OnLine Journal of Biological Sciences

Research Article

Molecular Characterization and in Silico Analysis of Phytochemical Compounds from Plants with Immunomodulatory Potential

Hermin Pancasakti Kusumaningrum[1], Rejeki Siti Ferniah[1], Siti Nur Jannah[2], Dea Kafita Sari[2], Khusnul Khotimah[2], Candra Wahyuningsih[3] and Azalia Puspa Herida[3]

[1]*Biotechnology Study Program, Faculty of Science and Mathematics, Diponegoro University, Semarang, Indonesia*
[2]*Biology Study Program, Faculty of Science and Mathematics, Diponegoro University, Semarang, Indonesia*
[3]*Marine Science Study Program, Faculty of Fisheries and Marine Science, Diponegoro University, Semarang, Indonesia*

Article history
Received: 05-12-2024
Revised: 20-05-2025
Accepted: 28-05-2025

Corresponding Author:
Hermin Pancasakti Kusumaningrum, Biotechnology Study Program, Faculty of Science and Mathematics, Diponegoro University, Semarang, Indonesia
Email: herminsakti@gmail.com

Abstract: The immune system works to protect the body against foreign antigens from microorganisms and abnormal cells. The use of herbal plants as immunomodulators are used to prevent disease attacks. TNFα and interleukin-6 are pro-inflammatory immune systems that cause disease in uncontrolled synthesis. Herbal plants as inhibitors of TNFα and interleukin-6 need to be identified molecularly to reduce the occurrence of poisoning. This research aims to determine the molecular characteristics of herbal plants based on ITS primers and in silico studies of plant phytochemical compounds as inhibitors of TNFα and interleukin-6 for immunomodulatory. The research was carried out by isolating and amplifying the DNA of herbal plants, identifying phytochemical compounds through literature study, PASS Prediction analysis and molecular docking. The results showed that the ITS fragment size of six herbal plants was 700 bp. PASS Prediction analysis on anti-inflammatories and immunomodulatory shows that zerumbone has a Par value of 0.831 and hexadecanoic acid has a Par value of 0.481. The results of molecular docking show that the compounds betulin and benzenedicarboxylic acid have the smallest binding affinity values, namely -9.0 and -8.1 kcal/mol when interacting with TNF alpha and interleukin-6 compared with adalimumab as positive control.

Keywords: Phytochemical, Plant, Immunomodulatory, Molecular Docking, ITS

Introduction

The immune system plays important role against foreign antigens from microorganisms (e.g., bacteria, viruses, fungi). The immune system is classified into two categories namely innate and adaptive immune responses (Ramasamy, 2022). In normal condition, the antigens are destroyed by the innate immune. If the innate immune fails to eliminate the invaders, the adaptive immune is initiated. The innate immune system includes skin, low stomach pH, body temperature, and tears while the adaptive immune is composed of T and B lymphocytes (Alanazi & Elyasa, 2023). The adaptive immune respond includes two categories namely humoral (antibody mediated immunity) and cellular mediated immunity (Abbas *et al*., 2021). The ways of response based on the type of organism. However, both of low immunity and high immunity have damaging effects on the body and cause autoimmune disease. The autoimmune disease occurs when the immune system accidentally targeting its own cells. The error of immune system targeting the antigens impacts the crucial organs such as heart and lungs. Several factors that affect dysregulated immunity are excessive exposure of heavy metals, pathogenic bacteria and other parasites, dietary imbalances, environmental pollution, and high stress (Zampieron & Kamhi, 2012; Liu *et al*., 2023).

Furthermore, the autoimmune disease consists of rheumatoid arthritis, multiple sclerosis, lupus, psoriasis, type 1 diabetes (Richard-Eaglin & Smallheer, 2018). Therapy for autoimmune disease requires large costs. Based on Statista, global autoimmune disease spending cost 149 billion dollars (Statista, 2024).

Drug of autoimmune disease become the mainly important part to medication and therapy in this problem. This is a challenge for researcher to develop or find new

drug candidates. The varieties of drugs that have properties to immune-suppressants, interferons, corticosteroids, and anti-inflammatory are approved to treat the autoimmune disease. These drugs work to regulates the immunity by inhibit regulation of the immune cell, gene expression, and synthesis of pro-inflammatory cytokines. The balance of pro-inflammatory (IL-2, IL-6, IL-8, IL-1β, IFN-γ, TNFα) and anti-inflammatory (IL-4, IL-10, TGFβ) cytokines influence the inflammation and homeostasis of immune response (Boshtam *et al*., 2017). However, the immune-suppressants drugs have several adverse effects if given in the long term such as metabolism disorders, toxicity, infection, and cancer (Ponticelli & Glassock, 2019).

Several research reported that dietary have a significant effect on inflammatory regulation. Especially, consuming herbal plants which have immunomodulatory impact can help improve anti- inflammatory immune system in body. Herbal plants such as lempuyang, gandasuli, brotowali, pacing pentul, mangkokan, and mimba are sources of phytochemical compound namely flavonoid, polyphenol, terpene, alkaloid and others. The herbal plants are identified by molecular characterization using Internal Transcribed Spacer (ITS) primer to determine the species and specific phytochemical compounds from each plant. These compounds have potential as pharmaceutical agents. However, these six herbal plants have not been widely identified as immunomodulatory or anti-inflammatories. The pro-inflammatory cytokine which was identified as protein targets are TNFα and interleukin-6. Inhibition both of the protein target is expected to increase the synthesis of anti-inflammatory cytokines. Therefore, the purpose of this study was to determine the molecular characteristics of phytopharmaceutical plants based on ITS primers and in silico studies of herbal plant phytochemical compounds as inhibitors of TNFα and interleukin-6 for immunomodulatory.

Materials and Methods

Materials

Six species were used in this research namely lempuyang (*Zingiber zerumbet*), gandasuli (*Hedychium coronarium*), brotowali (*Tinospora cordifolia*), pacing pentul (*Costus spicatus*), mangkokan (*Polyscias scutellaria*), and mimba (*Azadirachta indica*). All of the plants were found in Gunungpati, Central Java, Indonesia.

DNA Isolation

DNA isolation from six phytochemical plants was carried out using a modification of the CTAB (Hexadecyltrimethylammonium bromide) method according to Kusumaningrum *et al*. (2018). This method is a DNA isolation technique discovered by Ausubel *et al*. (1994). This method uses CTAB buffer which is a cationic detergent to destroy plant cells. The quality and quantity of plant DNA were then measured using Nanodrop 2000.

