

Effect of *Caesalpinia sappan* and *Annona muricata* isolates on VEGF-mediated angiogenesis in HeLa cells

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ABSTRACT

Angiogenesis plays an important role in the growth of cancer cells, including cervical cancer. The compounds in sappan wood (*Caesalpinia sappan* L.) and soursop leaves (*Annona muricata* L.) have potential anticancer properties. The objective of this study was to evaluate the effect of sappan wood and soursop leaf isolates on the cytotoxic activity and VEGF expression in HeLa cells. Sappan wood and soursop leaf isolates were obtained by column chromatography, while their characteristics were analyzed by Fourier transform infrared spectroscopy (FTIR) and liquid chromatography-high resolution mass spectrometry (LC-HRMS). The MTT assay was used to detect the cytotoxicity. The expression of VEGF was measured using immunohistochemical staining, while its interaction was studied using network pharmacology and molecular docking. The study identified that sappan wood isolate contains (3-Hydroxybenzyl) malonic acid and (+/-)-9-HpODE, while soursop leaf isolate contains (3-Hydroxybenzyl) malonic acid. Molecular docking data indicate that these compounds may suppress angiogenesis activity by inhibiting SRC, AKT1, EGFR, and VEGFR. Isolates of sappan wood and soursop leaves have IC₅₀s of 8.20 and 25.63 µg/ml. A combination of 1/8 sappan wood and 1/10 soursop leaves showed a strong synergistic interaction in causing cell death. VEGF expression in the treated samples was less than in the control cells. Thus, it can be concluded that the single isolate or combination of sappan wood and soursop leaf acts by suppressing VEGF expression and inhibiting the vascularization process.

INTRODUCTION

Cervical cancer ranks as the fourth most commonly diagnosed cancer and also the fourth leading contributor to cancer deaths in humans. It was predicted that in 2020, there would be 604,000 newly diagnosed cases and 342,000 mortalities around the world [1]. Cancer caused by human papillomavirus (HPV) is the second-highest case after breast cancer, suffered by many Indonesian women [2].

Angiogenesis becomes one of the hallmarks of cancer, providing a key function in tumorigenesis by producing new blood and lymphatic vessels [3]. Initially, tumors obtain nutrients and oxygen through diffusion from surrounding tissues, but as the tumors become larger, the oxygen supply decreases, creating a hypoxic microenvironment that stimulates excessive angiogenesis [4]. Promising therapies to terminate the proliferation of gynecologic cancers include blocking the activity of vascular endothelial growth factor (VEGF) in angiogenesis [5]. Anti-VEGFA therapy monoclonal antibodies, such as bevacizumab, showed efficacy in antiangiogenic treatment for both early and advanced cervical cancer [6]. However, it has various side effects, such as causing vasoconstriction, inducing high blood pressure, and proteinuria due to kidney damage [7].



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The use of natural medicines as herbal products is an alternative that is widely chosen because of their lower side effects. Sappan wood contains plenty of bioactive substances, such as brazilein, brazilin, and protosappanin A, which have antioxidant, anti-inflammatory, and anticancer activities [8]. Petroleum ether extract from sappan wood was also shown to reduce VEGF expression in liver cancer cell lines (HuH-7) [9]. Besides sappan wood, soursop leaves also have anticancer activity with bioactive acetogenins, alkaloids, and flavonoids [10]. Soursop leaf extract showed antiangiogenic activity in bovine aortic endothelial cell cultures [11]. Soursop leaf essential oil has been reported to decrease the VEGF levels in rodent breast cancer models [12]. Sappan wood and soursop leaves, or their combination, have potential as anticancers [13–15]. Therefore, this study aimed to evaluate the effect of sappan wood and soursop leaves and their combinations on the cytotoxic activity and VEGF expression in HeLa cells. This study also identified compounds in isolates and used an *in silico* approach to support research data.

MATERIALS AND METHODS

Preparation of sappan wood isolate

For preparing the isolate, Sappan wood (SW) was obtained from B2P2TOOT, Indonesia (TL.02.04/D.XI.5/16536.407/2023). Based on previous research [14], 200 g of the sappan wood was macerated with 2 L of methanol for 24 h. The filtrates were evaporated at 55 °C. The extract was diluted into methanol and partitioned with n-hexane (1:1, v/v). The resulting methanol phase was further partitioned with methanol, distilled water, and ethyl acetate (1:3:1, v/v/v), and the concentrated ethyl acetate phase. The active compounds from this phase were separated through column chromatography with a stationary phase of silica gel 60, while the mobile phase was mixed with chloroform, ethyl acetate, and methanol (1:1:1, v/v/v). Separation was performed by thin-layer chromatography (TLC) using a G254 silica plate and a mixture of chloroform and methanol solvent (10:2, v/v). Fractions with the same spot pattern were collected and purified by preparative TLC until a single spot was obtained as an isolate.

