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Identification of Secondary Metabolites in Ethyl Acetate Extract of Ki Encok Plant Roots *(Plumbago zeylanica* Linn) and In Silico Cytotoxicity Activity

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Abstract*:* The Ki Encok plant (*Plumbago zeylanica* Linn) has been extensively studied and is known to have cytotoxic activity. However, no research has yet investigated the compound content of the ethyl acetate extract from that plant. This study aims to identify the secondary metabolite content in the ethyl acetate extract of *Plumbago zeylanica* L. roots and to determine the potential of these secondary metabolites to bind with the Caspase-3 protein in silico. The ethyl acetate extract of Plumbago zeylanica roots was tested for its phytochemical properties and examined using GC-MS. The compounds found were then searched for their structures in Pubchem, tested in silico using the CB-Dock2 software, and evaluated for drug-likeness with SwissADME. Qualitative phytochemical tests indicate the presence of alkaloid and tannin compounds. The GC-MS test showed the presence of Plumbagin, Gamma sitosterol, and 4-ethoxybenzaldehyde. In the in silico test, docking between Gamma sitosterol, Plumbagin, and 4-ethoxybenzaldehyde with the target protein Caspase-3 has free binding energies of -8.5, -7.0, and -6.1. The Gamma sitosterol-Caspase-3 complex shows the best free-binding energy among the three ligands. Further in vitro or in vivo studies are needed to assess whether the interaction between Gamma sitosterol and Caspase-3 is inhibition or activation.

Keywords: ethyl acetate, gamma sitosterol, gas chromatography, *Plumbago zeylanica*

Abstrak: *Tanaman Ki Encok (Plumbago zeylanica Linn) telah banyak diteliti dan diketahui memiliki aktivitas sitotoksik. Namun, belum ada penelitian yang meneliti kandungan senyawa ekstrak etil asetat dari tanaman tersebut. Penelitian ini bertujuan untuk mengidentifikasi kandungan metabolit sekunder dalam ekstrak etil asetat akar Plumbago zeylanica L. dan mengetahui potensi metabolit sekunder tersebut untuk berikatan dengan protein Caspase-3 secara in silico.Ekstrak etil asetat akar Plumbago zeylanica diuji sifat fitokimia-nya dan diperiksa dengan GC-MS. Senyawa yang ditemukan kemudian dicari strukturnya di Pubchem, diuji secara in silico menggunakan software CB-Dock2, dan dinilai drug-likeliness dengan SwissADME. Uji fitokimia kualitatif menunjukkan adanya senyawa golongan alkaloid dan tannin. Uji GC-MS menunjukkan adanya senyawa Plumbagin, Gamma sitosterol, dan 4-etoksi benzaldehida. Dalam uji in silico, docking antara Gamma sitosterol, Plumbagin, dan 4-etoksi benzaldehida dengan protein target Caspase-3 memiliki free binding energy -8,5, -7,0, dan -6,1. Komplek Gamma sitosterol-Caspase-3 menunjukkan free binding energy yang paling baik di antara ketiga ligan. Diperlukan penelitian in vitro atau in vivo lanjutan untuk menilai apakah interaksi antara Gamma sitosterol dan Caspase-3 merupakan inhibisi atau aktivasi.*

Kata kunci: *etil asetat, gamma sitosterol, gas kromatografi, plumbago zeylanica.*

INTRODUCTION

Apoptosis is a programmed cell death that causes damaged cells to be removed from tissues (Bebars *et al*. 2017). Caspase stands for cysteine-aspartic protease. This enzyme is a proteolytic enzyme known to control cell death and inflammation. Caspase-2, -3, -6, -7, -8, -9, -10 are caspases involved in the apoptosis process. Caspases involved in apoptosis are

also divided into initiators and effectors based on the presence or absence of specific proteins at the N-terminus. Caspases that are initiators are Caspase-2, -9, -8, -10. Caspases that are executors are Caspase-3, -6, -7b (Shalini *et al*. 2015). The mechanism of apoptosis involving Caspase-3 is that DNA damage will activate p53 and stimulate the production of Bax/Bak. The presence of Bax/Bak will induce the release of Cytochrome C from mitochondria. The release of Cytochrome C will activate Caspase-9, which then activates Caspase-3 as an apoptosis effector and is involved in cytotoxic activity (Shalini *et al*. 2015).

Indonesian herbal plants contain various secondary metabolites that have cytotoxic activity. One of the herbal plants that has been studied for its cytotoxic effects is the Ki Encok plant (*Plumbago zeylanica*) because it is known that the roots of this plant are rich in Plumbagin (Purwoko *et al*. 2022[b]; Sundari *et al*. 2017) Plumbagin has antiproliferative effects on cancer cells (Ito *et al*. 2018).However, chloroform extract of *Plumbago zeylanica* roots did not significantly reduce Caspase-3 expression in Psoriasis model mice (Purwoko *et al*. 2022[a]).