PCR and Electrophoresis

PCR reaction uses materials consisting of plant DNA, PCR kit (MyTaq), 18S forward primer, 18S reverse primer and ddH_2O with a total volume of 25 µL. The PCR steps consist of pre-denaturation, denaturation, annealing, extension and post-extension stages. The annealing temperature is carried out at various temperatures at 50-56°C. The PCR cycle is carried out for 30 cycles. PCR products are visualized by electrophoresis in 1% agarose with 1 kb DNA ladder. The results of ITS fragment amplification are visualized using UV-TEC doc gel.

Sequencing

The ITS fragments of the six plants were searched for their base sequences using Sanger sequencing with the BigDye® Terminator v3.1 cycle sequencing device. The sequencing results were then used to determine species and phylogenetic relationships.

Phylogenetic Sequencing

Relationship analysis was done through sequence alignment using ClustalX program. The Phylogenetic tree was constructed through MEGA X Software with Neighbor-Joining: Jukes-Cantor Model tree (Kumar *et al*., 2018).

Receptor and Ligand Preparation

The structure of the TNFα target protein (PDB ID: 2AZ5) and interleukin-6 (PDB ID: 1P9M) was obtained from the RCSB protein database (https://www.rcsb.org/). Preparation of receptor was carried out using Biovia Discovery studio to remove water, ligand native, and other unused compounds. The phytochemical compound or ligand was obtained from GC-MS analysis and the structure was retrieved from Pubchem NCBI.

PASS Prediction

The SMILES structure of ligands was obtained from Pubchem NCBI (https://pubchem.ncbi.nlm.nih.gov/). Prediction of Activity Spectra for Substance (PASS) analysis was done through the Way2Drug Predictive Services Website (https://www.way2drug.com/passonline/). Furthermore, Pa value analysis of anti-inflammatory and immunomodulatory parameters was carried out (Dzufakar *et al*., 2023). Compounds that have immunomodulatory activity from the results of PASS analysis will proceed to molecular docking to determine their binding affinity values.

Molecular Docking

Molecular docking both of receptor and ligand were carried out using PyRx software. Binding affinity value and Root Mean Square Deviation (RMSD) are used as the main parameters. The molecular interaction between receptor and ligand was analyzed using Biovia Discovery Studio (Kusumaningrum *et al*., 2022).

Lipinski Rule of Five Tests and ADMET Analysis

All ligands were converted into pdb format using the PyMol program (Wahyuningsih *et al*., 2023).

The physicochemical test of the highest binding against TNFα and interleukin-6 of each candidate plant. The five Lipinski rules analyzed are molecular mass, high lipophilicity, hydrogen bond donors, hydrogen bond acceptors, and molar refractivity. Furthermore, Lipinski and ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) were analyzed using the pkCSM website (https://biosig.lab.uq.edu.au/pkcsm/prediction). structure is entered in the column provided to predict pharmacokinetic properties (Yasin *et al*., 2020; Kusumaningrum *et al*., 2022).

Results and Discussion

Molecular Characterization

This research identified six species of phytopharmaceutical plants from Indonesia consisting of lempuyang, gandasuli, brotowali, pacing pentul, mangkokan, and mimba. The DNA isolation of the six plants was successful with the DNA concentration of more than 100 ng/µL was obtained and a purity in the range of 1.69 to 2.09 as seen in Table 1.

Table 1: Quantitative test result of phytopharmaceutical plant DNA using nanodrop 2000

Sample name	Nucleic Acid Concentration (ng/µL)	λ260	λ280	λ260/280
Pacing Pentul	1048.3	20.97	10.11	2.07
Mangkokan	1787.1	35.74	19.51	1.83
Mimba	247.9	4.96	2.46	2.01
Lempuyang	690.5	13.81	7.50	1.84
Gandasuli	504.5	10.09	4.83	2.09
Brotowali	168.8	3.38	1.99	1.69

DNA concentration was determined using spectrophotometry at 260 nm absorption (λ260) and DNA purity free from protein and polysaccharide contamination was assessed through the λ260/280 and λ260/230 absorption ratios (Wilson & Walker, 2005). The quality of DNA is considered to be pure DNA based on test results using nanodrop at λ260/280 absorption ratio of 1.8-2.0 and with a DNA concentration above 100 ng/µL. The λ260/280 purity ratio is an important measure to estimate the level of polyphenol contamination in extracted DNA. The λ260/280 ratio that is far below 1.8 makes the extracted DNA unsuitable for further molecular investigations (Sambrook & Russell, 2001). DNA extraction that produces pure and high-quality DNA will make further molecular analysis easier. The DNA extraction process is an important start in molecular identification, especially in plants because each plant species has different levels of polysaccharides, polyphenols, and secondary metabolites which combine with nucleic acids during DNA isolation (Aboul *et al*., 2019). The CTAB (cetyltrimethylammonium bromide) protocol to lyse the plant cell and added the chloroform and isopropanol to extract the DNA. This method has been highly efficient to remove the contaminant like secondary metabolites. The purity and concentration of DNA affect the amplification processes with ITS primer. DNA with high purity result the clear sequences.

The extracted phytopharmaceutical plant DNA samples were amplified using ITS primers and analyzed using 1% (m/v) agarose gel electrophoresis. Visualization of PCR results can be seen in Fig. 1 on the electrophoresis gel with criteria: bright and thick of DNA band and there is no heavy smear.

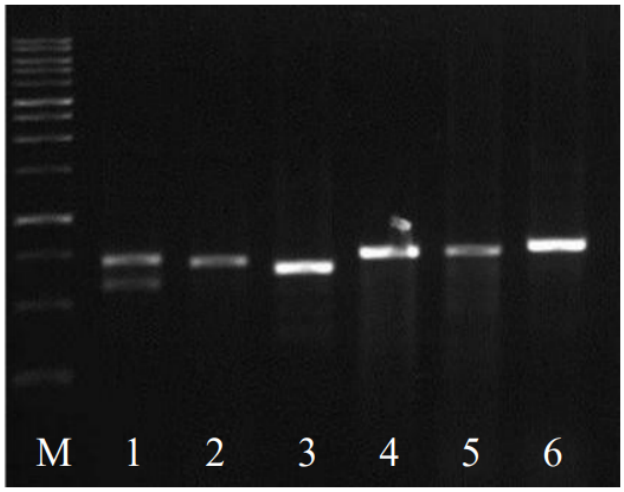

Fig. 1: Electrophoresis Visualization of ITS Fragment PCR Result: M) DNA ladder 1 kb, (1) Lempuyang, (2) Gandasuli, (3) Brotowali, (4) Pacing Pentul, (5) Mangkokan, (6) Mimba

