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The Potential of Paku Gajah (*Angiopteris evecta*) as Antitumor Through *In Vitro* and *In Silico* Studies

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ABSTRACT. Paku gajah (*Angiopteris evecta*) is one of the largest ferns which has been used empirically by the Dayak tribe of Kalimantan, Indonesia as a traditional medicine to treat various diseases, one of which is tumors. This research aims to determine the potential utilization of *A. evecta* stem extract as an antitumor by secondary metabolites analysis, toxicity and antitumor assay. The methods used in this study were gradual maceration using three solvents (*n*-hexane, ethyl acetate, and methanol), phytochemical screening, toxicity test, antitumor activity assay with the Alamarblue method, Liquid Chromatography-Mass Spectrometry (LC-MS) analysis, and molecular docking analysis. This study indicated that *A. evecta* stem extract contained secondary metabolites such as flavonoids, saponins, tannins, and steroids. The ethyl acetate and methanol were found as toxic extracts with LC_{50} 130.67 and 314.31 μg/mL, respectively. In line with the toxicity, the antitumor activity of the ethyl acetate extract was the highest with an IC_{50} of 240.94 μg/mL and phytochemical analysis revealed the presence of violanthin and angiopteroside in the extract. Molecular docking showed that the binding energy and inhibition constants of violanthin and angiopteroside against receptors were higher than standard ligand F82. The interaction between violanthin and the receptor results five hydrogen-bond (H-bond) with Lys920, Cys919, Asp1046, and Leu840, while the angiopteroside produces four H-bonds with Leu836, Leu834, and Arg831.

Keywords: Antitumor, molecular docking, paku gajah (*Angiopteris evecta*).

INTRODUCTION

Angiopteris evecta is a fern from the Marattiaceae family, which has local name paku gajah or paku raja. This plant is one of the largest ferns in the world (Hartini, 2015). This plant has been used empirically by the Dayak tribe of Kalimantan, Indonesia as a traditional medicine to treat various diseases, one of which is for the treatment of tumors (Noorcahyati, 2012).

Research on the bioactivity of *A. evecta* is quite limited. It was noted that there were only a few studies reporting the bioactivity of this plant, including antifungal (Khan & Omoloso, 2008), antibacterial (Mismawati et al., 2015), antituberculosis (Mohamad et al., 2011). The phytochemical isolated from its rhizomes, identified as angiopteroside, show activity as inhibitor of HIV 1 Reverse Transcriptase (Taveepanich et al., 2005). Best of our knowledge there has been no research regarding antitumor activity reported from this sample. Nevertheless, previous research conducted by Saleride et al. (2017) reported that *A. evecta* contain compounds from the class of flavonoids and tannins. Polyphenol group compounds including flavonoids and tannins have the potential as antitumor compounds because their cytotoxicity has been proven against various types of tumor cells (Greenwell & Rahman, 2015). On the other hand, research related to the antitumor bioactivity of plant extracts from the same genus, namely the species *Angiopteris ferox* Copel also showed good antitumor activity (Aisyah et al., 2020). The results of these two studies indicate that *A. evecta* may have the potential to inhibit tumor cell growth.

Tumors are pathological disorders of cell growth characterized by excessive or abnormal cell proliferation (Sinha, 2018). Malignant tumors are one of the leading causes of death worldwide (Ferlay et al., 2021). Malignant breast tumors are the most common malignant tumor diagnosed in women worldwide and the primary cause of death from malignant tumors (Smolarz et al., 2022).

Recently, treatment for tumors relies on immunotherapy, surgery, and chemotherapy which are relatively expensive and harmful due to the side effects (Sinha, 2018). Therefore, other alternative treatments are needed with relatively affordable costs

and do not cause adverse side effects to the body. One effort that can be done is to utilize natural-based medicines or medicinal plants.

Research on medicinal plants begins with extraction procedures which are an important step in processing bioactive compounds (Azwanida, 2015). Choosing extraction solvent can affect the type of compound extracted. Many studies have utilised various solvents for extraction and revealed differences in the chemical and bioactivity of extracts obtained (Asbanu et al., 2019; Istiqomah et al., 2021). In this study, we use three different extraction solvents such as *n*-hexane, ethyl acetate, and methanol to obtain a broad range polarity of compounds possibly contained in the sample

The potency of plants as antitumor is known through a series of tests, starting with toxicity tests and *in vitro* tests on tumor cells. According to Marliza & Oktaviani (2021), toxicity have a correlation with the cytotoxic power of antitumor compounds, so they are often used for initial screening to search for antitumor compounds. Specifically for breast antitumor studies, research by Eltayeb et al. (2017); Mallick et al. (2015); Nordin et al. (2018) used MCF-7 breast tumor cells in several plant extracts, so in this study, we use the cell line for antitumor assay. Moreover, a molecular docking analysis was performed to explore the interaction between compounds from extract that has strongest activity and the protein receptor involved in suppressing the growth of tumor cells. Accordingly, this research search for antitumor candidates by utilizing natural products *A. evecta* by applying *in vitro* analysis such as toxicity and cytotoxicity analysis, and *in silico* analysis through molecular docking simulation.

EXPERIMENTAL SECTION

Sample Preparation and Extraction

A. evecta stem was collected from Berau, East Kalimantan, Indonesia. The stems were cleaned, cut and dried at a room temperature. The sample was ground using 60 mesh sieve. Samples were weighed as much as 750 g and macerated using 1 L of *n*-hexane for 1 x 24 hours, then filtered to obtain filtrate and residue (the maceration was carried out repeatedly and controlled with TLC eluted by 10% ethyl acetate in *n*-hexane). All the filtrates obtained were combined and evaporated to obtain a thick extract. The residue was then macerated with ethyl acetate and methanol the same as the previous procedure.

Phytochemical Test

The extract of *A. evecta* were tested for phytochemical constituens to identify the contains of alkaloids, flavonoids, saponins, tannins, and steroids/terpenoids (Shaikh & Patil, 2020). The following was the complete procedure of phytochemical analysis carried out.