Preparation of soursop leaves isolate

Soursop leaves (SL) were obtained from B2P2TOOT, Indonesia, with no. voucher TL.02.04/D.XI.5/16536.408/2023. The isolation process, as described in a recent study [15], involved the extraction of soursop leaf powder with chloroform (1:10 b/v) via percolation. Chloroform extract was separated by column chromatography method, with a stationary phase of silica gel 60 and a mobile phase of chloroform. Furthermore, the separation was carried out by TLC on silica G254 plates with n-hexane and ethyl acetate solvents (8:2 v/v). Fractions with similar spot patterns were mixed and purified through preparative TLC until a single spot was obtained as an isolate.

Characterization of isolates by using FTIR and LC-HRMS

Isolate samples were separated from silica using methanol for sappan wood samples and chloroform for soursop leaf samples. The solutions were homogenized, filtered, and, before testing, smeared on KBr pellets, dried, and analyzed with FTIR in the 4000–400 cm⁻¹ wave range [16].

The isolated sample was loaded into a tube, and 1 mL of MeOH (MS grade) was added. The samples were extracted by vortexing for over 30 seconds and sonicating for 30

minutes. Then, samples were microcentrifuged for 5 min at 1400 x g. The supernatant was filtered with a 0.22 µm PTFE filter before LC-HRMS injection. MeOH served as the blank for metabolomic analysis. Results were processed using Compound Discoverer® software (Thermo Scientific, USA) [17].

Evaluation of ADME (Absorption, distribution, metabolism, and excretion), toxicology parameters, and network pharmacology construction

The compounds from LC-HRMS were identified with PubChem. These SMILES were used for ADME and Toxicology analysis with Swiss-ADME and pkCSM [18]. The targets of the compounds were generally analyzed using Swiss Target Prediction [19]. Targets of angiogenesis in cancer were obtained from Gencards [20]. The targets of the active compound and the common targets of angiogenesis were formed into a Venn diagram. The overlapping targets were analyzed by STRING with a medium score (>0.70). Centrality measures use topological measures, including node degree, betweenness centrality, and closeness centrality [21]. GO and KEGG were analyzed using DAVID with p-value <0.05 [22].

Molecular docking

Protein structures, SRC (1Y57), AKT1 (3O96), EGFR (1M17), VEGFR1 (3HNG), and VEGFR2 (3VHE), were received from the Protein Data Bank and pre-processed with PyMol 3.0.3. The active compounds of each isolate were obtained from the PubChem database in SDF format. Molecular docking was performed with PyRx 0.8 to estimate the binding affinity of the compounds with VEGF receptors. The root mean square deviation value used is less than three [23,24]. Compounds with the lowest binding affinity values were visualized using Discovery Studio v16.

Cell culture and cytotoxic assay

The Health Research Ethics Commission of RSUD Dr. Moewardi in Solo City granted ethical approval for this study (No. 2.115/VIII/HREC/2024). HeLa cells were received from Gadjah Mada University and cultured with RPMI medium (Gibco, USA). Cytotoxicity testing employed the MTT colorimetric assay [25] with various concentrations of 6.12, 12.5, 25, 50, and 100 µg/ml, in triplicate per isolate. The cells and media controls were administered with 100 µl of RPMI medium per well. The sample absorbance was analyzed at 595 nm by a microplate reader, and then the IC₅₀ value was calculated [15]. Additionally, combination cytotoxicity testing was conducted with dilution concentrations of ½, ¼, ⅛, and 1/10 of the IC₅₀ value, alongside 16 further concentration variations. The results of the single and combination-dose tests were utilized to generate the combination index [26]. Using a previous method [12], the absorption value of each well on the microplate of the MTT assay data was examined to find the percentage of viable cells. Furthermore, a linear regression equation was developed to estimate the relationship between concentration and cell viability. The variable Y was substituted with 50, so that the IC₅₀ value of variable X was obtained.

$$\% \text{ living cells} = \frac{\bar{X} \text{ treatment absorbance} - \bar{X} \text{ media control absorbance}}{\bar{X} \text{ cell control absorbance} - \bar{X} \text{ media control absorbance}} \times 100\%$$

The combination dose cytotoxic test was carried out by calculating the combination index (CI) with the same mode of action and different modes of action using the formula [13]. The acquired CI values were evaluated, as shown in Table 1 [26].

$$\text{Action Mode Same} = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2}$$

$$\text{Different Action Mode} = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2} + \frac{(D)_1 \times (D)_2}{(Dx)_1 \times (Dx)_2}$$

Notes: (D)₁ = concentration of SW isolate combination treatment

(Dx)₁ = concentration of SW isolate single treatment with response (D)₁

(D)₂ = concentration of SL isolate combination treatment

(Dx)₂ = concentration of SL isolate single treatment with response (D)₂

Table 1. Interpretation of CI values.