Table 2: Species identification based on BLAST analysis

Sample name	Scientific name	Query Cover	Percent Identity	Accession
Pacing Pentul	*Costus spicatus*	99%	97.33%	AY972903.1
Mangkokan	*Polyscias scutellaria*	97%	94.86%	AF229716.1
Mimba	*Azadirachta indica*	86%	88.45%	LC461769.1
Lempuyang	*Zingiber zerumbet*	94%	84.18%	KJ872294.1
Gandasuli	*Hedychium coronarium*	99%	90.23%	MF076969.1
Brotowali	*Tinospora cordifolia*	38%	63.46%	KC333652.1

Visualization of DNA amplification in ITS region revealed all samples had DNA band size around 700 bp measured based on 1 kb DNA marker. Annealing temperature in PCR process for each sample is 50°C for Pacing Pentul, 53°C for Mimba, 55°C for Mangkokan, 56°C for Lempuyang, 52°C for Gandasuli, and 53°C for Brotowali. ITS primer is widely used in phylogenic analysis of plant DNA. This is because the ITS region can easily be amplified to many copies and also promising DNA sequence for the development of a DNA barcode (Abdullah *et al*., 2016).

The DNA sequences amplified by ITS primers were matched with the genomic database available in the webpage of NCBI using BLASTn analysis. The results were found in Table 2 that DNA sequences matched the genomic DNA references with identity percentage >60%.

BLAST in NCBI is used to search sequence databases using query sequences to find the significance of matches, regions of similarity between base sequences, local alignments of varying lengths, and provides descriptive parameters and statistical evaluation of each match (Altschul *et al*., 1990).

Molecular identification using BLAST to identify matches usually based on query cover and percent identity. Query coverage is the percentage of the query sequence length that is included in the alignment, and the greater the query coverage and the better the match between the query with sequence database. Per. Identity is the highest percent identity for a set of align segments to the same subject sequence (Newell *et al*., 2013).

The sequences from sequencing process were aligned and a phylogenetic tree was formed to see the relationship between samples. Phylogenetic analysis based on the sequenced ITS regions were applied to the studied plant species. The phylogenetic tree resulted from using the neighbor-joining method with bootstrap 1000 revealed two clusters have been made from six sequence samples. Cluster 1 included two plant species, Gandasuli and Lempuyang supported with 97% bootstrap. Cluster 2 comprises two clades, Brotowali and Mimba supported with 74% bootstrap in clade 1. Clade 2, supported with 66% bootstrap comprises two species, Mangkokan and Pacing Pentul (Fig. 2).

Pacing Pentul was confirmed as *C. spicatus* (based on BLASTn and ITS gen) was grouped in the order of Zingiberales. As a group of Zingiberales order, Pacing Pentul has a main chemical compound of terpene, especially in sesquiterpene compound (Devendran & Ganesan, 2015). Mangkokan was clustered in the same clade as Pacing Pentul. Mangkokan was grouped in the order of Apiales that has terpene especially triterpenoid as the main chemical compound. It is clustered in the same clade as Pacing pentulin in the phylogenetic tree (Bhowon *et al*., 2022). Mimba was grouped in the order of Sapindales and Brotowali in the order of Ranunculales. Mimba has flavonoids such as probenazole with high concentrations founded in GC-MS analysis (Manandhar *et al*., 2019). Brotowali has d-Mannose and Melezitose with a high peak area of GC-MS analysis (Katara *et al*., 2021). Lempuyang and Gandasuli were in the same cluster in the phylogenetic analysis.

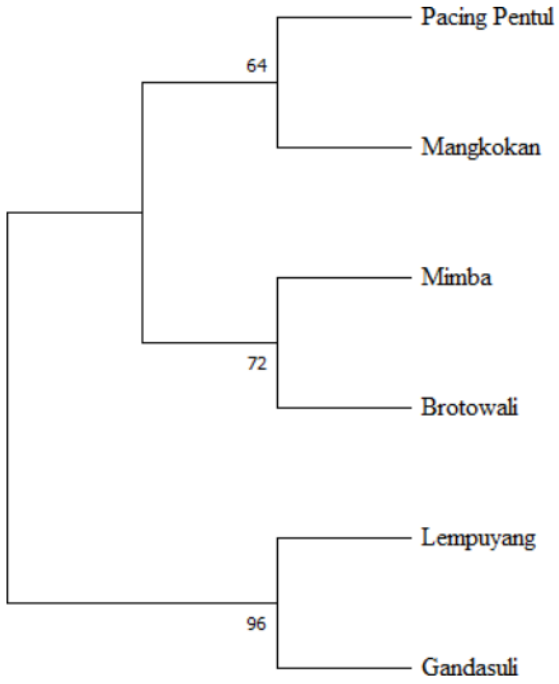

Fig. 2: Phylogenetic tree of six phytopharmaceutical plants using Neighbor-Joining

In the taxonomy system, Lempuyang and Gandasuli were grouped in the order of Zingiberales. The high chemical compound in Lempuyang was Zerumbone, while in Gandasuli it was Beta-pinene. Zerumbone and Beta-pinene were in the same group of terpenes in bioactive compounds in plant (Shanmugam *et al*., 2015; Akhtar *et al*., 2019).

Phytochemical compounds of herbal plants, literature study of phytochemicals compounds of six plants on Table 3 based on GC-MS analysis showed that some of the compounds have a high percentage. These compounds are used as a ligand or medication candidate that will be docked with protein (receptor). Pacing Pentul has very high eremanthin reaching 93.44% as antioxidant and anti-inflammatory (Devendran & Ganesan, 2015).

Phytochemical compounds are flavonoid, sesquiterpene, alkaloid, phenolic, and terpenoid. Moujir *et al*. (2020) and Albuquerque *et al*. (2021) reported that sesquiterpene and phenolic have cancer activity, anti-inflammatory, antifungal, anxiolytic, analgesic, and antitrypanosomal. This activity occurs because sesquiterpene and phenolic compounds can inhibit disease-target enzymes or proteins. Diterpenes are terpene compounds that have 20 isoprene units (C20), while sesquiterpenes have 15 (C15) (Mai *et al*., 2021). Both groups of compounds are produced naturally by plants with bioactivity and toxicity properties. The pharmacological activities from sesquiterpenes and diterpenes include antimicrobial, anti-neurotropic, antitumor, anti-inflammatory, and antiviral (Yin *et al*., 2019; Thawabteh *et al*., 2021).

PASS Prediction Analysis

Pharmacological prediction obtained from PASS database. This data showed the potential of the phytochemical compound as anti-inflammatory and immunomodulatory. Data of pharmacological are presented of Table 4.

