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Metabolite Profiling and Molecular Docking Phytoestrogen of 96% Ethanol Extract of S. Mutabilis Leaves Againts ER-β

Denis Mery Mirza[1*] Dian Novita Wulandari[1] Ricadonna Raissa[2] Ersanda Nurma Praditapuspa[3]

[1]Department of Pharmacy, Faculty of Medicine, Universitas Islam Malang, Malang, Indonesia
[2]Department of Pharmacology, Faculty of Veterinary, Universitas Brawijaya, Malang, Indonesia
[3]Department of Pharmacy, Faculty of Pharmacy, Universitas Hang Tuah, Surabaya, Indonesia
Email Corresponding : denismerymirza@gmail.com

Abstract

Aging is a natural multifactorial phenomenon experienced by women and characterized by the physical and mental decline. This phenomenon that occurs in women's bodies continues to increase year by year and in certain conditions can cause death. One of the causes is a decrease in estrogen hormone homeostasis in postmenopausal women. This condition can be overcome with phytoestrogen therapy obtained from efficacious natural ingredients such as S. mutabilis leaves. The main objective of this study was to obtain phytoestrogen from S. mutabilis leaves. 96% ethanol extract of S. mutabilis leaves was analyzed by metabolite profiling analysis using UHPLC-HRMS. The results of the analysis were followed by molecular docking simulations using PyRX 0.8 and Biovia Discovery Study 2019 to obtain compounds with phytoestrogenic activity against the ER-β receptor (PDB ID: 3OLS). Untargeted metabolite profiling analysis showed a total of 22 active compounds of 51 total compounds identified in the 96% ethanol extract of S. mutabilis leaves. Molecular docking simulations show that 96% ethanol extract of S. mutabilis leaves contains 3-O-Methyl-Quercetin and 5,7-dihydroxy-3-(4-hydroxyphenyl)- 6-methoxy-4H-chromen-4-one compounds which have the potential to provide phytoestrogenic activity when forming receptor ligand bonds with ER-β.

Keywords: Molecular Docking, Metabolite profiling, Phytoestrogen, S. mutabilis

INTRODUCTION

Aging in women is driven by chronic inflammation, DNA transcription failure, and disturbances in homeostasis (Villa et al., 2016; Wagner et al., 2016). The condition causes various degenerative disorders and in certain conditions cause death (Poulose and Raju, 2014; Wagner et al., 2016; Fougere et al., 2017). Research by Naja et al. (2017) shows that globally the population of older women in the world increases year by year, estimated to be no less than 1.5 billion in 2030 and predicted to be 2.1 billion by 2050 (Prince et al., 2015; Naja et al., 2017). This condition is is closely related to the decline of estrogen hormone homeostasis in postmenopausal women (Villa et al., 2016).

Estrogen hormone activity declines in both quality and quantity in postmenopausal women (Ji and Yu, 2015). This condition causes various adverse effects, including health problems (Cui et al., 2013). These health problems are generally addressed with estrogen hormone replacement therapy. However, long-term use can lead to other health problems, including embolism, stroke, cancer, and even death (Lee et al., 2013). One successful therapy is phytoestrogen therapy, derived from potent natural ingredients. Phytoestrogen compounds replace estrogen in the female body and provide estrogenic activity (Ma'arif et al., 2019; Mirza et al., 2021). One plant with the potential to be a source of phytoestrogens is *Stachytarpheta mutabilis*.

S. mutabilis is known as a medicinal plant used as a diuretic and to remove either kidney or bladder stones (Nasihah, 2021). Furthermore, *S. mutabilis* leaves are known to lower blood sugar

levels, act as antioxidants and anti-inflammatory agents (Kustini and Susila, 2019). These activities are thought to be due to the various compounds in *S. mutabilis* leaves, such as flavonoids, steroids, alkaloids, and saponins (Londonkar and Kesralikar, 2022). The various beneficial compounds in *S. mutabilis* have yet to be identified and flavonoids are predicted to be the secondary metabolites with recognized phytoestrogenic potential. Unfortunately, there is no study that has combined metabolite profiling and molecular docking targeting the estrogen receptor to characterize compounds, especially flavonoids in *S. mutabilis* leaves, as phytoestrogens. This gap became the research goal to identify the bioactive constituents of *S. mutabilis* and evaluate their potential as phytoestrogen sources. 96% ethanol was chosen because of its ability to extract a broad range of the compounds in the sample (Qodriah et al., 2023). UHPLC-HRMS was chosen because of its ability to identify the broad compound, followed by docking simulations against estrogen receptor-β (ER-β; PDB ID: 3OLS), one of the principal receptors mediating estrogenic activity.

