ORIGINAL ARTICLE

Activity study of *Kaempferia pandurata* Roxb. extract as antiestrogen receptor (-) breast cancer cell line 3,4-methylenedioxyamphetamine-MB-231 by molecular docking and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay

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Abstract

Aim: This research was determined the cytotoxic activity of extract the rhizomes of *Kaempferia pandurata as* anti-breast cancer in 3,4-methylenedioxyamphetamine (MDA)-MB-231 cell line by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and study of estrogen receptor (ER) negative with target vascular endothelial growth factor (VEGF) by molecular docking approach. **Materials and Methods:** The docking study was conducted using AutoDock Vina. The macromolecule was retrieved from protein data bank (PDB), with ID code 2OH4 and saved in PDB format. The ligands were prepared using Marvin Sketch and saved in.PDB format. The next step was *in vitro* assay of the extract against MDA-MB-231 cell line using MTT test. From this assay, we will get the half maximal inhibitory concentration (IC_{50}) value of extract. **Results:** Compounds pinostrobin and pinocembrin have the lowest Gibbs energy and Ki values, even lower compared to gossypol, which means that this molecule is most active when bound to VEGF. The hydrogen bonds that occur between the pinostrobin and the receptor are similar to the hydrogen bonds that are present between gossypol and its receptor, called Phe1045, Val846, Leu1033, and Cys917. This amino acid as active site of VEGF. The IC_{50} value of standard cisplatin, doxorubicin, and hexane extract was 18.4, 1.24, and 20.54 µg/ml, respectively. Hexane extracts of *K. pandurata* demonstrated antiproliferative activities. **Conclusion:** The extract rhizomes of *K. pandurata* with pinostrobin and pinocembrin chalcone as major compounds showed potent activity as anticancer against breast cancer cell line.

Key words: Anti-breast cancer, estrogen receptor negative, half maximal inhibitory concentration, *Kaempferia pandurata* Roxb., 3,4-methylenedioxyamphetamine - MB-231, molecular docking

INTRODUCTION

Breast cancer is one of the most common cancers among women. Approximately 1.67 million cases had been diagnosed in 2012. The deaths which were caused by breast cancer are approximately 522,000 cases worldwide.[1] Breast cancer development and progression are identified with molecular targets. Some of them are estrogen and the

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estrogen receptors (ERs).[2] Estrogens influence breast cancer development and progression by various methods. These methods are including stimulation of cell proliferation through ERα pathway, increasing rates of genetic mutations, or influencing DNA repair system.[3] ER (-) breast cancer cell means that it does not have receptors for estrogen. They are considered as hormone sensitive because they can respond to hormone therapies.[4] Meanwhile, 30% of all breast cancers are hormone insensitive (negative to ER) and is often referred to the term triple-negative breast cancer.[4] On the another hand, this breast cancer expresses vascular endothelial growth factor (VEGF) and is thus resistant to these therapies.[5]

Unfortunately, hormonal therapy is unlikely to work as a treatment for this kind of breast cancer, as they lack specific proteins or receptors as targets of specific therapy.[5,6] Furthermore, patients with triple-negative breast cancer, compared to non-triple-negative breast cancer, have poorer prognosis, higher risk of metastasis, and decreased response to neoadjuvant therapy in the first 3 years of disease progression.[7] There are still some options of treatments such as surgery, radiotherapy, and chemotherapy; however, they may pose threats such as drug resistance, tumor relapse, or post-treatment toxicity.[8,9]

Due to its limited options of therapeutic management, the use of alternative therapy is needed. *Kaempferia pandurata* is a herbal plant found in Southeast Asia and is already known for its anti-inflammatory, antitussive, and antidiuretic effects.[10] Studies have also shown *K. pandurata* having anticancer effects to many tumor cell lines including breast cancer and myeloma.[10] *K. pandurata* consists mostly of flavonoids and various essential oils as its bioactive compound, with pinostrobin as its most abundant flavonoid.[11,12] Pinostrobin itself is known to have apoptotic and anti-aromatase effect on breast cancer cell lines.[13]

The use of *in silico* in pharmacological researches has been used for more than a decade. *In silico* is able to do virtual screening based on the level of complex affinity indicated by the ability of active molecules to target both the nature and effect.[14] *In silico* can be used to demonstrate the binding of substances using molecular docking with molecule such as pinostrobin and other active compounds found in *K. pandurata* to macromolecule structure, including receptor. 3,4-Methylenedioxyamphetamine-MB-231 cell line expresses VEGF in cytokines which have an important role in angiogenesis.[15]

MATERIALS AND METHODS

Protein Preparation

The crystal structure of the target protein was retrieved from protein data bank (PDB) (2OH4) shown at Figure 1, and minimization of the protein was generated by python molecular viewer 1.5.6cr3. The protocol prepares the protein by inserting the added partial charges using Gasteiger method; add polar hydrogens in the protein and residue materials, such as water and ligand molecules, are removed before minimization.