Table 3: Phytochemical compound of plant based on GC-MS

Sample name	Compound based on GCMS	Percentage	Molecular formula	References
Pacing Pentul (*Costus spicatus*)	Eremanthin	93.44%	$C_{15}H_{18}O_2$	Devendran & Ganesan, 2015
	Isolongifolene	3.68%	$C_{15}H_{24}$	
	1,2-Ethanediol, monoacetate 1,2-	2.79%	$C_4H_8O_3$	
	Benzenedicarboxylic acid, diisooctyl ester	2.55%	$C_{24}H_{38}O_4$	
Mangkokan (*Polyscias scutellaria*)	Phytyl tetradecanoate	15.08%	$C_{34}H_{66}O_2$	Islam *et al.,* 2022
	Hexadecanoic acid, ethyl ester	9.49%	$C_{18}H_{36}O_2$	
	Tridecanoic acid, 12-methyl-, methyl ester	7.69%	$C_{15}H_{30}O_2$	
	Propane, 2-fluoro-2-methyl-	6.51%	C_4H_9F	
Mimba (*Azadirachta indica*)	Probenazole	36.46%	$C_{10}H_9NO_3S$	Manandhar *et al.,* 2019
	Etofenprox	17.38%	$C_{25}H_{28}O_3$	
	Cinerin 1	13.74%	$C_{20}H_{28}O_3$	
	Prohydrojasmon	11.81%	$C_{15}H_{26}O_3$	
Lempuyang (*Zingiber zerumbet*)	Zerumbone	58.44%	$C_{15}H_{22}O$	Akhtar *et al.,* 2019
	Alpha-Humulene	12.24%	$C_{15}H_{24}$	
	Camphene	5.36%	$C_{10}H_{16}$	
	Humulene epoxide	4.96%	$C_{15}H_{24}O$	
	Caryophylla-4 (14),8 (15)-dien-5.alpha.-ol	3.86%	$C_{15}H_{26}O$	
Gandasuli (*Hedychium coronarium*)	Beta-pinene	39.1%	$C_{10}H_{16}$	Shanmugam *et al.,* 2015
	1,8-Cineole	32.2%	$C_{10}H_{18}O$	
	Alpha-pinene	9.6%	$C_{10}H_{16}$	
	Alpha-Terpineol	4.9%	$C_{10}H_{18}O$	
	Limonene	3.1%	$C_{10}H_{16}$	
Brotowali (*Tinospora cordifolia*)	Melezitose	18.4	$C_{18}H_{32}O_{16}$	Katara *et al.,* 2021
	Betulin	8.4%	$C_{30}H_{50}O_2$	Naik *et al.,* 2014
	Palmitic acid	14.1%	$C_{16}H_{32}O_2$	
	Phytol	11.4%	$C_{20}H_{40}O$	

Based on Table 4, PASS prediction analysis was carried out on the phytochemical compound of *C. spicatus*, *P. scutellaria*, *A. indica*, *Z. zerumbet*, *H. coronarium*, and *T. cordifolia* have a potential as anti-inflammatory and immunomodulatory. The highest anti-inflammatory activity was found in zerumbone from Z. zerumbet with a Pa value of 0.831. The compound with the highest immunomodulatory activity is hexadecanoic acid from *P. scutellaria* with a Pa value of 0.481. Adalimumab as positive control has Pa value of anti-inflammatory less than the phytochemical compound from herbal plants. Experimentally, compounds with a Pa value of more than 0.7 have a high chance of finding activity or in vitro and in vivo can be potential by the results of in silico analysis. Compounds with $0.5 < Pa < 0.7$ have a small chance of finding activity, but there is a possibility that it does not match the PASS prediction results. While compounds with $Pa < 0.5$ have a small chance of finding activity, chemical entities have a high probability of being found (Lagunin *et al*., 2000).

Table 4: PASS prediction analysis

Sample name	Compound based on GC-MS	Anti-inflammatory (Pa)	Immunomodulator (Pa)
Positive control	Adalimumab	0.308	-
Pacing Pentul (*C. spicatus*)	Eremanthin	0.816	-
	Isolongifolene	0.287	-
	1,2-Ethanediol, monoacetate	0.754	0.393
	1,2-Benzenedicarboxylic acid, diisooctyl ester	0.411	0.360
Mangkokan (*P. scutellaria*)	Phytyl tetradecanoate	0.540	0.276
	Hexadecanoic acid, ethyl ester	0.600	0.481
	Tridecanoic acid, 12-methyl-, methyl ester	0.562	0.421
	Propane, 2-fluoro-2-methyl-	0.415	0.429
Mimba (*A. indica*)	Probenazole	0.266	-
	Etofenprox	0.300	-
	Cinerin 1	-	-
	Prohydrojasmon	-	-
Lempuyang (*Z. zerumbet*)	Zerumbone	0.831	0.219
	Alpha-Humulene	0.741	-
	Camphene	0.287	0.223
	Humulene epoxide	0.731	-
	Caryophylla-4 (14),8 (15)-dien-5. alpha.-ol	0.504	0.366
Gandasuli (*H. coronarium*)	β-pinene	0.611	0.326
	1,8-Cineole	0.327	-
	α-pinene	0.490	0.304
	α-terpineol	0.651	-
	Limonene	0.610	0.205
Brotowali (*T. cordifolia*)	Melezitose	0.265	0.343
	Betulin	0.629	-
	Palmitic acid	0.727	0.419
	Phytol	0.458	0.308

Molecular Docking Analysis between TNFα and Interleukin-6 With Phytochemical Compound

The TNFα target protein (PDB ID: 2AZ5) and interleukin-6 (PDB ID: 1P9M) from humans and the ligands can used phytochemical compounds from *C. spicatus, P. scutellaria, A. indica, Z. zerumbet, H. coronarium, T. cordifolia* and adalimumab as positive control. The 2AZ5 grid box is in dimensions (Angstrom) X: 71.6928, Y: 67.4813, and Z: 71.3257. The 1P9M grid box is in dimensions (Angstrom) X: 44.0390, Y: 36.2833, dan Z: 47.1135. The results of molecular docking of the TNFαand interleukin-6 receptor with phytochemical compounds are presented in Tables 5 and 6.

