Mahogany seeds extract showed potential immunomodulator by in-silico and in vivo testing

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Abstract. Environmental disturbances such as climate change affect human health both infectious and non-communicable disease. Immunity plays an important role in maintaining body health while the mahogany seed has been used empirically to increase immunity. This study aims to evaluate the potential of mahogany seed as an immunomodulator through an in-silico test by molecular docking and an in vivo test using a hemagglutination method. Docking simulation was done towards 16 bioactive contents using Protein Data Bank, PubChem, Marvinsketch, Chimera, Swissdock and pkCSM. The immunomodulatory effect of 96% ethanol extract was studied in 36 DDY mice, separated into 6 groups: normal, negative and positive groups, also mahogany seed extract with dose of 50mg/kgBW, 100mg/kgBW, and 200mg/kgBW). Cyclophosphamide was used to depress immune system and Sheep Red Blood Cells as antigen. The result for the in-silico study showed Gibbs energy of swietenin C and swietenin E more negative than tocilizumab and complied with Lipinski's rule. The result of the in vivo showed the average antibody titres of group I-VI were 0.00±0.00; 1.90±0.330; 4.31±0.631; 3.51±1.168; 3.71±0.631; 4.01±1.037, respectively. There was no significant difference between the mahogany seed extract treatments with the positive control ($p>0.05$). This study concludes that mahogany seed extract has immunomodulatory activity that can be utilized by the community in the prevention of various diseases.

1 Introduction

Climate change profoundly influences public health, affecting nearly the entire global population and accounting for around 10% of worldwide mortality. It affects human health through multiple pathways. Climate-related hazards pose direct dangers to well-being and survival, including extreme weather events, storms, rising sea levels, flooding, and droughts. In the realm of public health, direct repercussions encompass the aggravation of heat-related ailments, respiratory issues stemming from heightened air pollution, and the spread of infectious diseases as climate alterations reshape the habitats of disease-carrying vectors [1].

Indonesia has many medicinal plant resources that can be used to boost the immune system and have been traditionally used for generations. One part of the medicinal plant that

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has the potential to enhance the immune system (immune booster) is mahogany seeds. The purified mahogany seeds extract showed a potential antioxidant activity with IC50 33.86μg/mL [2] which considerable to support the immune system [3]

Mahogany trees are abundant in Indonesia, especially in the Sumatra region [4]. Mahogany seeds are easily obtained without damaging the plant and its habitat, and they contain secondary metabolites such as flavonoids, alkaloids, saponins, steroids, and triterpenoids, which are suspected to have immunomodulatory activity [5]. Research showed an increase in the number of eosinophils and lymphocytes, antibacterial activity, and antioxidant activity of mahogany extracts [5], [6], [7] A mahogany seed isolate contained 1,4-bis-3,4,5-trimethoxy-phenyl-tetrahydro-furo(3,4-c) furan showed a reduction on the malondialdehyde level, an oxidative stress biomarker [7]. However, further studies are still needed to identify the active compounds contained in mahogany seed extract. A testing to confirm the immune response in antibody formation also needed to strengthens the benefits of mahogany seeds as immunomodulators.

Hence, this research aims to identify bioactive compounds involved in immunological activity using the molecular docking method. Furthermore, determination of the potential immunomodulator activity of mahogany seed extract was performed by using the hemagglutination method to evaluate the ability to form antibodies as an immune response.

2 Material and Method

2.1 In Silico Study

2.1.1 Tools and Applications

Laptop with specifications Intel Core i5-8250U CPU @1.6GHz 1.8GHz, Windows 10, 4GB RAM. The applications used are Protein Data Bank (PDB), PubChem, Marvinsketch, Chimera, Swissdock webserver, and pkCSM.