CI Value	Interpretation
< 0,1	Very strong synergistic
0,1 – 0,3	Strong synergistic
0,3 – 0,7	Synergistic
0,7 – 0,9	Mild-moderate synergistic
0,9 – 1,1	Additive
1,1 – 1,45	Mild-moderate antagonistic
1,45 – 3,3	Antagonistic
> 3,3	Strong-very strong antagonist

Immunocytochemistry of VEGF

Immunohistochemical staining was performed with the VEGF-A (C1) antibody (Santa Cruz Biotechnology; sc-7269, Dallas, USA). VEGF expression was observed under light microscopy and measured semi-quantitatively by assessing the proportion of stained cells per 100 cells and the staining intensity. Five fields of view at 400x magnification were used to obtain the average amount of stained cells per hundred cells [27]. Semi-quantitative assessment [28] was interpreted based on the stained area [28] and staining intensity [29], using Table 2.

Table 2. Interpretation of semi-quantitative assessment of VEGF expression.

Proportion of cells stained	Score	Staining intensity	Score	Staining type	Total score
none	0	None	0	Negative staining	≤ 3
≤10 %	1	Weak (light yellow)	1	Positive staining	> 3
11-50 %	2	Medium (light brown)	2		
51-75 %	3	Strong (dark brown)	3		
>75 %	4				

Statistical analysis

Data cytotoxicity was analyzed using ANOVA one-way and Tukey's post hoc test with a level of significance at $p < 0.05$.

RESULTS

Secondary metabolites from sappan wood and soursop leaf isolates

Characterization of the isolates was carried out using FTIR and LC-HRMS. Each absorption at a certain wavenumber in FTIR describes the presence of a specific functional group [30]. The identification of functional groups of secondary metabolite compounds of sappan wood and soursop leaf isolates using FTIR is shown in Table 3. Interestingly, the (3-hydroxybenzyl) malonic acid was exclusively detected in soursop leaves, whereas (3-hydroxybenzyl) malonic acid and (+/-)9-HpODE were solely identified in sappan wood (Table 4).

Table 3. Characterization of sappan wood and soursop leaf isolates using FTIR.

Sappan wood isolate		Soursop leaf isolate	
Wavenumber (cm ⁻¹)	Functional group	Wavenumber (cm ⁻¹)	Functional group
3307,33	O-H	3019,84	=C-H alkene
2943,60	C-H aliphatic	1519,03	C=C aromatic, (lactam = lacton+amida)
2831,72			
1448,98	C=C aromatic	1423,65	C-H alkane
1416,82		1214,37	C-O ester
1115,32	C-O-C ether	1045,94	C-O alcohol
1022,37	C-O	743,67	C-H alkene
631,66	C-H aromatic	668,28	C-H aromatic

Table 4. Characterization of sappan wood and soursop leaf isolates using LC-HRMS.

No.	Name	Molecular mass	RT (min)	Chemical structure
Soursop leaves (SL)				
1.	(3-Hydroxybenzyl) malonic acid	210.05215	5.224	
Sappan wood (SW)				
1.	(3-Hydroxybenzyl) malonic acid	210.05215	5.224	
2.	(10E,12Z)-9-Hydroperoxy-10,12-octadecadienoic acid or (+/-)9-HpODE	312.22984	11.375	

Drug permeability of SW and SL isolates

The Lipinski rule was used to determine the level of drug permeability in the body. This rule consists of 4 criteria, including having a molecular weight (MW) ≤ 500 g/mol, $w\text{LogP} \leq 5$, hydrogen bond acceptor (nHA) ≤ 10 , and hydrogen bond donor (nHD) ≤ 5 . A molecule is predicted to have poor bioavailability if it violates two criteria from the Lipinski rule [31]. According to the ADME-Swiss analysis (Table 5), all detected compounds were accepted by the Lipinski rule.

Table 5. ADME and toxicology prediction.

