Revealing the Potential of Compounds in Sappan Wood as Cervical Cancer Metastasis Chemopreventive Agent With MMP9 Target

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Abstract

Matrix metalloproteinase-9 (MMP9) has an essential role in cervical cancer metastasis. Sappan wood extract (SWE) from *Caesalpinia sappan* contains metabolites that have pharmacological effects and can potentially inhibit metastasis by targeting the protein markers. This research aims to discover the potency of compounds in *C. sappan* as chemopreventive agents for metastasis in cervical cancer by targeting MMP9. SWE was obtained by maceration with methanol and analyzed using thin layer chromatography (TLC). *In vitro* cytotoxicity test of SWE on HeLa cells was performed by direct counting method. MMP9 expression profiles and survival rates in cervical cancer patients were explored through bioinformatics studies by the GEPIA database. The CMAUP and PubChem databases were used to obtain the metabolomic profile of SWE. SWE compounds' activities on target proteins were obtained through KNIME software, while its interaction with MMP9 was analyzed using molecular docking with MOE software. We obtained SWE with a yield of 9.7% w/w. The extract contains brazilin and is indicated by the spot appearance at Rf 0.375. The cytotoxicity of SWE against HeLa cells was considered potential as the IC_{50} value was 54.93 µg/mL. Based on the bioinformatics analysis, there is a significant difference in MMP9 expression between normal and cervical cancer tissue. The patient's survival probability decreased if MMP9 was overexpressed. The molecular docking results showed that active compounds of SWE bind to the MMP9 inhibition site with higher affinity compared to the native ligand. This study reveals that SWE potential to be developed as a chemopreventive agent through metastasis inhibition in cervical cancer by targeting MMP9.

Keywords: *Caesalpinia sappan L., metastasis, bioinformatics, molecular docking, MOE.*

INTRODUCTION

Cervical cancer occupies the second position as cancer with the highest prevalence in Indonesia, with 50% of sufferers experiencing death (Sulistyawati, *et al*., 2020). Approximately 50% of cervical cancer patients die from metastasis (Wright and Kuhn, 2012). Metastasis is the most significant cause of death in cancer patients, where the pro-

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cess is regulated by matrix metalloproteinase-9 (MMP9) (Yousef, *et al*., 2014). Matrix metalloproteinases (MMPs) are essential enzymes that help cancer cells metastasize, especially in extracellular matrix degradation and cancer cell invasion (Deryugina and Quigley, 2006). Seeing the critical role of MMP9 in the spread of cervical cancer cells to other tissues, this protein is a potential target for antimetastatic chemopreventive compounds.

Sappan wood (*Caesalpinia sappan* L.) is one of the plants containing potential compounds as chemoprevention that promise cytotoxic and anti-MMP activities in various highly metastatic cancer cell lines such as MDA-MB-231, 4T1, MCF-7/HER2, and T47D (Hsieh, *et al*., 2013; Handayani, *et al*., 2016; Haryanti, *et al*., 2017; Jenie, *et al*., 2020; Handayani, *et al*., 2020). The mechanisms, including down-regulates the expression of MMP9, MMP2, Rac1, and p120; inhibits the gelatin-degradation activity of MMP9 and MMP2, and inhibits cell migration and invasion. This research is intended to reveal the ability of compounds in *C. sappan* such as brazilin, sappanchalcone, brazilein, protosappanin, brazilane, sappanone, caesalpiniaphenol, 3-deoxysappanone, 3-deoxysappanchalcone, 3'-deoxy-4-omethylepisappanol as a supportive agent in inhibiting cervical cancer cell metastasis with specific targets on MMP9 using bioinformatics analysis and cytotoxicity assay of SWE against HeLa cell line.