2.1.2 Protein and Ligand Preparation

The search for the interleukin-6 receptor in the PDB was conducted using the keyword interleukin-6 (IL-6) and the protein code 1ALU was obtained, which will be used as the protein target. A total of 16 bioactive compounds contained in mahogany seeds, namely 3-O-acetylswietenolide, swietemahonin A, swietemahonin E, swietemahonin F, swietenin B, swietenin C, swietenin D, swietenin E, swietenin F, 3-O-tigloyl-6-O-oxogedunin, 3,6-0,0-diacetylswietenolide, swietenolide, and proceranolide. The 2D and 3D structures of the bioactive compounds from mahogany seeds and tocilizumab as a positive control were obtained from PubChem and Marvinsketch, and then prepared using Chimera.

2.1.3 Molecular Docking Protocol

The docking simulation was conducted between the 1ALU receptor and the test compound using SwissDock. The 1ALU receptor (.pdb) and the test compound (.mol2) were submitted to SwissDock. The xyz coordinates in this simulation are 2.226; -20.815; 9.030. The results of the docking simulation will be sent by Swissdock via the user's email. The analysis of the docking results is conducted on the cluster with the most negative Gibbs energy. The obtained Gibbs energy is compared with the positive control; if the compound has the same or more negative Gibbs energy than the positive control, it is predicted to be active on the receptor.

Then, a Lipinski analysis was conducted, where the test compounds that meet two out of four requirements in this study are recommended as immunomodulator candidates. The requirements are a molecular weight <500 Da, Log P <5, Hydrogen Bond Donor (HBD) <5, and Hydrogen Bond Acceptor (HBA) <10. Next, the candidate compounds were visualized using the Chimera application [8].

2.2 In Vivo Study

2.2.1 Equipment

Analytical balance, weighing scale, syringe, micropipette, 96 well plate, water bath, test tube, beaker, measuring cylinder, volumetric flask, round-bottom flask, evaporating dish, wooden clamp, stirring rod, filter paper, Erlenmeyer flask, filter paper, mouse cage, and oral and subcutaneous injection syringe.

2.2.2 Materials

Mahogany seed simplicia (*Swietenia mahagoni*), sheep red blood cells (SRBC), cyclophosphamide, commercial herbal products as positive control, aquadest, Alsever's solution, pyrogen-free phosphate saline, sodium carbonate solution, chloroform, acetic acid, amyl alcohol, methanol, petroleum ether, hydrochloric acid (HCl), lead solution, iron (III) chloride, Meyer reagent, FeCl3, and concentrated sulfuric acid.

2.2.3 Preparation of Mahogany Seed Extract

Mahogany seed extract was obtained from the Research Institute for Spices and Medicinal Plants (BALITRO), Bogor. Mahogany seeds were extracted using the kinetic maceration method with 96% ethanol as the solvent for 3 days with 3 times filtration. The solvent in the filtrate was removed using a rotavapor, resulting in a thick extract.

2.2.4 Phytochemical Screening

Alkaloid Identification: 1.0 mL of mahogany seed extract was added with NH_3, then ground and mixed with 5 mL of $CHCl_3$, and then filtered. The filtrate was added with 2M H_2SO_4 until an acid layer formed. The acid layer is pipetted and placed into 3 test tubes, then 2 drops of Meyer's reagent are added to the first tube, 2 drops of Wagner's reagent to the second tube, and 2 drops of Dragendorff's reagent to the third tube. White precipitate in the first test tube and a reddish-brown precipitate in the second and third test tubes, indicate alkaloids [9].

Flavonoid Identification: 1.0 mL of mahogany seed extract is taken, added with 2 drops of concentrated HCl, heated over a water bath, and left for 1 minute. The sample is declared to contain flavonoids if a red colour is formed. Tannin Identification: 1.0 mL of mahogany seed extract is added with 2 mL of methanol and filtered; the filtrate is then treated with 2 drops of $FeCl_3$. The sample is declared to contain tannins if a reddish-brown colour is formed. Phenolic Identification: A total of 5.0 mL of the sample is added with 3-4 drops of iron (III) chloride solution. The sample is declared to contain phenolic compounds if a blue-black colour is formed. Identification of Steroids/Triterpenoids: A volume of 1.0 mL of mahogany extract is added with 2 drops of anhydrous acetic acid and 2 drops of concentrated acetic acid. The sample is declared to contain steroids if a blue or green colour is formed and is declared to contain triterpenoids if a purple or orange colour is formed [10].