RESEARCH METHODS

Equipment and Materials

S. mutabilis powder purchased from UPT Laboratorium Herbal Materia Medika Batu; 96% Ethanol purchased from SMARTLab, Ultrasonic bath (Auguri 410TD), Rotary evaporator (Heidolph Hei-VAP Core G3/HL), Universal oven (Binder Type 115ED) at the Laboratorium Pusat Riset Kedokteran, Faculty of Medicine, Universitas Islam Malang (LPRK FK Unisma); Methanol pro LC/MS, 5 mL syringe, 0.22 µm millipore, 1.5 mL vial, Ultra High Performance Liquid Chromatography High Resolution Mass Spectoscopy (UHPLC-HRMS) System (Thermoscientific Dionex 3000 Ultimate) and personal computer at the Laboratorium Pusat Terpadu, Universitas Brawijaya Malang (LRT UB) then the target protein (ER-β) (PDB ID: 3OLS) obtained from PDB (https://rcsb.org)

Ultrasonic Assisted Extraction (UAE) of *S. mutabilis* Leaves

30 grams of *S. mutabilis* powder was weighed and dissolved in 500 mL of 96% ethanol. UAE was then performed in an ultrasonic bath (3 x 10 minutes/30°C). The filtrat was concentrated using a rotary evaporator (40 rpm/35°C) and oven (10% flap/40°C) to remove residual solvent.

Metabolite Profiling of 96% Ethanol Extract of *S. mutabilis* Leaves

Concentrated extract was dissolved in methanol pro LC-MS then filtered through a 0.22 µm Millipore filter to obtain a clear solution. The solution was transferred to a 1.5 mL vial and placed in the tray of the UHPLC-HRMS system. Samples were tested using PRM mode with a running time of 30 minutes to obtain compound names and similarity values from the mzCloud database. Excluded compounds those with a similarity value ≤85%, a retention time >20 minutes, a molecular weight >500 kDa, and compounds with the same name but with a lower similarity value (Liu et al., 2019; Castano-Ortiz et al., 2024).

Molecular Docking Approach for 96% Ethanol Extract of *S. mutabilis* Leaves

Compounds obtained from untargeted metabolite profiling were prepared using a modification of research method of Mirza et al. (2021). Compounds were prepared using the MarvinSketch plug-in SwissADME to obtain 2D compound structures. The 2D compound structures were prepared using Avogadro to obtain the most stable 3D structure geometry. Target proteins obtained from PDB (https://rcsb.org) were prepared using Biovia Discovery Studio Visualizer 2019. Preparation was carried out to separate the target protein from each internal ligand found in both. The test compounds prepared with Avogadro and the internal ligand were

subjected to molecular docking simulations using PyRX 0.8 to obtain binding affinity values. The results of the molecular docking simulations were followed by analysis of ligand-protein interactions and amino acid residues using Biovia Discovery Studio Visualizer 2019.

RESULT AND DISCUSSION

Extraction of *S. mutabilis* Leaves

Phytoestrogens include compounds from the phenolic and flavonoid groups, and its thermolabile compound. This characteristic makes UAE as the best extraction method to maximize phytoestrogen yield. UAE extraction widens the pores of the material, ultimately drawing the compounds out without heat (Boriz-enrique et al., 2017). A 96% ethanol solvent was chosen to maximize the extraction of flavonoid compounds from *S. mutabilis* leaves. This solvent is known for its ability to extract polar compounds, from simple phenolics to flavonoids (Lezoul et al., 2020). The resulting of this extraction is 96% ethanol extract of S. mutabilis leaves yielded 6.803%.