METHODS

Sappan Wood Extraction (SWE) by Maceration Method

Extraction is carried out in Citrus House, Cancer Chemoprevention Research Center (CCRC), Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia. The method was based on the previous reports of our research group (Handayani, *et al*., 2016; Haryanti, *et al*., 2017; Jenie, *et al*., 2020; Handayani, *et al*., 2020). Sappan wood simplicia was obtained from the Center for Research and Development of Traditional Medicinal and Medicinal Plants (B2P2TOOT) Tawangmangu, Jawa Tengah, Indonesia and has been determined to be a *Caesalpinia sappan* L. Before extraction, the sappan wood was ground to reduce the size of the sappan wood and sieved using a 40 sieved mesh to obtain a uniform size of sawdust. After that, the maceration process was carried out for 48 h using 100 grams of simplicia soaked in 1L methanol with constant stirring for 6 h/24 h. Then to optimize the extraction results, remaceration was carried out 1 time. After obtaining the volume of the macerate at the maceration and remaceration stages, it was followed by filtering the macerate with Whatman filter paper. The two filtrates were mixed and concentrated using a rotary evaporator at 100 rpm for 2 h at 55°C. The viscous extract was re-evaporated the solvent residue in the fume hood and weighed periodically until a constant weight was obtained. Yield percentage was obtained by dividing the final weight of the thick extract by the initial weight of 100 grams of powder multiplied by 100%.

Identification of Phytochemical Profile of Sappan Wood Extract (SWE)

The test was carried out at the Research Laboratory of the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia. Qualitative identification of the phytochemical profile of the active compounds of sappan wood was carried out using the Thin Layer Chromatography (TLC) to obtain a standardized extract. The comparison of a reference standard, brazilin (Sigma, St. Louis, USA), with a concentration of 1 mg/mL and SWE dissolved in methanol with a 6 mg/mL concentration were carried out on activated silica gel F254. Furthermore, the optimization method was carried out with a mobile phase combination using ethyl acetate: n-hexane (6:4 v/v) in the chamber to the elution limit on the plate. The elution results obtained were observed under visible light, UV light at wavelength 254 nm and 366 nm. The data was obtained in the form of Retention Factor (Rf), which is the result

of dividing the sample spot elution distance by the mobile phase elution distance and then compared with the standard Rf. If the same Rf is obtained, it can be concluded that the extract contains the same compounds as the standard.

Cell Culture

The HeLa cells were a collection of Cancer Chemoprevention Research Center (CCRC), Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia. Cells were cultured in the DMEM medium (Gibco, New York, USA) with the addition of 10% FBS (Gibco) and 1% penicillin-streptomycin (Gibco) in a 100 mm tissue culture dish. Cells were maintained in a 37°C incubator with 5% CO_2. Trypsin-EDTA (Gibco) was used to detach the cells upon confluence. The experimental assays were carried out at the Parasitology Laboratory of the Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Indonesia.

Cytotoxic Assay

We performed the cytotoxic assay by using a trypan blue exclusion test of cell viability. A day before the treatment, cells were transferred to 24 well-plate with a density of 10,000 cells/well. After the cells grow properly, each well-plate was treated with a series of concentrations of sappan wood extract (10, 25, 50, 100, 250, 500 μg/mL), then incubated for the next 24 h. Next, we harvested the cells using trypsin-EDTA. The culture medium was used to inactivate the trypsin. We took 10 μL of cells suspension and added 10 μL of 0.4% trypan blue (Sigma). Trypan blue will stain the non -viable cells because, in these cells, the membrane cell is not intact and permeable for trypan blue to stain the cells. Later, we counted the viable cells (unstained cells) under a light microscope. The number of the viable cells under samples-treatment then normalized with the number of untreated (control) cells and plotted in a graph (concentration vs. % of cell viability), then the IC_{50} was calculated. The potency of cytotoxicity is determined based on its IC_{50} (Weerapreeyakul, 2012), where the IC_{50} value 100–500 μg/mL is considered as moderate and IC_{50} value 10–100 μg/mL is considered as strong cytotoxicity.

Identification of the Metabolic Profile of the Active Compounds of Sappan Wood

Compounds contained in sappan wood were traced using the CMAUP database (http://bidd.group/CMAUP/), and the SMILES code of each compound was obtained by using the PubChem database. The results were arranged in Table 1. The data obtained were processed using KNIME.

