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In Silico Evaluation: Bioactive Compounds in Soursop Plant (*Annona muricata* L.) as Caspase-3 Inhibitor for Prostate Cancer

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Abstract

Background*: Prostate cancer has become one of the leading causes of death in men. Cancer patients often seek alternative treatments apart from chemotherapy, radiation therapy, and surgery. The use of medicinal plants in both preventive and curative actions in healthcare has been widely recognized. One of the plants known to have anticancer activity is the soursop leaf (Annona muricata L.).* ***Objective****: This study was conducted to explore the potential of active compounds contained in A. muricata as drug candidates for the inhibition of caspase-3 in silico.* ***Methods****: The research began with the prediction of Lipinski's Rule of Five and ADMET properties for the compounds found in A. muricata. The prediction process was followed by pharmacophore modelling and molecular docking simulations on caspase-3 (PDB: 1NME) as the target protein and 2-hydroxy-5-(2-mercapto-ethylsulfamoyl)-benzoic acid as the natural ligand using AutoDockTools 1.5.6.* ***Results****: Based on the molecular docking results, 22 test ligands were able to form bonds with the caspase-3 enzyme. The two best interactions were observed with the test ligands, Isolaureline and S-norcorydine, with binding energy values of -6.20 kcal /mol and -6.12 kcal /mol and inhibition constant values of 28.65 µM and 32.53 µM. In terms of receptor-target interactions, these two compounds also exhibited hydrogen bonding and van der Waals interactions similar to the natural ligand.* ***Conclusion****: The best bioactive compounds in A. muricata (Isolaureline and S-norcorydine) were predicted to have the ability to interact with caspase-3 and the potential to be used as prostate cancer drug candidates.*

Keywords*: Annona muricata, caspase-3, molecular docking, prostate cancer*

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INTRODUCTION

Cancer is a complex and life-threatening illness that has impacted the lives of countless individuals and families worldwide. It refers to a group of disorders characterized by abnormal cell growth and division within the body (Faubert *et al*., 2020). Cancer can manifest in a variety of organs and tissues, disrupting the body's normal functions and posing significant physical and emotional challenges. The cancer journey is often difficult, requiring courage, resilience, and unwavering support. The causes of cancer are multifaced, including genetic, environmental, and lifestyle factors. Inherited or acquired genetic mutations can disrupt cell function and increase the risk of cancer. Tobacco smoke, radiation, certain chemicals, and infectious agents are all carcinogens that can contribute to the development of cancer. Furthermore, lifestyle factors such as diet, physical activity, tobacco, and alcohol use may all affect the risk of developing certain types of cancer (Anand *et al.*, 2008).

Early detection of cancer is critical for successful treatment and improved outcomes. Regular screenings and self-examinations are critical for early detection because they allow medical professionals to identify abnormalities and initiate appropriate diagnostic procedures. Imaging scans, biopsies, and laboratory tests provide valuable insights into the presence, extent, and characteristics of cancer, allowing for more personalized treatment plans. It is a multidisciplinary approach to treatment that is determined by the type, stage, and location of the disease, as well as the individual's overall health. Surgery to remove tumors, radiation therapy to target and destroy cancer cells, and chemotherapy to kill cancer cells throughout the body. Besides, the use of drugs or treatments that specifically target specific molecules or pathways involved in the development and progression of cancer is known as targeted therapy (Shuel, 2022).

Related to cancer treatment and drug development, targeted therapy and molecular docking are inextricably linked. Molecular docking is a useful tool for discovering, designing, and optimising targeted therapies. It is a computational prediction and modelling of molecular interactions between molecules, such as drug candidates and their target proteins. By stimulating the interactions between drug molecules and target proteins, molecular docking allows researchers to predict and evaluate the effectiveness of potential drug candidates (Suhandi *et al*., 2022).

As cancer treatment requires the development of new medication, herbal medicine is a global icon of alternative medication much needed as new drugs. Plants with medicinal properties have been used to treat a variety of ailments since the dawn of time. The key characteristics were medicinal plant chemical compounds that can have a physiological effect on the human system (Ilango *et al*., 2022). *Annona muricata*, also known as soursop, has been studied for its potential as a source of anticancer compounds. Several studies have been carried out in laboratories to investigate the effects of various extracts and isolated compounds from *Annona muricata* on cancer cells. There have been reports of widespread use of *A. muricata, of* which 80.9% of patients with prostate, breast, and colorectal cancer for their malignancies (Foster *et al*., 2017). *A. muricata* is also known as one of the liver cancer therapies that been used by 14% Peruvian patients to treat their cancer-related symptoms (Rojas *et al*., 2018).

This study aimed to predict the cytotoxic activity, inhibition of proliferation, and apoptosis induction activity of *A. muricata* leaf extract targeting the caspase-3 pathway of prostate cancer. An increase in caspase-3 can cause certain proteins in cells to become activated, which can accelerate the process of apoptosis. Via the intrinsic mechanism, an increase in caspase-3 enzyme expression will accelerate the PC-3 prostate cancer cell line's in vitro turnover (Ismy *et al*., 2020).

MATERIALS AND METHODS

Materials

Caspase-3 structure, with PDB ID number "1NME," was retrieved from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) at www.rcsb.org for use in this investigation. The bioactive substances used in the test ligands came from the soursop plant (*Annona muricata* L.). The three-dimensional structure of a natural ligand and the test compounds, which include the reference medication Sorafenib, were retrieved from PubChem (pubchem.ncbi.nlm.nih.gov) created using the program ChemOffice 2010.

Tools

The computer used in this investigation was an Acer Swift SF314-56G with an Intel ® CoreTM i5-8265U processor, 4.00 GB of RAM, and a Windows 11 Home Single Language 64-bit operating system. The docking positions and interactions were visualized using the Biovia Discovery Studio Visualizer. The

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production of macromolecules and ligands, as well as the execution of docking studies, were done using AutoDock 1.5.6.

Method

Physicochemical and pharmacokinetic prediction

Physicochemical and pharmacokinetic predictions were conducted for both the test ligands. The ligand structures were obtained from the PubChem database (pubchem.ncbi.nlm.nih.gov) in (.sdf) format. The physicochemical predictions were performed using Lipinski's Rule of Five on the SwissADME website (http://www.swissadme.ch/). The pharmacokinetic predictions were carried out by submitting the structure of the test ligands to the PreADMET website (https://preadmet.webservice.bmdrc.org/).

Ligand preparation

For both test ligands, physicochemical and pharmacokinetic predictions were made. The ligand structures were downloaded in (.sdf) format from the PubChem database at pubchem.ncbi.nlm.nih.gov. On the SwissADME website (http://www.swissadme.ch), Lipinski's Rule of Five was used to make the physicochemical predictions. The test ligands' structures were entered into the PreADMET website (https://preadmet.webservice.bmdrc.org) to make pharmacokinetic predictions.

Receptor preparation

The Protein Data Bank's Caspase-3 3D structure (PDB ID: 1NME) was visualized using the BIOVIA Discovery Studio application. The protein chain was then isolated from the native ligand by removing those components as well as the water molecules surrounding the protein. The generated structure was stored as the receptor in the *pdb file format. In addition, the natural ligand's structure was recovered by eliminating the protein chain and saved in the *pdb file format. A software called AutoDockTools 1.5.6 was used to produce the native ligand as well as the protein. To proceed with this production, hydrogen atoms had to be added to the structure's polar side, Kollman charges had to be applied for the receptor, and Gasteiger charges for the native ligand.

Validation of the molecular docking method

The approach is validated to confirm that the docking parameters are appropriate for the docking process of the test ligand with the caspase-3 receptor. Re-docking, which includes inserting the dissociated native ligand back into the Caspase-3 receptor, is used to carry out this validation. A grid box with the dimensions 30 x 30 x 30 and the coordinates x = 42.197; y = 96.352; z = 24.611 is used in the re-docking procedure.

The Genetic strategy (GA) value is set to 10, and Lamarckian GA 4.2 is used as the docking strategy in the docking settings. The default settings are used for all other docking parameters.

The Root Mean Square Deviation (RMSD) is a parameter that can be taken into account in the re-docking outcomes. The RMSD value of 2.0 is considered acceptable (Istyastono, 2018).

Molecular docking

The reference drug and each of the twenty-two test compounds were docked against the Caspase-3 receptor after being optimized and produced. Using AutoDockTools 1.5.6, the receptor was created by separating it from its natural ligand and docking it in the same manner as in the technique validation.

Data analysis and visualization

The data presentation design in this study took into account various factors such as binding free energy, conventional hydrogen bonds, van der Waals bonds, and the number of hydrogen bonds. The BIOVIA Discovery Studio allows visualization of the receptor's complex conformations, ligand interactions, and contact amino acid residues in both two-dimensional (2D) and three-dimensional (3D) formats.

RESULTS AND DISCUSSION

Physicochemical and pharmacokinetic prediction

Physicochemical and pharmacokinetic prediction are two fields essential for drug discovery to explain how two chemical compounds interact in the human body. Physicochemical studies explain the physical and chemical properties of chemical compounds; therefore, pharmacokinetics predictions refer to scores of absorption, distribution, metabolism, excretion, and toxicity (ADMETox) predicted by how the chemical compounds work on the human body.

Lipinski's Rule of Five (Ro5) is one of the methods that can be used to predict the pharmacokinetics of drug-like chemical compounds designed for oral route administration. These rules are used to determine if chemical compounds have the potential to be drugs. The parameters include molecular weight (MW), partition coefficient (LogP), hydrogen bond donor (HBD), and hydrogen bond acceptor (HBA). The drug-like compound has a molecular weight under 500 Da. LogP value less than five represents hydrophobicity; no HBD is more than five, and no HBA is more than 10. (Chagas *et al.*, 2018).

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On the other hand, the parameters of ADMETox are HIA (Human Intestinal Absorption) and $CaCO_2$, which define the capability of oral and transdermal administration of drug candidates to absorb. PPB (Plasma Protein Binding) is defined as the capacity of a chemical compound to bind with blood protein. BBB (Blood-Brain Barrier) is defined as the capability of a chemical compound to pass through the blood-brain barrier and reach the brain. Mutagenicity and carcinogenicity are parameters that determine the toxicity properties of potential drug candidates (Afinasari *et al.*, 2022).

Lipinski's Rules of Five and ADMETox evaluations are used as an early-stage evaluations for drug discovery. From the Lipinski prediction results (Table 1), one out of 22 chemical compounds from *A. muricata* L. did not pass Lipinski's Rule of Five and from the predicted result of the ADMETox (Table 2). Seven compounds did not fit the HIA criteria; only one compound did not fit the CaCO2 criteria; eight compounds did not fit the PPB criteria; and only three compounds passed the BBB criteria. Six compounds were found to have no toxicity, either carcinogenicity or mutagenicity.

Ligand preparation

ChemDraw Pro 12.0 software was used to construct 2D structures for the 22 test ligands during the preparation process. Afterwards, their 3D structures were created, and Chem3D Pro 12.0 software was used to optimize the test compounds' molecular mechanics (MM) geometry for stability.

To create the most stable structure that closely resembles the existing natural compound structures, the total energy of the molecules was minimized during the geometry optimization process (Roy *et al.*, 2015). These findings led to the creation of the test compound's optimal structures.

Receptor preparation

X-ray crystal structure of human Caspase-3 (PDB ID: 1NME) was chosen because it originated from humans (*Homo sapiens*), had no mutation, had a good resolution (<2 Å), and had a natural ligand. The Caspase-3 receptor was prepared by separating the protein from its original ligand using BIOVIA Discovery Studio software, resulting in a space or pocket that would be utilized during the docking simulation. The native ligand's structure without the protein was also discovered, in addition to the protein structure having a pocket for the test ligand.

Table 1. Lipinski prediction results of bioactive compounds in soursop plant (*Annona muricata* L.)

Compound	Parameter Lipinski rule of five				Application of Lipinski's rule of five
	Molecular weight (<500 Da)	LogP (<5)	Hydrogen donor (<5)	Hydrogen acceptor (<10)	
Annoionol A	230.34	1.73	3	3	Passed
Annoionol B	244.33	0.81	4	4	Passed
Anomuricine	329.39	2.65	2	5	Passed
Anomurine	343.42	3.08	1	5	Passed
Anonaine	265.31	2.88	1	3	Passed
Asimilobine	267.32	2.65	2	3	Passed
Chlorgenic acid	354.31	-0.39	6	9	Passed
Coclaurine	285.34	2.36	3	4	Passed
Coreximine	327.37	2.40	2	5	Passed
Epicathecine	290.27	0.83	5	6	Passed
Isoboldine	327.37	2.45	2	5	Passed
Isolaureline	265.31	3.12	0	4	Passed
Liriodenine	275.26	2.88	0	4	Passed
Myricyl alcohol	438.81	10.52	1	1	Passed
Myristic acid	228.37	4.45	1	2	Passed
N-methylcoclaurine	299.36	2.59	2	4	Passed
Palmitic acid	256.42	5.20	1	2	Passed
Polyphenol	458.37	1.01	8	11	Didn't pass
Remerine	279.33	3.13	0	3	Passed
Reticuline	329.39	2.64	2	5	Passed
S-norcorydine	327.37	2.61	2	5	Passed
Xylopine	295.33	2.88	1	4	Passed

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Table 2. The predicted results of the ADMETox profile of bioactive compounds in soursop plant (*Annona muricata* L.)

Compound	Parameter						
	Absorption		Distribution		Toxicity		
	HIA (%) [HIA > 70%]	Caco-2 (nm/sec) [CaCO2 > 5 nm/sec)	PPB (%) [PPB < 90%]	BBB [LogD > 2]	Mutagen	Carcinogen	
						Mouse	Rat
Annoionol A	80.621107	21.3756	78.685043	1.83591	mutagen	negative	negative
Annoionol B	68.686646	21.159	55.761220	0.426248	non-mutagen	negative	negative
Anomuricine	93.529812	29.5702	78.913882	0.558569	non-mutagen	negative	positive
Anomurine	95.858409	51.8528	76.072725	0.112353	non-mutagen	negative	negative
Anonaine	96.493990	47.6818	65.565065	0.984994	mutagen	negative	negative
Asimilobine	93.174009	26.308	63.678123	2.4684	mutagen	positive	negative
Chlorgenic acid	24.404298	19.2384	39.507803	0.0370233	non-mutagen	positive	negative
Coclaurine	89.643482	7.29997	97.170383	0.693997	mutagen	negative	negative
Coreximine	93.267525	14.641	84.091816	0.726882	non-mutagen	positive	negative
Epicathecine	66.707957	0.56962	100.000000	0.394913	mutagen	negative	negative
Isoboldine	93.265891	24.5565	61.235563	1.51842	non-mutagen	negative	negative
Isolaureline	99.658001	56.6616	71.572095	2.38425	mutagen	negative	negative
Liriodenine	97.767428	28.1451	49.719419	0.809938	mutagen	negative	negative
Myricyl alcohol	100.000	51.3386	100.00000	0.0875288	non-mutagen	positive	negative
Myristic acid	97.848313	24.0726	100.000000	5.03596	mutagen	negative	positive
N-Methylcoclaurine	93.072310	13.5813	91.190030	1.64191	non-mutagen	negative	negative
Palmitic acid	98.297110	26.0735	100.000000	8.21885	mutagen	negative	positive
Polyphenol	20.712498	12.0421	100.0000	0.0875288	non-mutagen	negative	positive
Remerine	100.000	56.7725	74.322985	1.80495	mutagen	negative	negative
Reticuline	93.26485	12.2566	83.959605	1.00893	non-mutagen	negative	negative
S-norcorydine	95.533986	29.2909	56.866176	1.00154	non-mutagen	negative	negative
Xylopine	95.782499	49.1829	60.230097	0.850866	mutagen	negative	negative

(a) (b)

Figure 1. The three-dimensional (3D) structure of Caspase-3 without a ligand (a) and native ligand 2-Hydroxy-5-(2-mercapto-ethylsulfamoyl)-benzoic acid (b)

Water molecules (H2O) must also be removed during this procedure. To ensure that only amino acid molecules are present in Caspase-3 and that only these amino acid molecules will interact with the test ligand. Water molecules must be removed. Additionally, eliminating water molecules can maximize the interaction between the protein and test ligand (Lemmon & Meiler, 2013). Figure 1 depicts the 3D architectures of Caspase-3 with its natural ligand separated and without it.

Validation of the molecular docking method

To make sure the utilized approach satisfies the necessary standards and can be used for subsequent testing stages, the molecular docking method underwent validation. The Root Mean Square Deviation (RMSD) is the parameter used for validation. A metric called RMSD shows how much the protein-ligand interactions have changed between the crystal structure before and after docking. If the RMSD value is 2.0, the docking approach is regarded to be valid (Istyastono, 2018). The obtained RMSD value in this experimental technique validation is 1.85, demonstrating the validity of the docking approach used. The information is shown in Table 3. In Figure 2, which is based on the re-docking results, the interactions between the natural ligand and Caspase-3 are visualized in 2D and 3D. The ligand interacts with Caspase-3 through conventional hydrogen bonds, van der Waals bonds, Pi-Sulfur links, and Pi-Pi T-shaped bonds.

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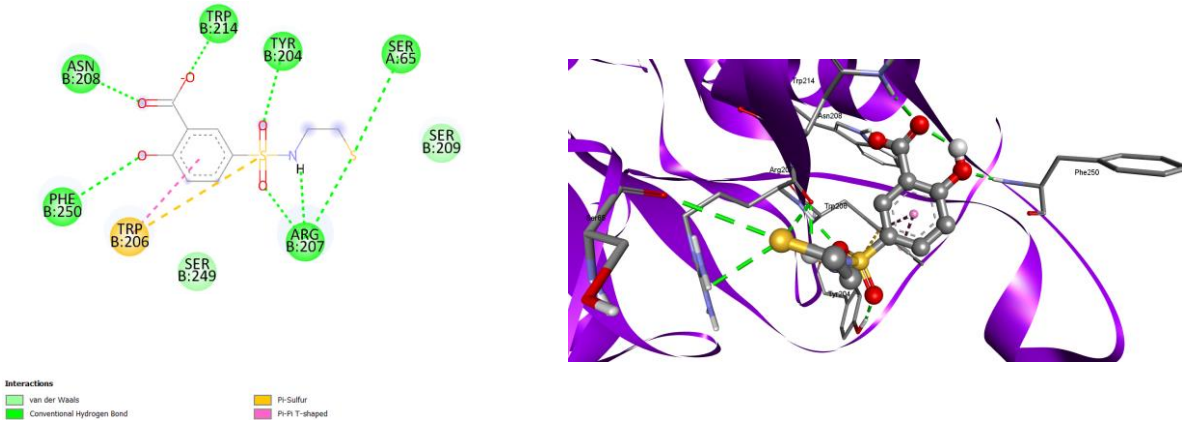
Table 3. The results of the method validation through re-docking with 2-Hydroxy-5-(2-mercapto-ethylsulfamoyl)-benzoic acid

PDB ID	Grid Box (x, y, z)	Validation		Binding Energy (kkal/mol)
		RMSD cluster (Å)	RMSD reference (Å)	
1NME	42.197 96.352 24.611	0.00	1.85	-5.28



(a) (b)

Figure 2. Visualization of the 2D interactions between Caspase-3 and the natural ligand (a) and visualization of the 3D interactions between Caspase-3 and the natural ligand (b)

Molecular docking

Using the same program, AutoDockTools 1.5.6, the test compounds continued to be docked to Caspase-3 after fulfilling the docking method's validation criteria. Since those coordinates corresponded to the region where the native ligand interacts with Caspase-3, they were modified to fit the grid box utilized during method validation when docking the test compounds on Caspase-3.

The procedure continued with docking the test compounds on 1NME using the same software, AutoDockTools 1.5.6, after witnessing the validated findings of the docking approach that satisfied the requirements. Since these coordinates indicated the site of the native ligand interaction with 1NME, they were modified to match the grid box utilized during method validation while docking the test compounds on 1NME. The test chemicals' docking with the 1NME receptor produced the following results: hydrogen bonding, inhibition constant, and binding energy.