Previous studies have not used ethyl acetate solvent to make *Plumbago zeylanica* root extract, so the secondary metabolite content is unknown. Identification of the compound content of an extract can be assessed by various methods such as thin-layer chromatography, high-performance liquid chromatography, liquid chromatography-mass spectrometry, and gas chromatography-mass spectrometry (GC-MS) (Nurulita *et al*. 2022; Yunianto *et al.* 2017). GC-MS is a physical technique for separating compounds that require volatile and thermally stable analytes so they can be identified and quantified (Rontani 2022). This study aims to identify the content of secondary metabolites in the ethyl acetate extract of *Plumbago zeylanica* roots and to determine the potential of these secondary metabolites to bind to Caspase-3 protein in silico.

MATERIALS AND METHOD

Plumbago zeylanica Plants

The roots of *Plumbago zeylanica* were obtained from the researcher's yard in Palembang. The roots were taken from plants that were at least 2 years old. The plants have been identified by botanists from the Biology Education Study Program, Faculty of Teacher Training and Education, Muhammadiyah University of Palembang, with letter number 141/Lab. Bio FKIP/X/2020. The wet weight of the roots after being washed with water was 210.53 g. The roots were then dried, obtaining a dry weight of 160.53 g. The clean and dry plant roots were blended and filtered into a fine powder.

Ethyl Acetate Extract of Plumbago zeylanica Roots (EAPZ)

Plumbago zeylanica root powder of 30 g was soaked in 300 ml of ethyl acetate pro analysis (Smartlab, Indonesia) for 24 hours in a closed jar. The solution was then filtered with Whatman filter paper number 42. The filtered results were then evaporated with a rotary evaporator at 60 °C. The extract was then stored in an open container in the refrigerator for 7 days until the extract thickened. The dry weight of the extract obtained was 0.02 g, and its yield was 0.07%. The EAPZ extract was then stored in a small pot with a lid in the refrigerator until used.

Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS examination of EAPZ extract was carried out at the DKI Jakarta Regional Health Laboratory using Agilent Technologies 7890 Gas Chromatograph with Auto Sampler and 5975 Mass Selective Detector and Chemstation data system (Agilent, California, US). Chemical analysis was conducted in an HP Ultra 2-capillary column (Agilent, California, US) with dimensions 30 m x 0.20 mm x 0.11 µm using *constant flow* mode 1.2 mL/minute. The injection volume was 5 µL, split 8:1. Injection port temperature 250°C, *ion source* 230°C, *interface* 280°C, *quadrupole* 140°C. The carrier gas used is Helium. The identified compounds are matched with EI MS library (mainlib) data from NIST/EPA/NIH.

Phytochemical Screening

Alkaloid

EAPZ extract was added with 10 ml of Chloroform and 10 ml of ammonia, then filtered into a test tube. The filtrate was then added with 10 drops of H_2SO_4. The solution was shaken and left to stand until two layers were formed. A total of ± 1 mL of the upper layer was transferred into another test tube, and Mayer's reaction was added. The result was positive if a white precipitate appeared in the tube (Abriyani dkk. 2022).

Tannin

The EAPZ extract was extracted with methanol in a test tube. The extraction results were then dipped with 2-3 drops of 5% $FeCl_3$ solution. Tannin was declared positive if a blue-green colour was detected (Abriyani dkk. 2022).

Flavonoid

EAPZ extract was extracted with methanol and heated in a test tube. Magnesium powder and concentrated HCl were added to the test tube. Flavonoids were declared positive if a pink colour was detected in the test tube (Abriyani dkk. 2022).

Saponin
EAPZ extract is put into a test tube and added to distilled water. Then, the test tube is shaken vigorously. Saponin is declared positive if stable foam is observed in the test tube tube (Abriyani dkk. 2022).

Phenolate
The EAPZ extract was extracted with methanol in a test tube. The extract was then dripped with 2-3 drops of 5% $FeCl_3$ solution. Phenolate was declared positive if a yellowish-green colour appeared in the tube.

Drug-Likeliness Screening

Drug-likeliness analysis was performed using the SwissADME program (http://swissadme.ch/) by entering the Simplified Molecular Input Line Entry Specification (SMILES) ligand. Drug-like property analysis produces a compound property score against Lipinski's rule of five, which includes the compound's molecular weight ≤of 500, log P partition coefficient value ≤of 5, number of hydrogen bond donors ≤of 5, and number of hydrogen bond acceptors ≤of 10 (Rowaiye *et al*. 2020).