Protein Expression and Survival Rate Analysis

The expression of MMP9 and its correlation with the survival rate of cervical cancer patients and MMP9 protein were traced through the database GEPIA and accessed through the page (http://gepia.cancer-pku.cn) (Tang, *et al*., 2017). The database uses CESC samples (cervical squamous cell carcinoma and endocervical adenocarcinoma. The correlation of the expression level of MMP9 and the patient's survival rate was used to determine the potential of MMP9 as a target protein in this study.

Prediction of Sappan Wood Compound Activity on Matrix Metalloproteinase-9 (MMP9)

The prediction model for the activities of the active compound of sappan wood was carried out using the KNIME application version 4.4.0 using TeachOpen computer-aided drug design (CADD) platform. The data of small molecules that have the type of activity as MMP9 inhibitor was obtained from the ChEMBL database with the target code CHEMBL321. We used the machine learning Random Forest (RF) Learner and Support Vector Machine (SVM) Classifier in this study. We chose the machine learner based on the results of the parameters, including the AUC value, Cohen's

kappa, and others. The higher the value, is expected the obtained results obtained are better and more accurate. After choosing the machine learner, we processed the SMILES code of the compounds in the sappan wood. Prediction models were used to find the most potent compound in the sappan wood to inhibit the target protein, MMP9. The higher the predicted value, the compound is expected to have better potential in inhibiting MMP9.

Molecular Docking and Interaction Analysis of Potential Compounds in Sappan Wood to Matrix Metalloproteinase-9 (MMP9)

Interaction between active compounds in sappan wood or native ligand of MMP9 with the MMP9 inhibitory active site was analyzed using Molecular Operating Environment (MOE) 2010. MOE is a molecular docking software licensed by the Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia, which is used to predict the affinity of compounds with the target protein binding sites and determine the orientation of the ligand at the binding site (Ray, *et al*., 2012). We obtained the structure of MMP9 protein (2OW1) from the PDB database at https:// www.rcsb.org/. We obtained a Root Mean Square Deviation (RMSD) value < 2 for all ligands based on the docking results, which means process docking is valid. The docking was done with the default in MOE 2010, *i.e.*, using placement Triangle Matcher, scoring London dG, and refinement forcefield. Prediction docking using MOE provides analytical parameters in bond energy, molecular docking score, and the distance between the molecules with the substrate and catalyst (Shin, *et al*., 2021). The method is considered valid if the RMSD value was less than 2 (RMSD < 2). We then analyzed the conformation of the lowest docking score compounds.



Figure 1. Sappan wood. (A) The simplicia of sappan wood. (B) The sappan wood extract (SWE), is obtained by maceration using methanol solvent.

RESULTS

Sappan Wood Extraction (SWE) by Maceration Method

Extraction was carried out by the maceration method with methanol as solvent. The yield of extract obtained from the maceration process was 9.7% w/w. SWE is a thick solid with light red to dark red color.

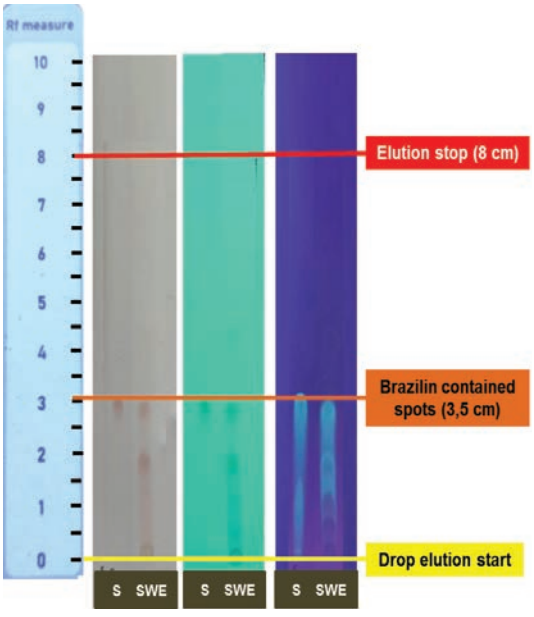


Figure 2. Identification of the phytochemical profile of sappan wood extract (SWE). The thin layer chromatography system used was ethyl acetate: n-hexane (6:4 v/v) as mobile phase and silica F254 as stationary phase. TLC profiles were observed at visible light, UV 254 nm, and UV 366 nm, respectively from left to right. The left lane was the reference standard (S), brazilin, (Rf 0.375) and the right lane was SWE (Rf 0.375).