(i) Alkaloids Test. Three test tubes were filled with the sample solution, and then a few drops of Dragendorff's, Mayer's, and Wagner's reagent were added into each tube. The presence of an alkaloid was indicated by an orange/red/yellow precipitate with Dragendorff's reagent, a yellowish cream-colored precipitate with Mayer's reagent, and a brownish red-colored precipitate with Wagner's reagent.

(ii) Flavanoids Test. The sample solution was put into test tubes then added a magnesium powder, and a few drops of concentrated HCl. Observe the reaction after 2 minutes. The presence of a flavonoid was indicated by a red color solution.

(iii) Terpenoids/Steroids Test. Test tubes containing the sample solution were filled with 0.5 mL of Lieberman Burchard's reagent. The presence of terpenoids was indicated by a red/purple color and a green/blue color for steroids after 5 minutes reaction.

(iv) Saponins Test. Test tubes containing the sample solution were filled with 0.5 mL of distilled water and shaken briskly. The appearance of foam lasting for thirty seconds suggested the presence of saponin.

(v) Tannins Test. Test tubes containing the sample solution were filled with 0.5 mL of distilled water, followed by a few drops of 1% $FeCl_3$. The precipitate in various colors (red, purple, green, blue, and black) was indicate the presence of the tannin compound.

Antitumor Assay with the Alamarblue Method Against MCF-7 Tumor Cells

MCF-7 cells were grown in DMEM supplemented with 10% FBS and 1% penicillin. Then incubated in an incubator at 37 °C and 5% CO_2 and subculture when nearly fused using 0.25% trypsin-EDTA. Cultured MCF-7 cells were grown in 96-well plates (3 × 103 cells/well for MCF-7) and 10 µL of trypan blue cell solution was added slowly using a pipette. the number of healthy cells was counted and the number of (viable) cells per mL was determined. Cells were cultured into 96 well plates, then incubated for 24 hours at 37 °C and 5% CO_2 gas. After that, the cells were treated with different extract concentrations for 48 hours. The media in each well was discarded. 100 μL of the mixed solution (9 mL of medium and 1 mL of presto blue reagent) was added to each well of the microplate and then incubated for 1-2 hours until a color change was seen. Furthermore, the absorbance was measured at a wavelength of 570 nm using a multimode reader.

LC-MS Analysis

The mobile phase solution was prepared by adjusting the mobile phase to 100 mL of acetonitrile and water at a ratio of 9:1. The column was prepared by pouring the buffer solution into the column until equilibrium was reached. The flow rate was set at 1 mL/min. Extract samples were injected into the LC-MS column. The mobile phase begins to flow until no sample remains or is bound in the column.

Molecular Docking

The three-dimensional structure of the MCF-7 breast cell protein (PDB ID: 6GQO) was downloaded

from the Protein Data Bank website (https://www.rcsb.org/structure). All residues in the protein structure were removed and prepared using the Dock Prep menu in Chimera software using the AM1-BCC semi-empirical method, while standard ligands from the structure of the protein complex were taken and prepared using the same method as the protein. Prepared proteins and ligands were saved in ".pdb" format (Pettersen et al., 2004).

Three-dimensional structures of violanthin and angiopteroside compounds were made using Avogadro software (Hanwell et al., 2012). Furthermore, the ligands were prepared by optimizing them using Chimera software. The optimization process uses the AM1-BCC semi-empirical method and was saved in ".pdb" format. The molecular docking process is carried out using AutoDock 4.2 software with the help of the AutoDockTools (Morris et al., 2009). Each ligand is anchored to the active site of the protein. Docking parameters are made by setting the grid box to 40 × 40 × 40 Å which is centre on the ligand, the coordinates of the ligand are adjusted so that they have the same coordinates as the standard ligand shape when complexed with the target protein and spacing of 0.375 Å then saved in ".gpf" format". The docking process is set to produce 10 conformations and runs at a maximum evaluation energy of 2.500.000. The sampling algorithm used is the Lamarckian genetic algorithm (Morris et al., 1998) to obtain binding energy data and predict the inhibition constant. The validation of the docking process was determined by observing the RMSD value of the standard ligand redocking with a value of no more than 2 Å. Docking results were observed using the Discovery Studio Visualizer software.

RESULTS AND DISCUSSION

Extraction

The extraction in this study was carried out by a multilevel maceration method using 3 solvents with different polarities.

The solvents used were *n*-hexane (nonpolar), ethyl acetate (semipolar), and methanol (polar). The purpose is that all the compounds in the sample can be extracted based on the polarity of each solvent. The maceration process was carried out repeatedly using the same solvent and controlled by TLC. The maceration is continued with the next solvent if the stain on the TLC plate has decreased. This is done to ensure that all compounds have been maximally extracted. Data from **Table 1** shows that the highest yield was obtained from the methanol extract, followed by the ethyl and, finally, the *n*-hexane extract of *A. evecta*. These results are consistent with research conducted by Rahmawati & Mustarichie (2018) which reported that the content of polar compounds in *A. evecta* extract is higher than semi-polar and non-polar compounds.

Phytochemical Test

The results of the respective phytochemical screening of *n*-hexane, ethyl acetate, and methanol extract are shown in **Table 2**. **Table 2** shows the different phytochemical screening results for each extract. Based on the results of the phytochemical screening, *n*-hexane extract contained steroids and saponins, ethyl acetate extract contained flavonoids, and methanol extract contained tannins, flavonoids, and saponins. This is consistent with research conducted by Asbanu et al. (2019); Aziz & Anggarani (2021); Edison et al. 2020; Widyasanti et al. (2019) regarding differences in solvent polarity affecting the type of secondary metabolites extracted.