In Silico

The ligands used are secondary metabolites from GC-MS, namely Plumbagin, Gamma sitosterol, and 4-ethoxy benzaldehyde. The structures of the three ligands were obtained from PubChem in .sdf format. The target protein used is Caspase-3 with the 3DEI protein structure in .pdb format obtained from www.rcsb.org. Docking simulations were performed using the online software CB-Dock2, available at https://cadd.labshare.cn/cb-dock2/php/index.php (Liu *et al.* 2022).

RESULT AND DISSCUSION

Ethyl acetate extract only showed positive values in the alkaloid and Tannin phytochemical tests (Table 1). The absence of flavonoids follows previous studies (Rajakrishnan *et al*. 2017). Phenolate will appear if the roots are extracted with ethyl acetate solvent (Rajakrishnan *et al*. 2017). However, phenolate did not appear in this study.

Gamma sitosterol and 4-ethoxy benzaldehyde were found in the ethyl acetate extract of *Plumbago zeylanica* roots (Table 2). Dhalani *et al*. 2020 also found gamma sitosterol in the petroleum ether extract of *Plumbago zeylanica* leaves. Gamma sitosterol is a non-polar substance (Dhalani *et al*. 2020). Gamma-sitosterol is known to have strong cytotoxic activity on MCF-7 breast and A549 lung cancer cells (Sundarraj *et al*. 2012). 4-ethoxy benzaldehyde, also known as Ethoxybenzaldehyde, has not been widely researched in the medical field, so there is no information regarding the benefits of this compound for human health.

Plumbagin can downregulate cyclin, cyclin-dependent kinase, and cyclin-dependent kinase inhibitors. This downregulation can stop the cell cycle in the G1 phase (Zhang *et al*. 2020). Plumbagin induces apoptosis by upregulating pro-apoptotic molecules such as Bax, Caspase-9, Caspase-6 and Caspase-3. Plumbagin causes oxidative damage and increases apoptosis by producing more reactive oxygen species. The apoptotic pathway inhibited by Plumbagin is the PI3K/AKT/mTOR pathway (Zhang *et al*. 2020)

GC-MS results showed that the ethyl acetate extract of the roots of the Ki Encok plant (*Plumbago zeylanica* L.) contained three compounds (Table 2), with the highest peak appearing being the Plumbagin compound (Figure 1). When tested by molecular docking, these three compounds showed a complex with Caspase-3 (Figure 2).

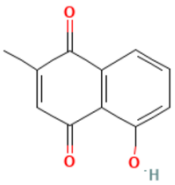
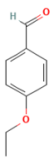
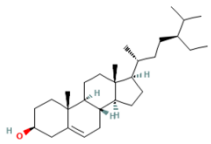
The interaction between the three ligands with the Caspase-3 protein showed the presence of hydrophobic bonds with various amino acid residues (Table 3). The number of residue interactions between the ligand and the protein reflects the stability of the ligand binding to the target protein (Fu *et al*. 2018). Gamma sitosterol showed 17 residue interactions with Caspase-3 protein, the largest of the three ligands. The free-binding energy value can indicate the number of interactions. The lower the free-binding energy, the more residue interactions occur (Iman *et al*. 2015).

The Gamma sitosterol and Caspase-3 complex showed the best free-binding energy. The lower the binding value, the stronger the bond. One of the shortcomings of in silico tests is the inability to show whether the ligand binding to the target protein will be inhibitory or activating. The free-binding energy between Gamma sitosterol and Caspase-3 in this study was higher than previous studies that obtained

Table 1. Phytochemical screening result

Group	Outcome
Alkaloid	+
Tannin	+
Flavonoid	-
Saponin	-
Phenolate	-

Table 2. Secondary metabolites in ethyl acetate extract of *Plumbago zeylanica* roots

No.	Name	Molecule Formula	Chemical structure	*Retention Time*	Area (%)
1	Plumbagin	$C_{11}H_8O_3$		24.900	93.97
2	4-ethoxy benzaldehyde	$C_9H_{10}O_2$		27.562	1.74
3	Gamma-sitosterol	$C_{29}H_{50}O$		40.877	1.83