Name	(+/-)-9-Hpode	(3-Hydroxybenzyl) Malonic Acid
MW (g/mol)	312.23	210.052
wLogP	5.35	1.32
nHA	4	4
nHD	2	2
TPSA (Å²)	66.76	74.60
Lipinski's violation	1	0
Bioavailability Score	0.55	0.85
Intestinal absorption (human)	89.14	94.406
VDss (human)	-0.794	-1.171
BBB permeability	-0.143	-0.018
CYP3A4 inhibitor	Yes	No
Total clearance	2.117	0.284
AMES toxicity	No	No

Network pharmacology of angiogenesis targets

The intersection of angiogenesis targets (4,826) and active compounds targets (191) contained 138 proteins (Figure 1A). Protein-protein interaction (PPI) network was formed with the core targets of SRC, AKT1, and EGFR (Figure 1B). The results of KEGG analysis showed that 132 pathways were identified, including the pathway in cancer, PI3K-AKT signaling pathway, and VEGF signaling pathway (Figure 1C). GO analysis resulted in 376 BPs, 46 CCs, and 110 MFs, with the top five results shown in Figure 1D.

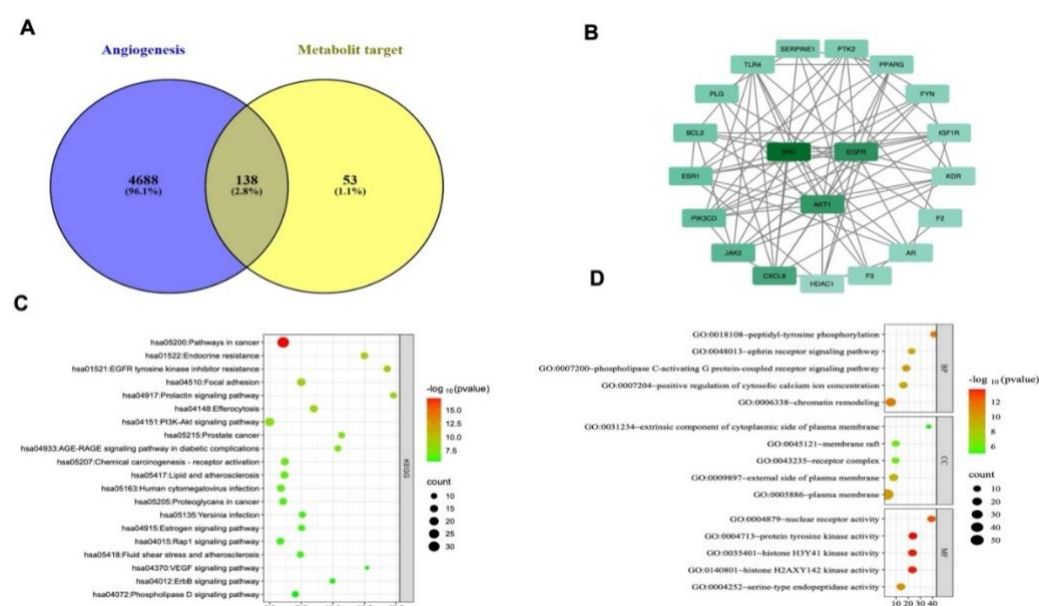


Figure 1. Network pharmacology of angiogenesis targets. A) Venn diagram of potential anti-angiogenesis isolate targets, B) Core PPI tissue targets, C) Top 20 pathway KEGG analysis results, D) GO analysis results including biological process (BP), cellular component (CC), and molecular function (MF) categories.

Interaction of SW and SL isolates with the targets of angiogenesis

The results of the interaction of active compounds with proteins are shown in Table 6, while the interactions are shown in Figure 2. The interaction of compounds with the core targets of PPIs showed that the interaction of (3-Hydroxybenzyl) malonic acid is greater than (+/-)-9-HpODE, except for AKT1. Nevertheless, all compounds were able to interact well with the target proteins as they had binding affinity values less than -5.0 Kcal/mol [32].

Table 6. Binding affinity score.

No.	Compounds	Binding affinity (kcal/mol)				
		SRC (1Y57)	AKT1 (3O96)	EGFR (1M17)	VEGFR-1 (3HNG)	VEGFR-2 (3VHE)
1.	(3-Hydroxybenzyl) malonic acid	-6.3	-7.2	-6.6	-7.3	-7.5
2.	(+/-)-9-HpODE	-5.7	-7.3	-6.1	-7.1	-7.4
3.	Inhibitor control	-8.7	-14.6	-6.9	-10.7	-12.6