Alkaloid test results with Mayer, Wagner, and Dragendorf reagents for all extracts did not show the presence of alkaloid compounds. The results of the flavonoid test showed ethyl acetate extract and methanol extract were positive, while the *n*-hexane extract was negative. A change in color to yellow indicates a positive result in the flavonoid test. Concentrated HCl will hydrolyse the flavonoid glycosides into flavonoid aglycones, forming a yellow complex with magnesium.

Table 1. Extracts yield

A. evecta Stem Extract	Sample Weight (g)	Dry Extract Weight (g)	Yield (%)
n-Hexane Extract	750	1.875	0.250
Ethyl Acetate Extract	748.125	4.7	0.628
Methanol Extract	743.425	5.675	0.763

Table 2. Phytochemical test results

Secondary Metabolites	*n*-Hexane Extract	Ethyl Acetate Extract	Methanol Extract
Alkaloid (Mayer)	-	-	-
Alkaloid (Wagner)	-	-	-
Alkaloid (Dragendorf)	-	-	-
Flavonoid	-	+	+
Terpenoid	-	-	-
Steroid	+	-	-
Tannin	-	-	+
Saponin	+	-	+

Description: (-) negative, (+) positive

The steroid and terpenoid tests on the three extracts showed negative results for terpenoids, while steroids were only positive for *n*-hexane extract. The positive result for steroids is indicated by the formation of a green color, whereas a red color indicates the presence of terpenoids. The reaction in this test begins with the process of acetylation of hydroxyl groups using acetic anhydride. The resulting compound will undergo conjugation extension which causes a red or green color. The tannin test gave positive results only for methanol extract. The positive result is indicated by a change in color to blackish brown. The tannins in this test will form complexes with Fe^{3+} ions from $FeCl_3$. Meanwhile, saponin tests for *n*-hexane extract and methanol extract showed positive results. This test gives a positive result if a stable foam with a height of 13 cm is formed. This foam is formed due to the presence of hydrophobic aglycones and hydrophilic glucose in saponins.

Toxicity Test

The brine shrimp lethality test (BSLT) is carried out through three stages, which are the preparation of shrimp larvae, sample preparation, and toxicity test. The shrimp larvae preparation stage uses a container equipped with an aerator and a lamp. The function of the aerator is to aerate or supply oxygen to the hatchery and light from the lamp is needed because Artemia salina shrimp are phototropic. Shrimp larvae are used for BSLT after the larvae are 48 hours old. *Artemia salina* Leach shrimp larvae grew very fast after 48 hours of age, so it was assumed to be abnormal cell growth, such as tumor cells. The next stage is sample preparation. At this stage, the sample was diluted into several concentration variations (1000, 500, 250, 62.5, 31.2, and 15.6 ppm) to see the effect of concentration on the toxicity of the extract in shrimp larvae and this measurement was carried out triple (three times) in order to obtain good statistical data. Negative controls were also made to see the effect of using solvents on the death of shrimp larvae. The solvent used is DMSO because it is safe and can dissolve almost all polar and non-polar compounds. The last stage was the toxicity test for each extract, and the following results were obtained (**Table 3**).

The extract is categorized as very toxic if it has LC_{50} values < 30 ppm, is categorized as toxic if it has LC_{50} values of 30-1000 ppm, and is categorized as non-toxic if it has LC_{50} values larger than 1000 ppm. The level of toxicity can also indicate the potential for its activity as an antitumor, where the lower the LC_{50} value, the more toxic a compound is, and it has the potential to act as an antitumor (Reymon et al., 2021). The data in **Table 3** shows the extract that has the highest LC_{50} value among the three extracts, namely ethyl acetate extract, in the toxic category, because the LC_{50} value is 30-1000 ppm. Methanol extract occupies the second position in the toxic category. The extract that has the lowest LC_{50} value is *n*-hexane extract in the non-toxic category because the LC_{50} value is larger than 1000 ppm. The difference in the results of the toxicity tests of the three extracts is in accordance with the research conducted by Asbanu et al. (2019) who reported that the choice of solvent type in multilevel maceration affects the components of the extracted compounds and affects their bioactivity. Based on the data from Table 3, it can be concluded that ethyl acetate extract and methanol extract are toxic and have the potential to antitumors.

Antitumor Assay with the Alamarblue Method Against MCF-7 Tumor Cells

Antitumor tests in this study used 2 selected extracts from the BSLT results, which have the potential as antitumor compounds, ethyl acetate, and methanol extract. This test is carried out using the Alamarblue test or the resazurin reduction test. According to Syahputra (2015), this method is often used in research related to cell activity and metabolism tests because it is easy to use, affordable, does not require special skills, tests can be carried out quickly for a large variety of samples, is not toxic, and is useful for determining cell growth rate. This test was performed to determine the cytotoxicity of the test sample against various tumor cells. The assay is based on the reduction of non-fluorescent blue resazurin to highly fluorescent pink resorufin by living cells. Non-viable cells rapidly lose the metabolic ability to reduce resazurin so that it does not produce a fluorescence signal (Nyaboke et al., 2018). The color change in the resazurin compound is carried out by enzymes present in cells in the mitochondria and cytoplasm (Syahputra, 2015). The results of the antitumor test are shown in **Table 4.**

Table 3. BSLT method toxicity test results

A. evecta Stem Extract	LC_{50} Value (μg/mL)	Toxicity Category
Methanol Extract	314.31	Toxic
Ethyl Acetate Extract	130.67	Toxic
n-Hexane Extract	1948.78	Non Toxic

Table 4. Antitumor test results

A. evecta Stem Extract	IC_{50} Value (μg/mL)	Category
Methanol Extract	>1000.00	Inactive
Ethyl Acetate Extract	240.94	Weak
Cisplatin	13.33	Active

The cytotoxic activity of the extracts that attack tumor cells can be classified into four based on the IC_{50} value. An extract is said to be very active if it has an IC_{50} value ≤ 20 µg/mL, moderately active if IC_{50} > 20-100 µg/mL, weak if IC_{50} > 100-1000 µg/mL, and is said to be inactive if IC_{50} > 1000 µg/mL (Nordin et al., 2018). The data in **Table 4** shows that IC_{50} ethyl acetate extract is in the weak category and methanol extract is in the inactive category. As for the positive control or comparator used in this test is cisplatin, which has an IC_{50} value in the active category for pure compounds.