The scoring function computation for the ligand conformation (I) inside the macromolecule/receptor under equilibrium conditions (conformational search) yields the binding energy. The Gibbs energy (G), also known as the binding energy, can be calculated from these variables using the equation [E+I] = [EI] (Limongelli, 2020). The binding energy reveals how well the test substances bind to the 1NME receptor. A lower value of the binding energy denotes a more solid interaction between the protein and the produced ligand (Manna *et al*., 2017). The strength of a compound's ability to prevent a receptor's action is shown by its inhibition constant. On the other hand, the strength of inhibitory potency is indicated by a smaller value of the inhibition constant (Garcia-Molina *et al*., 2022).

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Table 4. The docking results of bioactive compounds in the soursop plant (*Annona muricata* L.) against the Caspase-3 receptor

No.	Compound	Binding Energy (kcal/mol)	KI (µM)	Bonding with amino acids		
				Hydrogen bonds	Van der waals bonds	Other bonds
1	Native ligand	-5.28	134.89	ASN 208, TRP 214, TYR 204, SER65, ARG 207, PHE 250	SER 209, SER 249	TRP 206
2	Sorafenib	-5.97	42.26	ARG 207, PHE 250, PHE 252	HIS 121, THR 62, SER 249, ASN 208, TRP 217, TRP 206, SER 205	TYR 204, SER 251
3	Annoionol A	-4.99	221.09	TYR 204, TRP 214, GLU 248, PHE 250	ASN 208, ARG 207, SER 249, SER 251	TRP 206
4	Annoionol B	-5.75	61.28	PHE 256, SER 209	HIS 257, SER 251, ARG 207, LYS 210, ASN 208, PHE 250, TRP 214, SER 249	TRP 206
5	Anomuricine	-5.40	109.72	-	ASP 253, SER 251, TYR 204, TRP 206, ASN 208, PHE 250, ASP 253, SER 251	PHE 252, PHE 256
6	Anomurine	-5.40	19.78	SER 209, PHE 250, TYR 204	ASN 208, SER 65, ARG 207, SER 205, SER 251, SER 249	GLU 248, PHE 256, TRP 206, TRP 214
7	Anonaine	-5.97	41.97	TYR 204, PHE 250	ASN 208, TRP 214, SER 249, SER 251	TRP 206, ARG 207
8	Asimilobine	-5.78	57.73	ASN 208	SER 209, SER 249, TYR 204	TRP 214, PHE 250, TRP 206, SER 205, ARG 207
9	Chlorgenic acid	-5.65	72.61	GLU 248, PHE 250, TYR 204, ARG 207, SER 209	SER 65, ASN 208, TRP 214, SER 249, TRP 206	ARG 207
10	Coclaurine	-5.14	171.23	ASN 208, ARG 207	SER 251, PHE 250, GLU 248, TRP 214, PHE 247, SER 63, SER 65, SER 209	SER 249, TRP 206
11	Coreximine	-5.63	75.01	ARG 207, SER 209, TRP 214, PHE 250	ARG 63, ARG 64, SER 65, ASN 208, PHE 247, TYR 204	SER 249, GLU 248, TRP 206
12	Epicathecine	-5.23	146.95	ARG 207, SER 209, PHE 250	SER 65, ASN 208, TRP 214, GLU 248, SER 249, TRP 204	ARG 207, TRP 206
13	Isoboldine	-5.61	76.84	TRP 214, PHE 250	ASN 208, ARG 207, SER 209, SER 251, GLU 248, PHE 247	PHE 256, TRP 206, SER 249
14	Isolaureline	-6.20	28.65	ARG 207, ASN 208	SER 249, PHE 247, PHE 250	TYR 204, TRP 206, TRP 214, SER 251
15	Liriodenine	-5.97	42.20	TYR 204, ARG 207, PHE 250	TRP 214, ASN 208, SER 249, SER 251	TYR 204, ARG 207, TRP 206
16	Myricyl alcohol	-0.10	842.90	-	SER 65, SER 209, TYR 204, PHE 256, ARG 207, SER 251, ASN 208, PHE 250, SER 249, GLU 248, TRP 214	PHE 252, TRP 206

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17	Myristic acid	-3.38	3.31	ARG 207	GLU 248, ASN 208, SER 205, SER 249, PHE 250, TYR 204, SER 209	TRP 206, TRP 214
18	*N*-methylcoclaurine	-5.29	132.07	ARG 207, SER 206, PHE 250, GLU 248	ASN 208, SER 209, TRP 214, SER 249	TYR 204, ARG 207, SER 205, TRP 206
19	Palmitic acid	-3.37	3.36	ARG 207	TRP 214, SER 249, PHE 250, GLU 248, ASN 208, SER 209, TYR 204	TRP 206
20	Polyphenol	-5.7	63.80	PHE 250, SER 209, ARG 207, TYR 204	SER 249, SER 251, TRP 206, SER 65	ASN 208
21	Remerine	-5.38	113.09	PHE 250, ASN 208, SER 209, ARG 207, SER 65	SER 249, SER 251, PHE 256, TYR 204	TRP 206, TRP 214
22	Reticuline	-5.65	72.70	SER 209, SER 251	SER 65, TRP 206, SER 249, TRP 214	ARG 207, GLU 248, ASN 208, PHE 252, PHE 250
23	S-norcorydine	-6.12	32.53	PHE 250, GLU 248	ARG 207, ASN 208, SER 249, SER 251	TYR 204, TRP 206, TRP 214
24	Xylopine	-5.58	80.58	GLU 248, PHE 250	ARG 207, ASN 208, PHE 247	SER 249, TRP 214, TRP 206

Data analysis and visualization

Molecular docking is used to predict the effectiveness of a ligand in interacting with the target cell. In this study, twenty-four ligands were likely present in the *A.muricata* plant to see the effectiveness of its anticancer activity against the caspase-3 enzyme (PDB ID: 1NME). Caspase-3 plays a role as the major mediator of apoptosis activated.

The first parameter observed from the docking results is the binding energy. Analysis and interpretation of the energy were done to data provided by AutoDockTools 1.5.6. According to AutoDock, the binding energy is the sum of the intermolecular forces acting upon the receptor-ligand complex (Lin *et al.,* 2011). A good binding energy is represented by a more negative (lower) value. While a low binding energy suggests that the compound requires less energy during binding, indicating that a low binding energy signifies a greater potential for interaction and the formation of a strong bond with the target protein (Rena *et al.,* 2022). The second parameter is inhibition constant (KI). Inhibition constant indicates the concentration required by the ligand to inhibit the target protein. A good inhibition constant is represented by a smaller value of KI. (Rena *et al.,* 2022).

In addition to the energy binding value and inhibition constant value, the interaction between residues and ligands, such as hydrogen bonding, is also taken into consideration. Hydrogen bonds play a crucial role in protein structure because the stability of a protein's structure depends on hydrogen bonds (Suryani *et al.,* 2018). The binding location of the ligand on the protein is determined by the extent of residue-ligand interactions. A greater number of interacting residues leads to a stronger bond between the ligand and the protein. (Rena *et al.,* 2022).

A total of twenty-two test ligands from *A. muricata* under research were employed and docked onto the caspase-3 receptor. Each ligand produced conformation based on binding energy values, inhibition constant, and hydrogen bonding. Based on Table 4, the test ligands, Isolaureline and S-norcorydine, had a binding energy value of -6.20 kcal /mol and -6.12 kcal /mol and inhibition constant value of 28.65 µM and 32.53 µM. Those compounds have lower binding energy values (-5.28 kcal/mol) and inhibition constant (134.89 µM) from the native ligand. The visualization can be seen in Figure 3 and 4.

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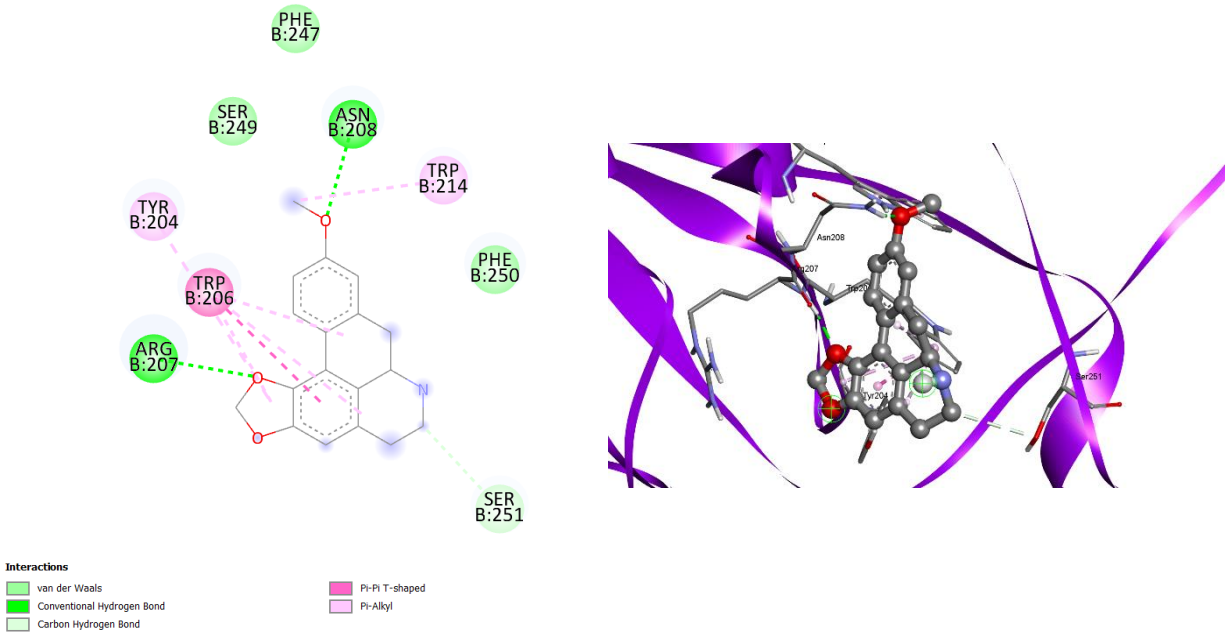
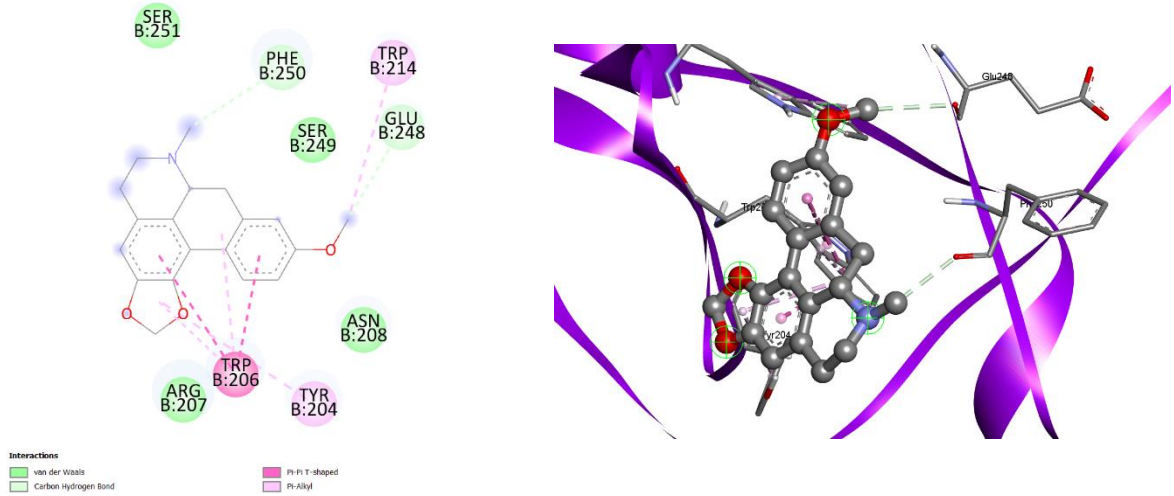


(a) (b)

Figure 3. Visualization of the 2D interactions between Caspase-3 and Isolaureline (a) and visualization of the 3D interactions between Caspase-3 and Isolaureline (b)



(a) (b)

Figure 4. Visualization of the 2D interactions between Caspase-3 and S-Norcorydine (a) and visualization of the 3D interactions between Caspase-3 and S-Norcorydine (b)

Table 4 shows that Isolaureline forms hydrogen bonds with the amino acid ARG 207 and ASN 208, and S-Norcorydine forms hydrogen bonds with the amino acid PHE 250 and GLU 248. Hydrogen bonding plays a major role in the stability of molecular interactions, resulting in an abundance of hydrogen bonds that increase the bond energy between the enzyme and the substrate (Arwansyah *et al.*, 2014). In addition, these two compounds formed amino acid residue interactions in the form of hydrophobic interactions; specifically, Van Der Waals bonds with the residue. Isolaureline form Van Der Waals bonds with the amino acid SER 249, PHE 247, and PHE 250. On the other hand, S-Norcorydine form Van Der Waals bonds with the amino acid ARG 207, ASN 208, SER 249, and SER 251. The results indicated that the bonds

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of the three compounds were nearly as strong as those of natural ligand–as observed from the numerous similarities in amino acid residues formed. Based on all the parameters, these two compounds (Isolaureline and S-norcorydine) have the potential as candidates for anticancer agents.

CONCLUSION

Twenty-two compounds from *A. muricata* were successfully docked and then analyzed based on their free energy binding and intermolecular interactions with the caspase-3 binding site. The results of molecular docking showed that two compounds, namely Isolaureline and S-norcorydine, showed the best results, giving the lowest binding energy value and lowest inhibition constant with the most preferred interaction. A further study can be conducted to investigate the anticancer activity of these compounds via in vivo and in vitro research.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.A., M.W.A.R., Z.A.H., S.L.R., H., N.P., A.P.A.P., D.L.A.; Methodology, A.A., M.W.A.R., Z.A.H., S.L.R., H., N.P., A.P.A.P., D.L.A.; Software, A.A., M.W.A.R., Z.A.H., S.L.R., H.; Validation, A.A.; Formal Analysis, A.A., M.W.A.R., Z.A.H., S.L.R., H.; Investigation, A.A., M.W.A.R., Z.A.H., S.L.R., H.; Resources, A.A., M.W.A.R., Z.A.H., S.L.R., H.; Writing - Original Draft, A.A., M.W.A.R., Z.A.H., S.L.R., H.; Writing - Review & Editing, A.A., M.W.A.R., Z.A.H., S.L.R., H.; Visualization, A.A., M.W.A.R., Z.A.H., S.L.R., H.; Supervision, A.A., M.W.A.R., Z.A.H., S.L.R., H., N.P., A.P.A.P., D.L.A.; Project Administration, A.A., M.W.A.R., Z.A.H., S.L.R., H., N.P., A.P.A.P., D.L.A.; Funding Acquisition, A.A., M.W.A.R., Z.A.H., S.L.R., H., N.P., A.P.A.P., D.L.A.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Standardization of Myristicin in Nutmeg (*Myristica fragrans* Houtt.) Fruit using TLC-Densitometric Method

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Abstract

Background*: Myristica fragrans Houtt. (Myristicaceae family), with the main content of myristicin, has been immensely used in herbal medicine. Standardization is essential to ensure the safety of natural extracts and the quality of herbal medicines using various chemical analysis techniques. Method validation is necessary to ascertain the reliability and reproducibility of the method. Myristicin is a member of the phenylpropene group, a natural organic compound found in small amounts in nutmeg fruit, which has pharmacological effects.* ***Objective:*** *This study aims to determine the myristicin content in nutmeg fruit using TLC-Densitometry.* ***Methods****: Determination of myristicin in nutmeg fruit extract was performed using TLC-Densitometry with silica GF_{254} as stationary phase, mobile phase n-hexane: ethyl acetate (8:2 v/v), and spot visualized at 285 nm. In this study, the content of myristicin in nutmeg fruit was determined using compendial methods (AOAC), thus requiring method verification with parameters including selectivity, linearity, precision, LOD, and LOQ.* ***Results****: The validation of this method showed good linearity and selectivity with y = 0.0001x + 0.0226 (r = 0.9996) and 1.53 (>1.5), respectively. The LOD and LOQ results were low with values of 0.11 µg/spot and 0.33 µg/spot, respectively. The percentage coefficient of variation for precision was below the requirement value of not more than 4%. The average myristicin content in nutmeg fruit extract was approximately 0.0017 ± 0.0003% (w/w).* ***Conclusion****: The developed method was valid and sensitive for the quantification of myristicin content in nutmeg fruit.*

Keywords*: densitometry, Myristica fragrans Houtt., myristicin, standardization, validation*

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INTRODUCTION

Method validation is an analytical technique based on laboratory experiments to demonstrate that the validation parameters satisfy the needs of the method's users and that the findings are nearly identical to the actual value and repeatable (Sugihartini *et al*., 2012). Quantitative analysis of substances or drug components in a biological sample, for example, in plants, must meet the requirements of the method validation. Therefore, method validation is crucial to determine and ensure the accuracy, specificity, reliability, and reproducibility of a method to be used for analytical purposes (United States Pharmacopeial Convention, 2004). Method validation parameters include accuracy, precision, specificity/selectivity, limit of detection (LOD), limit of quantification (LOQ), and linearity. If a standard method (Compendial method) is used, there is no need for method validation but verification. For data collected, data standardization will apply (Sudjarwo *et al*., 2019).

Standardization is necessary to guarantee herbal medicine's quality (Kadian *et al*., 2016). Standardization of herbal medicine is the process of comparing levels to features, constant factors, and qualitative and quantitative values that offer genuine assurance of safety, efficacy, quality, and repeatability (Kumari & Kotecha, 2016; Shulammithi *et al*., 2016; Butt *et al*., 2018). The standardization emphasized the determination of compounds with pharmacological effects, a process of ensuring quality assurance and good standards for both drugs and herbal products (Kunle, 2012).

The nutmeg plant (*Myristica fragrans* Houtt.), belonging to the *Myristicaceae* family, contains small amounts of naturally occurring chemical substances called myristicin, also known as phenylpropenes. Myristicin is known to be responsible for a disease's pharmacological properties or biological activity. The concentration of myristicin in nutmeg oil varies depending on the origin of the fruit, extraction technique, drying process, and part of the fruit used (Liunokas & Karwur, 2020).

In the standardization of myristicin, spectroscopic, TLC, and HPLC methods can be used (Naikodi *et al*., 2011). The TLC-Densitometry method, which is versatile enough to identify nearly every constituent in plants, is one of the most popular analytical techniques for examining the chemical components of plants. Considering the enormous demand for herbal medicines in the global market, many methods are well-developed for standardizing raw materials. This current study determined myristicin content using the compendial method (United States Pharmacopeial Convention, 2012; AOAC, 2019).

MATERIALS AND METHODS

Materials

Myristica fragrans Houtt. was obtained from Haruku Island, Oma Village, Central Maluku Regency, Maluku Province, Indonesia, and identified by Herbal Materia Medica Laboratory, Batu, Malang, East Java (067/259/102.20/2023). Myristicin standard (pharmaceutical grade, Chemfaces), technical ethanol 96%, pro-analytical ethanol (Merck), ethyl acetate (Merck), n-hexane (Merck), and Silica Gel TLC 60 F_{254} (Merck).

Instruments

This current study utilized several instruments which include OHAUS analytical balance, rotary vacuum evaporator (BUCHI), sonicator, 20×10×5 cm3 chromatography chamber (CAMAG), densitometer (CAMAG), UV CAMAG lamp, TLC Scanner 4 (CAMAG), Linomat 5 (CAMAG), and VisionCATS software (CAMAG).

Methods

Plant determination

Determination of the plant samples was carried out at the Herbal Materia Medica Batu Laboratory. Based on the letter number 067/259, 102.20/2023 issued in Batu on February 08, 2023, the plant used was *Myristica fragrans* Houtt from the *Myristicaceae* family.

Dried plant powder preparation and extraction

Nutmeg (*Myristica fragrans* Houtt.) fruit has been cleaned and washed, chopped into small pieces, dried by aerating at room temperature 15-30 ° C, and not exposed to direct sunlight. The dried plant then was ground or mashed to form a powder. The powder of nutmeg (*Myristica fragrans* Houtt.) was weighed as much as 200 grams and extracted with 96% ethanol (1:4) using the UAE (Ultrasonic Assisted Extraction) method for 45 minutes while occasionally shaking. Re-maceration was performed twice, using the same type and volume of solvent. A rotary vacuum evaporator concentrated the extract at 50–60°C until a thick extract was produced (Budiastra *et al*., 2013).