2.2.5 Hemagglutination Test

The immunomodulator activity test of mahogany seed extract in vivo used 36 male DDY strain mice divided into 6 groups, which were acclimatized for 1 week with standard feed and mineral water available ad libitum, storage room temperature maintained at 25±2°C, and a light-dark cycle of 12 hours each to adjust the circadian rhythm of the test animals. Body weight measurements of the test animals were conducted during the treatment. The treatment of the test animals can be seen in Table 1.

Table 1. Experimental animal treatments

Group	Treatment	Administration Time
I	Normal Saline Solution (NaCl 0.9%)	14 days
II	Cyclophosphamide 200mg/kgBW	Day 0
III	Cyclophosphamide 200mg/kgBW	Day 0
	Positive control 150mg/kgBW	14 days
IV	Cyclophosphamide 200mg/kgBW	Day 0
	S. mahagoni Extract 50mg/kgBW	14 days
	SRBC Antigen	Day 7
V	Cyclophosphamide 200mg/kgBW	Day 0
	S. mahagoni Extract 100mg/kgBW	14 days
	SRBC Antigen	Day 77
VI	Cyclophosphamide 200mg/kgBW	Day 0
	S. mahagoni Extract 200mg/kgBW	14 days
	SRBC Antigen	Day 7

The preparation of Alsever solution is done by dissolving 2 g of dextrose, 0.8 g of sodium citrate, 0.05 g of citric acid, and 0.42 g of NaCl in distilled water until the volume reaches 100 mL. Then, sterilization is carried out using an autoclave at a temperature of 115°C for 30 minutes[11].

Antigen is made using fresh sheep blood taken aseptically through the jugular vein. Fresh sheep blood is mixed with sterile Alsever solution, then centrifuged for 5 minutes, and the red blood cells that settle at the bottom of the tube are collected. The obtained sheep red blood cells (SRBC) were washed three times using pyrogen-free phosphate buffer (pH= 7.2). SRBC is stored in the refrigerator until it is time to use it [12], [13].

Antibody titre is determined by the hemagglutination technique of blood samples obtained from the orbital sinus, approximately 2 mL, then centrifuged at 3000 rpm for 20 minutes to get the serum. The testing began by numbering the 96-well U-bottom microplate from 1 to 8 for the dilution of mouse serum. In wells 2–8, 25 µL of phosphate buffered saline (PBS) and 50 µL of mouse serum were added to well 1. Dilution was performed by taking 25 µL of serum from well 1, adding it to well 2, and homogenizing it, repeating this process until well 8. Each well was added with 25 µL of 1% SRBC, homogenized, and stored at room temperature for 2 hours. The antibody titre value is determined from the highest dilution that still shows agglutination [14]. Example of reading hemagglutination titre and conversion value calculated using the formula 2 log[titer]+1, which is:

1. Normal Group (N) = 0, conversion value = 0
2. Control Group (-) = 2, conversion value = 1.60
3. Control Group (+) = 32, conversion value = 4.01
4. Dose 1 Group (D1) = 8, conversion value = 2.81
5. Dose 2 Group (D2) = 64, conversion value = 4.61
6. Dose 3 Group (D3) = 16, conversion value = 3.41

4 Result and Discussion

In silico research using the interleukin 6 (IL-6) receptor with PDB code 1ALU. IL-6 is a pro-inflammatory cytokine whose levels are known to increase in patients with severe Covid-19, becoming a major mediator of excessive inflammation and immune response, potentially leading to a cytokine storm. In severe Covid-19 patients, elevated IL-6 can be dangerous as it can cause severe inflammation and a decrease in immune system response [15].