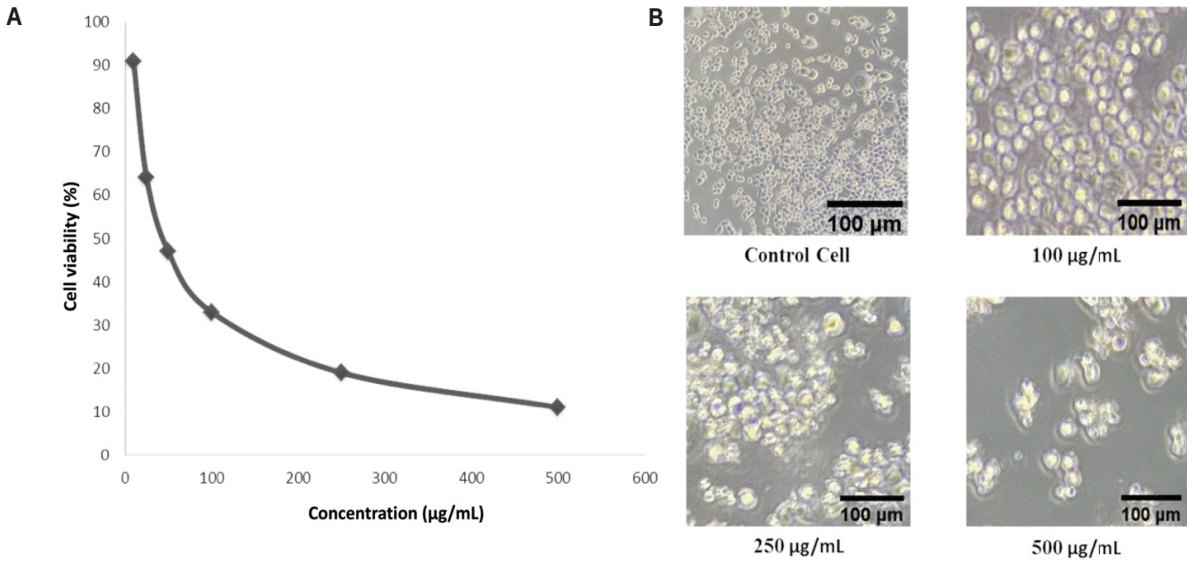


Figure 3. Cytotoxicity activity of sappan wood extract (SWE) against HeLa cells. (A) Cells treated with 10–500 µg/mL SWE. (B) Morphology of HeLa cells upon treatment of SWE for 24 h.

We performed the extraction based on Hung, *et al.* (2014) report, which shows that methanolic extract of *C. sappan* exhibits potent inhibitory activity against various cancers cell lines, especially against HeLa cells. Other studies from our research group also used methanolic extract in highly metastatic cancer cells (Handayani, *et al*., 2016; Haryanti, *et al*., 2017; Jenie, *et al*., 2018; Handayani, *et al*., 2020).

Identification of Phytochemical Profile of Sappan Wood Extraction (SWE)

We identified the phytochemical profile using TLC with brazilin as the reference standard. Analysis of the TLC was performed at visible light, UV 254 nm, and 366 nm (Figure 2). We observed a similar position between one of the spots from the extract and the reference standard (brazilin) at Rf 0.375. This result indicated that the methanolic extract of sappan wood contained brazilin.