Determination of moisture content

The thick extract of nutmeg fruit and dried plant powder was each weighed 1 gram in a preheated porcelain crucible with a lid at 105 °C for 3 hours until their constant weight was achieved. Subsequently, the materials in the crucible were flattened, dried at 105°C for 1 hour, and then weighed. The step was repeated

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twice until a constant weight of the heating product was obtained (no more than 0.25%) (Courtney, 2017; Sri *et al*., 2021).

Preparation of myristicin standard solution

The myristicin standard (10 mg) was dissolved in ethanol p.a in a 10 mL volumetric flask and added ethanol p.a until the limit mark reached 10 mL.

Sample solution preparation

The thick extract of nutmeg fruit (500 mg) was dissolved in ethanol p.a in a 5 mL volumetric flask.

Wavelength determination

Each blank, myristicin standard, sample, and sample solution with myristicin standard addition were photographed on the silica GF_{254} plates with a Linomat 5 applicator. The plate was developed using the selected mobile phase and observed at 200 - 400 nm wavelength region spectrum with a densitometer.

Method verification

Selectivity

Each 10 µL of blank solution, myristicin standard, sample solution, and myristicin standard-added sample solution were spotted on silica GF_{254} 6 × 10 cm TLC plates. The TLC plate was developed with the selected mobile phase; then, the chromatogram was observed with a densitometer, as well the Rf value and the degree of resolution (Rs) were calculated at the selected wavelength.

Limit of detection (LOD) and limit of quantification (LOQ)

An amount of 10 µL of blank solution and a series of myristicin standard solutions with an increasing concentration were spotted on the silica GF254 TLC plate. The plates were developed with a selected mobile phase, and the areas were observed at a selected wavelength. The standard deviation (σ) of the blank area and the linear regression equation (y = bx + a) between the weight of the myristicin standard solution (µg) and the response area, slope (b) were calculated (Bhardwaj *et al*., 2020):

$$LOD = 3.3\ \sigma/b \quad (1)$$

$$LOQ = 10\ \sigma/b \quad (2)$$

Linearity

Myristicin standard series solution was photographed with equal volume on silica gel GF254 TLC plates with selected mobile phase and then observed at a selected wavelength with the densitometer.

Precision

Ten spotting points were made on a silica GF_{254} TLC plate, each being bottled with 60 µL of a 1,000 µg/mL myristicin standard solution. The TLC plates were developed with the selected mobile phase. Using a densitometer, the average area of the spots was calculated at the selected wavelength, standard deviation (SD), and coefficient of variation (C.V).

Determination of myristicin content in nutmeg (*Myristica fragrans* Houtt.) extracts

The thick extract of nutmeg fruit (0.5 g) was weighed, and myristicin standard solution of varying weight was added. Then, 50 µL of each solution was bottled onto silica GF254 TLC plates using Linomat 5. A constant application rate of 100 nL/s was maintained, with a distance of 15 mm between each band. The slit dimension on the densitometer used was 10 × 0.4 mm, and the scanning speed used was 100 mm/s. The TLC plates were developed with the selected mobile phase. The area was observed at the selected wavelength on the densitometer. This level was determined with as many as four replicates in the same way (AOAC, 2019; Kai & Province, 2008; United States Pharmacopeial Convention, 2012).

RESULTS AND DISCUSSION

Preparation of nutmeg (*Myristica fragrans* Houtt.) fruit extract

Out of 15 Kg (wet weight) nutmeg (*Myristica fragrans* Houtt.), 1,437 grams were dried plants, and they were then mashed and obtained 1,337 grams. After that, 200-gram powder was macerated with 800 mL of ethanol. After it was concentrated with a rotary vacuum evaporator, 35.469 grams (17.7%) of thick nutmeg meat extract (*Myristica fragrans* Houtt.) was obtained.

Determination of moisture content

Based on the values of water content in dried plants and extracts of nutmeg (*Myristica fragrans* Houtt.) fruit was 9.8% (w/w) and 6.22% (w/w), respectively (Table 1). These results proved that the dried plant and extracts met the water content requirements (not more than 16.0%) (Courtney, 2017; Sri & Anggelina, 2021). If the water content exceeds the requirement, microorganisms might contaminate the dried plants and extracts. The contamination happens because microorganisms can use the amount of free water as a breeding source (Sri & Anggelina, 2021).

Determination of maximum wavelength

The spectra of the blank, myristicin standard solution, sample solution, and sample solution added to the myristicin standard had a maximum wavelength of 285 nm with a pH of 5. Thus, the selected wavelength was 285 nm (Figure 1). Other researchers reported a wavelength of 254 nm, but no pH value was reported

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(Naikodi *et al*., 2011). The difference in wavelength and spectrum will change at different pH conditions. The higher the pH, the larger the wavelength (red shift) (Balashov *et al*., 1991; Suharyani *et al*., 2021). The selection of different wavelengths could be adjusted according to the analytical needs.

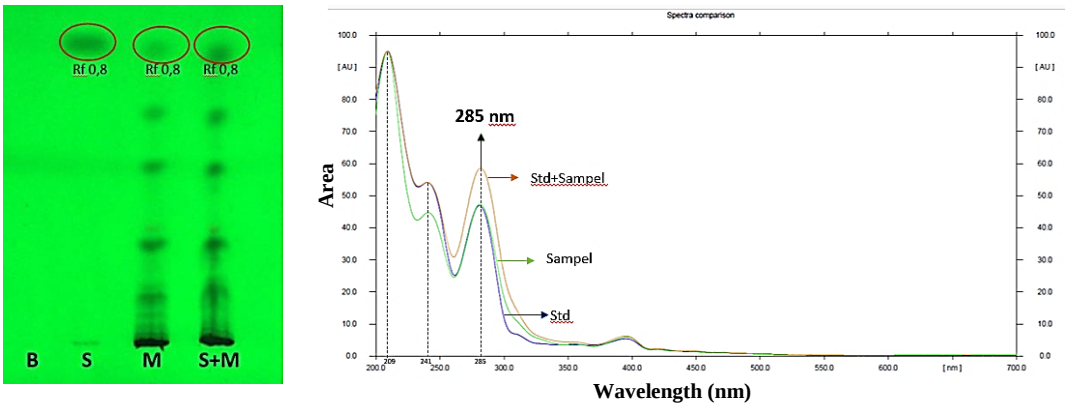
Table 1. Water content results

Material	Sample Weight (gram)	Sample Weight After Heating (gram)	Moisture Content (% *w/w*)
Nutmeg fruit dried plant	1.0044	0.9060	9.80
Nutmeg fruit extract	1.0071	0.9445	6.22

Method verification

Selectivity

The selectivity with the degree of resolution parameter (Rs) was done using the selected mobile phase. The ratio of n-hexane: ethyl acetate (8:2; v/v) showed a degree of resolution (Rs) ≥ 1.5 in each solution (Table 2 and Figure 2). This value proves that the analyte was well-separated from other components. Other researchers using petroleum ether: dichloromethane (30:70; v/v) mobile phase obtained resolution values of 8.5 and 9 (Parsley *et al*., 2014). The distinction was influenced by the different of mobile phase systems. If the mobile phase system is unalike, the polarity will be different (Sudjarwo *et al*., 2019).



(a) **(b)**

Figure 1. UV stain spectra 254; B: blank spectrum; S: standard myristicin; M: nutmeg fruit extract; S + M: extract + standard myristicin (a), standard myristicin spectrum profile at 200-400 nm wavelength (b)

Table 2. Results of Rf-value and Rs-value of myristicin stain

Mobile Phase	Solution Type	R_f-Value	R_s-Value
n-hexane:ethyl acetate (3:2; v/v)	a. Myristicin standard solution	0.91	-
	b. Nutmeg (*Myristica fragrans* Houtt.) fruit extract solution	0.98	1.60
	c. Nutmeg (*Myristica fragrans* Houtt.) extract solution that has been diluted with myristicin standard solution	0.98	1.30
n-hexane:ethyl acetate (8:2; v/v)	a. Myristicin standard solution	0.81	-
	b. Nutmeg (*Myristica fragrans* Houtt.) fruit extract solution	0.82	1.70
	c. Nutmeg (*Myristica fragrans* Houtt.) extract solution that has been diluted with myristicin standard solution	0.86	1.53
Methanol:ethyl acetate (2:8; v/v)	a. Myristicin standard solution	0.97	-
	b. Nutmeg (*Myristica fragrans* Houtt.) fruit extract solution	0.96	1.20
	c. Nutmeg (*Myristica fragrans* Houtt.) extract solution that has been diluted with myristicin standard solution	0.96	1.20

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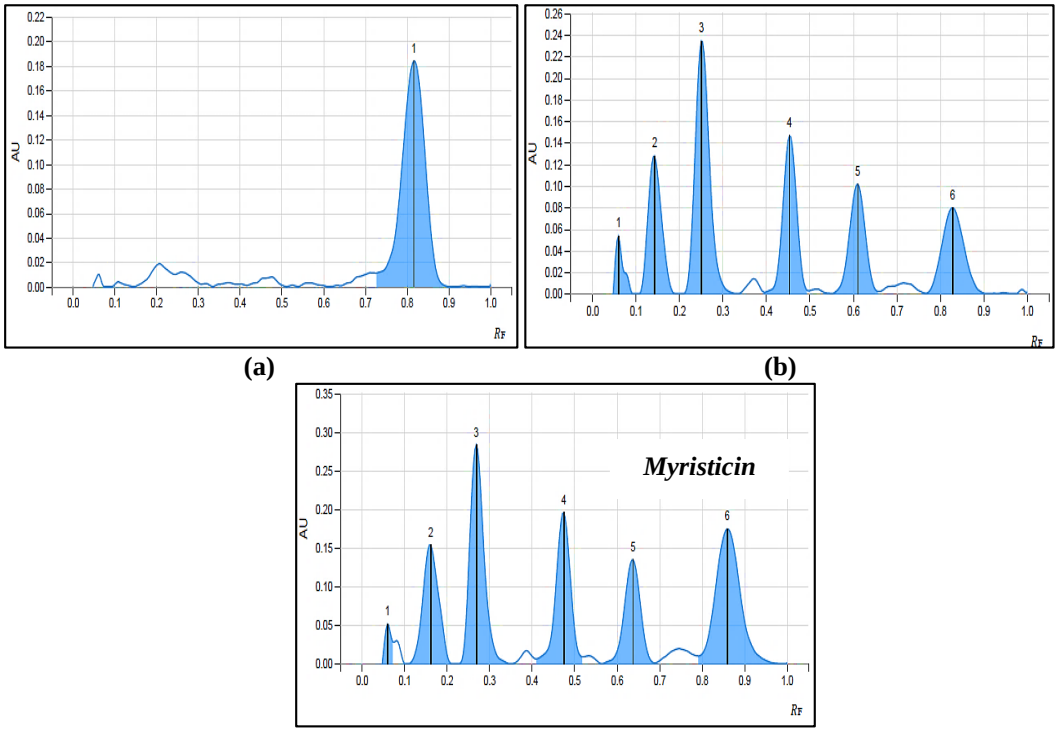

Figure 2. Chromatogram of standard myristicin (a), chromatogram of nutmeg (*Myristica fragrans* Houtt.) fruit extract (b), chromatogram of nutmeg (*Myristica fragrans* Houtt.) fruit extract added with standard myristicin with mobile phase n-hexane: ethyl acetate (8:2)

LOD (Limit of Detection) and LOQ (Limit of Quantification)

In determining LOD and LOQ, myristicin standard series solutions with concentrations 2 µg to 6 µg were used and then photographed on TLC plates with the same volume of 10 µL. The SD value of the blank was 0.00003346, and the regression equation of the myristicin standard was y = 0.001x + 0.00005 (r = 0.9998), with LOD and LOQ results obtained of 0.11 µg/spot and 0.33 µg/spot, respectively. The TLC-densitometry method developed was sensitive because the LOD and LOQ values were low. The lowest amount of analyte in the sample that can still be detected is indicated by LOD. On the other hand, LOQ indicates the lowest analyte concentration in the sample that can be accurately and precisely identified using quantitative means (Fatmawati & Herlina, 2017). In another study, the LOD and LOQ were obtained at 0.1 µg/spot and 0.4 µg/spot, respectively, for compounds of the *Myrtaceae* family (Rastogi *et al*., 2008). If modest LOD and LOQ values are acquired, it is more sensitive since the difference in LOD and LOQ depends on the accuracy of the study (Table 3).

Linearity

The linearity of myristicin is found from equation y = 0.0001x + 0.0226 (r = 0.9996) (Figure 3). This shows a linear relationship between the weight of the myristicin standard in the bottled (µg) and the response area. The myristicin standard calibration curve provides a good linearity value, and the determination of levels with the calibration curve is guaranteed to be correct (Fatmawati & Herlina, 2017). The linearity from the *Myrtaceae* family found by other researchers showed y = -592.632 + 6.341x (r = 0.9939) (Rastogi *et al*., 2008). The difference depends on the accuracy of the study.

Precision

The precision obtained for the average myristicin area is the coefficient of variation (C.V) of 0.326% (Table 4). This value still met the requirements of C.V (not more than 4%) (Birmingham *et al*., 2021). Another study obtained a precision of 0.76% (Rastogi *et al*., 2008). This difference occurs due to the researchers' different expertise, resulting in different accuracy.

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Table 3. LOD and LOQ results

Bottling Amount of Blank (µL)	Area
10	0.00001
10	0.00000
10	0.00000
10	0.00006
10	0.00008
Standard Vial Amount (µg)	
2	0.00212
3	0.00312
4	0.00415
5	0.00515
6	0.00624

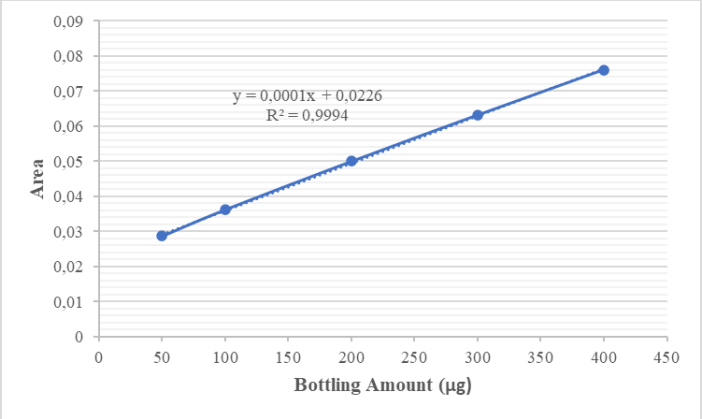

Figure 3. Linearity of *myristicin* standard solution

Table 4. Precision results

Bottling Amount (µg)	Area
60	0.03750
60	0.03848
60	0.03930
60	0.03971
60	0.03988
60	0.03856
60	0.03772
60	0.03734
60	0.03683
60	0.03583
Average (X)	0.03811
Standard Deviation (SD)	0.00124
C.V	0.326

Determination of *myristicin* content in nutmeg (*Myristica fragrans* Houtt.) fruit extracts

The application of the TLC-densitometry method for the determination of myristicin content in nutmeg fruit extract was carried out by the compendial method (United States Pharmacopeial Convention, 2012; AOAC, 2019). Based on the results in Table 5, the average myristicin content in the fruit extract sample of nutmeg is 0.0017 ± 0.1632% (w/w). In another study, the myristicin content in nutmeg was reported to be 109.28% (µg) (Naikodi *et al*., 2011). The differences in myristicin content are attributed to variations in plant parts, sampling locations, genetic factors, growing environments, cultivation practices, and harvest times (Zarshenas *et al*., 2013; Mustafa *et al*., 2017). In this study, nutmeg fruit flesh was used, while in other studies, the aril (mace) part was utilized. As reported by Gayathri and Anuradha (2015), the total phenol content in the fruit was lower than that in the seeds and aril of nutmeg. Another possibility is caused by the use of different methods and solvents.

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Table 5. Myristicin content in nutmeg (*Myristica fragrans* Houtt.) fruit extract

Replication	Weigh (gram)	Obtained (gram)	% (*w/w*)
1	0.5000	0.00000700	0.0014
2	0.5099	0.00000767	0.0015
3	0.5199	0.00000833	0.0016
4	0.5324	0.00001133	0.0021
		Average	0.0017
		Standard Deviation (SD)	0.0003
		C.V.	0.1632

CONCLUSION

The developed method was valid and sensitive for the quantification of myristicin content in nutmeg fruit, producing myristicin in the standardized nutmeg (*Myristica fragrans* Houtt.) fruit extract of 0.0017 ± 0.0003% (*w/w*).

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AUTHOR CONTRIBUTIONS

Conceptualization, SJ.; Methodology, SJ., SK.; Software, D.E.E.; Validation, SJ., SK.; Formal Analysis, D.E.E.; Investigation, D.E.E.; Resources, D.E.E., SJ., SK.; Writing - Original Draft, D.E.E.; Writing - Review & Editing, SJ., SK.; Visualization, D.E.E.; Supervision, SJ., SK.; Project Administration, D.E.E., SJ., SK.; Funding Acquisition, SJ., SK.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Skin Penetration of Corn Silk (*Zea mays* L.) Transdermal Patch on Wistar Mice Skin Using Franz Diffusion Cell

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Abstract

Background*: Corn silk (Zea mays L.) contains many active compounds, especially the flavonoid quercetin which has pharmacological activity as an antihyperlipidemic agent by reducing cholesterol and triglyceride levels in the body. Antihyperlipidemic treatment by oral route, such as statin drugs, has the disadvantage of experiencing a first-pass effect in the liver, which reduces the bioavailability of the drug. In addition to avoiding the first-pass effect, transdermal patches can improve patient compliance because they are easy to use.* ***Objective****: This study aims to optimize the transdermal patch formula of corn silk extract and test the penetration of the optimum formula by in vitro.* ***Method****: Optimization of the formula using the Regular Two-Level Factorial Design method on Design Expert®. This study used 2 factors, namely HPMC with a concentration of 3%-4% and PVP with a concentration of 1%-2%. The optimum formula obtained was subjected to in vitro penetration test using Franz diffusion cell.* ***Results****: Based on the results of factorial design analysis, the optimum formula of transdermal patches is at HPMC and PVP concentrations of 3.49% and 1% with moisture content, moisture uptake, percentage of elongation, and folding endurance respectively of 7.79%, 4.19%, 13.26% and 470.58 fold. The optimum formula of corn silk extract transdermal patch preparation also had an optimum percent cumulative amount of penetrated flavonoids of 96.06% and flux of 6.17 µg/cm^2.hour at 3 hours.* ***Conclusion****: Transdermal patch dosage of corn silk extract with HPMC and PVP concentrations of 3.49% and 1% proved to have good characteristics and penetration rate.*

Keywords*: franz diffusion, optimization, transdermal patch, Zea mays L.*

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INTRODUCTION

Due to abnormal fat metabolism or function, hyperlipidemia is characterized by elevated levels of total cholesterol, triglycerides, low-density lipoprotein (LDL), and decreased levels of high-density lipoprotein (HDL) (Mala *et al*., 2019). Eating disorders, obesity, the genetic disease familial hypercholesterolemia (FH), and diabetes are the causes of hyperlipidemia (Yao *et al*., 2020). Corn silk (*Zea mays* L.) is a natural constituent with antihyperlipidemic properties that can be used to treat this disease.

Corn silk contains secondary metabolites such as alkaloids, steroids, carotenoids, saponins, anthocyanins, phenolics, and flavonoids (Al-Oqail *et al*., 2019; Kim *et al*., 2019; Yucharoen *et al*., 2023). The pharmacological activities of corn silk include anti-inflammatory, anti-diabetic, antioxidant, anti-bacterial, anti-fatigue, anti-depressant, and antihyperlipidemic (Hasanudin *et al*., 2012; Linard De Carvalho *et al.*, 2019; Wang & Zhao, 2019; Limmatvapirat *et al.*, 2020; Lapčík *et al.*, 2023). Research conducted by Wang *et al.* (2017) proved that corn silk thick extract at 400 and 800 mg/kgBW could significantly reduce total cholesterol, triglyceride, and LDL levels in rat test animals. This activity relates to the flavonoid compound in corn silk, namely quercetin. Research conducted by Yucharoen *et al.* (2023) proved that ethanol thick extract of corn silk contains 4.71 ± 0.79 mg quercetin equivalent/g extract. By inhibiting intestinal cholesterol absorption by reducing the expression of the epithelial cholesterol transporter, quercetin can function as an antihyperlipidemic. Niemann-Pick C1-like 1 (NPC1L1) inhibits the formation of lipid oxidative stress, enhances PPAR expression, and inhibits macrophage-modified LDL oxidation by decreasing the content. LDL particles contain -tocopherol (Agung, 2021; Fukaya *et al*., 2021; Yi *et al*., 2021).