TNFα is pleotropic cytokines work to influence tumor cells growth, invasion, metastasis, and induces cytotoxic cell death (Lee *et al*., 2019). Structure of TNFα is homotrimer protein consisting of 157 amino acids that produced by macrophages, T-lymphocytes, and natural killer cells (Horiuchi *et al*., 2010). In other hand, this type of cytokines was identified as a major regulator of inflammatory responses and involved in the autoimmune disease (Jang *et al*., 2021). Receptor TNFα and ligand was docked especially in active site of protein. The amino acid which arrangement the binding site was interacted with the phytochemical compound as inhibitor. The interaction between receptor and ligand as inhibitor will prevent the native ligand or substrate to bind with active site and occurred a general reaction. Molecular docking simulation will show the binding affinity value to identify the level inhibition produced. The lowest binding affinity value indicates the smallest energy used by the receptor to interact with the ligand, so the reaction occurs spontaneously. This value also affects the stability of ligand and receptor interactions (Kellenberger *et al*., 2008). Docking between TNFα and phytochemicals compound from six plants performed that betulin, 1,2-Benzenedicarboxylic acid, and eremanthin have lower binding affinity value namely -9.1, -8.0, and -7.9 Kcal/mol, respectively. This value means that the three phytochemicals compound inhibit TNFα with the lowest energy. Adalimumab, a commercial drug used to inhibit TNFα, had a binding affinity less negative than these three phytochemical compounds from herbal plants. Reaction of the ligand against TNFα affected TNFα pathway and decrease pro-inflammatory immune system in the body.

Interleukin-6 is a type of adaptive immune system that produced by T cells, B cells, monocytes, endothelial cells, fibroblasts, mesangial cells, adipocytes, and keratinocytes. Structurally, IL-6 consisting of 185 amino acid polypeptides of 22 kDa (Kishimoto, 2010; Scheller *et al*., 2011). IL-6 receptor has two modes of action, namely cis and trans.

Cis-signaling mediated IL-6 affecting homeostatic and anti-inflammatory. Transsignaling of IL-6 possesses detrimental pro-inflammatory effects (Petes *et al*., 2018). Inhibition of IL-6 give several effects such as decreasing pro-inflammatory immune system and reducing the progression of cytokine storm (Copaescu *et al*., 2020; Kishimoto, 2021). The phytochemical compounds from six herbal plants as candidates to treat the immune system disease with inhibit of IL-6 pathway. Betulin, 1,2-Benzenedicarboxylic acid, and eremanthin have less energy to inhibit IL-6 receptor than adalimumab showed from smallest binding affinity value, namely -8.1, -7.6, and 7.0 Kcal/mol, respectively. Tran *et al*. (2023) stated that more negative the docking score indicates stronger binding both of ligand and protein target. This interaction was affected to increase anti-inflammatory immune system and protect body from inflammation.

Based on the Figs. 3-6, interaction both of receptor and ligand in the binding site will be produced amino acid residues. The amino acid residues act as the amino acid that arrangement the binding site and place to substrate or inhibitors to interacted with receptor. Several ligands bonded with the same of amino acid residues. This indicated the type or name of amino acid residues which is arrangement the binding site. Visualization of interaction between adalimumab, eremanthin, 1,2-Benzenedicarboxylic acid diisooctyl ester, and betulin with TNFα showed that TYR D:151, GLY C:121, LEU C:57, TYR D:59, TYR D:119, LEU B:94, and LEU D:55 bonded with the compounds.

Table 5: Binding affinity value ligand and TNFα

Sample name	Compound based on GCMS	Binding affinity value (Kcal/mol) TNFα	Amino acid residues
Positive control	Adalimumab	-8.0	TYR A:151, GLY A:121, TYR B:151, TYR A:119, TYR
Pacing Pentul (*C. spicatus)*	Eremanthin	-7.9	TYR D:151, GLY C:121, LEU D:57, LEU C:57, TYR D:59, TYR D:119
	Isolongifolene	-7.1	TYR B:119, TYR B:59, TYR B:151
	1,2-Ethanediol, monoacetate	-3.7	GLY C:121, TYR D:151
	1,2-Benzenedicarboxylic acid, diisooctyl ester	-8.0	LEU B:94, LEU D:55, TYR B:59
Mangkokan (*P. scutellaria*)	Phytyl tetradecanoate	-6.1	TYR C:151, TYR D:59, LEU C:57
	Hexadecanoic acid, ethyl ester	-5.7	ARG D:82
	Tridecanoic acid, 12-methyl-, methyl ester	-5.2	TYR D:151, GLY C:121, LEU D:120, SER D:60, TYR C:151, TYR C:119, TYR D:59, TYR C:59
	Propane, 2-fluoro-2-methyl-	-3.3	TYR59, GLY A:121
Mimba (*A. indica)*	Probenazole	-6.4	LEU C:120, GLY C:121, TYR D: 59, TYR D:151, TYR D:119, TYR C:119
	Etofenprox	-7.0	LEU D:55, ILE B:155, LEU A:57, TYR B:59, LEU B:57, TYR B:119
	Cinerin 1	-7.3	LEU D:57, LEU C:57, TYR C:119,
	Prohydrojasmon	-6.2	GLY C:121, TYR D:151, TYR C:59, LEU C:57, LEU D:57, TYR D:59
Lempuyang (*Z. zerumbet)*	Zerumbone	-7.3	TYR B:59, LEU B:57, TYR B:119 TYR B:199, TYR
	Alpha-Humulene	-7.1	A:119, LEU B:57, TYR B:59
	Camphene	-5.5	TYR B:59, TYR B:151, TYR B:119
	Humulene epoxide	-7.4	TYR C:119, TYR D:119, TYR D:59, TYR D:151
	Caryophylla-4 (14), 8 (15)-dien-5.alpha.ol	-7.2	GLY C:121, TYR D:151, TYR D:59, LEU D:57
Gandasuli (*H. coronarium*)	β-pinene	-5.6	TYR B:59, TYR A:119, TYR B:119, TYR B:151
	1,8-Cineole	-5.9	TYR B:59, TYR B:119
	α-pinene	-5.9	TYR D:151, TYR C:119, TYR D:59, TYR D:119
	α-terpineol	-5.9	TYR B:151, GLY A:121, TYR B:59, TYR B:119, TYR A:119
	Limonene	-5.6	TYR D:119, TYR D:59, LEU D:57, LEU C:57
Brotowali (*T. cordifolia*)	Melezitose	-7.1	GLU B:127, GLN B:125, SER D:95, ARG B:82, ASN D:92, ASN D:92, ARG B:82
	Betulin	-9.1	TYR B:119, TYR A:119, LEU D:55
	Palmitic acid	-5.2	TYR A:151, LEU A:120, TYR B:59, LEU A:57, TYR B:119
	Phytol	-5.5	LEU D:120, SER D:60, ILE D:155, LEU C:57, LEU D:57, TYR D:59, TYR C:59, TYR D:119

