a higher inflammation inhibition value compared to the single extract from pomelo peel. However, the combination of both extracts, specifically in the 1S1J treatment group, demonstrated superior inflammation inhibition compared to all other treatment groups (Figure 3). This indicates that the combination of salak pondoh seed extract and pomelo peel extract enhances the anti-inflammatory effects, as assessed by the albumin denaturation assay.

In the cytotoxicity testing, both the single extract of salak pondoh seeds and the combination extracts significantly reduced cell viability compared to the untreated control (Figure 5). The treatments S, 1S3J, and 1S1J exhibited cytotoxic effects and fell within the category of moderate cytotoxicity. Thus, these three treatments have potential as adjunct therapies for cervical cancer.

Apoptosis and Inhibition of HeLa Cell Proliferation Testing

Cancer cell death can occur through apoptosis or necrosis. However, apoptosis is a preferable mechanism for cancer cell death compared to necrosis. The single extract from pomelo peel (J) exhibited the highest rate of cell death via apoptosis (Figure 5). In addition to inducing apoptosis, the single extract from pomelo peel (J) also caused necrotic cell death, which was even greater than that induced by doxorubicin. Nevertheless, the combination of pomelo peel extract with salak pondoh seed extract significantly reduced the percentage of necrotic cell death. The optimal combinations were found to be 1S1J and 1S3J.

Thus, the bioactive compounds in the extracts of salak pondoh seeds and pomelo peel demonstrated synergistic effects, as the combined extracts were able to suppress necrotic cell death while promoting apoptosis. The HeLa cell proliferation assay showed that all treatment groups effectively inhibited wound closure. In general, the combination of salak pondoh seed extract and pomelo peel extract was more effective at inhibiting wound closure compared to the positive control, doxorubicin, although this difference was not statistically significant (Figure 6). The inhibition of wound closure indicates a reduction in proliferation among HeLa cells. The 1S1J combination emerged as the most optimal and potential antiproliferative agent against HeLa cells. This finding is consistent with the *in silico* results, where the bioactive compounds in both extracts were shown to inhibit proliferation through the suppression of the PI3K pathway.

Principal Component Analysis (PCA)

The combination of salak pondoh seed extract and pomelo peel extract at a 1:1 ratio exhibits the highest anti-inflammatory activity compared to other treatments. Furthermore, this combination shows moderate toxicity toward cervical cancer cells (HeLa). The 1S1J treatment also demonstrates the ability to induce apoptosis, reduce cell death due to necrosis, and inhibit the migration and proliferation of HeLa cells. These effects are influenced by the different types of phytochemical compounds synthesized and their biomedical activities. Salak pondoh seed extract has anti-inflammatory activity, while pomelo peel extract exhibits antiproliferative properties. Both extracts also possess the ability to induce apoptosis. Therefore, the combination of these two extracts is necessary to maximize anti-inflammatory and antiproliferative effects, as well as apoptosis induction. This is supported by Belhouala et al. (2024), who reported that the combination of extracts from *Bryonia dioca*, *Aristolochia longa*, *Telephium imperiati*, and *Evernia prunastri* exhibited higher anticancer activity against HT-29, PC-3, and A-549 cell lines compared to individual extracts. This combination indicates a synergistic mechanism among the phytochemical constituents that enhances anticancer and antiangiogenic activities while reducing toxicity.

The mechanism of action of the combination of salak pondoh seed extract and pomelo peel extract as an anticancer adjuvant includes the inhibition of the PI3K protein produced by cancer cells. PI3K is an enzyme that plays a crucial role in promoting the proliferation and migration of cancer cells while inhibiting their apoptotic capabilities. Therefore, the inhibition of PI3K leads to a reduction in both proliferation and migration activities of cancer cells, while simultaneously inducing apoptotic activity. Additionally, the combination of extracts also demonstrates the ability to inhibit the COX-2 enzyme, resulting in a reduction of inflammatory activity (Song et al., 2020; Tan et al., 2023).

CONCLUSION

The extracts of salacca seed (*Salacca zalacca*) and pomelo peel (*Citrus maxima*) exhibit potential as adjunctive agents in cervical cancer therapy. Their combination in a 1:1 ratio, demonstrates higher efficacy compared to their individual extracts, especially in inhibiting inflammation, proliferation, and metabolism of HeLa cells. Further studies are needed regarding the optimal concentration of this 1:1 combination to maximize its inhibitory effects without adversely affecting overall cellular response.

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