The morphological images of MCF-7 cells also support the data from the antitumor test results after being treated using positive samples and controls with various concentration variations, which can be seen in Figures **1-3. Figure 1** shows the morphology of MCF-7 cells after being treated with positive control, which is cisplatin with several concentration variations, from the highest concentration to the lowest. **Figure 1** shows that untreated tumor cells have an irregular shape due to abnormal cell division or proliferation. According to Haryanti et al. (2017), antitumor compounds or agents inhibit proliferation and induce cell death. One of the markers and differentiating categories of cell death is morphological changes followed by changes in biochemical functions, such as cell metabolism. Morphological changes that occur in treated cells are early markers of apoptosis induction. Induction of apoptosis results in cell cycle disruption, which will continue to inhibit the proliferation process. As seen in **Figure 1**, the higher the concentration of cisplatin given, the fewer the number of tumor cells that develop. Tumor cells began to decrease significantly at a concentration of about 12.50 µg/mL. **Figure 2** shows the morphology of MCF-7 cells after being treated with ethyl acetate extract with different concentrations. It shows that the higher the concentration of ethyl acetate extract given, the fewer the number of tumor cells that develop. Tumor cells began to decrease significantly at concentrations around 250 µg/mL.

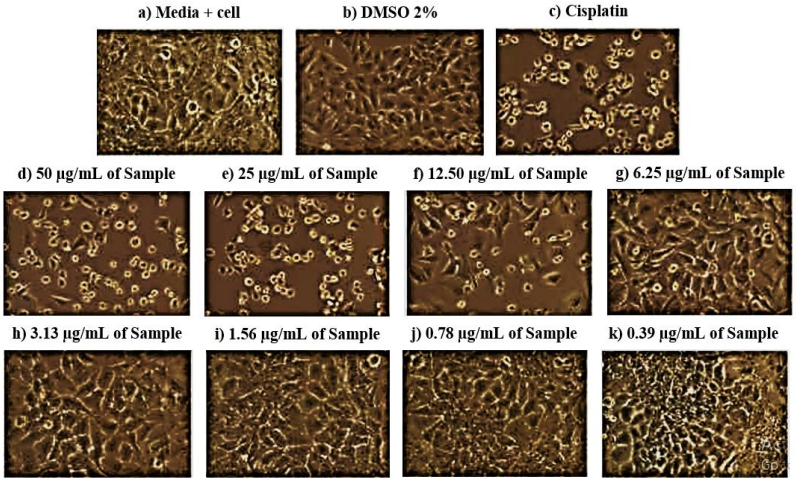


Figure 1. MCF-7 cell morphology from antitumor test results using cisplatin

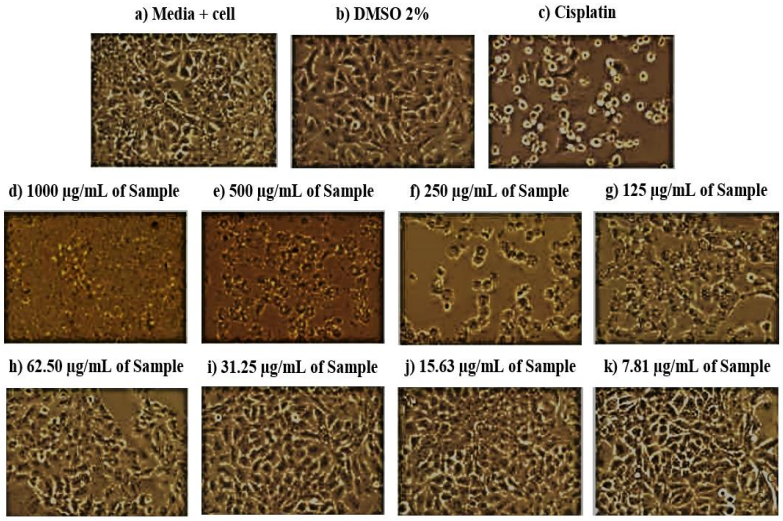


Figure 2. MCF-7 cell morphology from antitumor test results using ethyl acetate extract

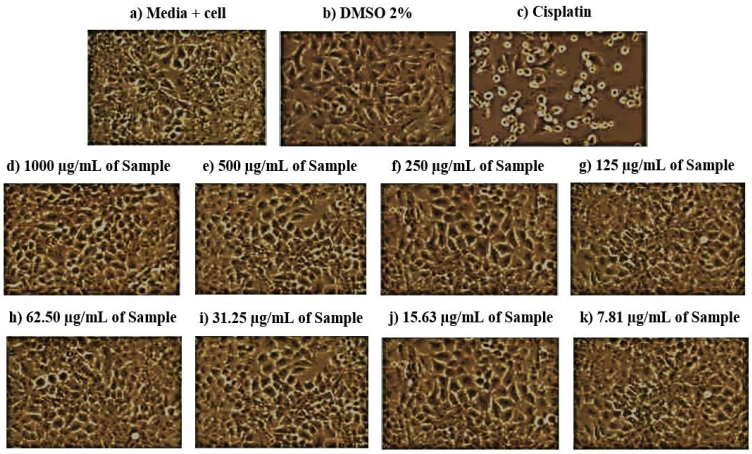


Figure 3. MCF-7 cell morphology from antitumor test results using methanol extract

Figure 3 illustrates the morphology of MCF-7 cells after being treated with methanol extract with various concentrations, from the highest concentration to the lowest. Figure 3 shows that there was no change or inhibition of tumor cell growth starting from a concentration of 7.81-1000 µg/mL. This can be seen from the number of irregularly shaped cells.