Table 6: Binding affinity value ligand and Interleukin-6

Sample name	Compound based on GCMS	Binding affinity value (Kcal/mol) IL-6	Amino acid residues
Positive control	Adalimumab	-7.0	PRO B:139, PRO B:141, LEU B:92
Pacing Pentul (*C. spicatus)*	Eremanthin	-6.3	LEU B:165, LEU B:158, ALA B:58, MET B:161, LEU B:62
	Isolongifolene	-3.9	THR B:137, ALA B:135, ASP B:134
	1,2-Ethanediol, monoacetate	-8.1	THR B:43, LYS B:46, LEU B:39, PHE B:105, LEU B:167, LEU B:101, ARG B:104
	1,2-Benzenedicarboxylic acid, diisooctyl ester	-4.9	LYS B:120, PRO B:141
Mangkokan (*P. scutellaria*)	Phytyl tetradecanoate	-5.6	ARG B: 104, LEU B:101, LEU B:39, LEU B:167, PHE B:105, ALA B:121
	Hexadecanoic acid, ethyl ester	-4.5	VAL B:96, ASN B:144, LEU B:92, ILE B:136, LYS B:120, ILE B:123
	Tridecanoic acid, 12-methyl-, methyl ester	-3.6	VAL B:85, ILE B:88, ALA B:130
	Propane, 2-fluoro-2-methyl-	-6.1	CYS B:73, ARG B:179, SER B:176, PHE B:74, PHE B:78
Mimba (*A. indica)*	Probenazole	-6.2	THR B:138, ILE B:136, PRO B:139, PRO B:141, LEU B:92
	Etofenprox	-6.4	THR B:138, ASN B:144, ILE B:136, LEU B:92. LYS B:120, ILE B:123
	Cinerin 1	-4.9	ASN B:60, THR B:162, GLN B:154, MET B:161, ALA B:58, LEU B:165, LEU B:151, LYS B:150
	Prohydrojasmon	-5.9	ASN B:144, VAL B:96, PRO B:141, LEU B:92
Lempuyang	Zerumbone	-5.7	PRO B:141
(*Z. zerumbet)*	Alpha-Humulene	-4.7	LEU B:62, LEU B:158, ALA B:58, MET B:161, LEU B:165
	Camphene	-5.9	ILE B:123, LEU B:92, PRO B:141
	Humulene epoxide	-6.3	GLU B:99, PRO B:141, LEU B:92
	Caryophylla-4 (14), 8 (15)-dien-5.alpha.ol	-4.8	PRO B:141, LEU B:92, PRO B:139
Gandasuli	β-pinene	-4.8	THR B:138, PRO B:139, PRO B:141, LEU B:92
(*H. coronarium*)	1,8-Cineole	-4.8	PRO B:139, PRO B:141, LEU B:92
	α-pinene	-5.1	ASN B:144, LEU B:92, PRO B:141, VAL B:92
	α-terpineol	-4.8	PRO B:139, PRO B:141, LEU B:92
	Limonene	-5.7	GLU B:99, VAL B:96, THR B:138, ASN B:144
Brotowali	Melezitose	-7.6	GLN B:127, PRO B:141, LEU B:92, ILE B:136 ASN
(*T. cordifolia*)	Betulin	-4.5	B:144, THR B:138, PRO B:141, VAL B:96
	Palmitic acid	-5.2	ILE B:136, LEU B:92, ILE B:123, LYS B:120
	Phytol	-7.0	PRO B:139, PRO B:141, LEU B:92

In other hands, interaction the four compounds with IL-6 have visualization which shows the amino acid residues, namely PRO B:139, PRO B:141, LEU B:92, THR B:43, LYS B:46, LEU B:39, PHE B:105, LEU B:167, LEU B:101, ARG B:104, GLN B:127, and ILE B:136. Stability both of ligand and receptor effected by hydrogen bond, electrostatic interaction, hydrophobic, van der Waals, and steric factor (Fakih, 2020). The six plants and the phytochemical compound are commonly found in nature. Molecular docking results must be studied further in vitro and in vivo to obtain valid results. Apart from that, further identification can be carried out to determine the side effects of phytochemical compounds on the human body or other subjects before they are used as anti-TNF alpha and interleukin-6 inhibitors.

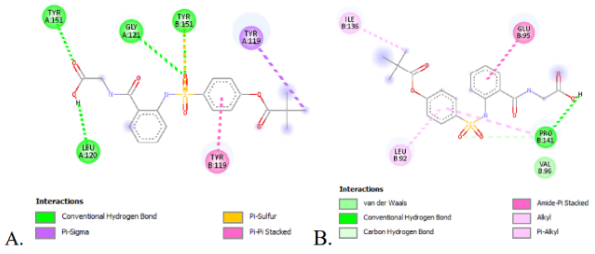

Fig. 3: Visualization of interaction adalimumab (positive control) and receptor (A. TNFα, B. Interleukin-6)

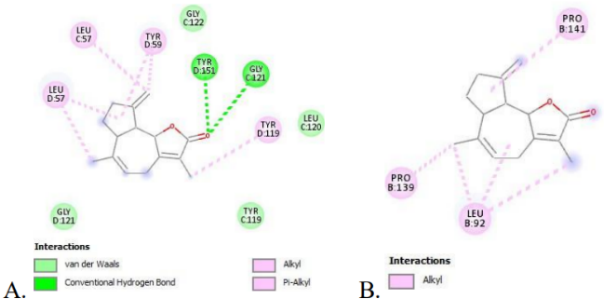

Fig. 4: Visualization of interaction eremanthin and receptor (A. TNFα, B. Interleukin-6)

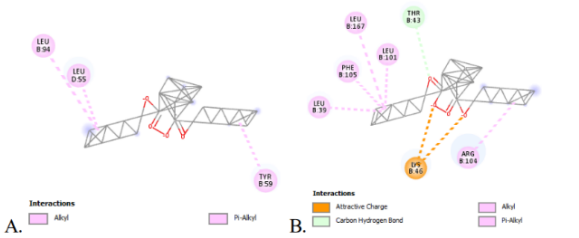

Fig. 5: Visualization of interaction 1,2-Benzenedicarboxylic acid, diisooctyl ester and receptor (A. TNFα, B. Interleukin-6)

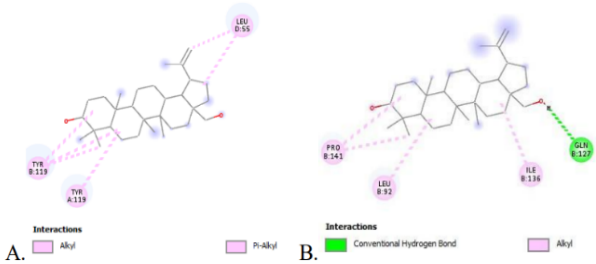

Fig. 6: Visualization of interaction betulin and receptor (A. TNFα, B. Interleukin-6)

Conclusion

Molecular characterization of lempuyang, gandasuli, brotowali, pacing pentul, mangkokan, and mimba based on BLAST showed that the sample which used are C. spicatus, P. scutellaria, A. indica, Z. zerumbet, H. coronarium, and T. cordifolia. Zerumbone and hexadecanoid acid have the highest anti- inflammatory and immunomodulatory properties. Inhibition of proinflammatory immune system are TNFα and IL-6 with the stronger bond carried out betulin and 1,2-Benzenedicarboxylic acid diisooctyl ester compounds.