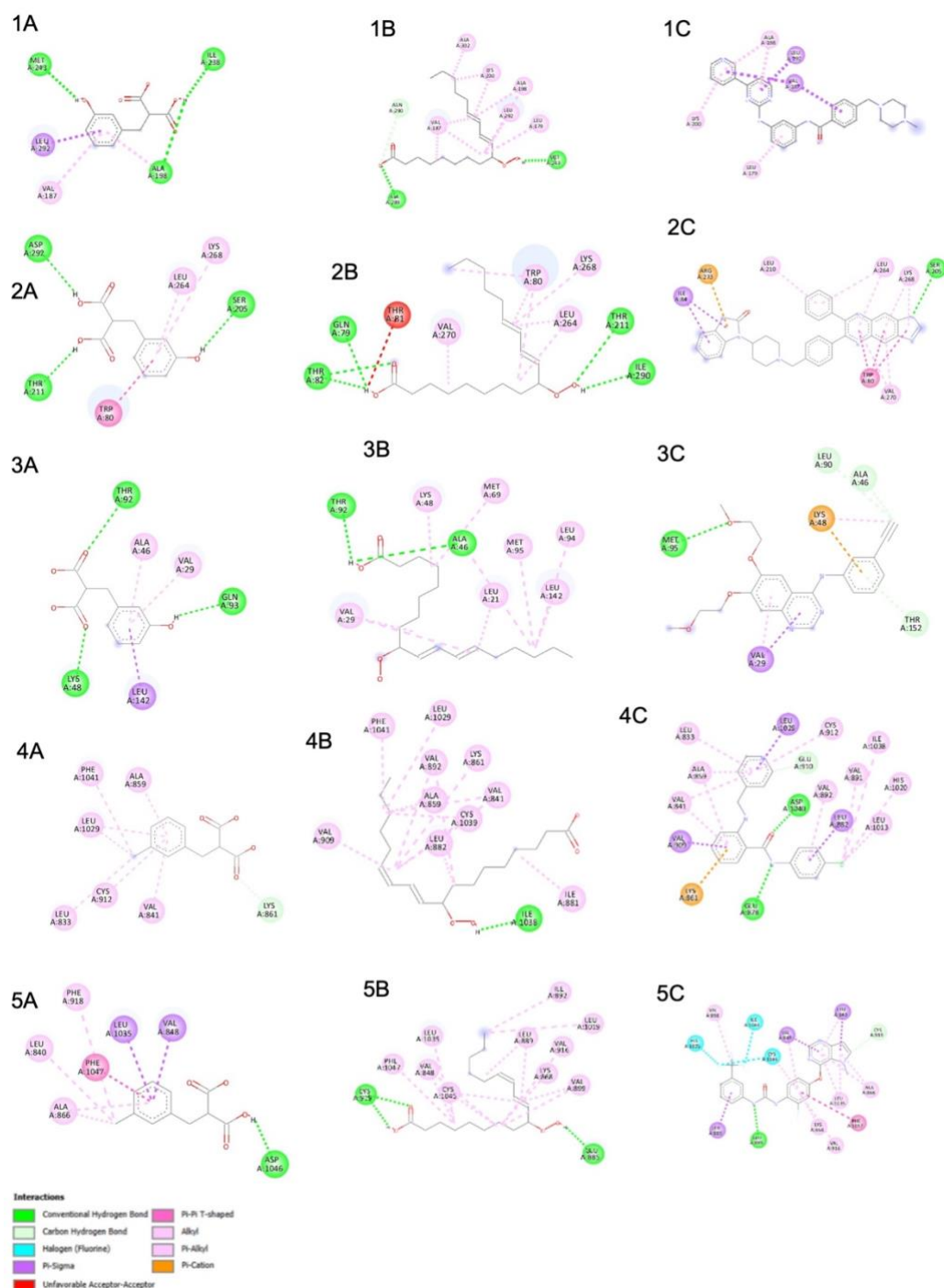


Figure 2. Interaction of protein 1) SRC, 2) AKT1, 3) EGFR, 4) VEGFR-1, and 5) VEGFR-2 with active compounds, including A) (3-Hydroxybenzyl) malonic acid, B) 9-HpODE, and C) Inhibitor control, respectively.

SW and SL isolates are toxic to HeLa cells

The inhibitory concentration (IC_{50}) value is the concentration that inhibits the growth of cancer cells by 50% and shows a compound's potential toxicity to cells. The IC_{50} value is determined from the linear regression curve equation between the percentage of cell viability and log concentration. The linear equation obtained is used to calculate the IC_{50} value [33]. Figure 3 shows that the viability of HeLa cells was best at the lowest concentration (6.12 $\mu\text{g/ml}$) and lowest at the highest concentration (100 $\mu\text{g/ml}$) in the case of both isolates.