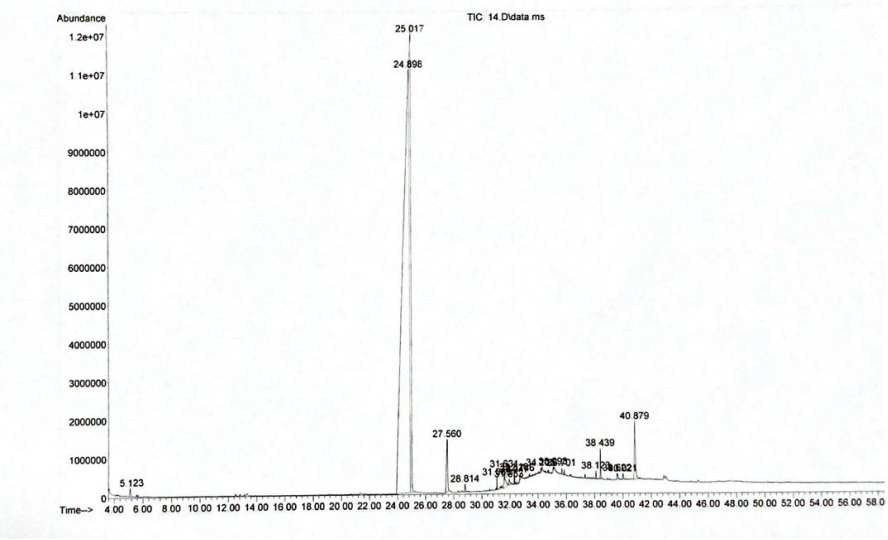


Figure 1. GC-MS chromatogram of ethyl acetate extract of *Plumbago zeylanica* roots. The highest peak indicates the compound Plumbagin.

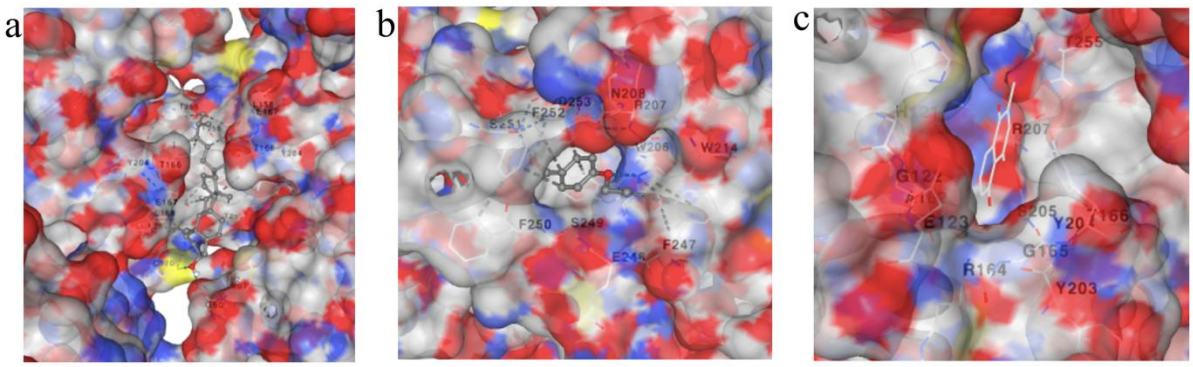


Figure 2. Docking poses of interactions between (a) Gamma sitosterol and Caspase-3, (b) 4-ethoxy benzaldehyde and Caspase-3, and (c) Plumbagin and Caspase-3.

Table 3. Docking results between ligand and Caspase-3

Ligand Name	*Pocket*	Vina score	Cavity volume	*Center* (x, y, z)	*Docking size* (x, y, z)	Residues interactions
Gamma Sitosterol	C1	-8.5	4206	-50, 17, -31	35, 25, 35	Chain A: Thr166, Glu167, Leu168, Cys170, Tyr204, Thr255, Phe256, Lys259 Chain C: Gly60, Met61, Thr62, Thr166, Glu167, Leu168, Tyr204, Thr255, Phe256
Plumbagin	C1	-7.0	4206	-50, 17, -31	35, 29, 35	Chain A: Thr255 Chain C: Thr62, His121, Gly122, Glu123, Aka162, Arg164, Gly165, Thr166, Tyr203, Tyr204, Ser205, Arg207
4-ethoxy benzaldehyde	C1	-6.1	4206	-50, 17, -31	35, 29, 35	Chain A: Ser251, Phe252, Asp253 Chain C: Trp206, Arg207, Asn208, Trp214, Phe247, Glu248, Ser249, Phe250

Table 4. Drug likeness test results of ligand compounds

Compound Names (Pubchem ID)	Molecule weight (g/mol)	*Log P*	H-bond donor	H-bond acceptor
Gamma sitosterol (457801)	414.71	7.24	1	1
Plumbagin (10205)	188.18	1.72	1	3
4-ethoxy benzaldehyde (24834)	150.17	1.79	0	2

a figure of -7.4, which used Caspase-3 with PDB ID 1NME (Febrina *et al.* 2021).

The results of the drug-likelihood test showed that Gamma sitosterol did not meet all Lipinski criteria, namely the log *P value* (Table 4). This needs to be considered if Gamma sitosterol is targeted to become a drug candidate in the future.

CONCLUSION

The secondary metabolite compound in the ethyl acetate extract of Plumbago zeylanica L. roots, namely Gamma sitosterol, is recommended as a ligand for the Caspase-3 protein. However, Gamma sitosterol does not meet Lipinski's criteria as a good drug candidate. Therefore, further in vitro and in vivo tests are needed to assess Gamma sitosterol as a good drug candidate and to determine whether the ligand will be an inhibitor or activator for Caspase-3.

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