In this study, 16 bioactive compounds contained in mahogany seeds and tocilizumab were used as a positive control. Tocilizumab is an IL-6 inhibitor that is related to the body's immune system [16], [17]. The docking simulation results showed that swietenin C, swietenin E, and 3-O-tigloyl-6-O-acetylswietenolide have Gibbs energies of -7.39 kcal/mol, -7.69 kcal/mol, and -7.81 kcal/mol respectively, as shown in Table 2. The Gibbs energies of these bioactive compounds are equal to or more negative than tocilizumab (-7.39 kcal/mol), indicating they are predicted to have immunomodulatory activity.

Table 2. Results of compound docking test with 1 ALU receptor

No	Compound Name	Estimated ΔG (kcal/mol)	Prediction
-	Tocilizumab (reference standar)	-7.39	Inactive
1.	3-O-acetylswietenolide	-7.08	Inactive
2.	Swietemahonin A	-7.29	Inactive
3.	Swietemahonin E	-7.38	Inactive
4.	Swietemahonin F	-7.11	Inactive
5.	Swietenin B	-7.29	Inactive
6.	**Swietenin C**	**-7.39**	**Active**
7.	Swietenin D	-7.21	Inactive
8.	**Swietenin E**	**-7.69**	**Active**
9.	Swietenin F	-7.39	Inactive
10.	**3-O-tigloyl-6-O-acetylswietenolide**	**-7.81**	**Active**
11.	6-O-acetylswietenolide	-7.29	Inactive
12.	6α-acetoxygedunin	-7.12	Inactive
13.	7-deacetoxy-7-oxogedunin	-6.77	Inactive
14.	3,6-0,0-diacetylswietenolide	-7.37	Inactive
15.	Swietenolide	-7.20	Inactive
16.	Proceranolide	-6.85	Inactive

After obtaining the docking results, a Lipinski analysis was conducted on the three compounds. This aims to see the similarity of the test compound with drug molecules, thereby increasing the possibility of being developed as a drug candidate. 3-O-tigloyl-6-O-acetylswietenolide does not meet Lipinski's criteria due to its molecular weight (>500 D) and Log P being too high (>5).

Molecular weight is related to the compound's ability to penetrate cell membranes, while Log P is related to lipophilicity; the higher the Log P, the more lipophilic the compound, leading to greater retention in tissues and an increased risk of toxicity. HB and HBA relate to the interaction between the ligand and the target. The stronger the binding, the higher the risk of toxicity [8]. In this study, swietenin C and swietenin E meet Lipinski's criteria, making them potential candidates for development as immunomodulators, as shown in Table 3. Both compounds were visualized with Chimera to observe the interactions that occur and which amino acids are involved. In swietenin C, there are 2 hydrogen bonds with the amino acids ASN63 and LEU64, while the hydrophobic interactions occur with the amino acids ASN61, LEU57, LEU62, LEU64, GLU58, GLU69, SER169, PRO65, LYS64, LYS66, LYS86, LYS70, and ALA86. There are 3 hydrogen bonds in swietenin E with the IL-6 receptor through the amino acids LEU62, LYS66, LYS86, while its hydrophobic interactions involve the amino acids LYS54, LYS66, LYS70, ALA56, ALA68, GLU55, GLU63, PHE94, ASP71,

SER169, MET67, ASN61, ASN63, PRO65, LEU57, and LEU64. The visualization results of swietenin C and swietenin E are presented in Table 4 and Figure 1.