Metabolite Profiling of 96% Ethanol Extract of *S. mutabilis* Leaves

Flavonoid compounds in 96% ethanol extract of *S. mutabilis* leaves were analyzed using a UHPLC-HRMS instrument. This instruments has an advantage of comprehensively mapping the compounds contained in 96% ethanol extract of S. mutabilis leaves. This advantage is achieved through the use of a Compound Library on UHPLC-HRMS instruments such as mzCloud (Madala et al., 2016; Mirza, 2021). The analysis results showed 51 compounds with a running time of 30 minutes based on the Total Ion Chromatogram (TIC) results (Figure 1).

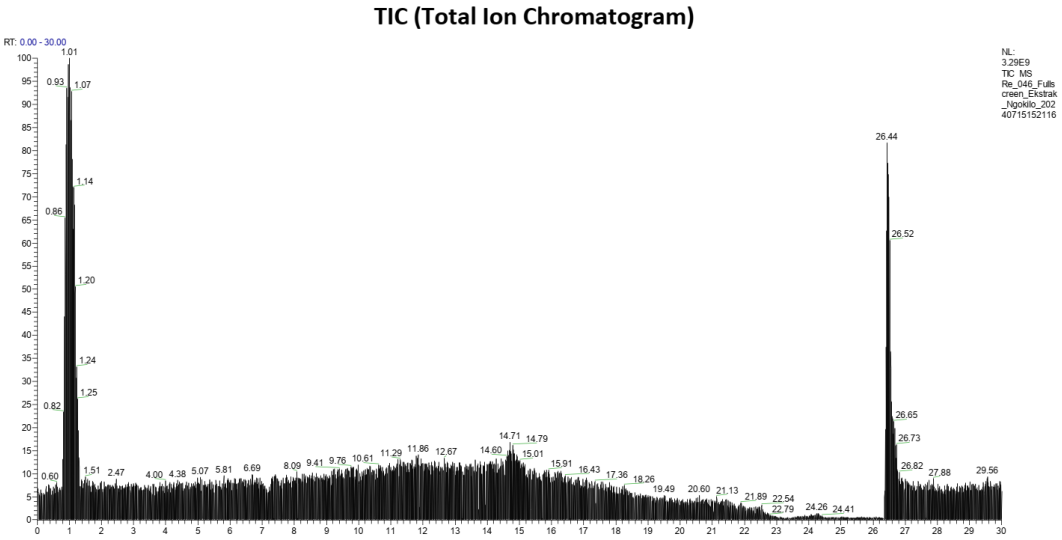

Figure 1. Total Ion Chromatogram of 96% ethanol extract of *S. mutabilis* leaves

Further analysis of the compounds shown in Figure 1 was carried out by eliminating compounds that were included in the exclusion compounds. Compounds that were included in the inclusion compounds (Table 1) were then analyzed by molecular docking simulations.

Table 1. Inclusion Compounds of 96% Ethanol Extract of S. mutabilis Leaves as Results of UHPLC-HRMS Analysis

No	Name	Formula	Calc. MW	RT [min]	Area (Max.)	mzCloud Best Match
1	Choline	C5H13NO	103.09973	1.023	6.57E+09	98.1