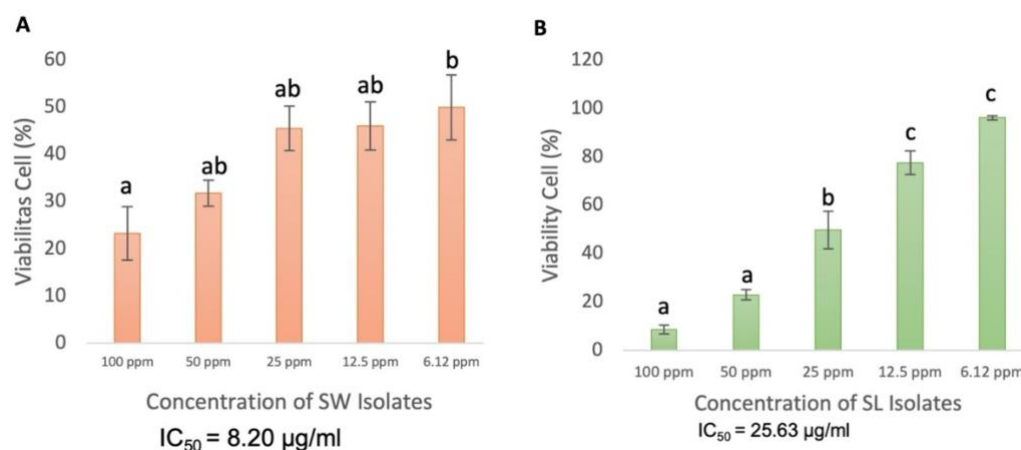


Figure 3. Cell viability at a single dose of A) Sappan wood (SW) and B) Soursop leaf (SL) isolates. *Values with the different superscripts (a-c) are significantly different ($p > 0.05$).

SW and SL isolates exhibited a synergistic effect on cytotoxicity

The combination index is the degree of interaction effect between two components combined in a certain dose and is related to the efficacy of the drugs or components [34]. Table 7 presents the results of evaluating the combination index of the cytotoxic impact. The 1/8 SW-1/10 SL combination showed a strong synergistic effect, indicating that mixing these isolates may boost the cytotoxic effect.

Table 7. Combination index results of cytotoxic effects.

Combination	Same action mode	Different action mode	Category
$\frac{1}{8}$ SW- $\frac{1}{4}$ SL	1.03	1.06	Additive
$\frac{1}{8}$ SW- $\frac{1}{8}$ SL	0.53	0.55	Synergistic
$\frac{1}{8}$ SW- $\frac{1}{10}$ SL	0.25	0.25	Strong synergistic

Combined effect of SW and SL isolates on the expression of VEGF

Immunocytochemistry was conducted to determine the effectiveness of sappan wood isolates and soursop leaves in inhibiting VEGF protein expression, which plays a role in the angiogenesis process. VEGF staining is essentially cytoplasmic, but the nucleus can also be stained [35]. A qualitative analysis of VEGF protein inhibition is presented in Figure 4, while the semi-quantitative assessment of VEGF expression is detailed in Table 8.

Activity of VEGF-A as a marker of angiogenesis that promotes tumor vascularization [36]. The results indicate that the control cell had positive staining, showing that VEGF was present and not blocked. The treatment with the sappan wood and soursop leaf isolates showed negative staining, indicating a reduction and blocking of VEGF-A.

Inhibition of the protein eliminates the supply of nutrients and oxygen from cancer cells by decreasing and inhibiting the development of blood vessels [37].

Table 8. Immunocytochemical observation of VEGF.

Samples	Average stained cell proportion score	Average staining intensity score	Average final score
Control cell	2	2	4
SW isolate	1	2	3
SL isolate	1	2	3
1/8 SW-1/10 SL	1	2	3