Oral antihyperlipidemic drugs, such as statins, can be well absorbed, but they may undergo a first-pass effect in the liver, thereby decreasing drug bioavailability (Korani *et al*., 2019). Therefore, a transdermal patch was devised using corn silk extract. Transdermal delivery has advantages such as preventing the first-pass effect, increasing bioavailability, and increasing patient compliance due to its ease of use (Tijani *et al*., 2021). The active substance is released on the surface of the epidermis by diffusion through the corneal layer into the dermis, allowing it to enter the systemic circulation and be delivered to the target organ (Arum *et al*., 2022). A polymer is an essential component in the production of transdermal patches. This study utilized Hydroxy Propyl Methyl Cellulose (HPMC) and Polyvinyl Pyrrolidone (PVP) as polymers. The concentrations of HPMC and PVP used were 3% to 4% and 1% to 2%, respectively. The polymer was optimized using the Regular Two-Level Factorial Design method on Design Expert® software because this method is simple and easy to analyze the influence of independent variables, as in the research of Pratiwi *et al*. (2020). Polymers can affect drug discharge from the matrix of a transdermal patch. The medication release capability of the matrix of a transdermal patch can be evaluated in vitro using the Franz Diffusion Cell method.

Based on the description above, research was conducted on "Penetration of Transdermal Patch Preparations of Corn Silk Extract (*Zea mays* L) in the Skin of Wistar Rats Using the Franz Diffusion Cell Method". Corn silk (*Zea mays* L.) was extracted using the maceration method with 96% ethanol as the solvent. Optimization of a formula utilizing the Regular Two-Level Factorial Design method in Design Expert® software. This research uses factors A (HPMC) and B (PVP). The corn silk (*Zea mays* L.) transdermal patch was evaluated to obtain the optimum formula and in vitro diffusion test to describe the ability of the drug substance to penetrate after being released from the preparation.

MATERIALS AND METHODS

Materials

The materials used in this research were corn silk (Zea mays L.), Wistar male white rats (Local, Indonesia), AlCl3 (Merck®, Indonesia), sodium acetate (Merck®, Indonesia), methanol (Merck®, Indonesia), ethanol 96 % (Merck®, Indonesia), phytochemical screening reagent, NaOH (Merck®, Indonesia), KH2PO4 (Merck®, Indonesia), HPMC (MakingCosmetic®, USA) with a viscosity of 83.92 Poise, PVP (JH Nanhang Life Sciences Co., Ltd, China), propylene glycol (DOW®, Indonesia), Dimethylol-dimethyl (DMDM) hydantoin (PA Chemical, Indonesia), distilled water (Local, Indonesia), and silica (Local, Indonesia).

Tool

The tools used in this research were an oven (IMU55L), glass jar (DLX Glass®), grinder (MKS-ML500), digital scale (NewTech Electronic Balance®), magnetic stirrer (IKA® C-MAG HS 4), Franz diffusion cell (Kalfaro), test tube (Pyrex®), beaker (Pyrex®), 100-1000 µl micro pipette (Dragon Lab®), aluminum foil (Best Fresh®), filter paper (Sumber Ilmiah Persada),

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micrometer screw (Tricle Brand®), stative (Trivi®), clamp (Trivi®), rotary evaporator (Dragon Lab®), and desiccator (Duran®).

Method

Preparation of corn silk extract

The maceration procedure was used to extract 1 kilogram of powdered corn silk simplicia. The samples were macerated with 96% ethanol at a 1:10 (w/v) ratio and remacerated with the same ratio. This mixture is thoroughly agitated, sealed, and left for 72 hours. Remaceration is performed for 48 hours (Widyaningrum *et al*., 2020). The filtrate was filtered using filter paper and evaporated at 50°C using a rotary evaporator until a viscous extract was obtained. Equation 1 is utilized to determine the extract's percentage yield.

$$\% \text{ Yield Extract} = \frac{\text{Weight of Extract}}{\text{Weigh of Simplicia}} \times 100\% \quad \text{..... [1]}$$

Phytochemical screening

Phytochemical screening includes qualitative examination of flavonoids, alkaloids, terpenoids/steroids, tannins and saponins (Ministry of Health of the Republic of Indonesia, 2017).

Determination of total flavonoid content

Total flavonoid contents were determined using a modified aluminum chloride colorimetric method using quercetin as a standard solution (Limmatvapirat *et al*., 2020). Quercetin was used as a standard. The quercetin concentration used to create the calibration curve is 20-60 ppm. Then, 5 mL of quercetin and corn silk extract samples were reacted with 0.1 mL of 0.01 mol/L aluminum chloride and 0.1 mL of sodium acetate. The mixture was then left at ambient temperature for 10 minutes, and the absorbance value at 430 nm was determined using a UV-Vis spectrophotometer.

Transdermal patch formulation

The formulation of the transdermal patch is based on Yusuf's (2020) research with minor modifications. This research altered the concentration of the active substance and polymer. Utilizing a 2^2-factorial design with two factors and two levels, the polymer was optimized using HPMC and PVP as the two factors and 3-4% and 1-2% as the two levels. The formula was presented in Table 1.

Preparation of transdermal patches

As mass 1, HPMC was dispersed in a 1:1 mixture of ethanol and distilled water as the solvent. Mass 2 of PVP was dispersed in ethanol. Then, mass 1 and mass 2 are thoroughly combined until uniform. In ethanol, the extract was dissolved. While agitating, the extract solution, propylene glycol, and DMDM (Dimethylol-dimethyl) hydantoin were added to the polymer solution. The mixture was then poured into a petri dish coated with liquid paraffin and desiccated for 24 hours at 42^{o}C (Putri *et al*., 2019).

Table 1. The 2^2-factorial design for transdermal patch formulation

Ingredients	Formula			
	F1	F2	F3	F4
Corn Silk Extract (mg)	200	200	200	200
HPMC*	3	4	3	4
PVP*	1	1	2	2
Propylene Glycol (mL)	0.11	0.11	0.11	0.11
DMDM Hydantoin (mL)	0.01	0.01	0.01	0.01
Distilled Water (mL)	15	16	12	13
Ethanol (mL)	ad 50	ad 50	ad 50	ad 50

*The ratio was calculated to a total of 400 mg polymer

Table 2. Criteria for Optimum Formula

Parameters	Goals
Thickness	Minimum
% Moisture Content	In range
% Moisture Uptake	Minimum
% Elongation	Maximum
Weight Uniformity	Minimum
Folding Endurance	Maximum

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Evaluation of transdermal patches

Organoleptic

Organoleptic analysis involves observing the patch's form, odor, color, and surface condition (Indrawati *et al*., 2022).

Thickness

The thickness of a patch is measured with a screw micrometer or a vernier caliper. The data obtained was then calculated as an average value (mean ± standard deviation) (Hashmat *et al*., 2020).

Percentage of moisture content

The patch weight was measured and then placed in a desiccator for 72 hours. After 72 hours, the weight was measured again (Budhathoki *et al*., 2016). The percentage of moisture absorption capacity is calculated based on equation 2.

$$\% \text{ Moisture content} = \frac{\text{Initial Weight - Final Weight}}{\text{Initial Weight}} \text{ x } 100\% \quad \text{..... [2]}$$

Percentage of moisture uptake

The patch weight was measured and then stored in a desiccator for 24 hours. After 24 hours, the patch weight was measured again (Budhathoki *et al*., 2016). The percentage of moisture uptake is calculated based on equation 3.

$$\% \text{ Moisture uptake} = \frac{\text{Final Weight - Initial Weight}}{\text{Initial Weight}} \text{ x } 100\% \quad \text{..... [3]}$$

Percentage of elongation break test

The patch is clamped between the top and bottom material clamps by applying a load or force. The final length can be seen if the patch is torn (Panda, 2022).The elongation percentage is calculated based on equation 4.

$$\% \text{ Elongation} = \frac{\text{Final length - Initial length}}{\text{Initial length}} \text{ x } 100\% \quad \text{..... [4]}$$

Weight Uniformity

Patch weights were weighed using an analytical balance. For each 3 patches, the average weight, standard deviation and % CV were calculated (Panda, 2022).

Folding Endurance

The patch is folded repeatedly in the same position until it tears. The number of folds is a value of fold resistance (Budhathoki *et al*., 2016).

Irritation Test

The skin irritation test was conducted on 15 volunteers by placing a patch on the back of their hand and observing for 24 hours for indicators of redness, erythema, and edema (Wahyuni *et al*., 2023).

Determination of optimum formula

The optimum formula is determined using Design Expert® based on a desirable value close to 1. The criteria for determining the optimum formula can be seen in Table 2.

Franz diffusion penetration test

The penetration test was conducted using Franz diffusion cells and the back skin of a mice. Between the donor and receptor compartments was mice epidermis. The patch is applied directly to the epidermis. As a diffusion medium, 50 ml of phosphate buffer solution at pH 7.4 was added to the receptor compartment. At a temperature of 37 ± 0.5°C and a speed of 200 rpm, tests were conducted. At 0, 15, 30, 45, 60, 90, 120, 150, and 180 minutes, 3 mL of solution was pipetted from the receptor compartment and replaced with 3 mL of fresh medium. The sample was subsequently filtered and placed in a 10 mL vial. (Putri *et al*., 2019). Using a UV-Vis spectrophotometer, the flavonoid concentrations of the samples were determined. Based on equation 5, the cumulative quantity of flavonoids that penetrate is calculated.

$$Q = \frac{Cn.V + \sum_{i=0}^{n-1} C.\,S}{A} \quad \text{..... [5]}$$

Note

Q	:	the cumulative amount of flavonoids penetrated (µg/cm^2)
Cn	:	flavonoid concentration at the nth hour
$\sum_{i=0}^{n-1} C$	:	total flavonoid concentration in the first sample collection until before the nth hour
V	:	Franz diffusion cell volume (mL)
S	:	sampling volume (mL)
A	:	membrane area (cm^2)

Next, the flavonoid penetration level (flux) was calculated using Fick's law (equation 6).

$$J = \frac{Q}{t.\,A} \quad \text{..... [6]}$$

Note

J	:	flux of flavonoid penetration rate (µg/cm^2.hour)
Q	:	the cumulative amount of flavonoids passing through the diffusion membrane (µg/cm^2)
t	:	time (hour)
A	:	membrane area (cm^2)

Data analysis

Formula design and evaluation data analysis were carried out using the Factorial Design method of Design Expert® software.

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RESULTS AND DISCUSSION

Corn silk extract

The corn silk extract produced in this study was brownish green, had a distinctive odor, and was thick, yielding 9.79%. Figure 1 depicts the organoleptic results of corn silk extract. The percentage yield results acquired differ from previous research conducted by Fajrina *et al.* (2021), which found a 23.65% yield. Various factors, including the extraction process and the vicariance of secondary metabolite compounds due to disparities in growing locations, contributed to the differences in yields. The solvent employed can influence the efficacy of an extraction procedure. This study utilized a 96% ethanol solvent. Flavonoids are compounds with limited water solubility and greater solubility in organic solvents such as methanol and ethanol (Mubarokah *et al*., 2023). A 96% ethanol has a water content with a small concentration of 4%. The extraction process in this study is similar to that of Fajrina *et al*. (2021), who used ethanol as a solvent and the maceration method, so the differences in results are likely due to the distinct growing locations. This statement is proven by research conducted by Utomo *et al*. (2020), which demonstrates that differences in the height of the same plant will provide significant differences in the content of secondary metabolites. However, the yield obtained in this study still satisfies the requirements of the Indonesian Herbal Pharmacopoeia for extract yield percentage, which is at least 7.2% (Ministry of Health of the Republic of Indonesia, 2017).

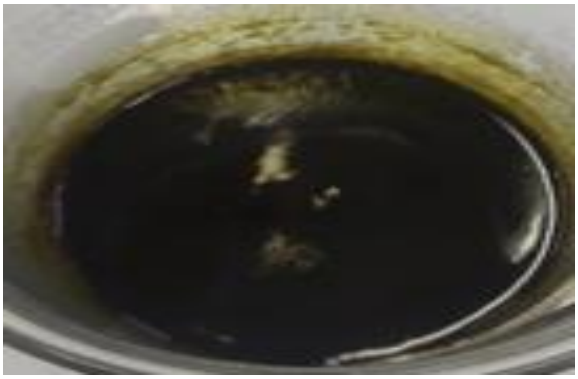

Figure 1. Corn silk extract

Phytochemical screening

The objective of phytochemical screening is to identify the phytochemical class of compounds present in corn silk extract. Various chemical reagents are utilized in the analysis technique for phytochemical screening. Table 3 displays the results of the phytochemical screening of corn silk extract.

Table 3. Phytochemical screening result

Compound	Observation result	Conclusion
Alkaloids	White precipitate	Positive (+)
Flavonoids	Yellowish-red color	Positive (+)
Tannins	Greenish-black color	Positive (+)
Terpenoids	Bluish-green color	Negative (-)
Steroids	Bluish-green color	Positive (+)
Saponins	Permanent foam	Positive (+)

Table 3 demonstrates that corn silk extract contains phytochemical compounds including alkaloids, flavonoids, tannins, steroids, and saponins. According to research conducted by Limmatvapirat *et al.* (2020), corn silk extract includes alkaloids, flavonoids, tannins, steroids, and saponins. Corn silk extract's potential as an antihyperlipidemic agent will be determined by the phytochemical compounds it contains.

Many studies have proven the effect of phytochemical compounds on antihyperlipidemic activity. Research conducted by Islam *et al.* (2021) found that *Rhizoma coptidis*, containing a combination of five main alkaloids, could reduce the accumulation of lipids and cholesterol in HepG2 cells and control the levels of total cholesterol, triglycerides, LDL-c, and HDL-c in hamsters experiencing hypercholesterolemia. Flavonoids have been studied extensively for their ability to act as antihyperlipidemia by inhibiting intestinal cholesterol absorption by reducing the expression of the epithelial cholesterol transporter Niemann-Pick C1-like 1 (NPC1L1), inhibiting the formation of lipid oxidative stress, increasing PPAR expression, and inhibiting the oxidation of macrophage-modified LDL by reducing the α-tocopherol content of LDL particles (Agung, 2021; Fukaya *et al*., 2021; Mulyani, 2020; Yi *et al*., 2021). Research conducted by Issac *et al.* (2018) proved that giving tannins of 25 and 50 mg/kg BW p.o. for 35 days was able to reduce LDL and VLDL levels and increase HDL levels in mice that had been induced by streptozotocin-nicotinamide. Saponin compounds have also been proven to be able to act as antihyperlipidemia. Research conducted by Elekofehinti *et al.* (2013) demonstrated that the use of 20-100 mg/kgBW of saponin for 21 days was able to reduce glucose, TC, TG, and LDL levels and increase HDL levels in rats induced by alloxan. Research conducted by Wang *et al.* (2017) also proved that corn silk extract at 400 and 800 mg/kgBW could significantly reduce total cholesterol, triglyceride, and LDL levels in rat test animals.

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Total flavonoid content (TFC)

Using the regression equation of the quercetin standard calibration curve, namely y = 0.0114x – 0.0024 with a correlation coefficient of 0.9994, the total flavonoid content in the extract was determined. According to Table 4, corn silk extract contained 36.6 ± 0.003 mg RE per gram of extract. This very high total flavonoid content is consistent with the findings of Singh *et al.* (2022), who reported that corn silk extract contained multiple flavonoid compounds in the form of maysin, apigmaysin, 3-methoxymaysine, ax-4-OH maysin, and isoorientin-2"-O-a-L-rhamnoside.

Transdermal patch of corn silk extract

The transdermal patch containing corn silk extract was manufactured using solvent casting. The solvent casting method was selected because it is straightforward and uncomplicated to implement (Borbolla-Jiménez *et al*., 2023). The evaluation results of the four transdermal patch formulations are presented in Table 5.

According to Table 5, both F1 and F2 satisfy the patch thickness requirements of 0.15 to 0.2 mm. The thickness of the transdermal patch impacts the drug's release, permeation, retention, and diffusivity through the epidermis (Latif *et al*., 2022). All formulations satisfy the moisture absorption test requirements of 3.52–9.79% and the drying shrinkage test requirements of 10%. When the percentage of moisture absorption and drying shrinkage is low, the patch is more stable and microbial contamination is minimized (Fuziyanti *dkk*., 2022). All formulations pass the test requirements for percentage of elongation > 5% and folding endurance > 200 times. The research by Omar *et al*, (2019) and Pal *et al*, (2023) found that a high percentage of elongation and folding resistance indicates that the patch has excellent mechanical properties and elasticity so that it does not tear easily when applied to the skin. Using Design Expert, the influence of the HPMC and PVP factors on the observed response will be determined based on the measured characteristics of the transdermal patch preparation.

Design expert analysis

Optimization of model fitting

Model fitting analysis is the first stage in the optimization procedure. Model fitting analysis is carried out to determine which parameters can be continued for the optimization process. With parameters such as R^2, adjusted R^2, predicted R^2, adequate precision, and p-value, the DoE method of model fitting can yield accurate prediction results. Each response model satisfies the criteria. If R^2 is greater than 0.70, the difference between adjusted R^2 and predicted R^2 should not exceed 0.2, the adequate precision value should be greater than 4, and the p-value should be less than 0.05 (Apriani *et al*., 2023). Table 6 displays the results of the six measured responses' model-fitting analysis.

Table 4. Total flavonoid content in corn silk extract

Replication	Absorbance	Mean Absorbance ± SD	CV%	TFC/g (mg) ± SD
1	0.441			
2	0.416	0.415 ± 0.003	0.008	36.6 ± 0.003
3	0.418			

Table 5. Transdermal patch evaluation results

Parameter	Formula			
	F1	F2	F3	F4
Organoleptic	Smooth, thin, pale yellow, slightly sticky, typical smell of corn silk extract	Smooth, thin, pale yellow, slightly sticky, typical smell of corn silk extract	Smooth, thin, pale yellow, slightly sticky, typical smell of corn silk extract	Smooth, thin, pale yellow, slightly sticky, typical smell of corn silk extract
Thickness (mm)	0.158 ± 0.019	0.158 ± 0.004	0.133 ± 0.004	0.148 ± 0.025
Moisture Content (%)	8.422 ± 0.366	7.118 ± 1.369	11.503 ± 1.097	5.826 ± 0.097
Moisture Uptake (%)	5.614 ± 0.244	2.681 ± 0.108	7.200 ± 1.297	2.913 ± 0.048
Elongation (%)	16.049 ± 1.008	10.309 ± 0.948	11.592 ± 0.5394	11.969 ± 0.375
Weight Uniformity (%)	0.041 ± 0.009	0.004 ± 0.037	0.004 ± 0.03	0.042 ± 0.001
Folding Endurance	431 ± 4.899	512.333 ± 5.558	311 ± 3.742	384.333 ± 5.437
Irritation Test	No irritation	No irritation	No irritation	No irritation

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Table 6. Model Fitting Result

Responses	Parameter				
	R^2	*Adjusted* R^2	*Predicted* R^2	*Adequate Precision*	*p-value*
Thickness	0.2906	0.0246	-0.5961	2.2116	0.4065
% Moisture Content	0.7572	0.8516	0.7572	10.9547	0.0003
% Moisture Uptake	0.9244	0.8961	0.8299	11.8126	<0.0001
% Elongation	0.8844	0.8410	0.7399	2.2116	0.0004
Weight Uniformity	0.4127	0.1924	-0.3215	3.1183	0.2124
Folding Endurance	0.9954	0.9937	0.9897	57.3938	<0.0001

*The result indicates that there is a significant influence on the response ($p < 0.05$)

Based on Table 6, the parameters of thickness and weight uniformity do not meet the requirements for a good model, in contrast, the parameters of moisture content, moisture uptake, percentage of elongation and folding endurance which show good model results. These four parameters have R^2 values greater than 0.7, ranging from 0.7572 to 0.9954, indicating that between 75.72 and 99.54 percent of the data obtained is influenced by the factors used, namely HPMC (A), PVP (B), and the interaction between the two factors (AB). While the remainder is an estimate of error for each parameter. The difference between the adjusted R^2 and predicted R^2 of the four parameters is also less than 0.2, indicating that the data analyzed and the data predicted by the Design Expert system are comparable. The adequate precision value of the four parameters is greater than 4, indicating that the model is resistant to arising disturbances. The p-values of the four parameters also indicate that the factors used have a significant effect on the observed parameters ($p<0.05$). Based on the result, these four parameters can be utilized for response analysis and formula optimization.