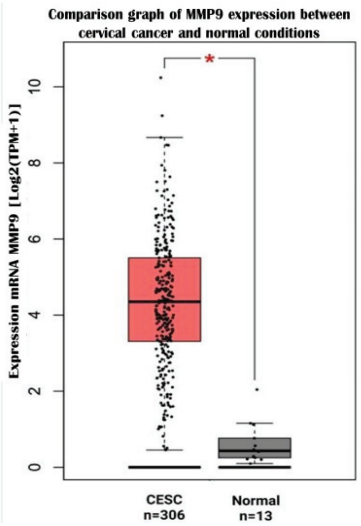


Figure 4. Matrix metalloproteinase-9 (MMP9) protein expression profile in cervical cancer and normal conditions. The symbol (*) indicates a significant difference in MMP9 protein expression between normal and cervical cancer conditions.

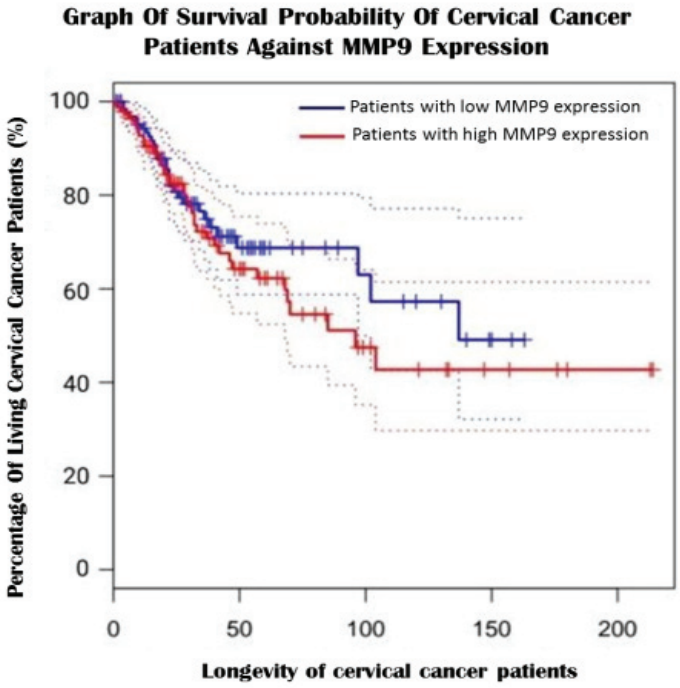


Figure 5. Correlation graph of Matrix metalloproteinase-9 (MMP9) protein expression level versus survival rate of cervical cancer patients. The data was obtained from GEPIA database. The red line shows patients with MMP9 overexpression and the blue line indicates patients with low MMP9 expression.

Cytotoxicity of Sappan Wood Extract (SWE) Against HeLa Cells

We performed a cytotoxic assay as described in the Method section. We treated the cells with a series concentration of SWE at 10, 25, 50, 100, 250, and 500 µg/mL. Based on the results (Figure 3), we calculated the IC_{50} and obtained the value of 54.93 µg/mL. According to Ueda, *et al.* (2000), an IC_{50} value less than 100 µg/mL is considered to have cytotoxicity potential.

Matrix Metalloproteinase-9 (MMP9) Protein Expression Profile in Normal and Cervical Cancer Cells

MMP9 protein plays an essential role in the invasion and metastasis of cancer cells. Analysis with the GEPIA database showed a significant difference in MMP9 expression in normal cells (13 samples) compared to CESC cancer cells (cervical squamous cell carcinoma and endocervical adenocarcinoma) (306 samples) (Figure 4).

Table 1. Metabolomic profile of sappan wood active compounds.

Chemical	SMILES
Brazilin	C1C2=CC(=C(C=C2C3C1(COC4=C3C=CC(=C4)O)O)O)O
Sappanchalcone	COC1=C(C=CC(=C1)O)C(=O)C=CC2=CC(=C(C=C2)O)O
Brazilein	C1C2=CC(=C(C=C2C3=C4C=CC(=O)C=C4OCC31O)O)O
Protosappanin A	C1C(=O)COC2=C(C=CC(=C2)O)C3=CC(=C(C=C31)O)O
Brazilane	C1C2COC3=C(C2C4=CC(=C(C=C41)O)O)C=CC(=C3)O
Sappanone A	C1C(=CC2=CC(=C(C=C2)O)O)C(=O)C3=C(O1)C=C(C=C3)O
Caesalpiniaphenol D	C1=CC(=C(C=C1C(O)OC2=C(C=CC(=C2)O)C=O)O)O
3-Deoxysappanone B	C1C(C(=O)C2=C(O1)C=C(C=C2)O)CC3=CC(=C(C=C3)O)O
3-Deoxysappanchalcone	COC1=C(C=CC(=C1)O)C(=O)C=CC2=CC=C(C=C2)O
3'-Deoxy-4-O-Methylepisappanol	COC1C2=C(C=C(C=C2)O)OCC1(CC3=CC=C(C=C3)O)O

Table 2. Parameters of the machine learner.