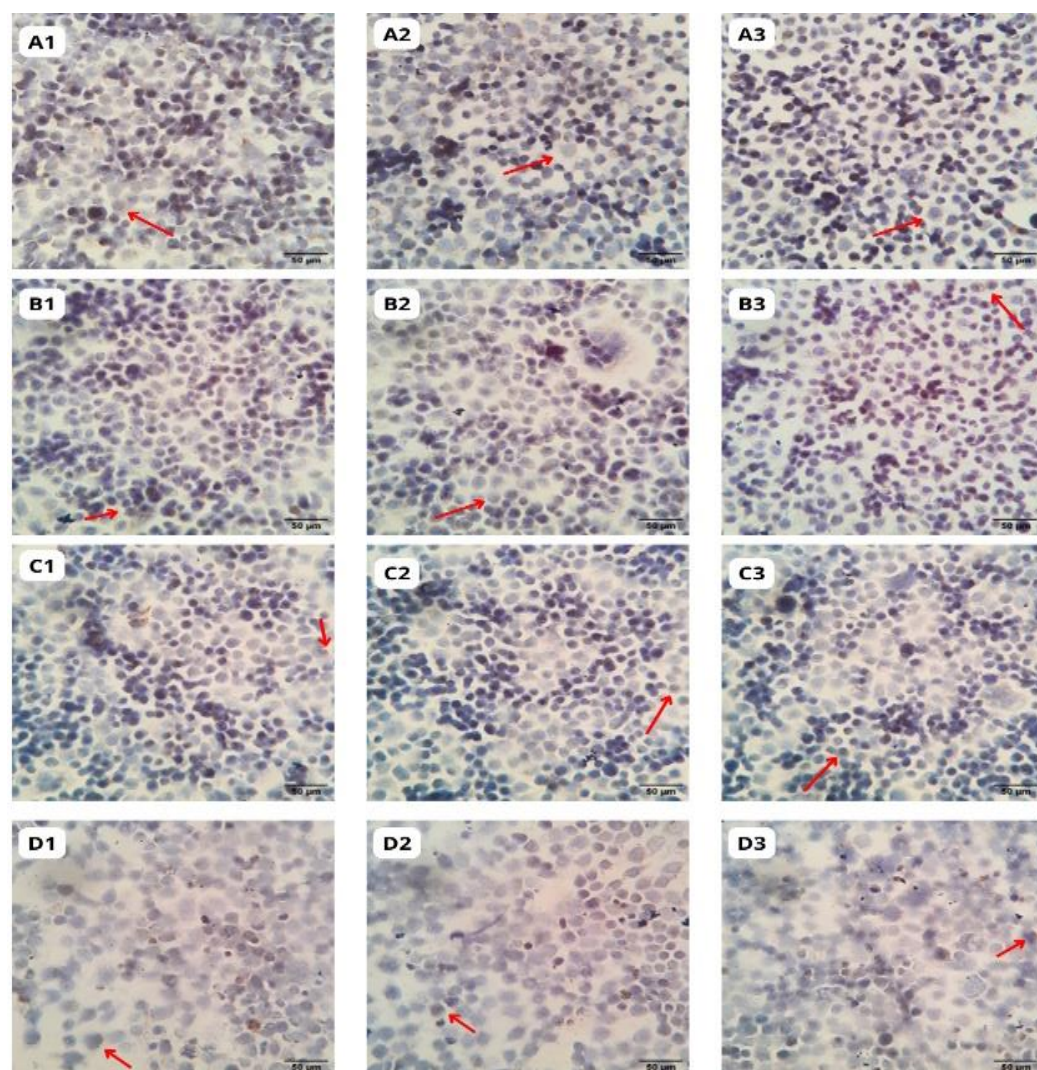


Figure 4. VEGF immunocytochemistry in (A1-A3) cell control, (B1-B3) SW isolate, (C1-C3) SL isolate, (D1-D3) 1/8 SW-1/10 SL; brown cell cytoplasm marked with red arrows.

DISCUSSION

In this study, LC-HRMS analysis found that sappan wood and soursop leaf have similar active compounds, specifically (3-hydroxybenzyl) malonic acid. The sappan wood isolate has (+/-)-9-HpODE, which includes linoleic acid metabolites [38]. Linoleic acid can induce apoptosis, inhibit invasion and metastasis, and stop the cell cycle in breast cancer cells [39]. This is consistent with the findings of the cytotoxicity test in this investigation.

ADME and toxicology studies show that the compounds have high intestinal absorption (>70%), meaning they have good oral bioavailability. The steady state volume of distribution (VD_{ss}) showed a negative value, which means the distribution of the compound is limited. BBB permeability showed a negative value, so the compounds had limited access to the brain. Additionally, (+/-)-9-Hpode was an inhibitor of CYP3A4. CYP3A4 is an enzyme in drug metabolism that causes drug-drug interactions [18]. Compounds that do not inhibit CYP3A4 have a better metabolic profile than CYP3A4 inhibitor compounds. All the compounds could be eliminated from the body, with (+/-)-9-Hpode having the highest elimination rate compared to the other compounds. Additionally, the compounds did not have mutagenic effects. Compounds with good bioavailability can be absorbed and distributed in the body, increasing the likelihood of interacting with target proteins.