Acknowledgment

This research was funded by Hibah Riset Madya from Faculty of Science and Mathematics Diponegoro University Year 2023 number SPK: 92/UN7.F8/HK/2023 which is gratefully acknowledged.

Funding Information

This article is part of a research entitled “Penambatan Molekuler dan Karakterisasi Molekuler Fitofarmaka Indonesia Berpotensi Immune Booster”.

Author’s Contributions

Hermin Pancasakti Kusumaningrum: Generating original ideas, writing manuscripts, designing research, reviewing research implementation, writing and discussing research results, checking and approving final drafts, and coordinating research.

Dea Kafita Sari: Collected data in the field and laboratory, data analysis, and experimental development. Rejeki Siti Ferniah: Supervised laboratory work and designed the research, methodology, and analysis.

Azalia Puspa Herida: Assisting in the preparation of experimental designs, assisting in data analysis, preparing figures and tables.

Candra Wahyuningsih: Analyzed the data, prepared figures and/or tables, sample collection, prepared drafts.

Khusnul Khotimah: Performed the experiments, analyzed the data, prepared figures and/or tables.

Siti Nur Jannah: Conceived and designed the experiments.

Ethics

This article is entirely original and includes never-before seen content. The corresponding author attests that the work has been read and approved by all other authors and that there are no ethical omissions.

References

Abbas, A. K., Lichtman, A. H., & Pillai, S. (2021). *Cellular and molecular immunology* (E-book). Elsevier.

Abdullah, F. I., Lee, S. C., Zaidah, R., Azman, A. S., & Wagiran, A. (2016). Plant genomic DNA extraction for selected herbs and sequencing their internal transcribed spacer region amplified by specific primers. *Natural Product Communications, 11*(10), 1491–1496.

Aboul-Maaty, N. A. F., & Oraby, H. A. S. (2019). Extraction of high-quality genomic DNA from different plant orders applying a modified CTAB-based method. *Bulletin of the National Research Centre, 43*(25). https://doi.org/10.1186/s42269-019-0066-1

Akhtar, N. M. Y., Jantan, I., Arshad, L., & Haque, M. A. (2019). Standardized ethanol extract, essential oil and zerumbone of *Zingiber zerumbet* rhizome suppress phagocytic activity of human neutrophils. *BMC Complementary Medicine and Therapies, 19*, 331. https://doi.org/10.1186/s12906-019-2748-5

Alanazi, H. H., & Elfaki, E. (2023). The immunomodulatory role of *Withania somnifera* (L.) dunal in inflammatory diseases. *Frontiers in Pharmacology, 14*, 1084757. https://doi.org/10.3389/fphar.2023.1084757

Albuquerque, B. R., Heleno, S. A., Oliveira, M. B. P., Barros, L., & Ferreira, I. C. (2021). Phenolic compounds: Current industrial applications, limitations and future challenges. *Food & Function, 12*(1), 14–29.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology, 215*(3), 403–410.

Bhowon, M. G., Lee, S. C., Shobha, J., Ayeshna, D. S., & Sabina, J. L. (2022). Morphology, biological and chemical profiling of three *Polyscias* species endemic to Mauritius. *International Journal of Plant-Based Pharmaceuticals, 2*(2), 239–251. https://doi.org/10.29228/ijpbp.6

Boshtam, M., Asgary, S., Kouhpayeh, S., Shariati, L., & Khanahmad, H. (2017). Aptamers against pro- and anti-inflammatory cytokines: A review. *Inflammation, 40*(1), 340–349.

Copaescu, A., Smibert, O., Gibson, A., Phillips, E. J., & Trubiano, J. A. (2020). The role of IL-6 and other mediators in the cytokine storm associated with SARS-CoV-2 infection. *Journal of Allergy and Clinical Immunology, 146*, 518–534. https://doi.org/10.1016/j.jaci.2020.07.001

Devendran, G., & Ganesan, S. (2015). Phytochemical analysis of leaf extract of plant *Costus spicatus* by GCMS method. *Journal of Drug Delivery and Therapeutics, 5*(4), 24–26.

Dzufakar, A. B. P., Wahyuningsih, C., Khotijah, P. R. S., Maharani, N. A., Dion, R., Putri, F. N., & Pujiyanto, S. (2023). Antioxidant potential of *Erythrina subumbrans* leaves extract from Semarang, Central Java to prevent acne. *AIP Conference Proceedings, 2738*(1).

Fakih, T. M. (2020). Dermaseptin-based antiviral peptides to prevent COVID-19 through in silico molecular docking studies against SARS-CoV-2 spike protein. *Pharmaceutical Sciences and Research, 7*(4), 65–70.

Horiuchi, T., Mitoma, H., Harashima, S. I., Tsukamoto, H., & Shimoda, T. (2010). Transmembrane TNF-α: Structure, function and interaction with anti-TNF agents. *Rheumatology, 49*(7), 1215–1228.

Islam, M. A., Zilani, M. N. H., Biswas, P., Kan, D. A., Rahman, M. H., Nahid, R., & Hasan, M. N. (2022). Evaluation of in vitro and in silico anti-inflammatory potential of selected medicinal plants of Bangladesh against cyclooxygenase-II enzyme. *Journal of Ethnopharmacology, 285*, 114900.

Jang, D. I., Lee, A. H., Shin, H. Y., Song, H. R., Park, J. H., Kang, T. B., … Yang, S. H. (2021). The role of TNF-α in autoimmune disease and current TNF-α inhibitors in therapeutics. *International Journal of Molecular Sciences, 22*(5), 2719.

Katara, A. H., Naveen, K. G., & Mukul, M. (2021). Separation and identification of antidiabetic compounds in *Tinospora cordifolia* extract and Ayurvedic formulation Guduchi Satva by GCMS and FTIR. *International Journal of Pharmaceutical Sciences and Drug Research, 13*(2), 183–189.