Machine Learner	Overall Accuracy	Cohen's Kappa	AUC
Random Forest (RF)	84.47%	0.619	0.904
SVM (Support Vector Machine Classifier)	66.96%	0.000	0.844

Based on this analysis, MMP9 was expressed 16 times higher in cervical cancer than in normal conditions. Therefore, it can be concluded that MMP9 is overexpressed in cervical cancer.

Correlation of Matrix Metalloproteinase-9 (MMP9) Expression Level and Cervical Cancer Patient's Survival Rate

The sample used is CESC (cervical squamous cell carcinoma and endocervical adenocarcinoma) data because these two types of cervical cancer are the most common (American Cancer Society, 2020). The correlation graph (Figure 5) of MMP9 expression level and Cervical Cancer Patient's Survival Rate showed a drastic decrease every month in high expression of MMP9 compared to low expression of MMP9. This data suggested that cervical cancer patients with MMP9 protein overexpression have a lower probability of survival than cervical cancer patients with low expression of MMP9 protein.

Identification of the Metabolomic Profile of the Active Compounds of Sappan Wood

The metabolomic profile of sappan wood (Table 1) was obtained through the database CMAUP, which was then selected based on the completeness of the data with potential activity to inhibit cancer cell metastasis. Furthermore, we obtained the SMILES code of the compounds by using PubChem.

Prediction of Active Compounds of Sappan Wood on Matrix Metalloproteinase-9 (MMP9)

Prediction model used to obtain a predictive value of the active compound activity of sappan wood as MMP9 inhibitor is RF. AUC value and overall accuracy RF was higher than SVM, with an AUC value of 0.904 and overall accuracy of 84.47% (Table 2). Therefore, in this study, we used RF as the machine learner.

The predicted values were obtained in tabular form and sorted from the compounds with the most prominent activity scores according to the potential order of the active compounds of sappan wood in inhibiting MMP9 protein (Table 3). Based on the results of Random Forest Learner, it is known that the highest predictive value was sappanchalcone and 3-deoxysappanchalcone. However, this does not rule out the possibility of other compounds inhibiting MMP9 with stronger ability than native ligand and existing patented MMP9 inhibitors.

Table 3. Prediction model of active compounds of sappan wood against matrix metalloproteinase-9 (MMP9).

Chemical	Prediction Activity RF
Sappanchalcone	0.89
3-Deoxysappanchalcone	0.89
Caesalpiniaphenol D	0.88
3-Deoxysappanone B	0.88
Protosappanin A	0.87
Sappanone A	0.87
Brazilein	0.86
Brazilane	0.81
Brazilin	0.8
3'-Deoxy-4-O-Methylepisappanol	0.77

Table 4. Affinity and RMSD value of results molecular docking.

Ligand	Docking score (kcal/mol)	RMSD (Å)
Caesalpiniaphenol D	-13.6828	1.3003
3-Deoxysappanone B	-13.4690	1.0496
Sappanchalcone	-13.0813	0.9106
3-Deoxysappanchalcone	-12.3484	1.7322
Brazilane	-12.1367	1.0187
3'-Deoxy-4-O Methylepisappanol	-11.7254	0.8469
Brazilin	-11.6196	1.9053
Brazilein	-11.3290	0.8418
Sappanone A	-10.9593	1.2945
Protosappanin A	-10.6561	0.9036
7MR (native ligand)	-10.4626	1.9725

Interaction and Affinity Analysis of Potential Compounds in Sappan Wood to Matrix Metalloproteinase-9 (MMP9)

The results of molecular docking showed that the value of docking score from all active compounds of sappan wood is lower than the (2R)-2-amino-3,3,3-trifluoro-N-hydroxy-2-{[(4-phenoxyphenyl)-sulfonyl]-methyl}propanamide (native ligand, 7MR) as presented in Table 4. Therefore, the active compounds of sappan wood have the potential to bind to the MMP9 protein inhibition site with better affinity than the native ligand and a patented MMP9 inhibitor. All docking results are valid because the RMSD value was less than 2 (RMSD < 2).