Soursop leaf isolate has a higher IC₅₀ value than sappan wood isolate. Sappan wood isolate is classified as very toxic, while soursop leaves are moderate or moderately toxic. A compound's cytotoxic activity increases as its IC₅₀ value decreases [40]. The IC₅₀ value of soursop leaf isolated is 8.20 µg/ml, which is lower than the ethanol extract of soursop leaves, which is 35.51 µg/ml [41]. Meanwhile, sappan wood isolate (25.63 µg/ml) also has a lower IC₅₀ value than the ethyl acetate fraction of sappan wood (65 µg/ml) [42].

A combined dosage cytotoxicity test of sappan wood isolate and soursop leaves was performed to assess the combination's effect on HeLa cells [14]. This study found an additive response at 1/8 SW-1/4 SL, a synergistic response at 1/8 SW-1/8 SL, and a strong synergist response at 1/8 SW-1/10 SL. The best dose combination in this study is 1/8 SW - 1/10 SL because it has a strong synergistic response. This indicates that mixing the two isolated dosages will enhance the cytotoxic impact. This is reflected in the cell viability value of the combination dose (7.39%), which is lower than the single dose of 1/8 sappan wood isolate of 52.65% and 46.91% at a single dose of 1/10 soursop leaf isolate.

Vascular endothelial cells consist of VEGF receptors that promote angiogenesis. The interaction of VEGF-A and VEGFR-1 promotes distant metastasis, whereas the interaction between VEGF-A and VEGFR-2 stimulates VEGF signaling pathways, resulting in tumor development through increased cell proliferation [43]. Thus, inhibiting the binding process will impede the angiogenesis process. (3-Hydroxybenzyl) Malonic acid showed the strongest inhibitory effects on both VEGFR, with binding affinity values of -7.3 kcal/mol (VEGFR-1) and -7.5 kcal/mol (VEGFR-2). There are three types of VEGFR inhibitors: type I, which targets the ATP-binding pocket; type II, which targets the hydrophobic pocket closest to the ATP-binding site; and type III, which is a covalent inhibitor with irreversible binding to cysteine at specific sites [44]. Inhibitor type I binds to VEGFR-2 and occupies hydrophobic pocket I (GLY992, LEU840, and PHE918), while inhibitor type 2 binds in hydrophobic pocket II (ILE892, ILE1044, LEU889, and VAL898) [45]. (3-hydroxybenzyl) Malonic acid is classified as a type I VEGFR-2 inhibitor, while (+/-)-9-HpODE is classified as a type II VEGFR inhibitor type II in blocking the activity. Inhibition of VEGFR-1 occurs by blocking the ATP binding

site with the main amino acid residue of LYS861 [46]. It also binds to (3-hydroxybenzyl) malonic acid and (+/-)9-HpODE, inhibiting angiogenesis by blocking the interaction of VEGFR-1 and VEGF-A. This also supports the observation on VEGF-A expression that a single isolate treatment and the combination of sappan wood and soursop leaves can decrease VEGF-A activity. VEGF-A's significance as an angiogenesis marker that promotes tumor vascularization [36]. Inhibition of the protein eliminates the supply of nutrients and oxygen from cancer cells by decreasing and inhibiting the development of blood vessels [37].

In the present study, both *in silico* prediction and *in vitro* assessment of SW and SL isolates were conducted using HeLa cells, primarily to examine cytotoxic responses and VEGF expression. However, the current findings provide only a partial insight into the anticancer activity of these isolates. Further research should specifically investigate their selectivity and confirm their biological activity *in vivo*, utilizing organism-level responses to validate their therapeutic potential.

CONCLUSIONS

The sappan wood isolate contains (3-hydroxybenzyl) malonic acid and (+/-)9-HpODE melanoic acid, while the soursop leaf isolate contains (3-hydroxybenzyl) malonic acid. Both compounds *in silico* have the potential to inhibit VEGF activity. Sappan wood isolate and soursop leaf isolate had IC₅₀ of 8.20 and 25.63 µg/ml, respectively. In addition, the combination of 1/8 SW - 1/10 SL had a strong synergistic impact. The expression of VEGF in the treated samples was less than the controls. Therefore, it is concluded that the single isolate or combination of sappan wood and soursop leaf acts by suppressing VEGF expression and inhibiting the vascularization process.

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AUTHOR CONTRIBUTIONS

OPA, ANA, and EH created outlines and drafted the article. LNF, SSD, ANA, and WMR executed the experiments and analyzed the results. The manuscript's original draft was written by LNF and WMR. OPA, ANA, and EH reviewed the scientific substance of the article. The final submitted version of the article was reviewed and approved by all authors.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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