Based on the IC_{50} value and cell morphology images, the ability of the two extracts belongs to the weak antitumor group to be inactive against MCF-7 cells. Between the two extracts it is known that ethyl acetate extract has higher antitumor activity than methanol extracts antitumor activity. These results could be due to differences in the content of compounds in each extract, as it is known that the results of the phytochemical screening showed that ethyl acetate extract contains flavonoids.

The content of flavonoids in ethyl acetate extract is thought to play a role in its antitumor activity. This agrees with an *in vitro* study conducted by Abotaleb et al. (2019); Chirumbolo et al. (2018); Rodríguez-García et al. (2019) who reported that compounds belonging to the flavonoid group have strong antitumor activity. In an *in vivo* study regarding the structure-activity relationship for flavonoids as antitumor agents conducted by Golonko et al. (2023); Lankala et al. (2022); Xie et al. (2015) obtained information that the hydroxyl groups of flavonoids and their substituents are in certain positions which determine how strong their activity as an antitumor is. Hydrophobic substituents such as phenyl groups, alkylamino chains, alkyl chains, and heterocyclic groups containing nitrogen or oxygen usually increase the activity of all types of flavonoids. However, methylation of the hydroxyl groups generally decreases its activity. According to Golonko et al. (2023), flavonoids work as antitumor through various mechanisms ranging from counteracting tumor-triggering free radicals such as reactive oxygen species (ROS), playing a role in arresting the cell cycle, inducing apoptosis, autophagy, and suppressing tumor cell proliferation and invasion.

Toxicity Test

The results of chemical content analysis using the LC-MS instrument for the most active extract from the antitumor test, which is ethyl acetate extract, are shown in **Figure 4**. The peak height on the chromatogram indicates the relative abundance of a compound in the extract, so the higher the peak, the more abundant the amount in the ethyl acetate extract injected into the chromatography. Based on the liquid chromatography (LC) chromatogram in **Figure 4**, it can be seen that the peak with the highest peak intensity is at a retention time of 15.30 minutes. The peak has mass spectrum (MS) data in **Figure 5**.

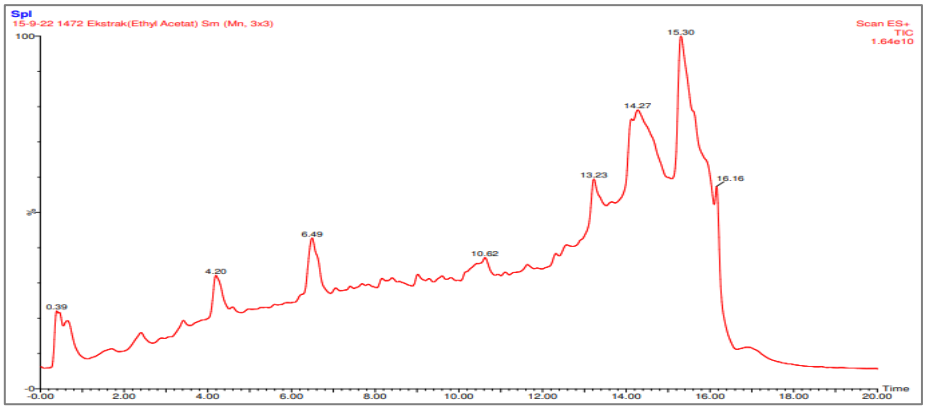


Figure 4. Ethyl acetate extract chromatogram

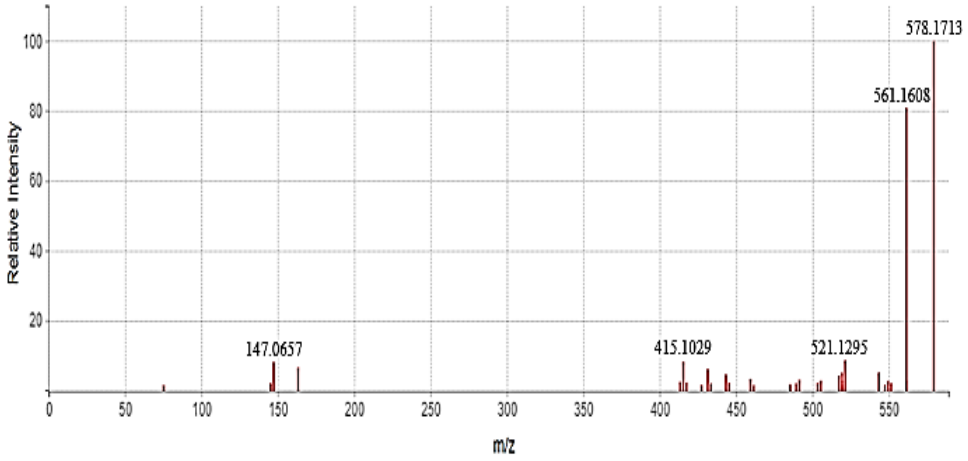


Figure 5. MS chromatogram of retention time peak 15.30 minutes

The MS data in **Figure 5** shows the presence of a compound with a molecular weight or *m/z* of around 578. The compound that is estimated to have the same *m/z* as the data is violanthin. Violanthin is a flavone C glycoside compound which is a flavone substituted for hydroxyl groups at positions 5, 7, and 4, beta-D-glucopyranosyl at position 6 and 6-deoxy-alpha-L mannopiranosil at position 8. This compound was previously isolated from the *A. evecta* plant by Wallace et al. (1981). Violanthin is reported to have several bioactivities, such as anti-cholinesterase activity (Dung et al., 2015) and antioxidant activity (Vukics et al., 2008). As for the fragmentation pattern of this compound, it is in accordance with the MS data fragmentation pattern in **Figure 6**.