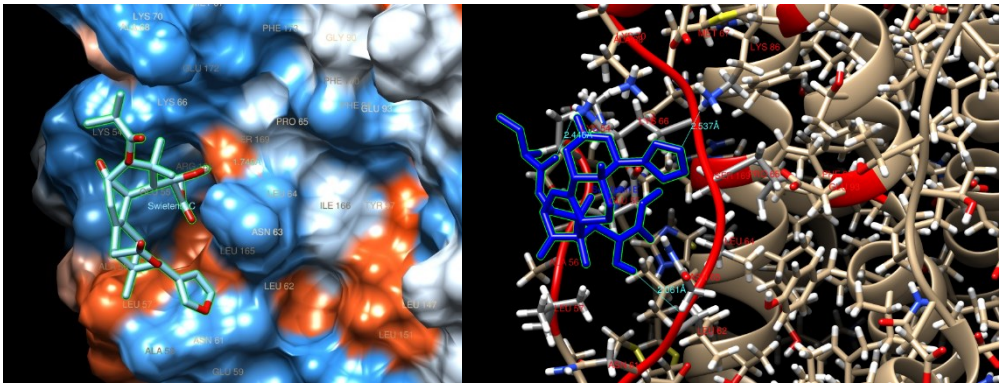
Table 3. Results of lipinski analysis

No.	Compound Name	Lipinski Prediction				Requirement
		MW	LogP	HBD	HBA	
1.	Swietenin C	556.5	3.97	1	9	**Qualified**
2.	Swietenin E	570.7	4.50	1	9	**Qualified**
3.	3-O-tigloyl-6-O-acetylswietenolide	610.7	5.07	0	10	Unqualified

Note: MW (molecular weight), HBD (hydrogen bond donor), HBA (hydrogen bond acceptor)

Table 4. Bonds formed in Swietenin C and Swietenin E

No.	Compound Name	Hydrogen Bond	
		Amino Acid Residue	Distance (Å)
1.	Swietenin C	ASN63	2,364
		LEU64	1,746
2.	Swietenin E	LEU62	2,061
		LYS66	2,446
		LYS86	2,537



a. Swietenin C b. Swietenin E

Figure 1. Visualization of Swietenin C and Swietenin E

The primary phytoconstituents of methanolic and aqueous extracts of mahogany seeds include tannins, alkaloids, saponins, terpenoids, anthraquinones, cardiac glycosides, and volatile oils. Limonoids swietenolide and 2-hydroxy-3-O-tigloyl swietenolide were extracted from the methanolic extracts of the seeds, exhibiting antibacterial properties. Meanwhile, 28 tetranotriterpenoids linked to swietenine and swietenolide were recovered from the ether extract. Among these chemicals, swietemahonins A, D, E, and G, along with 3-O-acetyl-swietenolide and 6-O-acetyl-swietenolide, inhibited platelet activating factor [18]. Other study showed that the principal ingredient of mahoni is switenine; nevertheless, the seeds also contain a significant amount of lipids. Switenine has been studied for its potential to reduce cholesterol levels, triglycerides, and liver glycogen [2].

The results of the in vivo test using the hemagglutination method are shown in Figure 2. To observed the antibodies titre, $1x10^8$ SRBC/mL is required. In this study, SRBC was immunized at a concentration of $1.025x10^8$ cells/mL, which was able to form antibodies in mice. Hemagglutination occurs due to the binding between SRBC (antigen) and antibodies formed by the immune system in mice. The antibody with a greater ability to bind to SRBC

is IgM, related to its size and high valency (pentamer), which can be observed with the presence of agglutination [14].

Antibody titer readings using a 96-well U-bottom plate can facilitate the observation of agglutination formed at the bottom of the plate. A positive result is indicated by the distribution of agglutinated cells in the liquid within the well, while a negative result is indicated by the presence of a precipitate in the form of a dot at the bottom of the well (Figure 2). The dilution results and antibody titers in the test animals are shown in Table 5.