Optimization of Responses Analysis

Response analysis was carried out to see the effect of HPMC concentration (A), PVP (B), and the interaction of the two factors (AB) on the parameters of moisture content, moisture uptake, elongation, and folding endurance. The results of the response analysis can be seen in Table 7 and Figure 2.

According to Table 7, HPMC concentration (A) has a significant effect on moisture content, moisture uptake, percentage of elongation, and folding endurance, whereas PVP concentration (B) has a significant effect on moisture uptake, percent elongation, and folding endurance, and the interaction of the two factors (AB) has a significant effect on moisture content and percentage of elongation ($p<0.05$). Figure 2, a depiction of the normal plot of residuals, also supports these findings. The factor points outside the straight line indicate that the factor has both positive and negative influences. This positive and negative influence can be observed by locating the point within the positive or negative region. In addition, it can be determined from the coefficient notation whether or not there is a negative indication. Positive influence indicates that the higher the concentration of the factor employed, the greater the resulting response value. Conversely, negative influence means that the observed response value decreases as the concentration of the factor used increases.

Tabel 7. Results of responses analysis

Parameter		*Intercept*	A (HPMC)	B (PVP)	AB (*Interaction*)
% Moisture Content	*Coefficient*	8.21736	-1.74488	0.447208	-1.09339
	p-value		0.0001*	0.1226	0.0029*
	% *contribution*		61.1718	4.0183	24.0201
% Moisture Uptake	*Coefficient*	4.6023	-1.80495	0.454317	-0.3385
	p-value		< 0.0001*	0.0449*	0.1147
	% *contribution*		84.15	5.3314	2.95966
% Elongation	*Coefficient*	12.4087	-1.41228	-0.770592	1.45784
	p-value		0.0009*	0.0241*	0.0008*
	% *contribution*		37.4216	11.1412	39.8754
Folding Endurance	Coefficient	409.667	38.6667	-62.00	-2.00
	p-value		< 0.0001*	< 0.0001*	0.2872
	% *contribution*		27.8537	71.6132	0.0745195

*The result indicates that there is a significant influence on the response ($p<0.05$)

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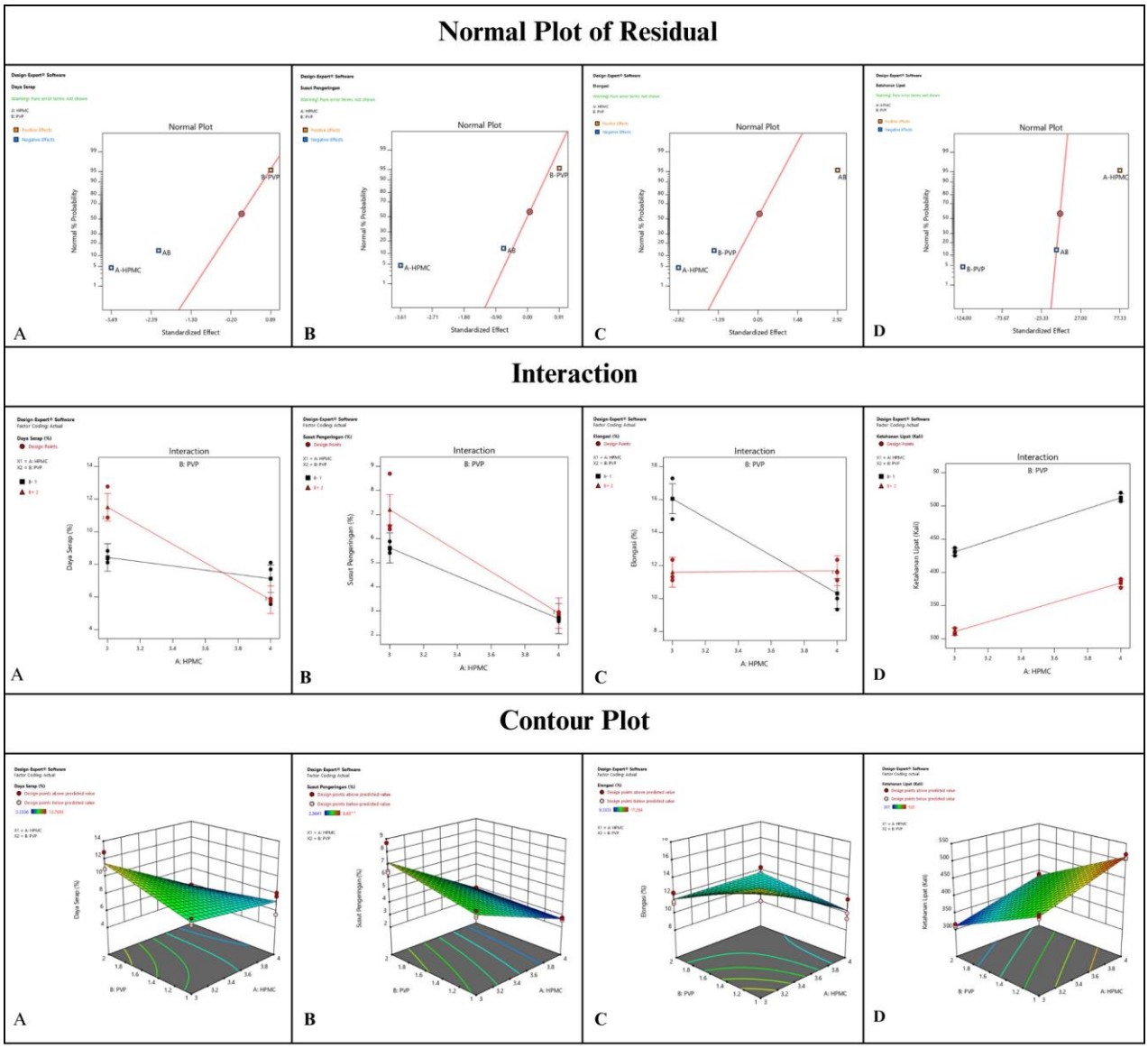

Figure 2. Normal graph plot of residual, interaction, and contour plot of moisture content (A), moisture uptake (B), elongation (C), and folding endurance (D)

Table 8. Optimum formula result

HPMC	PVP	Moisture Content	Moisture Uptake	Elongation	Folding Edurance	*Desirability*
3.487	1	7.788	4.187	13.256	470.582	0.913

The interaction between HPMC and PVP (AB) only affected moisture content and percentage of elongation significantly. This is evident in the % contribution of the AB factor to the moisture content parameter and the percentage of elongation that contributes more than 20%. The moisture uptake and folding endurance parameters are not substantially affected by the AB factor because the % contribution of the AB factor is much less than the estimated % error for these parameters, which are 7.5589% and 0.4585% respectively. Figure 2 also illustrates the interaction between the two variables (AB). Interaction graph in which the intersections of lines represent the interaction between factors A and B at both low and high concentrations. Figure 2's Contour Plot graph also depicts the predicted response area based on the used HPMC and PVP concentrations.

Based on the % contribution value in Table 7, the HPMC (A) factor has the greatest influence on moisture content and moisture uptake with a % contribution value of 61.1718% and 84.15%, respectively. The PVP (B) factor has the greatest influence on folding endurance with a % contribution value of 71.6132%, and the interaction between the two factors (AB) had the

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greatest effect on % elongation at 39.8754%. Figure 3 demonstrates that HPMC has a negative influence on the moisture content and moisture uptake parameters, as depicted by the normal plot of residual graph. This is possible because, unlike PVP, HPMC is not hygroscopic (Rowe *et al*., 2009). The higher the concentration of HPMC, the lower the resultant patch's moisture content and moisture uptake. PVP has a positive effect on the absorption capacity and drying shrinkage parameters due to the hygroscopic properties (Abdul *et al*., 2021). Contour Plot graph in Figure 3 corroborates this. According to the evaluation results in Table 5, PVP has a substantial effect on the parameters describing the resistance to folding. The hygroscopic nature of PVP has a negative effect on folding endurance because the resulting patch is still moist. During testing, wet patches will tear and break more readily. Nonetheless, according to the results of this study's folding endurance evaluation, all formulations met the evaluation criteria of > 300 folding cycles (Simaremare *et al*., 2022). The percentage of elongation is influenced not only by a single factor, but also by the interaction between the two factors, as depicted in Figure 2's interaction graph. The slope angle of the two lines indicates that the strong interaction between HPMC and PVP has a considerable impact, which is corroborated by the % contribution data from the interaction between the two factors (AB), which can be found in Table 7. This interaction exerts a positive influence, such that the greater the interaction between the two parameters, the greater the percentage elongation of the preparation. The interaction between PVP polymer and HPMC can increase the preparation's percentage elongation (Jayaprakash et *al*., 2010; Magfirah & Utami, 2022).

Optimum formula

Determining the optimum formula using Design Expert® software with response criteria according to the target in Table 2 by removing the parameters of patch thickness and weight uniformity due to having model fitting results that do not meet the requirements. Determining the optimum formula is done by looking at the desirability value, which is close to 1, which indicates that the formula is most comparable to the desired criteria. The optimum formula for corn silk extract transdermal patch preparation is at the HPMC : PVP concentration, 3.49 : 1, with a desirability value of 0.913. The characteristic results of the transdermal patch preparation in the optimum formula can be seen in Table 8.

Franz diffusion penetration of transdermal patch

Using a Franz diffusion cell device, the optimal formula penetration test was conducted to ascertain the amount of flavonoid compounds that penetrated through the skin during a specific time interval from the corn silk extract transdermal patch. Mice skin from the Wistar strain is used as the membrane because its permeability value is comparable to that of human skin, at 103.08 cm/hour x 10^{-5} and 92.27 cm/hour x 10^{-5}, respectively (Chandra, 2019). The skin membrane of the Wistar strain mice used was 1.76 cm^2. Before using animals for experimentation, a code of ethics with the number 022307099 must be obtained from the Ahmad Dahlan University Research Ethics Committee. Table 9 and Figure 3 display the penetration test results of the optimum transdermal patch formulation.

According to Table 9 and Figure 3, the cumulative value of flavonoids that penetrated that can penetrate rat skin is 32.59 µg/cm^2 at 3 hours. After 3 hours, a controlled release occurred. Probably due to the matrix-type formulation of the patch, in which the HPMC and PVP polymers bind to the active ingredient of the corn silk extract and regulate the rate of drug release (Das *et al*., 2022). According to Sivasankarapillai *et al.* (2021), the matrix-type patch has an intracellular penetration pathway that progressively traverses the stratum corneum, hair follicles, and sweat glands. These two routes will ultimately lead to the blood vessels. HPMC with a predominating hydrophilic composition absorbs water upon contact with phosphate buffer liquid at pH 7.4 and swells to form a gel with pores capable of releasing active substances. In contrast to PVP, which has the property of being readily wetted and thus can increase the release of active substances, HPMC has properties that limit the release of active substances (Aung *et al*., 2020). In addition, as an enhancer, propylene glycol can increase the rate of flavonoid penetration in corn silk extract (Carrer *et al*., 2020). By increasing the mobility of lipid molecules, propylene glycol increases the solubility of the active substance and the permeability of the stratum corneum, thereby increasing the penetration of the active substance into the epidermis layers (Haque & Talukder, 2018; Kis *et al*., 2022).

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Table 9. Cumulative amount and flux penetration of extract corn silk transdermal patch

Time (hour)	Cumulative Amounts of Flavonoids (µg/cm^2)	Flux (µg/cm^2.hour)
0	3.50	0
0.25	18.56	42.18
0.5	18.77	21.33
0.75	21.32	16.15
1	23.72	13.48
1.5	24.03	9.10
2	25.69	7.30
2.5	27.84	6.33
3	32.59	6.17

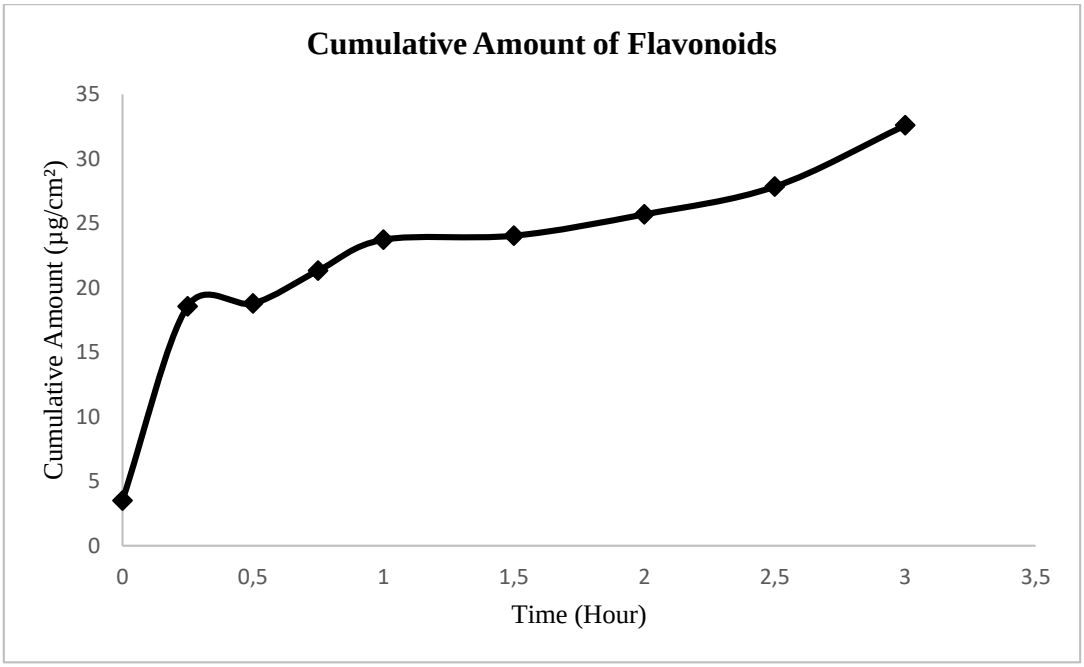

Figure 3. Cumulative amounts of flavonoids graph

CONCLUSION

The concentration of HPMC and PVP affects the evaluation of patch formulations, specifically the moisture content, moisture uptake, elongation percentage, and folding endurance. The HPMC:PVP ratio of 3.487 : 1 yielded the optimum formula based on the optimization of the factorial design analysis of the preparation evaluation response. The optimal formulation for the transdermal patch containing corn silk extract had a high cumulative rate of penetration and flux.

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AUTHOR CONTRIBUTIONS

Conceptualization, D.F.A.; Methodology, A.D.L.; Software, E.H.; Validation, E.F.A.; Formal Analysis, N.A.M.; Investigation, V.A.R.; Resources, E.H.; Data Curation, N.A.M.; Writing - Original Draft, A.D.L.; Writing - Review & Editing, E.F.A.; Visualization, V.A.R.; Supervision, E.F.A.; Project Administration, E.F.A.; Funding Acquisition, D.F.A.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Pharmacy Students' Readiness for Offline Learning in The New Normal Transmission of COVID-19: A Cross-Sectional Study

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Abstract

***Background**: Pharmacy students consist of undergraduate and professional pharmacy students. They are candidates for future pharmacist health workers who require practical experience. Offline learning with hands-on practice methods in health facilities supports their professional skills. **Objective**: This study aimed to determine pharmacy students' knowledge and attitude toward implementing offline learning methods during the new normal era. **Methods**: This study involved pharmacy students from Indonesia who were asked to participate in an e-questionnaire about the vaccination program, COVID-19 health protocols, pharmacist competence, and attitude toward implementing offline learning. The students' scores were based on their knowledge and attitude. Statistical analysis was performed to compare the scores between the two groups, and a correlation test was conducted to assess the relationship between the students' knowledge and attitudes. **Results**: A total of 652 pharmacy students were divided into two groups, undergraduate and professional pharmacy students, in a 3:1 ratio. About 74.6% of undergraduate students and 78.5% of professional pharmacy students had good and moderate knowledge. The level of knowledge was not different between the two groups ($p=0.602$; $p>\alpha$). Professional pharmacy students were more ready to engage in offline learning compared to undergraduate students ($p=0.001$; $p<\alpha$). However, there was a relatively low correlation between knowledge and attitudes, with $r = 0.079$ ($p=0.043$; $p<\alpha$). **Conclusion**: Professional pharmacy students are more ready for offline learning methods than undergraduate students, particularly in hands-on field practice.*

***Keywords**: COVID-19, learning, offline, pharmacy students, practice*

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INTRODUCTION

The coronavirus (COVID-19) pandemic swept across the globe, including Indonesia, disrupting all activity sectors, including education. Even though the majority of schools have reopened, the educational system is still recuperating and reviewing the lessons. The pandemic affected over 1.5 billion students, including vulnerable young learners. Indonesia had to close schools for a total of 77 weeks, which is a relatively high number in the total duration of school closures compared to other Southeast Asian countries (e.g. Malaysia 61 weeks, Thailand 52 weeks, Vietnam 31 weeks, Cambodia 64 weeks, etc.) (UNESCO, 2022).

In March 2020, the Indonesian Ministry of Higher Education issued a distance learning or e-learning policy due to the COVID-19 pandemic. Based on the Joint Decree (SKB) of the Four Ministers (the Minister of Education, Culture, Research, and Technology, the Minister of Religious Affairs, the Minister of Health, and the Minister of Home Affairs of the Republic of Indonesia), blended learning was implemented for the academic year 2020/2021. Face-to-face learning was only allowed in schools, colleges, and universities located in the yellow and green zones (SKB, 2020). In the Odd Semester of 2022/2023, learning is encouraged to be carried out face-to-face while implementing health protocols for COVID-19 (e.g. always wearing a mask, washing hands with soap or sanitiser, avoiding shaking hands or direct contact, and keeping a distance and avoid crowds) (Kemendikbud RI, 2022).

In Indonesia, pharmacy higher education is divided into two levels: the undergraduate pharmacy with a length of study of 4 years, graduates with a Bachelor of Pharmacy degree, and the professional education program with a length of study of 1 year, graduates with a pharmacist degree. The undergraduate program provides knowledge-based education, while the pharmacy professional program is focused on developing skills and experiential learning. After completing the pharmacy professional program, students are required to pass a national examination in order to obtain a license to practice as pharmacists. This national exam consists of two types: computer-based test (CBT) and Objective Structured Clinical Examination (OSCE). The CBT evaluates students' knowledge, while OSCE assesses their skills and attitudes (Mohamed *et al*., 2020).

During the pandemic, several adjustments have been made, with the hope that all programs will run smoothly and achieve their goals. According to the Global Competency Framework (GbCF), graduates of pharmacy education should possess four competency clusters, which are (1) pharmaceutical public health, (2) pharmaceutical care, (3) professional character, and (4) organization and management (FIP, 2020). These clusters require four elements, namely knowledge, attitudes, skills, and behaviour (Engle *et al*., 2020). While distance learning (online learning) can be used to acquire knowledge during the pandemic, it may not be as effective as face-to-face learning (offline learning). On the other side, developing attitudes, skills, and behaviour requires offline activities. In a US hospital, the pharmacy residency program was modified during the pandemic to ensure that pharmacy residents' learning experience was not disrupted and the rotation target was met. The program was carried out in three scenarios, for illustrates: (1) hybrid learning that combined remote and on-site drug dispensing practice, (2) remote learning for ward rounds with the team, and (3) on-site learning for emergency services. Nevertheless, to implement such programs, besides the availability of human resources, adequate supporting facilities are also required (Danelich *et al*., 2021).

Many people have concerns about implementing offline learning activities due to the risk of COVID-19 transmission. Students, parents, lecturers, educational institutions, and clinical sites are all worried about achieving learning targets while keeping everyone safe. Although 55% of lecturers had a positive attitude toward remote education (online learning), they were unsure whether the attainment of learning outcomes would improve or worsen (Safwan *et al*., 2022). The majority of medical students believe that online learning is beneficial, but the pandemic has limited their opportunities to become specialists. Moreover, they fear being infected with SARS-CoV-2 (Turana *et al*., 2022). Online learning is less effective in developing skills, knowledge, and interaction levels (AlQhtani *et al*., 2021).