Active Site Prediction

The minimized protein is further taken for binding site detection which will be very useful in active site. Grid box of VEGF (2OH4) at X=50, Y=50, Z=50 with center ligand at X=3.173, Y=33.766, Z=17.175. This study has been used to know the important residues in the target protein which is responsible for ligand binding present in the active site under active site prediction.[16]

Ligand Preparation

The structure of compound is shown in Tables 1 and 2. The compounds were made in two-dimensional (2D) using Marvin Sketch 15.1.19 software and saved in 3D structure

Table 1: Secondary metabolites with chalcone compounds of extract *K. pandurata*

Compounds	R1	R2	R3	R4
1	OMe	OH	OH	H
2	OH	OH	OH	H
3	OMe	OH	OH	OH
4	OH	OMe	OH	H
5	OMe	OMe	OH	OH
6	OH	H	OH	H
7	OMe	OH	OH	H

K. pandurata: *Kaempferia pandurata*

Table 2: Secondary metabolites with flavanones compounds of *K. pandurata* compounds

Compounds	R1	R2	R3	R4	R5
8	OMe	OH	H	H	H
9	OH	OH	H	H	H
10	OMe	OMe	H	H	H
11	OH	OMe	H	H	H
12	OMe	OH	H	H	OH
13	OH	OMe	H	H	OH
14	OMe	OH	H	H	OH
15	OMe	OMe	H	H	OH
16	OMe	OMe	OMe	H	H
17	OMe	OMe	H	H	OMe
18	OMe	OH	H	H	OMe
19	OMe	OMe	H	OMe	OMe
20	OMe	OH	OMe	H	OMe
21	OMe	OH	OMe	OMe	OMe

K. pandurata: *Kaempferia pandurata*

in PDB format. The ligands then being optimized using AutoDock tools to fix the charge, added the hydrogen and minimizing energy. 3D structure then saved in PDB partial charge atom type (.QT) format.

Molecular Docking Simulation of *K. pandurata* Compounds

The preparation of protein coordinates was saved as PDB files. 3D structure (PDB 2OH4) was taken from the research collaboratory for structural bioinformatics PDB (http://www.rcsb.org/pdb).[17] Molecular docking experiment is performed using AutoDock Vina program (Vina, The Scripps Institute) to dock the aryl eugenol derivatives to binding site of the B-cell lymphoma 2.[18] The AutoDock Vina tools are used to add partial charges using Gasteiger method and to arrange the polar hydrogens in the protein. Energy minimizations were performed for 1000 iterations until reaching a convergence and the conjugate gradient algorithm with a convergence criterion of 0.01 kcal/mol A. The ligands are set to have flexible torsion angles at all rotatable bonds, while the protein is prepared as a rigid structure. Both protein and ligand are saved as output PDBQT files. For specific docking *K. pandurata* compounds onto VEGF protein, the grid box volume was adjusted to 40 × 40 × 40 Å in the X-, Y-, and Z-axes, respectively, with grid sizes have a space up to 1 Å. AutoDock Vina employs an idealized active site ligand as a target to generate putative poses of molecules.

In vitro Assay

The cell lines MDA-MB-231 were purchased from PVRKP Laboratory, Faculty of Medicine, Universitas Indonesia (UI). Cell lines were prepared and cryopreserved using reagents such as dimethyl sulfoxide (DMSO) (Merck) which preserve the cell during freezing. DMSO is toxic at room temperature. The freezed ampoule is brought to room temperature by slow agitation (thawing). The freezed cryovials plunged into the water bath and are rapidly thawed until it gets liquefied. Solution centrifuged with saline for 10 min to remove DMSO. The saline is discarded and aliquot is taken for cell counting, cell viability, and subculturing. 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay is a quantitative colorimetric assay for measuring cellular growth, cell survival, and cell proliferation based on the ability of living cells. The assay was carried out using MTT (Merck).[12-15] After treatment with the plant extracts, the cells were pooled together and the remaining attached cells were detached from the culture plates by exposure to trypsin-EDTA (GIBCO, USA). The resultant cells were then stained with trypan blue at the concentration of 0.2%. Then, the trypan blue-excluded viable cells were counted using a microplate reader under microscope. Finally, the absorbance was monitored by a microplate reader at a wavelength of 570 nm. The percentage of viable cells was plotted versus the concentration of the test compound. The concentration used to determine 50% of the half maximal inhibitory concentration (IC50) using linear regression analysis.