We performed the interaction analysis with 3D and 2D visualization using MOE software to observe the binding environment between the active compounds of sappan wood and MMP9 protein, as shown in Figures 6A and 6B, respectively. The analysis of the sappan wood's active compounds showed the presence of identical amino acid residues (Arg 424, His 401, and Tyr 423) as in the native ligand (Figure 6B). Thus, it is suggested that the active compounds of sappan wood interacted with MMP9 inhibition sites with better affinity than the native ligand and involved the same amino acids as the native ligand.

DISCUSSION

Previous research revealed that the main compound of sappan wood, namely brazilin, had migration-inhibiting activity in cancer cells (Hsieh, *et al*., 2013). In our study, qualitatively, it was confirmed that the sappan wood extract contained brazilin, as seen in the similarity of Rf in the elution results of the sappan wood extract and the reference standard (brazilin), which was 0.375. Brazilin and brazilein isolated from *Caesalpinia sappan* L. inhibit cancer cell growth through induction of apoptosis, cell cycle arrest, and inhibition of migration in cancer cells (Kim, *et al*., 2012; Tao, *et al*., 2013; Hsieh, *et al*., 2013; Handayani, *et al*., 2016; Handayani, *et al*., 2017; Jenie, *et al*., 2020). Previous studies have focused more on the main compounds of sappan wood, namely brazilin and brazilein. However, there are still other compounds whose effectiveness has not been explored to inhibit metastasis, so further exploration is needed. Our observation on the cytotoxicity of HeLa cervical cancer showed the extract potency with an IC_{50} value of 54.93 g/mL.

MMPs are essential proteins that aid tumor cells during metastasis, especially in the extracellular matrix degradation and invasion of cancer cells. MMP2 and MMP9 are MMP subtypes that correlate