Offline learning activities are essential, but they require strict adherence to health protocols to ensure the safety of all personnel involved in teaching and learning (PBM) and their families. This study was conducted to determine the level of knowledge of Indonesian pharmacy students on vaccination, COVID-19 health protocols, pharmacist competence, attitudes toward COVID-19 prevention and protocols, and the readiness of pharmacy students to resume offline learning in the new normal era.

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MATERIALS AND METHODS

Study design

The design of this study is a cross-sectional study. Primary data were collected from the pharmacy students' answers through non-probability sampling.

Setting

This study was conducted in October 2021 and received approval by the ethics committee board of the Faculty of Pharmacy, Universitas Airlangga (number 43/LB/2021). Primary data were collected by completing answers to questionnaires. The questionnaires were distributed online via Google Forms. Participants provided informed consent before taking the questionnaires.

Participants

The respondents met the criteria: (1) Indonesian undergraduate or professional pharmacy student, (2) currently active for learning in a pharmacy study program (not on leave/semester off), (3) who voluntarily completed a survey. Participants who did not complete the survey were excluded.

Study instrument

The questionnaires consist of respondents' identity, i.e., name, age, gender, address, current level of education (undergraduate or professional pharmacy student), and origin of an educational institution. In addition, the questionnaires have 15 questions each for knowledge (using the Guttman scale) (Appendix 1) and attitudes (using the Likert scale) (Appendix 2) related to vaccination, the COVID-19 health protocol, pharmacist competency, and the readiness of the students and their families for offline learning.

Data analysis

Each correct question for knowledge is one point, and the score range is 0-15. All of the questions are two choices: true or false. In addition, the score range for attitude is 5-75, with the possible score for each question being 1, 2, 3, 4, and 5. Furthermore, the mean score for knowledge and attitudes is categorized into three categories: good (76-100%), moderate (56-75%), and poor (<56%).

Respondents who are in the good and moderate categories are considered knowledgeable. The characteristics of the data were analyzed using univariate data analysis. The Mann-Whitney Test was used to compare differences in gender, age, and different scores of knowledge and attitudes between two groups. In addition, the Spearman Test was used to determine the correlation between the knowledge and attitudes of the respondents.

RESULTS AND DISCUSSION

Participants

A total of 660 participants accessed the questionnaire, and 652 participants were willing to be respondents and filled out the entire questionnaire. Eight participants declined to take part in the study. The distribution of respondents can be seen in Table 1. The respondents were primarily undergraduate pharmacy students. The majority were female, with 414 (84.7%) people in the undergraduate students and 140 (85.9%) people in the professional pharmacy students. However, there was no difference between the gender and the educational level of the two groups ($p>0.05$). Most of the respondents were in the age range of 17-22 years old, and there is a difference between ages and respondents' educational levels ($p<0.05$).

Knowledge of participants

The knowledge level of the respondents can be seen in Table 2 and Appendix 1. The respondents have moderate-good knowledge about the benefits of vaccination, COVID-19 vaccination, and COVID-19 health protocols, except in understanding the type of immunization and type of COVID-19 vaccines (questionnaires no 1 & 3) in poor categories. In addition, the study found that pharmacist competency knowledge is poor and requires further improvement. Overall, the knowledge levels of vaccines, pharmacist competency, and health protocols for undergraduate and professional pharmacy students were moderate to good, accounting for 74.6% and 78.5%, respectively, and no difference in knowledge level between both groups ($p>0.05$).

Table 1. Respondents characteristics

Characteristics	Level of Education		p-value
	Undergraduate (n= 489)	Professional program (n= 163)	
Gender			
Male	75 (15.3)	23 (14.1)	0.704
Female	414 (84.7)	140 (85.9)	
Age			
17-22	448 (91.6)	51 (31.3)	0.001
23-28	41 (8.4)	112 (68.7)	

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Table 2. Pharmacy students' knowledge about vaccination, COVID-19 health protocols, and pharmacist competency

Level of Education	Knowledge Category			p-value
	Good	Moderate	Poor	
Undergraduate (n=489)				
Frequency	68	297	124	
Percentage (%)	13.9	60.7	25.4	0.602
Professional program (n=163)				
Frequency	16	112	35	
Percentage (%)	9.8	68.7	21.5	

Table 3. Pharmacy students' attitudes toward vaccination, COVID-19 health protocols, and their readiness for offline learning

Level of Education	Attitude Category			p-value
	Good	Moderate	Poor	
Undergraduate (n=489)				
Frequency	341	147	1	
Percentage	69.7	30.1	0.2	0.001
Professional program (n=163)				
Frequency	134	29	0	
Percentage	82.2	17.8	0	

Attitude of participants

Table 3 shows the distribution of the respondents based on their level of attitude. Undergraduate and professional pharmacy students' attitudes were mainly in the good attitude category, with 341 (69.7%) undergraduate students and 134 (82.2%) professional students. Only one person (0.2%) in the undergraduate students had a poor attitude category. There was a difference between the attitudes of undergraduate and professional pharmacy students ($p < 0.05$).

Correlation of knowledge and attitude participants

A Spearman test was performed to establish the correlation between the knowledge and attitudes of the respondents. The results showed a p-value of 0.043 ($p<0.05$), indicating a significant correlation. However, the correlation coefficient (z) was only 0.079, indicating a very weak correlation between knowledge and attitude. Despite this, the respondents' knowledge still had an impact on their attitudes.

Discussion

This study was conducted to determine the level of Indonesian pharmacy students' knowledge about vaccination, COVID-19 prevention and protocols, pharmacist competency, attitudes towards, and the readiness of pharmacy students for offline learning in the new normal era. Offline learning is face-to-face learning carried out by pharmacy students after a period of distance learning due to large-scale social restrictions (Indonesian: pembatasan sosial berskala besar (PSBB)). There are still barriers to implementing offline learning, while offline learning is necessary for competency achievement (Turana *et al*., 2022). Information related to student knowledge for prevention, health protocols for COVID-19, and attitudes toward offline learning is needed.

From this study, female respondents dominate both undergraduate and professional pharmacy students. This aligns with the statement that 70% of health workers worldwide are women (WHO, 2019). Of health workers in Indonesia, especially general practitioners, pharmacists, and nursing professionals, more than 60% are female (Efendi & Kurniati, 2020). Meanwhile, the age difference in this study is due to differences in educational levels, where professional pharmacy students will be older than undergraduate students.

Students of pharmacy are prepared to become change agents by acquiring the necessary knowledge, skills, attitudes, and values to meet complex demands, including human, technology, and data literacies (Mohamed *et a*l., 2020).

The knowledge levels regarding vaccinations, health protocols, and pharmaceutical competencies for undergraduate and professional pharmacy students are moderate to good, 74.6% and 78.5%, respectively (Table 2). Undergraduate and professional pharmacy students also have good attitudes categories for being willing to COVID-19 and, understand the importance of vaccination and health protocol, but they are more prominent in professional pharmacy students (82.2%) compared to undergraduate students (69.7%) (Table 3). There was no significant difference in knowledge between the two groups ($p=0.602$), while attitudes had a significant difference ($p=0.001$). The Spearman test results show that there is a significant relationship

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between knowledge and attitude (p=0.043). However, the correlation coefficient (z) is only 0.079, indicating a very weak correlation between knowledge and attitudes. The attitudes towards preventing the spread of COVID-19 and following health protocols are very positive. Both undergraduate and professional pharmacy students are willing to be vaccinated, understand the importance of vaccination, and have been implementing health protocols such as wearing masks and washing hands. They are also aware of how to behave in crowded environments to minimize the risk of infection. A large percentage of undergraduate students (83%) and professional pharmacy students (93.3%) expressed the need for offline learning. However, a positive attitude toward readiness for offline learning is more pronounced in the group of professional pharmacy students (84,7%) compared to undergraduate students (67,9%) (questionnaires no.7 and 8 in Appendix 2). Therefore, strengthening education and motivation for offline learning needs to be carried out, especially for professional pharmacy students. Professional students need face-to-face learning, especially skill development and direct practice in the field to support national exam preparation (CBT & OSCE). One of the learning outputs of professional pharmacy students is required to pass the national exam to obtain a license or legal permit to practice as a pharmacist (Mohamed *et a*l., 2020).

During the COVID-19 pandemic, pharmacists, pharmacy technicians, residents, and intern students played a crucial role in providing essential patient services and contributing to public well-being in various settings such as hospitals, clinics, and community pharmacies in high- and low-middle-income countries (Goff *et al*., 2020). It is essential to adopt technology to ensure the safety of patients and health workers. Using virtual presence during limited ward rounds can reduce the transmission of the virus while still allowing for ongoing service responsibility. Accordingly, offline learning can be safely implemented by following the appropriate protocols (Goff *et al*., 2020; Mohamed Ibrahim *et al*., 2021).

From this study, pharmacy students received good support from their families to carry out offline learning. However, support for offline learning is also more significant from the families of professional pharmacy students (82,8%) than undergraduate students (66,9%) (questionnaire no.9 in Appendix 2). Some parents still prefer online learning, so it is essential to educate and communicate with them about COVID-19 prevention, learning protocols during pandemics, and the importance of offline learning to fulfil pharmacy competencies. Similar results regarding the willingness of parents in Indonesia to support the implementation of offline learning for their children in early education were reported by Sutini et al (2022). It was found that parents with elementary school children in Indonesia have good knowledge, beliefs, and attitudes to supporting their children to take part in offline learning during the COVID-19 pandemic (Sutini *et al*., 2022).

CONCLUSION

The level of knowledge regarding vaccination, COVID-19 prevention and protocols, and pharmacist competency was not different between undergraduate and professional students. However, there are differences in their attitudes, with professional pharmacy students being more ready for offline learning methods than undergraduate students.

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AUTHOR CONTRIBUTIONS

Conceptualization, B.S., D.M.; Methodology, B.S., D.M., M.R.; Software, D.M., M.R., F.D.; Validation, B.S., D.M., M.R.; Formal Analysis, D.M., M.R., F.D.; Investigation, B.S., D.M., M.R., E.M., E.L., E.N.; Resources, B.S., D.M., M.R., E.M., E.L., E.N.; Data Curation, D.M., M.R., F.D.; Writing - Original Draft, B.S., D.M., F.D.; Writing - Review & Editing, B.S., D.M., F.D.; Visualization, F.D.; Supervision, B.S.; Project Administration, D.M.; Funding Acquisition, B.S.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Appendix 1. Frequency and percentage of the correct answers related to pharmacy students' knowledge

No	Questions	Level of Education	
		Undergraduate pharmacy (n=489)	**Professional pharmacy (n=163)**
1	Vaccination is a process for getting passive immunity.	147 (30.1)	39 (23.9)
2	Convalescent plasma therapy is a process to get ready-to-use antibodies.	427 (87.3)	140 (85.9)
3	The Sinovac COVID-19 vaccine is the whole virus vaccine category that weakened.	107 (21.9)	38 (23.3)
4	The Astra Zeneca's COVID-19 vaccine is a category viral vector vaccine.	424 (86.7)	147 (90.2)
5	The efficacy of the Sinovac vaccine in Indonesia is reported to be 65%, which means there will be a 65% reduction in cases in the vaccinated population compared to the placebo group	373 (76.3)	110 (67.5)
6	The characteristics of the subjects and placebo influence the vaccine efficacy value.	425 (86.9)	152 (93.3)
7	Storage of Astra Zeneca vaccine at -20^{o}C.	282 (57.7)	114 (69.9)
8	Vaccination is needed to obtain immunity, preventing the spread of infectious diseases.	451 (92.2)	145 (89.0)
9	Dispensing pharmacy is included in pharmaceutical supplies management.	431 (88.1)	123 (75.5)
10	Aseptic dispensing is included in the pharmaceutical supplies management	78 (16.0)	44 (27.0)
11	Being able to develop practices that are beneficial to society and gain national and international recognition are two of the competencies that new graduate pharmacists must have.	49 (10.0)	14 (8.6)
12	The highest transmission of COVID-19 is caused by the 20-34-year-old group.	367 (75.1)	124 (76.1)
13	Prevention COVID-19 disease can minimized by using two layers of masks, namely surgical masks and cloth masks.	442 (90.4)	159 (97.5)
14	Washing hands after removing a mask does not reduce the risk of preventing infection the COVID-19.	371 (75.9)	122 (74.8)
15	Maintaining a distance of around 50cm is one of the strategies for preventing COVID-19 disease.	314 (64.2)	106 (65.0)

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Appendix 2. Frequency and percentage related to pharmacy students' attitudes

No	Statements	Level of Education									
		Undergraduate pharmacy (n=489)					Professional pharmacy (n=163)				
		Strongly Agree	Agree	Slightly Agree	Disagree	Strongly Disagree	Strongly Agree	Agree	Slightly Agree	Disagree	Strongly Disagree
1	To reduce the risk of COVID-19 infection, I am willing to vaccinate.	382 (78.1)	85 (17.4)	22 (4.5)	0 (0.0)	0 (0.0)	143 (87.7)	18 (11.0)	2 (1.2)	0 (0.0)	0 (0.0)
2	I am ready to communicate the importance of vaccination in preventing the spread of COVID-19.	280 (57.3)	167 (34.2)	34 (7.0)	6 (1.2)	2 (0.4)	99 (60.7)	59 (36.2)	5 (3.1)	0 (0.0)	0 (0.0)
3	I recommend vaccination to my family members.	319 (65.2)	133 (27.2)	33 (6.7)	4 (0.8)	0 (0.0)	132 (81.0)	25 (15.3)	5 (3.1)	1 (0.6)	0 (0.0)
4	In my family, there are family members who are not willing to be vaccinated.	34 (7.0)	56 (11.5)	109 (22.3)	87 (17.8)	203 (41.5)	6 (3.7)	16 (9.8)	23 (14.1)	28 (17.2)	90 (55.2)
5	I prevent the transmission of COVID-19 by always implementing the COVID-19 protocols.	371 (75.9)	104 (21.3)	11 (2.2)	2 (0.4)	1 (0.2)	129 (79.1)	27 (16.6)	6 (3.7)	1 (0.6)	0 (0.0)
6	I implement health protocols in the main family.	264 (54.0)	154 (31.5)	51 (10.4)	13 (2.7)	7 (1.4)	87 (53.4)	50 (30.7)	21 (12.9)	3 (1.8)	2 (1.2)
7	To achieve pharmacist competency, I need offline learning.	258 (52.8)	148 (30.3)	66 (13.5)	9 (1.8)	8 (1.6)	111 (68.1)	41 (25.2)	10 (6.1)	0 (0.0)	1 (0.6)
8	I am ready to do offline learning.	158 (32.3)	174 (35.6)	118 (24.1)	27 (5.5)	12 (2.5)	93 (57.1)	45 (27.6)	21 (12.9)	3 (1.8)	1 (0.6)
9	Currently, my parents (family) allow me to do offline learning.	157 (32.1)	170 (34.8)	108 (22.1)	39 (8.0)	15 (3.1)	82 (50.3)	53 (32.5)	23 (14.1)	3 (1.8)	2 (1.2)
10	I feel safe not to wash my hands after removing the mask.	27 (5.5)	17 (3.5)	54 (11.0)	124 (25.4)	267 (54.6)	6 (3.7)	8 (4.9)	17 (10.4)	41 (25.2)	91 (55.8)
11	I have to do six steps of washing my hands to make sure they are clean.	331 (67.7)	125 (25.6)	31 (6.3)	2 (0.4)	0 (0.0)	116 (71.2)	39 (23.9)	8 (4.9)	0 (0.0)	0 (0.0)
12	I feel that coming to a dinner gathering with friends or family will be safe as long as the air ventilation is open.	57 (11.7)	128 (26.2)	195 (39.9)	75 (15.3)	34 (7.0)	19 (11.7)	42 (25.8)	58 (35.6)	36 (22.1)	8 (4.9)
13	Online learning can improve all my skills as a future pharmacist.	87 (17.8)	108 (22.1)	163 (33.3)	79 (16.2)	52 (10.6)	37 (22.7)	20 (12.3)	40 (24.5)	40 (24.5)	26 (16.0)
14	I feel safe around healthy-looking people.	134 (27.4)	135 (27.6)	131 (26.8)	51 (10.4)	38 (7.8)	43 (26.4)	27 (16.6)	51 (31.3)	28 (17.2)	14 (8.6)
15	I feel that to minimize crowds with other people, I don't need to exercise.	19 (3.9)	37 (7.6)	128 (26.2)	155 (31.7)	150 (30.7)	5 (0.8)	6 (3.7)	56 (34.4)	51 (31.3)	45 (27.6)

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Formulation and SPF Value Evaluation of Sunscreen Spray Gel Containing Lime Peel Extract (*Citrus aurantifolia*)

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Abstract

Background*: High exposure to sunlight has adverse effects on the skin. Lime peel contains more than 60% flavonoids, presenting the potential to function as a sunscreen due to the presence of conjugated aromatic benzene groups, capable of absorbing UV-A or UV-B rays from the sun. To prevent skin damage, lime peel extract is formulated into a spray gel, as it has the ability to dry rapidly, enhancing overall comfort for consumers during application.* ***Objective****: To determine the influence of variation concentration of lime peel extract in the sunscreen spray gel on its physical properties and in vitro SPF value.* ***Methods****: Lime peel crude extract was obtained using 70% ethanol and formulated into a sunscreen spray gel at concentrations of 5%, 10%, and 15%. The spray gel formulation was evaluated for its physical quality and SPF value.* ***Results****: The variation in extract concentration has a statistically significant effect on the physical properties of the preparation and SPF values (P<0.05). The physical stability conditions in each formula (F1, F2, and F3) meet the requirements of the spray gel preparation in terms of pH, viscosity, spreading test, drying time test, and adhesion test. The spray gel preparations F1 (5%), F2 (10%), F3 (15%) each have SPF values of 20, 25, and 35 respectively.* ***Conclusion****: The spray gel formulations in F1 (5%), F2 (10%), and F3 (15%) are physically stable and have moderate to high SPF values, with F3 (15%) having the highest SPF value of 35.*

Keywords*: Citrus aurantifolia, spray gel, topical sunscreen formulation*

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INTRODUCTION

Sun exposure has harmful effects on the skin due to UV radiation from UV-A and UV-B rays *(D'Orazio et al*., 2013). The human skin has natural defense mechanisms against sunlight, including sweating, melanin production, and thickening of the stratum corneum (Kalangi, 2014). Sunscreen formulations are commonly used to protect the skin by blocking UV rays (Dutra *et al*., 2004).

Lime peel contains over 60% flavonoids and has potential as a sunscreen agent (Pratiwi *et al*., 2017). The flavonoid compounds represent the principal secondary metabolites found in lime peel, belonging to the group of phenolic compounds capable of absorbing UV-A and UV-B rays from the sun. To confirm the UV absorption capability of lime peel, this study conducted a total phenol assay on the extract before it was formulated.(Andy Suryadi *et al*., 2021). Previous studies have demonstrated the sunscreen activity of lime peel extract at various concentrations, with SPF values ranging from 4.4 to 40.15 (Yasin, 2017). Gel, cream, and lotion formulations containing lime peel extract also showed sunscreen activity, with SPF values ranging from 11.36 to 20.68, 12.01 to 18.57, and 11.27 to 19.44, respectively (Kularti, 2019; Nafisah, 2019; Zuhroh, 2019). These findings highlight the potential of lime peel as a sunscreen agent.

To further explore the properties of lime peel extract-based sunscreen, research is needed on its physical characteristics, stability, and Sun Protection Factor (SPF). The study aims to develop a sunscreen spray gel formulation using lime peel extract (*Citrus aurantifolia*) as the active ingredient. The choice of a spray form is based on its ability to provide concentrated content that dries quickly, offering a convenient and pleasant user experience.

MATERIALS AND METHODS

Material

Lime (*Citrus aurantifolia*), filter paper, distilled water (Smart-Lab), absolute ethanol (Smart-Lab), Folin-Ciocalteau reagent, aquabides (Onemed), carbopol 940 (Newman Chemicals), HPMC, propylene glycol (DOW Chemical Pacific), methyl paraben (Salicylates and Chemicals), propyl paraben (Salicylates and Chemicals), triethanolamine (TEA) (Emplura), Na_2CO_3, and gallic acid (Sigma-Aldrich).