The peak at a retention time of 6.49 minutes has the following mass spectrum (MS) data (**Figure 7**). The MS data in **Figure 7** shows the presence of a compound with a molecular weight or *m/z* of around 290. The compound that is estimated to have the same *m/z* as the data is angiopteroside. Angiopteroside is an O-glycosyl compound. This compound is a glycoside in which the sugar group is attached via one carbon to another via an O-glycosidic bond. Angiopteroside was previously isolated from the rhizome of *A. evecta* by Taveepanich et al. (2005). This compound was reported to have specific antitumor activity against lung tumor cells, inhibition of HIV 1 Reverse Transcriptase (Taveepanich et al., 2005), and antiadipogenic (Lamichhane et al., 2020). As for the fragmentation pattern of this compound, it is in accordance with the MS data fragmentation pattern in **Figure 8**.

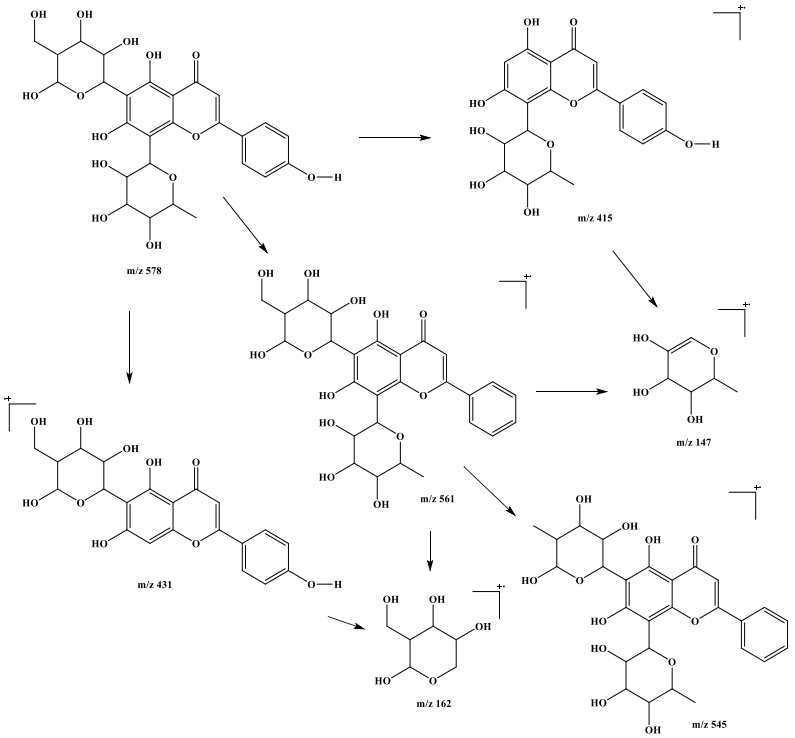


Figure 6. Fragmentation pattern of violanthin

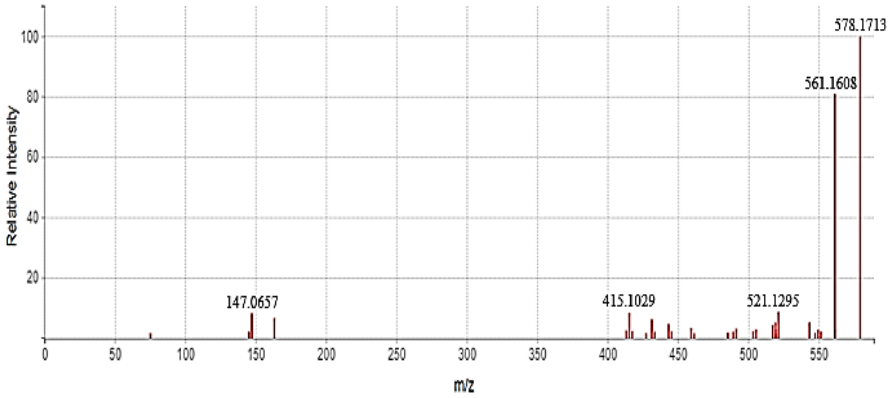


Figure 7. MS Chromatogram of retention time peak 6.49 minutes

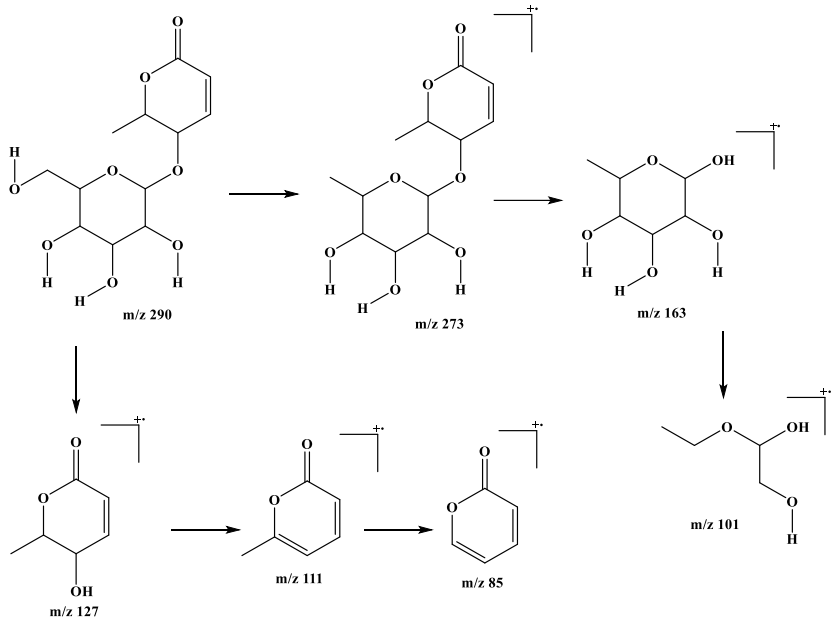


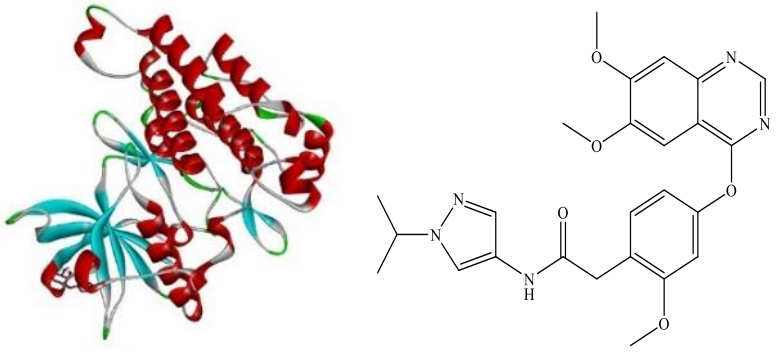
Figure 8. Fragmentation pattern of angiopteroside