2	Betaine	C5H11NO2	117.07875	1.186	3.78E+09	98.5
3	DL-β-Leucine	C6H13NO2	131.09433	0.973	2.86E+08	90.2
4	5-hydroxy-2-(4-hydroxy phenyl)-6-methoxy-7-{[(2S,3R,4S,5S,6R)-3,4,5- trihydroxy-6-(hydroxy-methyl) oxan-2-yl]oxy}-4H-chromen-4-one	C22H22O11	462.11461	8.23	2.19E+08	99.2
5	5,7-dihydroxy-3-(4-hydroxy phenyl)-6-methoxy-4H-chromen- 4-one	C16H12O6	300.0624	10.169	1.13E+08	98.8
6	α-Eleostearic acid	C18H30O2	278.22365	17.173	9.14E+07	95.8
7	9-Oxo-10(E),12(E)-octadeca dienoic acid	C18H30O3	294.21846	17.84	6.40E+07	97.1
8	Sedanolide	C12H18O2	194.13002	15.305	5.60E+07	87.1
9	5-hydroxy-2-(4-hydroxy phenyl)-6-methoxy-7-{[(2S,3R,4S,5S,6R)-3,4,5- trihydroxy-6-(hydroxy methyl)oxan-2-yl]oxy}- 4H-chromen-4-one	C22H22O11	462.11421	0.835	5.36E+07	99.2
10	4-Indolecarbaldehyde	C9H7NO	145.05251	7.805	4.71E+07	97.2
11	5,7-dihydroxy-2-(4-hydroxy-3-{[(2S,3R,4S,5S,6R)-3,4,5- trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}phenyl)-3-methoxy-4Hchromen-4-one	C22H22O12	478.10964	7.532	4.09E+07	96.1
12	(+)-ar-Turmerone	C15H20O	216.15071	17.562	2.84E+07	98.8
13	3-Methoxy-5,7,3',4'-tetra hydroxy-flavone	C16H12O7	316.05708	8.99	2.45E+07	97.6
14	(2R)-5-hydroxy-7-methoxy-2- phenyl-3,4-dihydro-2H-1-benzopyran-4-one	C16H14O4	270.08812	16.285	1.61E+07	95.7
15	Apigenin	C15H10O5	270.0519	11.009	1.49E+07	98.8
16	Nicotinamide	C6H6N2O	122.04791	1.429	1.47E+07	94.6
17	6-O-Methylscutellarin	C22H20O12	476.09407	8.319	1.42E+07	97.8
18	Linoleoyl ethanolamide	C20H37NO2	323.28121	19.208	1.38E+07	96.9
19	Apigetrin	C21H20O10	432.10423	8.07	1.24E+07	99.3
20	Kuromanin	C21H20O11	448.09914	7.317	9.84E+06	99.5
21	D-(+)-Proline	C5H9NO2	115.06323	0.849	4.89E+06	89.7
22	Adenosine	C10H13N5O4	267.0956	1.454	4.38E+06	99.8

Compounds included in the exclusion criteria such as compound with %similarity value of <85%. Based on the study by Castano-Ortiz et al. (2024), compounds with a value of <85% indicated a low degree of similarity and were suspected to be degradants. These degradants or impurities could be phthalate derivatives or plasticizers in the matrix being analyzed. These impurities could enter the sample due to the use of a 0.22µm millipore to form a true sample solution for UHPLC-HRMS (Mirza, 2021). Interestingly, compounds with different %similarity and retention time values were also found. Compounds with a high %similarity value under these conditions tend to be selected because they have a higher degree of confidence (Castano-Ortiz et al., 2024). This process was carried out to increase the validity of the metabolite profiling results. Retention time was also an exclusion criterion, where compounds with a retention time >20 minutes were eliminated. The results of research by Liu et al (2019) showed that compounds with a retention time > 20 minutes had a % similarity value < 80%.

Molecular Docking Simulation of 96% Ethanol Extract of *S. mutabilis* Leaves

Molecular docking simulations of 22 compounds of 96% Ethanol Extract of *S. mutabilis* leaves were performed to observe the compounds' ability to bind to therapeutic targets such as ER-β (PDB ID: 3OLS) obtained from the RCSB Protein Data Bank (https://rcsb.org). The ER-β protein was prepared and internally validated with PyRX 0.8 to obtain optimal prediction results (Fardhani et al., 2024). The internal validation results of the 3OLS protein showed a RMSD of 1.284 Å, indicating that both 3OLS and the Vina plug-in of PyRX 0.8 are suitable for use in the molecular docking stage of this study (Mirza et al., 2021; Fardhani et al., 2024). Compounds in the 96% ethanol extract of S. mutabilis leaves demonstrated potential estrogenic activity. Compounds such as 3-O-Methyl -Quercetin and 5,7-dihydroxy-3-(4-hydroxyphenyl)-6-methoxy-4H-chromen-4-one were observed to bind Glu305, Arg346 and His475 with hydrogen bonds on ER-β (Figure 2).