Tools

Oven (Memmert), grinder, dehydrator, hotplate (Philips), rotary evaporator (Heidolph), water bath (Memmert), furnace, calipers, analytical balance (Mettler Toledo), UV-Vis spectrophotometer (Shimadzu UV-1280), pH meter (Mettler Toledo), ultrasonic cleaner (Branson), viscometer (Brookfield LV), and magnetic stirrer (C-MAG HS 7 IKA).

Method

Preparation of simplisia

The lime samples used were initially determined at the Ecology and Biosystematics Laboratory, Biology Department, Faculty of Science and Mathematics, Diponegoro University, Semarang, to ensure that the lime used in the research is *Citrus aurantifolia*. The ripe lime fruits (*Citrus aurantifolia*), which were dark green in color, were sorted when wet, then thoroughly washed with running water to remove any dirt attached to them. Subsequently, the lime peels were separated from the fruit using a lime peeler. The herbal material was then dried using a dehydrator at a temperature of 50ºC for one week to obtain thoroughly dried herbal material. The dried herbal material was ground into a fine powder using a grinder.

Extraction

The extraction method used was maceration using 70% ethanol. A total of 1000.27 grams of powdered herbal material was soaked in a covered container with 10 liters of 70% ethanol solvent in a ratio of 1:10 (herbal material to solvent weight) for 24 hours, with 10 minutes of stirring each day. The maceration process was repeated three times using 5 liters of 70% ethanol solvent in a ratio of 1:5. The macerate was filtered using filter paper and then evaporated under a pressure of 75 mbar and a temperature of 50°C using a rotary evaporator. The resulting liquid extract was concentrated using a water bath at a temperature of 50°C until it thickened into a paste-like consistency (Zuhroh, 2019).

Determination of total phenolic content

Preparation of gallic acid stock solution

A total of 10 mg of gallic acid was dissolved in 10 mL of analytical grade ethanol to create a 1000 ppm gallic acid stock solution. Serial dilutions of the gallic acid stock solution were made to obtain final concentrations of 5, 10, 20, 30, and 40 ppm.

Preparation of 7.5% Na_2CO_3 solution

A total of 7,5 grams of Na_2CO_3 were weighed and dissolved in 100 mL of distilled water (Andriani & Murtisiwi, 2018).

Determination of operating time (OT)

A total of 300 µL of a 30 ppm gallic acid solution was mixed with 1.5 mL of Folin-Ciocalteau reagent, stirred, and left undisturbed for 3 minutes. Then, 1.2 mL of a 7.5% Na_2CO_3 solution was added, thoroughly

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mixed, and allowed to stand at room temperature throughout the operating time. The absorbance of the solution was measured at λ765 nm within the range of 0-60 minutes, and the point at which the solution reached a stable absorbance was determined as the operating time (Andriani & Murtisiwi, 2018).

Determination of maximum wavelength

A total of 300 µL of a 30 ppm gallic acid solution was mixed with 1.5 mL of Folin-Ciocalteau reagent, stirred, and left undisturbed for 3 minutes. Then, 1.2 mL of 7.5% Na_2CO_3 solution was added, thoroughly mixed, and allowed to stand for the operating time (30 minutes) at room temperature. The absorbance of the solution was measured within the wavelength range of 600-850 nm to determine the maximum wavelength (Andriani & Murtisiwi, 2018).

Measurement of standard gallic acid solutions

A total of 300 µL of each solution with concentrations of 5, 10, 20, 30, and 40 ppm was taken and mixed with 1.5 mL of Folin-Ciocalteau reagent. The mixture was stirred and left for 3 minutes. Then, 1.2 mL of a 7.5% Na_2CO_3 solution was added, and the solution was thoroughly mixed until homogenous. It was then left at room temperature for the operating time (30 minutes). The absorbance of the solution was measured at the gallic acid maximum wavelength. A gallic acid calibration curve was constructed based on the measured absorbance (Andriani & Murtisiwi, 2018).

Determination of total phenolic content

The lime peel extract was prepared at a concentration of 1000 ppm by weighing 10 mg of the extract and dissolving it in 10 mL of analytical grade ethanol. The extract solution was further diluted with analytical grade ethanol to a concentration of 100 ppm. Then, 300 µL of the diluted extract solution was mixed with 1.5 mL of Folin-Ciocalteau reagent, shaken, and left to stand for 3 minutes. After that, 1.2 mL of 7.5% Na_2CO_3 solution was added, followed by thorough mixing and incubation at the operating time (30 minutes). The absorbance was measured at the maximum wavelength (745.8 nm). The obtained phenolic content was recorded as the mg equivalent of gallic acid per gram of sample. The measurement was performed three times, and the total phenolic content was calculated using the appropriate formula (Andriani & Murtisiwi, 2018).

$$\text{Total Phenolic Content} = \frac{\text{C x V x FP}}{\text{g}}$$

Note: C = concentration (mg/mL; V = volume of extract (ml); FP = dilution factor; g = the weight of sample used (gram)

Formulation of spray gel preparation

The formulation of lime peel extract spray gel can be seen in Table 1. Methyl paraben and propyl paraben were dissolved in propylene glycol. Carbopol was dispersed in hot distilled water until homogeneous, then triethanolamine was added, and the mixture was homogenized with a combination of methyl paraben. HPMC was gradually dispersed into a beaker containing hot distilled water and stirred until homogeneous. The carbopol mixture was poured into the HPMC and sonicated until a homogeneous solution was obtained. Lime peel extract was dispersed in distilled water and sonicated until a homogeneous extract solution was obtained. The extract was added to the HPMC and carbopol mixture, followed by the addition of distilled water to a total volume of 100 mL, and sonicated for 5 minutes. The preparation was filled into spray containers (Suyudi, 2014).

Testing of physical properties of the preparation

The physical properties of lime peel (*Citrus aurantifolia*) spray gel formulation were tested using the following methods for each observed formula with 3 replicates.

Table 1. Formula of Sunscreen Spray Gel with Lime Peel Extract (*Citrus aurantifolia*)

Materials	Function of Materials	Concentration of Materials (*b/v* %)			
		K-*	F1	F2	F3
Lime peel extract	Active ingredient	-	5	10	15
Karbopol 940	Gelling agent	1	1	1	1
HPMC		2	2	2	2
Propylene Glycol	Humectant	15	15	15	15
Methyl paraben	Preservative	0.18	0.18	0.18	0.18
Propyl paraben		0.02	0.02	0.02	0.02
Triethanolamine	Alkalizing agent	qs	qs	qs	qs
Distilled water ad	Solvent	100	100	100	100

*K- = Negative Control (without addition of lime peel extract)

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Organoleptic test

Organoleptic observations were conducted by visually assessing the appearance of the formulation, including color, odor, clarity, homogeneity, separation, and any other changes that may occur after preparation (Depkes RI, 2020).

pH test

The pH of the spray gel formulation was measured using a pH meter. pH examination was performed to ensure that the pH value of the formulation falls within the required range for topical preparations (4.5-6.5) to prevent irritation (Depkes RI, 2020).

Viscosity test

A sample of the formulation (100 mL) was taken and placed in a Brookfield viscometer with spindle number 61 at a speed of 12 rpm. The viscosity reading was recorded once the value on the viscometer stabilized (Depkes RI, 2020).

Spreadability test

The formulation was sprayed onto a plastic film at a distance of 5 cm, and the spreadability of the formulation was measured using a caliper. The parameter used for measurement was the diameter (Depkes RI, 2020).

Drying time test

The formulation was sprayed onto the inner forearm of a volunteer at a distance of 5 cm. The time required for the formulation to dry was measured using a stopwatch and recorded (Hayati, R. et al., 2019).

Adhesion test

For adhesion testing, the formulation was applied to the inner side of the lower arm of a volunteer by spraying it at a distance of 5 cm. If the spray gel droplets dripped within 10 seconds, it was evaluated as dripping; if the droplets did not drip within 10 seconds, it was evaluated as adhering (Depkes RI, 2020)..

Stability testing of the preparation

The preparation was placed at a cold temperature (4±2°C) for 24 hours, followed by exposure to a hot temperature (40±2°C) for 24 hours (1 cycle). The testing was performed for 6 cycles, and the physical changes of the spray gel preparation were observed at the beginning and end of each cycle, including organoleptic evaluation, pH measurement, viscosity determination, spreading ability, drying time, and adhesive properties

SPF value testing of the preparation

The determination of the SPF value of lime peel extract begins with weighing each formulation, including F1 (5%), F2 (10%), F3 (15%), positive controls (NIVEA® sunscreen spray SPF 30, Wardah® UV Shield Essential Sunscreen Gel SPF 30, and Emina® Sun Battle SPF 30), and negative control (formulation without extract), amounting to 1 gram. The correction factor (CF) is determined by measuring the absorbance of the positive controls, which have known SPF values. Each weighted formulation is combined with 50 mL of 70% ethanol and sonicated for 15 minutes. The sonicated formulation is transferred to a 100 mL volumetric flask and filled with 70% ethanol up to the mark. The formulation is then filtered using filter paper, and the first 10 mL of the filtrate is discarded. An aliquot (filtered formulation) of 100 µL is pipetted into a 25 mL volumetric flask and diluted with 70% ethanol up to the mark. Subsequently, the absorbance is measured using a UV-Vis spectrophotometer. The absorbance spectrum of the sample in solution form is obtained at wavelengths ranging from 290 to 320 nm with a 5 nm interval, using 70% ethanol as the blank. The absorbance values for each concentration are recorded and used to calculate the SPF value (Dutra et al., 2004). The SPF calculation according to the Mansur equation is as follows, with EE x I representing a constant factor.

$$SPF = CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times abs(\lambda)$$

Note: CF= Correction Factor; EE= Erythema Effect; I= Intensity of sunlight; abs = sample absorbance

Data Analysis

The physical test data and SPF values of the preparations were analyzed using One-way ANOVA with a significance level of $p<0.05$. If the significance value is <0.05, then the test is continued with a post-hoc test. Additionally, a paired t-test was conducted with a significance level of $p<0.05$ to assess the physical stability of the preparations by comparing the physical conditions at cycles 0 and 6.

RESULTS AND DISCUSSION

Extraction

The extraction in this study was performed using the maceration method, which is simple, does not involve heating, and does not require special equipment. The material was soaked to break the cell walls and membranes through a pressure difference. Secondary metabolites in the cytoplasm are dissolved in the organic solvent (Ditjen POM, 2000). The extraction of phenolic compounds was conducted using a mixture of 70% ethanol and water. Ethanol 70% has the appropriate polarity for extracting flavonoids and tannins. Additionally, it has low toxicity and readily evaporates (Djarot et al., 2019). Stirring was performed to achieve concentration equilibrium. The re-maceration process was employed to extract any remaining compounds in

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the residue after solvent saturation (Andriani & Murtisiwi, 2018). The filtrate was concentrated using a water bath at 50°C, resulting in a concentrated extract weighing 185.9 grams with a yield of 18.58%. The yield value is related to the content of bioactive compounds in the raw material. A higher yield corresponds to a higher desired substance content (Ditjen POM, 2000).

Determination of total phenolic content

The determination of total phenolic content was performed using the Folin-Ciocalteau reagent. Phenolic compounds can react with this reagent to form a solution with measurable absorbance. The Folin-Ciocalteau reagent oxidizes the hydroxyl groups of phenolic compounds, forming a blue-colored complex. This reaction proceeds slowly under acidic conditions, Na_2CO_3 was added during the test to create a basic environment and accelerate the reaction (Andriani & Murtisiwi, 2018).

The standard solution used was gallic acid, which is a simple, natural, and stable phenolic compound. During the reaction, the hydroxyl groups in the phenolic compounds react with the Folin-Ciocalteau reagent, forming a blue-colored molybdenum-tungsten complex. The intensity of the blue color increases with the concentration of phenolate ions formed. In other words, the higher the concentration of phenolic compounds, the more phenolate ions will reduce the heteropoly acid (phosphomolybdate-phosphotungstate) to form the molybdenum-tungsten complex, resulting in a darker color (Andriani & Murtisiwi, 2018).

The absorbance measurements of the gallic acid standard solution were used to construct a calibration curve. The curve, shown in Figure 1, follows a linear equation y = 0.0111x + 0.2391 with a correlation coefficient (R) of 0.9778. This curve was used to determine the phenolic content of the sample. The average total phenolic content of lime peel extract was 34.8845 ± 0.6511 mg GAE/g, indicating that each gram of lime peel extract is equivalent to 34.8845 mg of flavonoid.

Testing of physical properties of the preparation

To achieve good and acceptable pharmaceutical formulations in society, the physical properties and stability of the preparations must be examined. Physical properties serve as determinants of the quality of pharmaceutical preparations. The physical characterization tests include organoleptic evaluation, pH, viscosity, spreadability, drying time, and adhesion. The statistical analysis results for the pH, viscosity, spreadability, drying time, and adhesion tests showed $p > 0.05$ in the Shapiro-Wilk test of normality, indicating that the data are normally distributed. Levene's test results also showed $p > 0.05$, indicating that the data are homogeneously distributed. In the One-Way ANOVA analysis, a p-value of less than 0.05 was obtained, indicating that the variation in lime peel extract concentrations has a statistically significant effect on the tested physical properties. Subsequently, a post-hoc analysis was conducted to examine the differences in the tested physical property values among the different formulas. The test results revealed a p-value of less than 0.05, indicating that there are statistically significant differences in pH, viscosity, spreadability, drying time, and adhesion values among the different concentrations of the extract. The results of physical property tests for the three formulas can be observed in Tables 2 and 3.

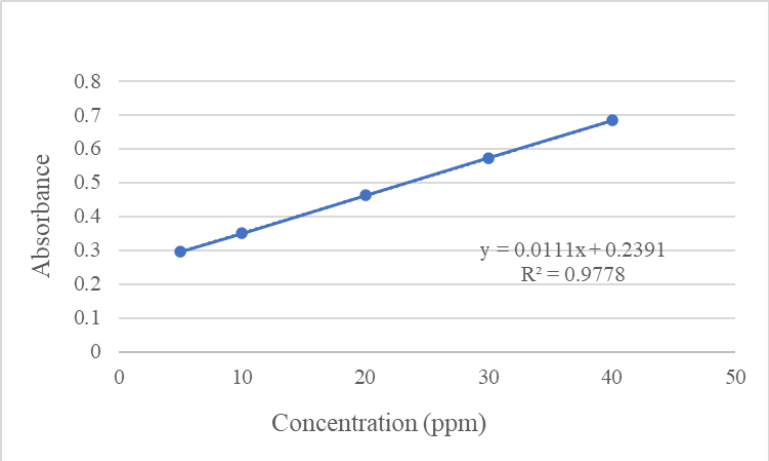

Figure 1. Gallic Acid Standard Curve

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Tabel 2. Organoleptic test results of sunscreen spray gel formulation with lime peel extract

Formula	Organoleptic			
	Form	**Odor**	**Color**	**Homogeneity**
F1 (5%)	Liquid	Lime smell	Yellow – brown	Homogeneous
F2 (10%)	Liquid	Lime smell	Brown	Homogeneous
F3 (15%)	Liquid	Lime smell	Dark brown	Homogeneous

Table 3. Results and significance test of sunscreen spray gel formulation with lime peel extract

Test	Formula	Results ± SD	Requirement	Sig. ANOVA ($p < 0.05$)
pH Test	F1 (5%)	5.3 ± 0.03	4.5 – 6.5	0.000
	F2 (10%)	5.2 ± 0.05		
	F3 (15%)	5.0 ± 0.05		
Viscosity Test	F1 (5%)	82.74 ± 1.57	< 150 cP	0.000
	F2 (10%)	112.07 ± 1.40		
	F3 (15%)	131.28 ± 1.78		
Spreadibility Test	F1 (5%)	6.4 ± 0.03	5 -7 cm	0.000
	F2 (10%)	5.8 ± 0.07		
	F3 (15%)	5.3 ± 0.07		
Drying Time Test	F1 (5%)	1.3 ± 0.08	< 5 minutes	0.000
	F2 (10%)	2.5 ± 0.06		
	F3 (15%)	3.4 ± 0.11		
Adhesion Test	F1 (5%)	36 ± 1	> 10 seconds	0.000
	F2 (10%)	61 ± 2		
	F3 (15%)	82 ± 2		

Organoleptic test

The three formulas have the same color and homogeneity, but they differ in consistency and color. A good formulation is characterized by a pleasant odor, attractive color, good consistency, and homogeneity. The consistency of the spray gel preparation is in liquid form, in accordance with its definition, which is one form of gel formulation development, which is a water-based phase system comprising at least 10% to 90% of the formulation's weight. The term 'spray' is defined as a composition that can be dispensed from its applicator, such as an aerosol or spray pump. A homogeneous formulation refers to a preparation that does not contain coarse particles, has evenly dispersed particles, and has a uniform color (Salwa et al., 2020). Although the preparation is in liquid form, the consistency of each formula is different. Formula III has the thickest consistency because it has the highest concentration of lime peel extract, which is 15%. This result proves that the higher the concentration of the extract, the thicker the resulting formulation, with a more intense color pigmentation.

pH test

The obtained results indicate that an increase in the concentration of lime peel extract has an effect on the pH value of the formulation, causing it to decrease. This is due to the higher concentration of salicylic acid, amino acids, citric acid, and vitamin C in the lime peel extract. As a result, the pH value of the formulation decreases. All formulations have met the requirement for a good pH value, which is in line with the pH of the skin ranges from 4,5 to 6,5. If the spray gel formulation is too acidic, it may cause skin irritation. On the other hand, if the pH of the formulation is too alkaline, it may lead to dryness of the skin (Hayati, R. et al., 2019).

Viscosity test

The obtained results indicate that an increase in the concentration of lime peel extract affects the viscosity value of the formulation, leading to an increase in viscosity. This is because higher concentrations of lime peel extract result in a thicker formulation. Viscosity also influences the spreadibility, drying time, and adhesion of the resulting formulation (Lachman et al., 2008). All formulations have met the requirement for a good viscosity value for the spray gel formulation, which is below 150 cP (Hayati, R. et al., 2019).

Spreadibility test

The results obtained indicate that as the concentration of lime peel extract increases, the spreadibility value of the formulation decreases. This is because higher concentrations of lime peel extract lead to a thicker formulation, reducing its ability to spread. Consequently, the opportunity for the active ingredients to come into contact with the skin diminishes, resulting in a decrease in the effectiveness of the formulation when applied topically (Jawa La et al., 2020). All

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formulations demonstrated a spreading pattern when sprayed and met the requirement for an ideal spreadibility value for the spray gel formulation, which is 5-7cm (Depkes RI, 2020).

Drying time test

The results obtained indicate that as the concentration of lime peel extract increases, the drying time of the formulation also increases. This is because higher concentrations of lime peel extract result in a thicker formulation, which requires more time to dry. All formulations have met the requirement for a good drying time value for the spray gel formulation, which is less than 5 minutes to prevent stickiness on the skin and provide comfort for the consumer when applied (Hayati, R. et al., 2019).

Adhesion test

The results obtained indicate that as the concentration of lime peel extract increases, the adhesion value of the formulation also increases. This is because higher concentrations of lime peel extract result in a thicker formulation, leading to a longer adhesion time and increased release of active ingredients. A sunscreen formulation is expected to adhere to the skin for a longer period of time to provide prolonged protection against ultraviolet radiation (Hana Shovyana & Karim Zulkarnain, 2013). All formulations can be considered to adhere well to the skin as long as the formulation droplets do not drip from the skin within less than 10 seconds (Hayati, R. et al., 2019).

Stability testing of the preparation

The entire sunscreen spray gel formula is stored at a cold temperature of 4°C ± 2°C for 24 hours and at a high temperature of 40°C ± 2°C for 24 hours (1 cycle). After that, a physical stability test is conducted for 6 cycles. The results of the physical stability test for the sunscreen spray gel formulation can be seen in Tables 4 and 5.

The statistical analysis results indicate that if the p-value is greater than 0.05 in the paired t-test, there is no significant difference or physical stability in the tested sample. However, if the p-value is less than 0.05 in the paired t-test, it indicates a significant difference or physical instability in the tested sample.