RESULTS AND DISCUSSION

Structure-based drug design begins with the identification of a molecular target such as a protein. This structure of protein used as a targeted for the drug discovery of a lead compound. The compounds are constructed for their fit in the active site of VEGF and functional group interactions. The selected docked conformations of chemical compounds from extract *K. pandurata* into VEGF receptor binding site are shown in Figures 2 and 3.

The docked conformations of VEGF are shown in Figure 4, this active site of VEGF revealed that all ligands were located in the binding energy is the primary parameter which is generated as a result of molecular docking. It gives us the idea of strength and affinity of the interaction between the ligand and the receptor. The higher the binding energy of a complex, the weaker the interaction of ligand-protein interactions. Docking studies were performed to evaluate the effect of ligands on the various protein receptors.

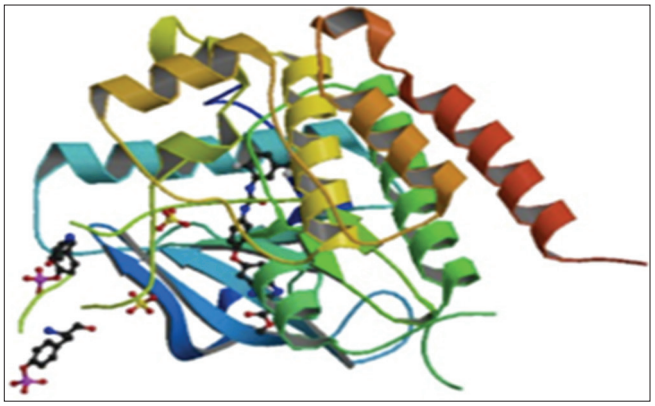

Figure 1: Structure three-dimensional vascular endothelial growth factor vascular endothelial growth factor structure 2OH4

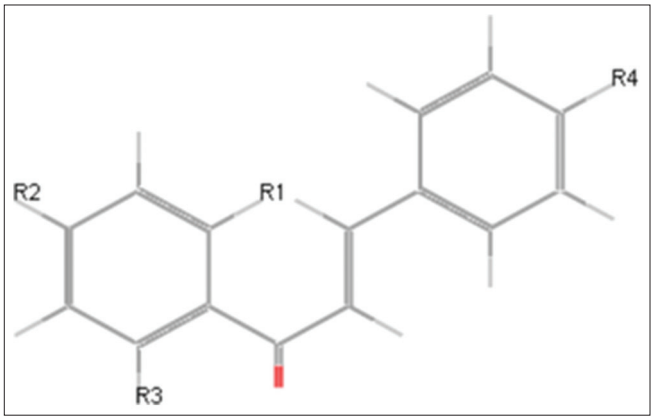

Figure 2: Chalcone-based structure

The result of the docking simulation of compounds can be shown in Table 3. Indicator from docking simulations can be seen by comparing the value of the Gibbs energy (ΔG). Docking study of *K. pandurata* compounds was showed that pinocembrin and pinostrobin have negative ΔG (kcal/mol) –11.3562 and –10.1539, respectively, and Ki value 352.23 nm and 386.61 nm, respectively. This energy binding lowest than other compounds, as found in our study; thus, pinocembrin and pinostrobin displayed much better binding than all compounds. The binding energies of the test ligands and the control have been depicted in Table 3. The complex of compounds with VEGF at binding site is shown in Figure 5.

In silico docking indicated many interactions with active site of VEGF. The best score from the best pose for each compound was taken and compound to the scores of the other compounds. The compounds which show the highest negative ΔG (kcal/mol) score show that it has the capacity to bind strongly with the protein. Docked conformers of all the molecules were analyzed for the presence of similar interactions [Figure 5]. In compounds pinostrobin and pinocembrin, the presence of hydroxyl phenyl substitutions found to push the hydrophilic head portion toward the hydrophobic region and orient differently in the pocket. Due to this, the H-bonding interaction of VEGF with Phe1045, Val846, Leu1033, Cys917, Cys1043, and Phe916 was totally absent [Table 4].

The docking results shown in Table 3, compounds pinostrobin and pinocembrin have the lowest ΔG and Ki values, even lower compared to gossypol, which means that this molecule is most active when bound to VEGF. The hydrogen bonds that occur between the pinostrobin and the receptor are similar to the hydrogen bonds that are present between gossypol and its receptor, called Phe1045, Val846, Leu1033, and Cys917. This amino acid as the active site of VEGF (2OH4).