Molecular Docking

The VEGFR2 receptor kinase, identified by the PDB code 6GQO, was utilized in the molecular docking analysis. The selection of the VEGFR2 kinase receptor was based on its overexpression in both primary and metastatic breast tumor, which plays a role in tumor metastasis, angiogenesis, and carcinogenesis. VEGFR2 represents a crucial pathway involved in tumor angiogenesis. Thus, blocking or inhibiting this pathway would lead to an effective response against angiogenesis and tumor growth (Abdel-Mohsen et al., 2020). This aligns with the findings of a recent study by Ramzan et al. (2023) which employed molecular docking to evaluate the antitumor potential of benzothiazinone compounds targeting the VEGFR2 protein.

The 3D structure of the VEGFR2 receptor kinase (**Figure 9a**) of PDB is in the form of a complex with a ligand, so separation is necessary in order to obtain an independent structure of the VEGFR2 receptor kinase protein and a standard ligand structure. The standard ligand available is 2-[4-(6,7-dimethoxyquinazolin-4-yl)oxy-2-methoxy-phenyl]-{N}-(1-propan-2-ylpyrazol-4 yl)etanamide marked with the symbol F82. The use of F82 as a standard ligand in VEGFR2 docking was also carried out by Ramzan et al. (2023). The structure of the standard ligand F82 can be seen in **Figure 9b**.

The docking process begins with a standard ligand redocking procedure to validate the docking program and calculate the energy value so that it can be implemented in violanthin and angiopteroside compounds. The parameter used to observe the redocking results is the root mean square deviation (RMSD) value. RMSD is a quantity that expresses the deviation of a state compared to its initial state, in this case, the position, location, interaction with the target protein, and other physicochemical properties of the standard ligand. The RMSD value must be less than 2 Å, because the higher RMSD value, the more the redocking results deviate from the initial state of the ligand (Rollando, 2018). The redocking results showed 10 conformations with different RMSD values (**Table 5**).



(a) (b)

Figure 9. 3D structure of VEGFR2 protein (a) and standard ligand F82 (b)

Table 5. RMSD value of redocking analysis of standard ligand F82

Conformation	RMSD Value (Å)
1	3.22
2	0.81
3	1.03
4	3.41
5	3.66
6	3.72
7	3.69
8	3.60
9	0.97
10	1.27

Based on **Table 5**, conformation 2 is the conformation with the lowest RMSD value, so the docking method on the design compound will follow the conformational redocking method 2. Comparison of the initial structure and conformation 2 is carried out in 3D to clarify the visualization of the overlapping of the two molecules. The overlap of the standard ligands from the redocking results can be seen in **Figure 2**.

Figure 10 shows that the overlap between the standard ligands and conformation 2 is good, this is in accordance with the low RMSD values obtained. In addition, a bond energy of -11.73 kcal/mol was also obtained. The docking process was continued for violanthin and angiopteroside compounds. Table 6 shows the results of the docking of the two compounds with the lowest energy values of all the conformations and provides information regarding the interaction of hydrogen bonds between VEGFR2 protein residues and ligands. Visualization of the interactions in two dimensions (**Figure 11**) and three dimensions (**Figure 12**) was made to clarify the hydrogen bonding interactions.

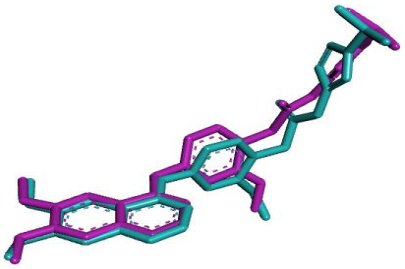


Figure 10. Overlapping of standard ligands (light blue) and conformation 2 (purple color) as a result of redocking

Table 6. Docking results of violanthin and angiopteroside compounds

Compound (PubChem CID)	Inhibition Constant (mM)	Binding Energy (kcal/mol)	H-bond Interaction Residue
Violanthin (442665)	0.00266	-7.6	Lys920, Cys919, Asp1046, Leu840
Angiopteroside (101512362)	0.97	-4.11	Leu836, Leu834, Arg831