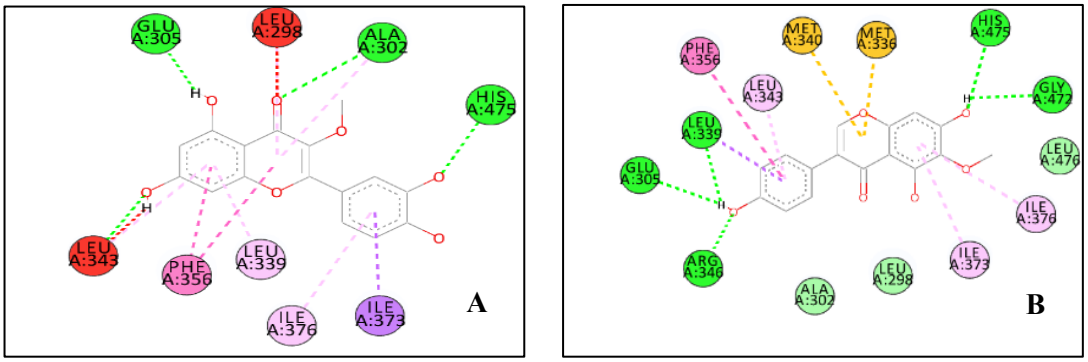

Figure 2. Interaction of compounds in 96% extract of *S. mutabilis* leaves against ER-β

(A) 3-O-Methyl-Quercetin

These three amino acids have been observed in several studies as amino acids predicted to be responsible for estrogenic agonist activity (Mirza et al., 2021). The results of the analysis of compound interactions with the ER-β protein further showed that the compound 5,7-dihydroxy-3-(4-hydroxyphenyl)-6-methoxy-4H-chromen-4-one was able to provide the best estrogenic activity compared to other compounds because it had a binding affinity of -7.9Å and a pharmacophore distance of 11.478Å (Table 2).

Table 2 Molecular Docking Simulation of Phytoestrogen Compounds from 96% Ethanol Extract of S. mutabilis Leaves Against ER-β Protein

Compound	Amino Acid Residue	*%Similarity* (%)	*Binding Affinity*	Pharmacophore Distance
3-O-MethylQuercetin	Ala302; Glu305; His475 (Hydrogen) Leu339; Ile376(Pi Alkyl) Phe356 (Pi T Shaped) Ile373 (Pi-sigma) Leu298; Leu343 (Unfavorable)	33,33	-4,6	9,698
5,7-dihydroxy-3-(4-hydroxyphenyl)-6-methoxy-4H-chromen-4-one	Glu305; Arg346; His475 (Hydrogen) Gly472 (Carbon-Hydro) Leu298; Ala302; Leu343 (Pi Alkyl) Phe356 (Pi T Shaped)	66,67	-7,9	11,478
17 β-Estradiol (Native Ligand)	Glu305; Arg346; His475 (Hydrogen) Leu298; Ala 302; Met336; Leu339; Leu343; Ile373 Ile376; Leu476 (Pi Alkyl) Phe356 (Pi T Shaped)	100	-10,6	11,182

NB: Bold represent amino acid residues that in the same location and bond type as the native ligand (17β-Estradiol).

This binding affinity value of 5,7-dihydroxy-3-(4-hydroxy phenyl)-6-methoxy-4H-chromen-4-one is higher than of 3-O-Methyl-Quercetin. This value indicates that compound requires lower free energy and tends to be more stable when binding to ER-β than other compounds. This finding is supported by the pharmacophore distance of 5,7-dihydroxy-3-(4-hydroxy phenyl)-6-methoxy-4H-chromen-4-one, which is close to that of 17β-Estradiol (10.926 Å). This value shows that the compound 5,7-dihydroxy-3-(4-hydroxy phenyl)-6-methoxy-4H-chromen-4-one has estrogenic activity close to the compound 17β-Estradiol on the ER-β protein (Siswandono and Soekardjo, 1995; Mirza *et al*., 2021).

CONCLUSION

The 96% ethanol extract of *S. mutabilis* leaves contains 3-O-Methyl-Quercetin and 5,7-dihydroxy-3-(4-hydroxyphenyl)-6-methoxy-4H-chromen-4-one which has the potential to provide phytoestrogenic activity when forming receptor ligand bonds to ER-β. Its compound should be confirmated with other method to support. Both compounds need further confirmation using other methods to strengthen their potential as phytoestrogen source from *S. mutabilis*.

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