Based on the results of the silico study, and confirmed using an *in vitro* test by MTT. The cytotoxic activity of the hexane, EtoAc, and aqueous extracts of *K. pandurata* on MDA-MB-231 cells from human breast cancer was investigated *in vitro* by MTT. The results showed decreased cell viability and cell growth inhibition in a dose-dependent manner. The IC_{50} value of standard cisplatin, doxorubicin, and hexane extract was 18.4, 1.24, and 20.54 μg/ml, respectively shown at Table 5. Hexane extracts of *K. pandurata* demonstrated antiproliferative activities. Many studies in the past have assumed that the free

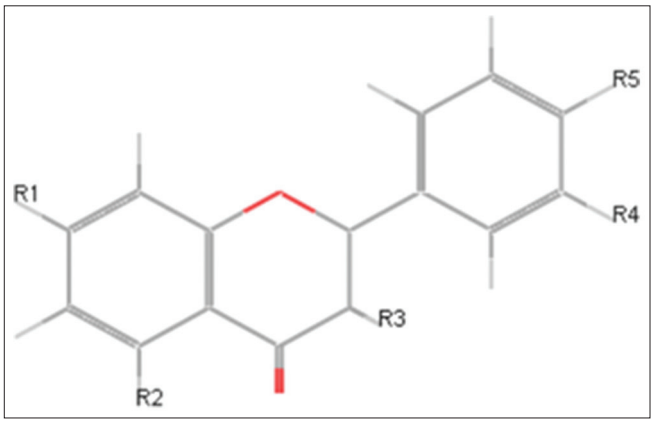

Figure 3: Flavanones based structure

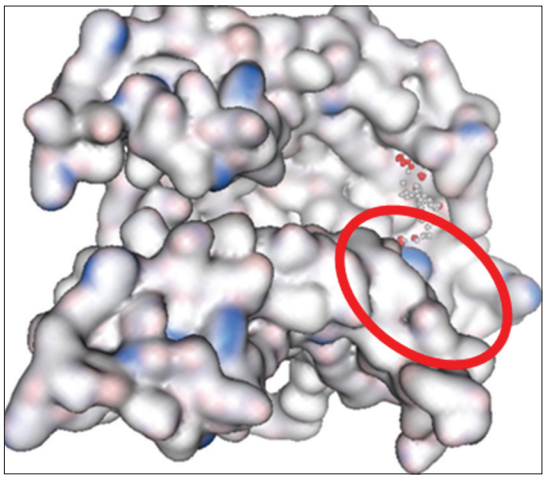

Figure 4: Active site (red cycle) of vascular endothelial growth factor structure 2OH4

Table 3: Docking of complex VEGF with compounds of extract *K. pandurata*

Compounds	ΔG	Ki (nm)
Cardamonin	–7.5632	649.24
Pinocembrin chalcone	–10.0353	402.10
Helichrysetin	–7.3485	675.30
2,6-dihydroxy-4-methoxychalcone	–9.2911	436.12
Flavokawain C	–7.5517	653.20
2’,4’,6’-trihydroxychalcone	–6.3856	776.32
Uvangoletin	–6.5829	723.50
Pinostrobin	–11.3562	352.23
Pinocembrin	–10.1539	386.61
5,7-dimethoxyflavanone	–5.2453	872.19
Alpinetin	–4.1366	934.67
Sakuranetin	–6.4356	759.43
7,4’-dihydroxy-5-methoxyflavanone	–5.4340	834.29
Tectochrysin	–5.8918	812.12
5,7-dimethoxyflavone	–4.3127	901.11
5-hydroxy-3,7-dimethoxyflavone	–6.2244	783.94
5,7,4’-trimethoxyflavone	–5.5111	821.02
5-hydroxy-7,4’dimethoxyflavone	–6.2281	782.19
5,7,3’,4’-tetramethoxyflavone	–5.0942	876.04
5-hydroxy-3,7,4’trimethoxyflavone	–6.9210	690.97
5-hydroxy-3,7,3’,4’tetramethoxyflavone	–6.7875	711.05
Gossypol	–11.0842	362.21

VEGF: Vascular endothelial growth factor, ΔG: Gibbs energy, *K. pandurata*: *Kaempferia pandurata*