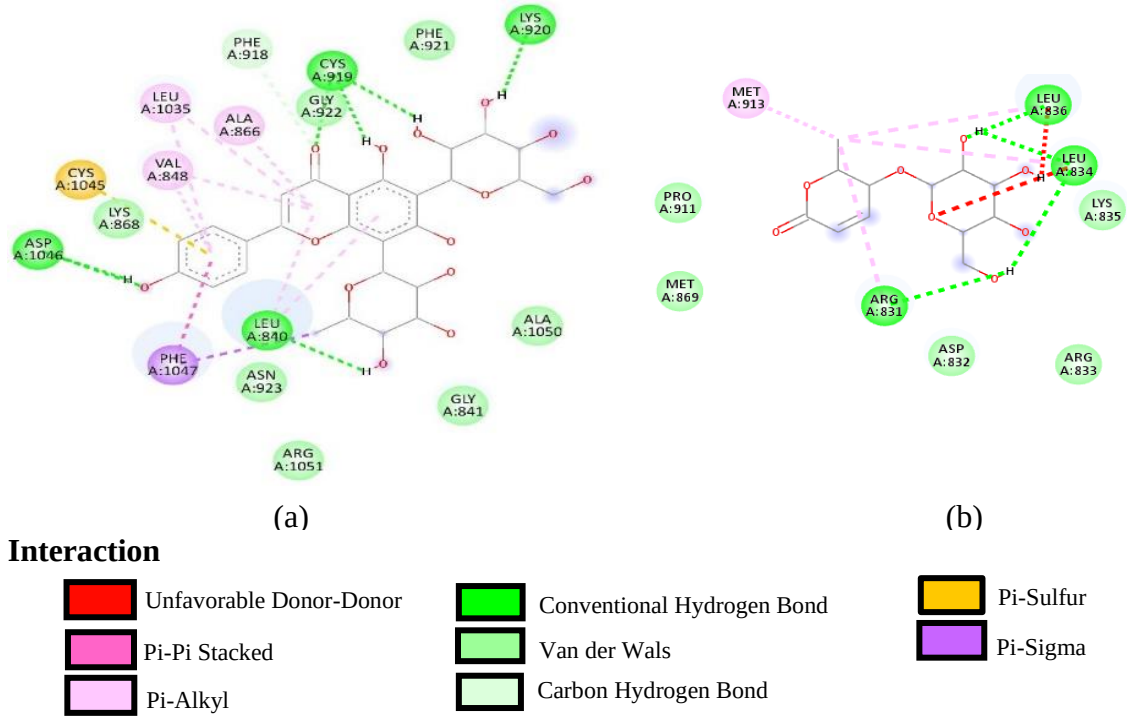


Figure 11. 2D visualization of target protein interactions with violanthin (**a**) and angiopteroside (**b**) ligands

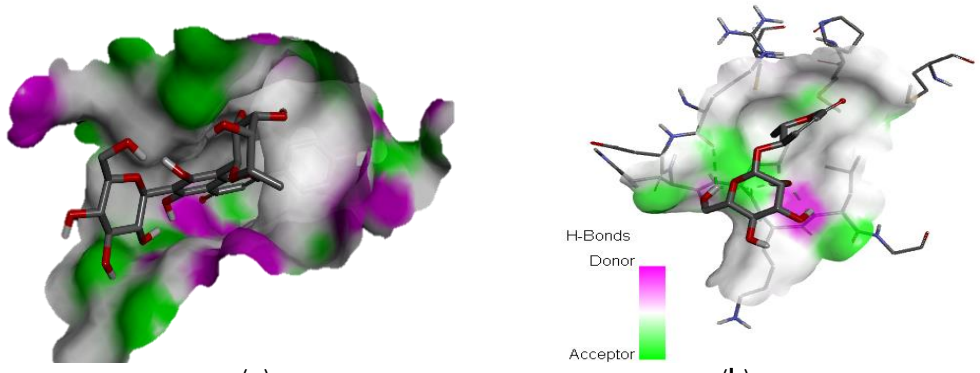


Figure 12. 3D visualization of target protein interactions with violanthin (**a**) and angiopteroside (**b**) ligands

The inhibition constant is a value that indicates the ability of a compound to inhibit the action of its receptors, the smaller the value, the higher the inhibition of the compound. The value of the inhibition constant is also aligned or directly proportional to the binding energy. Binding energy is the energy required for a ligand to be able to interact and form various types of bonds (one of which is hydrogen bonding which plays an important role) with the receptor at the binding site, the smaller the value of the binding energy, the less energy is required for the compound to interact with the target so that it is also more stable the complex formed (Hartanti et al., 2022). Based on **Table 6**, the violanthin compound has the lowest binding energy and inhibition constant of the two compounds, which were docked with 5 hydrogen bond interactions, namely between the hydrogen atom of Cys919 and carbonyl oxygen, Cys919 oxygen atom with 2 hydrogen atoms from the hydroxyl group, oxygen atom Lys920 with a hydrogen atom from the hydroxyl group, Leu840 oxygen atom with a hydrogen atom from the hydroxyl group and between the oxygen atom from Asp1046 and a hydrogen atom from the hydroxyl group (**Figure 11a**). The binding energy value and inhibition constant of violanthin obtained were still higher when compared to the standard ligand F82. As for the results of the docking of the angiopteroside compound, the inhibition value and bond energy were higher compared to violanthin and the standard ligand F82 as well as the interaction of 4 hydrogen bonds, namely the oxygen atom Leu836 with a hydrogen atom from the hydroxyl group, the oxygen atom Leu834 with 2 hydrogen atoms from the hydroxyl group and between the oxygen atom of Arg831 and the hydrogen atom of the hydroxyl group (**Figure 11b**).

The 3D visualization in **Figure 12** shows a section of the receptor topography with regions that can act

as hydrogen donors (marked in pink) and regions that can act as hydrogen acceptors (marked in green). The topographical representation of these interactions supports the interactions visualized in 2D diagrams (**Figure 11**). The topography only states the probability of interaction, but there is not necessarily an interaction that is formed, this could happen because the distance needed to form bonds is quite far, there are steric obstacles, high energy requirements in forming bonds and so on.

CONCLUSIONS

This study indicates that *A. evecta* can be a source of bioactive compounds that have the potential to be developed as antitumor candidates. This can be seen in the ethyl acetate extract which demonstrates both cytotoxicity and toxicity, with LC_{50} and IC_{50} values of 130.67 and 240.94 μg/mL, respectively. Although the extract's activity data did not fit into the strong cytotoxic category, phytochemical analysis using LC-MS revealed the presence of compounds with a variety of bioactivities, including violanthin and angiopteroside. Molecular docking simulation confirmed the compound's potential as a breast antitumor candidate. The compounds were able to interact with amino acids on the active side of the target protein with binding energies of -7.6 and -4.11 kcal/mol, respectively.

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