Kellenberger, E., Schalon, C., & Rognan, D. (2008). How to measure similarity between protein ligand-binding sites? *Current Computer-Aided Drug Design, 4*(3), 209.

Kishimoto, T. (2010). IL-6: From its discovery to clinical applications. *International Immunology, 22*, 347–352. https://doi.org/10.1093/intimm/dxq030

Kishimoto, T. (2021). IL-6: From arthritis to CAR-T-cell therapy and COVID-19. *International Immunology, 33*, 515–519. https://doi.org/10.1093/intimm/dxab011

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution, 35*(6), 1547–1549.

Kusumaningrum, H. P., Budiharjo, A., Suprihadi, A., Eshananda, Y., Fadillah, A., & Pangestuti, D. R. (2018). Characterization of *Citrus* sp. from Parang Island Karimunjawa based on morphology, DNA barcoding and nutritional analysis. *International Journal of Genetics and Molecular Biology, 10*(3), 26–38.

Kusumaningrum, H. P., Ferniah, R. S., Jannah, S. N., Kurniawati, M. B., Afifah, A., Sumbodo, Y. M., & Eshananda, Y. (2022). Relationship between phylogeny of *Apium* and *Foeniculum* plants from Central Java, Indonesia, and their secondary metabolite potency against COVID-19 protease. *Macedonian Journal of Medical Sciences, 10*(A), 1234–1241.

Lagunin, A., Stepanchikova, A., Filimonov, D., & Poroikov, V. (2000). PASS: Prediction of activity spectra for biologically active substances. *Bioinformatics, 16*(8), 747–748.

Lee, E., Ouzounova, M., Piranlioglu, R., Ma, M. T., Guzel, M., Marasco, D., & Korkaya, H. (2019). The pleiotropic effects of TNFα in breast cancer subtypes is regulated by TNFAIP3/A20. *Oncogene, 38*(4), 469–482.

Liu, L., Jiao, Y., Yang, M., Wu, L., Long, G., & Hu, W. (2023). Network pharmacology, molecular docking and molecular dynamics to explore immunomodulatory mechanisms of deer antler. *International Journal of Molecular Sciences, 24*, 10370. https://doi.org/10.3390/ijms241210370

Mai, J., Li, W., Ledesma-Amaro, R., & Ji, X. J. (2021). Engineering plant sesquiterpene synthesis into yeasts: A review. *Journal of Agricultural and Food Chemistry, 69*(33), 9498–9510.

Manandhar, S., Binita, M., & Ram, L. S. (2019). Antioxidant, antibacterial and GC-MS analysis of methanolic extract of *Azadirachta indica* Juss. *The Pharma Innovation Journal, 8*(12), 178–180.

Moujir, L., Callies, O., Sousa, P. M., Sharopov, F., & Seca, A. M. (2020). Applications of sesquiterpene lactones: A review of success cases. *Applied Sciences, 10*(9), 3001.

Naik, D., Dandge, C., & Rupanar, S. (2014). Chemical composition and antioxidant activity of essential oil from *Tinospora cordifolia* leaf. *Journal of Essential Oil Bearing Plants, 17*(2), 228–236.

Newell, P. D., Fricker, A. D., Roco, C. A., Chandrangsu, P., & Merkel, S. M. (2013). A small-group activity introducing the use and interpretation of BLAST. *Journal of Microbiology & Biology Education, 14*(2), 238–243. https://doi.org/10.1128/jmbe.v14i2.637

Petes, C., Mariani, M. K., Yang, Y., Grandvaux, N., & Gee, K. (2018). IL-6 inhibits IL-27- and IL-30-mediated inflammatory responses in human monocytes. *Frontiers in Immunology, 9*, 256.

Ponticelli, C., & Glassock, R. J. (2019). Prevention of complications from conventional immunosuppressants: A critical review. *Journal of Nephrology, 32*, 851–870.

Ramasamy, R. (2022). Innate and adaptive immune responses in the upper respiratory tract and the infectivity of SARS-CoV-2. *Viruses, 14*, 933.

Richard-Eaglin, A., & Smallheer, B. A. (2018). Immunosuppressive/autoimmune disorders. *Journal of Clinical Nursing, 53*(3), 319–334.

Sambrook, J., & Russell, D. W. (2001). *Molecular cloning: A laboratory manual.* Cold Spring Harbor Laboratory Press.

Scheller, J., Chalaris, A., Schmidt-Arras, D., & Rose-Jones, S. (2011). The pro- and anti-inflammatory properties of IL-6. *Biochimica et Biophysica Acta, 1813*, 878–888.

Shanmugam, P. V., Yadav, A., & Chanotiya, C. S. (2015). Enantiomer differentiation of key volatile constituents from *Hedychium coronarium*. *Journal of Essential Oil Research, 27*(2), 101–106.

Statista. (2024). Global autoimmune disorders spending from 2011 to 2023. https://www.statista.com/statistics/1233691/autoimmune-treatment-spend-worldwide/

Thawabteh, A. M., Thawabteh, A., Lelario, F., Bufo, S. A., & Scrano, L. (2021). Classification, toxicity and bioactivity of natural diterpenoid alkaloids. *Molecules, 26*(13), 4103.

Tran, Q. H., Nguyen, Q. T., Tran, T. T. N., Tran, T. D., Le, M. T., Trinh, D. T. T., & Thai, K. M. (2023). Identification of small molecules as potential IL-6 inhibitors: A multi-computational investigation. *Molecular Diversity, 27*(5), 2315–2330.

Wahyuningsih, C., Dion, R., Putri, F. N., Dzufakar, A. B. P., Khotijah, P. R. S., Maharani, N. A., & Kusdiyantini, E. (2023). Potency of secondary metabolites in *Erythrina subumbrans* leaf as lipase inhibitor. *AIP Conference Proceedings, 2738*(1).

Wilson, K., & Walker, J. (2005). *Principles and techniques of biochemistry and molecular biology.* Cambridge University Press.

Yasin, S. A., Azzahra, A., Ramadhan, N. E., & Mylanda, V. (2020). Studi penambatan molekuler dan prediksi ADMET senyawa bioaktif jamu Indonesia terhadap SARS-CoV-2 main protease. *Berkala Ilmiah Mahasiswa Farmasi Indonesia, 7*(2), 24–41.

Yin, T., Zhou, H., Cai, L., & Ding, Z. (2019). Non-alkaloidal constituents from *Aconitum*: A review. *RSC Advances, 9*, 10184–10194.

Zampieron, E. R., & Kamhi, E. J. (2012). Natural support for autoimmune and inflammatory disease. *Journal of Restorative Medicine, 1*(1), 38–47.