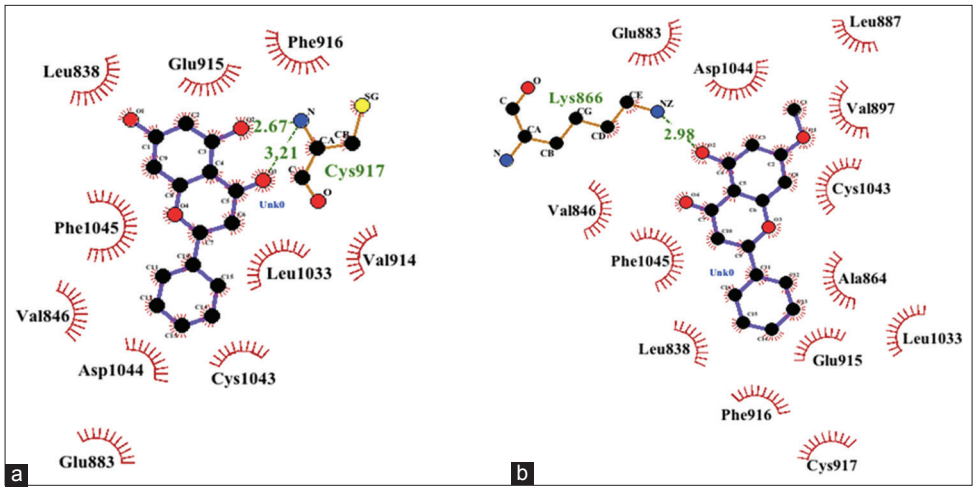

Figure 5: (a and b) Complex of compounds pinocembrin and pinostrobin with vascular endothelial growth factor

Table 4: Interaction of three hit compounds of extract *K. pandurata* with VEGF receptor

Amino acid	Native ligand	Pinocembrin	Pinocembrin chalcone	Pinostrobin
Leu1017	v			
Asp1044	v (HB)	v	v	v
Val897	v			v
Val914	v	v	v	
Phe1045	v	v	v	v
Val846	v	v	v	v
Leu1033	v	v	v	v
Gly920	v			
Ala864	v		v	v
Phe919	v			
Lys918	v			
Glu915	v	v	v (HB)	v
Leu838	v	v		v
Cys917	v (HB)	v (HB)	v (HB)	v
Cys1043	v	v	v	v
Glu883	v (HB)	v		v
Ile1042	v			
Leu887	v			v
Phe916	v	v	v	v
Asn921				
Arg1030				
Arg1049				
Gly839				
Lys866				v (HB)
Interaction	20	11	10	14

VEGF: Vascular endothelial growth factor, *K. pandurata*: *Kaempferia pandurata*

hydroxyl groups of the flavonoids and other polyphenols are necessary for biological effects.[19]

VEGF is a key regulator of developmental, physiological, and pathological neovascularization, including tumor growth and metastasis.[20,21] This growth factor consists of a family of proteins generated from a single gene by alternative splicing of the primary transcript.[22] VEGF isoforms consist of proteins 121, 145, 165, 189, and 206 amino acids. The presence or absence of genomic exons 6 and 7 determines whether these isoforms

Table 5: MTT assay extract of *K. pandurata* against MDA-MB-231 cell line

Citotoxicity	Extract hexane	Extract EoAc	Cisplatin	Doxorubicin
IC_{50}	20.54±1.5	70.32±2.1	18.4±1.7	1.24±1.2

IC_{50}: Half maximal inhibitory concentration, MDA-MB: 3,4-methylenedioxyamphetamine-MB, *K. pandurata*: *Kaempferia pandurata,* MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

are secreted in soluble forms (VEGF 121 and VEGF 165). VEGF 189 contributes to mammary tumor growth through both angiogenic and non-angiogenic functions associated with an autocrine effect on breast cancer cells.[23]

The docking results agreed well with the observed *in vitro* data, which showed that VEGF inhibitory activity of pinostrobin (–11.3562 K Cal/mol and Ki 352.23 nm) was higher than those of other compounds. Our investigations show that cisplatin has good inhibitory activity on VEGF and this can be helpful for further investigations. The docking results data support the inhibitory activity of pinocembrin and pinostrobin.

CONCLUSION

VEGF induced colonization of the lymph node, skeletal tissues, lungs, and brain, with all clones, as described for breast cancer MDA-MB-231 cell line. The results of silico and *invitro* showed that *Kaempferia pandurat* Roxb extract has the potential to be antiestrogen receptor through the VEGF mechanism. The flavonoids compound dominates the composition of the *K. pandurata* Roxb. and pinocembrin and pinostrobin potent as anticancer activities to breast cancer cell line.

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