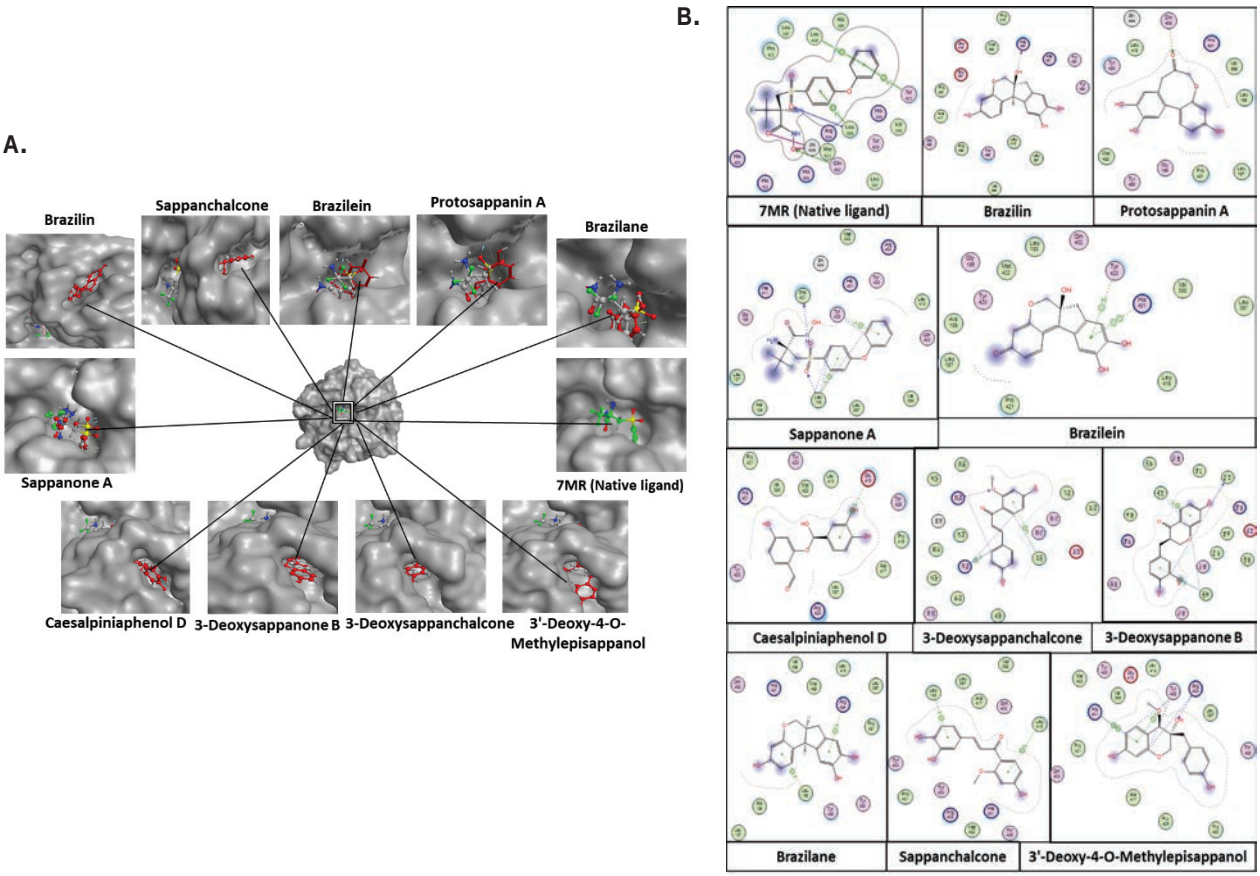


Figure 6. Interaction and affinity analysis of potential compounds of sappan wood extract (SWE) against matrix metalloproteinase-9 (MMP9). (A) 3D visualization. (B) 2D visualization.

with advanced tumor stage, increased metastasis, and poor prognosis (Deryugina and Quigley, 2006). Therefore, to verify that the MMP9 protein plays a critical role in the induction of cervical cancer, the differences in the expression of MMP9 protein in normal and cervical cancer cells or CESC were analyzed, and survival rate MMP9 in cervical cancer patients with database GEPIA. The results showed a significant difference between normal cells and CESC. Cervical cancer patients with overexpression of MMP9 protein have a lower probability of survival than cervical cancer patients induced by less MMP9 protein expression.

The metabolomic profile of sappan wood whose activity has the potential to inhibit cervical cancer cells and the metabolite data obtained include brazilin, sappanchalcone, brazilein, brazilane, protosappanin A, sappanone A, caesalpiniaphenol D, 3- deoxysappanone B, 3-deoxysappanchalcone, and 3'-deoxy-4- O-methylepisappanol used for further analysis. The potential bioinformatics test of the active compounds of sappan wood in inhibiting MMP9 protein was carried out with two software, KNIME, and MOE. Based on KNIME analysis, we predicted the activity of the active compounds of sappan wood as an MMP9 inhibitor. The prediction model proved valid because the AUC value was 0.904, and overall accuracy was 84.47%. Furthermore, based on the Random Forest Learner analysis, the highest predictive value was obtained from issappanchalcone and 3-deoxysappanchalcone, with a predictive value of 0.89. Molecular docking

using MOE software suggested that all the active compounds of sappan wood tend to bind to MMP9 protein with better affinity than the native ligand. The score docking lower levels and 2D visualization analysis of the active compound of sappan wood showed the presence of binding to the same amino acid residues environment as the native ligand (Arg 424, His 401, and Tyr 423). These results indicated that the active compounds of sappan wood have a stronger binding affinity with the MMP9 inhibition site.

CONCLUSION

Sappan wood contains a variety of active compounds that have the potential to be developed as a supportive therapeutic agent to inhibit metastasis in cervical cancer with a protein target of MMP9.

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