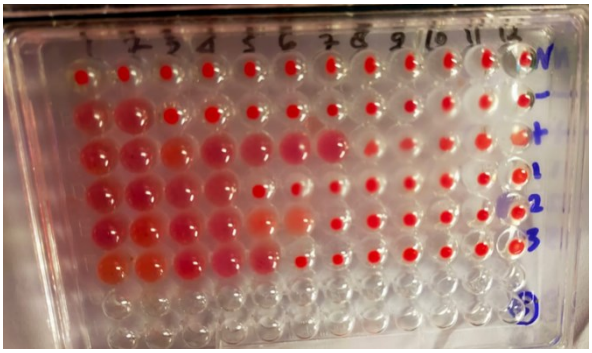


Figure 2. Results of antibody titer dilution

Table 5. Dilution and antibody titer in test animals

Experimental Animals	Dilution and Antibody Titer in Experimental Animals											
	Control N		Control (-)		Control (+)		D1		D2		D3	
	P	T	P	T	P	T	P	T	P	T	P	T
1.	0	0	4	2.2	64	4.6	256	5.8	32	4.0	256	5.8
2.	0	0	2	1.6	16	3.4	16	3.4	8	2.8	32	4.0
3.	0	0	4	2.2	128	5.2	16	3.4	16	3.4	32	4.0
4.	0	0	2	1.6	32	4.0	8	2.8	16	3.4	16	3.4
5.	0	0	4	2.2	64	4.6	8	2.8	32	4.0	8	2.8
6.	0	0	2	1.6	32	4.0	8	2.8	64	4.6	16	3.4

Note: D1 (S. mahagoni extract dose 50 mg/kgBW); D2 (S. mahagoni extract dose 100 mg/kgBW); D3 (S. mahagoni extract dose 200 mg/kgBW); P: Dilution; T: Antibody titer

The antibody titers formed in groups I–VI were 0.00±0.00; 1.90±0.330; 4.31±0.631; 3.51±1.168; 3.71±0.631; 4.01±1.037, respectively. Statistical analysis was conducted to assess the significance of the mean antibody titers between groups using ANOVA and the difference between two groups using Fisher's LSD as shown in Table 6. There is a significant difference between group II and the treatment group ($p<0.05$). This indicates that the administration of commercial herbal product and mahogany seed extract provides an optimal increase in antibody titers. There is no significant difference between the mahogany seed extract treatment group and the positive control group ($p>0.05$). This shows that mahogany seed extract (S. mahagoni) with three different doses has the same effectiveness as commercial herbal product (positive control), a phytopharmaceutical that is effective as an immunomodulator.

Table 6. Results of dilution and antibody titer in test animals

Group	Antibody titre	I	II	III	IV	V	VI
I	0.00±0.000	-	0,000*	0,000*	0,000*	0,000*	0,000*
II	1.90±0.330	-	-	0,000*	0,001*	0,000*	0,000*
III	4.31±0.631	-	-	-	0,072	0,173	0,359
IV	3.51±1.168	-	-	-	-	0,645	0,360

V	3.71±0.631	-	-	-	-	-	0,646
VI	4.01±1.037	-	-	-	-	-	-

Note: *the results of the statistical analysis show a significant difference

Mahogany seeds contain the largest secondary metabolites of flavonoids and alkaloids. In general, the flavonoid group can enhance the immune response by modulating oxygen and nitrogen radicals, increasing antibody production, and exhibiting cytotoxic activity against tumor cells [19]. Swietenin C and swietenin are flavonoid compounds that show binding with the IL-6 receptor, thus functioning as anti-inflammatory agents and having the potential to be further developed into immunomodulators. Swietenia mahagoni Jacq includes the terpene component swietenine, which also exhibits antidiabetic action [20].

5 Conclusion

Mahogany seeds in silico and in vivo studies showed an immunomodulatory activity. The in-silico study found swietenin C and swietenin E contained in mahogany seeds can bind to the interleukin-6 receptor, while the in vivo study showed that the antibody titre in the treatment group was not significantly different from the positive control.

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