Tabel 4. Organoleptic stability test results of sunscreen spray gel formulation with lime peel extract

Formula	Cycle	Organoleptic			
		Form	Odor	Color	Homogeneity
F1 (5%)	0	Liquid	Lime smell	Yellow – brown	Homogeneous
	6	Liquid	Lime smell	Yellow – brown	Homogeneous
F2 (10%)	0	Liquid	Lime smell	Brown	Homogeneous
	6	Liquid	Lime smell	Brown	Homogeneous
F3 (15%)	0	Liquid	Lime smell	Dark brown	Homogeneous
	6	Liquid	Lime smell	Dark brown	Homogeneous

Table 5. Results and significance of the physical stability test for the sunscreen spray gel formulation

Test	Formula	Results ± SD		Requirement	Sig (2-tailed) (p < 0.05)
		Cycle 0	Cycle 6		
pH Test	F1 (5%)	5.3 ± 0.03	5.3 ± 0.02		0.053
	F2 (10%)	5.2 ± 0.05	5.1 ± 0.05	4.5 – 6.5	0.095
	F3 (15%)	5.0 ± 0.05	4.9 ± 0.06		0.057
Viscosity Test	F1 (5%)	82.74 ± 1.57	81,96 ± 1.52		0.003
	F2 (10%)	112.07 ± 1.40	111.30 ± 1.59	< 150 cP	0.020
	F3 (15%)	131.28 ± 1.78	130.04 ± 1.71		0.004
Spreadibility Test	F1 (5%)	6.4 ± 0.03	6.4 ± 0.03		0.015
	F2 (10%)	5.8 ± 0.07	5.9 ± 0.09	5 -7 cm	0.020
	F3 (15%)	5.3 ± 0.07	5.4 ± 0.05		0.044
Drying Time Test	F1 (5%)	1.3 ± 0.08	1.2 ± 0.07		0.011
	F2 (10%)	2.5 ± 0.06	2.5 ± 0.05	< 5 minutes	0.006
	F3 (15%)	3.4 ± 0.11	3.3 ± 0.11		0.005
Adhesion Test	F1 (5%)	36 ± 1	32 ± 2		0.024
	F2 (10%)	61 ± 2	53 ± 2	> 10 seconds	0.001
	F3 (15%)	82 ± 2	73 ± 2		0.015

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Table 6. Results of correction factor (CF) for positive control of sunscreen formulation

Positive Control	SPF	Correction Factor (CF)	Results ± SD
NIVEA®	30	46.96	
Wardah®	30	49.28	46.49 ± 3.05
Emina®	30	43.24	

Organoleptic test

The organoleptic properties observed in the formulation include the form and consistency, color, odor, and homogeneity of the spray gel. The results of the organoleptic testing of sunscreen spray gel formulations F1, F2, and F3 indicate that there were no changes in odor, color, and form of the formulation, and no visible phase separation throughout the 6 cycles of storage, both at cold and high temperatures. This indicates that the spray gel formulation exhibits good stability in organoleptic tests over the 6-cycle storage period.

pH test

The obtained results indicate that the formulation is stable, but there is a decrease in pH during the stability test. Changes in pH values during storage indicate reactions or damage to the components within the formulation, resulting in an increase or decrease in pH value (Barel et al., 2009). This can occur due to oxidation reactions on the carboxylic acid groups of the acid compound in the extract, leading to the addition of hydrogen atoms and a decrease in pH value. Additionally, the use of transparent packaging is another factor contributing to the instability of the pH in the formulation as it allows light to interact and cause degradation reactions of secondary metabolites in the formulation (Tranggono & Latifah, 2007). This can be addressed by storing the formulation in a place that is not exposed to light and at an appropriate temperature. The choice of packaging should be tailored to the properties of the active substance and should protect the product from external influences. The use of buffers is also necessary in the formula to maintain the stability of the pH.

Viscosity test

The obtained results indicate that the formulation is physically unstable in terms of viscosity, but it still meets the viscosity acceptance criteria for a spray gel. The decrease in viscosity can be attributed to storing the formulation at high temperatures, which causes the active molecules in the formulation to move, weakening the intermolecular interactions and resulting in a decrease in viscosity (Putra et al., 2014). Choosing an ideal storage temperature is important to maintain the viscosity stability of the formulation. Stability testing of the viscosity of the spray gel is crucial to ensuring that the formulation remains easy to spray through the applicator and adheres to the skin.

Spreadibility test

The obtained results indicate that the formulation is physically unstable in terms of spreading power, but it still meets the acceptance criteria for spreading power in a spray gel. This is due to a decrease in viscosity after storage, resulting in the weakening of the gel matrix's strength in the formulation, which leads to an increase in the spreading power of the formulation (Putra et al., 2014).

Drying time test

The obtained results indicate that the formulation is physically unstable in terms of drying time, but it still meets the acceptance criteria for drying time in a spray gel. This is due to a decrease in viscosity after storage, resulting in the formulation becoming more watery, which leads to a faster drying time (Hayati, R. et al., 2019).

Adhesion test

The obtained results indicate that the formulation is physically unstable in terms of adhesion power, but it still meets the acceptance criteria for adhesion power in a spray gel. This is due to a decrease in viscosity after storage, resulting in the formulation becoming more watery, which leads to a decrease in the adhesion power of the formulation (Hayati, R. et al., 2019).

SPF value testing of the preparation

The determination of the correction factor (CF) value in this study was done by measuring the absorbance of sunscreen products with known SPF values to ensure the calculation of SPF based on the formula. The absorbance values were then processed using the Mansur equation to determine the CF value used to account for the spectrophotometry and solvent usage (Allen & Ansel, 2014). The positive control sunscreen products used in this study included NIVEA® sunscreen spray SPF 30, Wardah® UV Shield Essential Sunscreen Gel SPF 30, and Emina® Sun Battle SPF 30. The results of the Correction Factor (CF) for the Positive Control of the Sunscreen Formulation can be seen in Table 6.

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Table 7. Results and significance of spf testing for the spray gel

Formula	Results ± SD	SPF Categories	Sig. ANOVA (p < 0.05)
F1 (5%)	20 ± 0.2	Medium	
F2 (10%)	25 ± 0.4	Medium	0.000
F3 (15%)	35 ± 0.1	High	

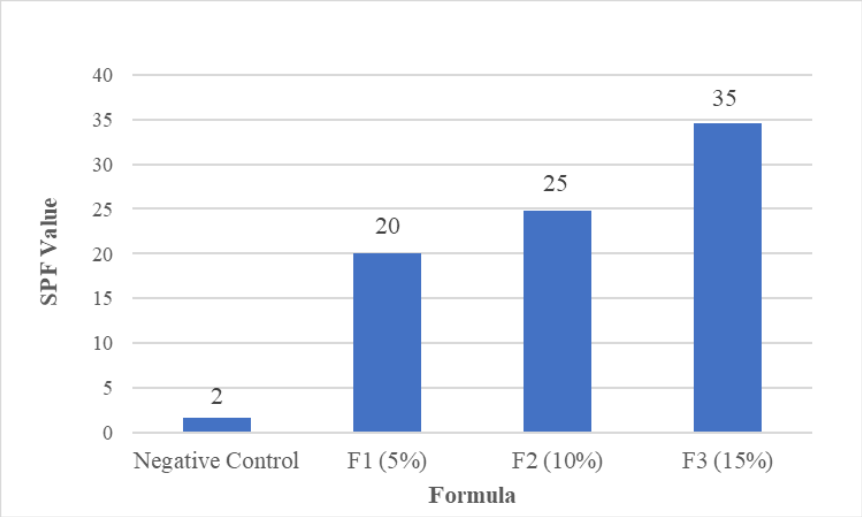

Figure 2. Graph of variation in extract concentration against spf value

The selection of three different products with known SPF values as positive controls aimed to validate the chosen method for this study. The average CF value obtained from these three products was 46.4945. This CF value would then be used to calculate the SPF value of the samples tested in this study. The SPF value testing on the negative control (formulation without extract) yielded an SPF value of 1,6687. The SPF value of the negative control indicated that the polymer in the gel spray without extract had no significant effect on the SPF value of the resulting gel spray formulation. The results of the SPF value testing for the gel spray formulation can be seen in Table 7, along with the graph showing the variation of extract concentration on the SPF value in Figure 2.

In accordance with the data presented in Table 7, the SPF values derived from the three formulations are classified within the medium to high range. This classification is established according to the protection range defined by the Indonesian Food and Drug Monitoring Agency (BPOM). SPF values within the range of ⩾6 - <15 are categorized as low, ⩾15 - <30 as moderate, ⩾ 30 - <50 as high, and ⩾ 50 are classified as very high (BPOM RI, 2020). F1 and F2 belonged to the moderate protection category against UV rays, while F3 belonged to the high protection category. Increasing the extract concentration enhanced the SPF value in the sunscreen gel formulation due to the higher phenolic compound content in the formulation (Zuhroh, 2019). The sunscreen activity of the formulation was attributed to the presence of phenolic compounds in the lime peel extract, which had conjugated aromatic benzene groups capable of absorbing UV-B rays that can be harmful to the skin. Higher SPF values indicate longer protection against UV rays (Dutra et al., 2004).

The statistical analysis showed a p-value > 0.05 in the Shapiro-Wilk normality test, indicating a normal data distribution. Levene's test yielded a p-value > 0.05, indicating a homogeneous distribution of data. The One-Way ANOVA analysis resulted in a p-value < 0.05, indicating that the variation in lime peel extract concentrations significantly affected the SPF values of the formulations. Further post-hoc statistical analysis was conducted to determine the differences in SPF values between each formula. The test results obtained a p-value <0.05, indicating statistically that each concentration of the extract in every formula produces significantly different SPF values. This is because higher extract concentrations result in higher SPF values for the formulation.

To achieve the desired sun protection factor (SPF) value, the sunscreen gel spray formulation should be applied evenly at a rate of 2 mg/cm^2 (Diffey, B., 2000). The average surface area of the human face is approximately 3.5% of the total skin surface area (Liu, Y. et al., 2008). Therefore, an estimated 1.12 grams of

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sunscreen are needed to cover the entire facial surface. The spray gel formulation for each formula is then weighed to determine the appropriate volume, and a spray test is conducted at a distance of 20 cm, resulting in 3-4 sprays to achieve the sunscreen dosage corresponding to the SPF value on the potentially exposed skin area.

CONCLUSION

The conclusion of this study is that the variation in the concentration of lime peel extract affects the physical properties and SPF value of the sunscreen gel spray formulation with a value of $p < 0.05$. Each formulation of the spray gel demonstrates good physical stability in viscosity, spreadibility, drying time, and adhesion tests. The SPF values derived from F1(5%), F2 (10%) dan F3 (15%) are classified within the medium to high range, with F3 (15%) having the highest SPF value of 34.64, classified as providing high protection against UV-rays.

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AUTHOR CONTRIBUTIONS

Conceptualization, E.R.S., F.I.W., E.V.R.; Methodology, E.R.S., F.I.W., E.V.R.; Software, E.R.S., F.I.W., E.V.R.; Validation, E.R.S., F.I.W., E.V.R.; Formal Analysis, E.R.S., F.I.W., E.V.R.; Investigation, E.R.S., F.I.W., E.V.R.; Resources, E.R.S., F.I.W., E.V.R.; Data Curation, E.R.S., F.I.W., E.V.R.; Writing - Original Draft, E.R.S., F.I.W., E.V.R.; Writing - Review & Editing, E.R.S., F.I.W., E.V.R.; Visualization, E.R.S., F.I.W., E.V.R.; Supervision, E.R.S., F.I.W., E.V.R.; Project Administration, E.R.S., F.I.W., E.V.R.; Funding Acquisition, E.R.S., F.I.W., E.V.R.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Effect of Suruhan Leaves (*Peperomia pellucida* L. Kunth) Extract on Triglyceride Blood Level in Diabetic Rats

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Abstract

Background*: Diabetes Mellitus causes complications, such as hypertriglyceridemia. Indonesia has biological wealth diversity that can be exploited in alternative medicine. One of which is Suruhan plants. Flavonoid contents in the plant extract can normalize blood triglyceride levels.* ***Objective****: This study aims to determine the effect of the Suruhan extract (Peperomia pellucida L. Kunth) on blood triglyceride levels in alloxan-induced diabetic white rats.* ***Methods:*** *The induction process used alloxan at a dose of 150mg/kgbw intraperitoneally to 12 rats. The rats were divided into 5 research groups, namely normal rats, diabetic rats, and diabetic rats were given various doses of extract. The treatment was carried out for 14 days. Blood samples for triglyceride examination were taken at the end of the study.* ***Results****: blood triglyceride levels were obtained in the normal group (127.67 mg/dl); and diabetic control group (395.67mg/dl); the dose group was 20mg/kg BW (216mg/dl); the dose group was 40 mg/kg BW (159.33 mg/dl) and the dose group was 80 mg/kg BW (143.33 mg/dl) in the statistical test with one way ANOVA ($p < 0.05$) obtained significance with a value of $p = 0.000$.* ***Conclusions****: There is an influence of plant extracts (Peperomia pellucida [L.] Kunth) administration on the blood reduction of triglyceride levels in diabetic white rats induced by alloxan.*

Keywords*: diabetes mellitus, suruhan plants (Peperomia pellucida [L.] Kunth), triglyceride*

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INTRODUCTION

Diabetes Mellitus is a metabolic disorder caused by pancreatic damage, resulting in the inability to produce the hormone insulin (*Petersmann et al*., 2019). Insulin is a hormone responsible for regulating the balance of sugar in the blood (Latres *et al*., 2019). Moreover, individuals with diabetes mellitus can experience increased free fatty acids in the blood, parallel to the fluctuation of blood glucose levels (Sobczak *et al*., 2019). The elevated levels of free fatty acids in the blood can lead to reduced tissue sensitivity to insulin *(Kojta et al*., 2020). This mechanism explains the correlation between cholesterol levels and diabetes (Wardani *et al*., 2015).

In 2021, the International Diabetes Federation (IDF) stated that the estimated number of people worldwide suffering from diabetes had reached 643 million by 2030 and this number is projected to reach 783 million by 2045 (International Diabetes Federation, 2021). Meanwhile, the projected population of Indonesia by 2030 suggests that around 21.3 million individuals will be afflicted with diabetes, placing the country in the 6th position globally, after India, China, the United States, Pakistan, Brazil, and Mexico (Infodatin, 2020).

According to data collected by the Jakarta Primary Non-Communicable Disease Risk Factors Surveillance in 2006, it was reported that cases of dyslipidemia occurring in Type 2 Diabetes (DMT2) patients had higher percentages: 67.7% increase in total cholesterol, 54.9% increase in triglycerides, 36.8% decrease in HDL, and 91.7% increase in LDL (Perkeni, 2019). Dietary control remains one of the most desirable avenues for the prevention and management of chronic degenerative diseases such as diabetes. Various plants have been employed in traditional medicine to treat diabetes (Kumar *et al*, 2021).

Meanwhile, according to a previous study, antihyperglycemic effect ethanol extract from the suruhan leaves (*Peperomia pellucida* L. Kunth) at doses of 56 mg/kg BW, 112 mg/kg BW, and 168 mg/kg BW have been proven to reduce blood glucose levels and improve liver cell damage in male mice (*Mus musculus*) induced with alloxan (Islamy, 2019). The other study reported that suruhan extract at doses of 20 mg/kgBW, 40 mg/kgBW, and 80 mg/kgBW reduced blood glucose in rats sucrose-induced (Salma *et al*., 2013). Polyphenol and flavonoid compounds in plants *Peperomia pellucida* have strong antioxidant and α-amylase inhibitory activity (Men *et al*., 2022). The enzyme α-amylase plays a role in breaking down starch into maltose and alpha-amylase reciprocally inhibits insulin production (Pierzynowski *et al*., 2023).

Alloxan is one of the drugs used to induce diabetes experimentally because of damage to pancreatic beta cells and damage to insulin work which can trigger hyperglycemia (Ibrahim *et al*., 2023). Alloxan accumulates in pancreatic beta cells and generates reactive oxygen species, has a significant necrotizing, and reduces the number of β-cells resulting in insulin deficiency (Lenzen, 2008; Radenković *et al*., 2016).

This research was conducted to determine the influence of the ethanol extract from the suruhan leaves on blood triglyceride levels in rats induced with alloxan. This study was based on the correlation between diabetes mellitus and blood cholesterol and lipid condition. This study also explored the dose-dependent effects of suruhan leaf extract on blood glucose and triglyceride levels in alloxan-induced diabetic rats.

MATERIALS AND METHODS

Tools

The tools used include a syringe (Onemed 3ml, Indonesia), analytical scale (Precisa, Switzerland), digital scale (SF 400, China), measuring cylinder, test tube, beaker glass, erlenmeyer flask, rotary evaporator (RV 10 digital V, USA), semi-auto chemistry analyzer photometer (Sunostik SBA 733), glucose test meter, and glucose meter strips (GlucoDR, Korea), EDTA tube (K2 3ml, China), stirring rod, spoon, oral probe, cage, rat feeding, and drinking area.

Materials

The materials used include suruhan leaves powder from the Research Institute for Spices and Medicinal Plants (BALITTRO) Bogor-West Java with determination at Indonesian Institute of Sciences (LIPI) with reference number 465/IV/DI.01/3/2021. Ethanol 96% (Unbranded, Indonesia), alloxan monohydrate (Sigma Aldrich, Germany), triglyceride reagent (Sigma Aldrich, Germany), Sprague-Dawley rats (BPOM, Indonesia), aquadest (Unbranded, Indonesia), sodium carboxymethyl cellulose (Na CMC, Wealthy, Indonesia), sodium chloride 0.9%, NaCl 0.9% (Wida, Indonesia).

Animals

Fifteen male rats (*Rattus norvegicus*) of the Sprague-Dawley strain, aged three months, weighing approximately 150-250 grams. The sample size in this study was calculated using the formula minimum and maximum sample sizes for analysis of variance (ANOVA) Design (Arifin & Zahiruddin, 2017). The Sprague-Dawley rats used as test animals must be

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healthy and obtained from the Experimental Animal Facility of The National Agency of Drugs and Food Control (BPOM), the Republic of Indonesia. The rats were maintained properly and according to the applicable ethical guidelines.

Method

Alloxan Preparation

In this study, a dose of 150 mg/kgbw of alloxan in 0.9% NaCl solution was used (Ibrahim *et al*., 2023). During the induction process, a total of 15 healthy rats were first acclimated for 7 days. This was followed by the alloxan induction phase on rats that had been fasted for 18 hours. The rats were weighed to determine the amount of alloxan to be administered to each rat, with a dosage of 150 mg/kg BW. Subsequently, the rats' blood glucose levels were examined through the caudal vein using a glucometer, two hours after the blood glucose level examination. Subsequently, alloxan induction was administered to the rats intraperitoneally, once at the initial stage of the study. Blood glucose level was checked within 72 hours following the induction procedure to as certain the degree of glucose elevation, indicating a successful induction.

Preparation of Suruhan Extracts

The plant material used is the powder of the Suruhan leaves plant *(Peperomia pellucida* L. Kunth*)*. The parts utilized were the leaves and stems in good condition, free from damage and mold. The powdered plant material was then extracted using the maceration method with 96% ethanol (Abubakar & Mainul, 2021). A total of 500 grams of powdered material were dissolved in 96% ethanol with solvent replacement every 24 hours for 3 days. After that, the residue was filtered. Then the residue was macerated again with ethanol 96% for 2 days and filtered as the mixing filtrate. Finally, the filtrates were mixed and then evaporated with a rotary evaporator at 60°C to obtain a thick extract. Subsequently, in the concentrated extract, organoleptic assessments were conducted, and the chemical content of the extract was identified through a flavonoid content test. A plant extract suspension was prepared with a mixture of 1% Na CMC (sodium carboxymethyl cellulose) in a total volume of 3 ml for each dose of 20 mg, 40 mg, and 80 mg (Salma *et al*., 2013).

Flavonoid Screening

The presence of flavonoids in the ethanol extract of Suruhan was identified using the Willstatter test (Rao *et al*., 2016; Thi *et al*., 2020). A crude extract of 5 ml was dissolved in ethanol and filtered. Added 0.1 g of magnesium powder (Mg), 1 ml HCL and 2 ml of amyl alcohol. Shake the mixture and allow it to separate. The presence of red, yellow, or orange coloration in the amyl alcohol layer indicates the presence of flavonoids.

Experimental Design

After induced diabetes with alloxan and blood glucose analysis at level >200 mg/dL, the rats were randomly divided into five groups (n =3 per group as follows; Group 1 represented the normal group, not induced and without treatment. Group 2 served as the DM control, induced of alloxan without treatment. Group 3 was induced and treated with 20 mg/kgbw of Suruhan leaves extract. Group 4 was induced and treated with 40 mg/kg BW of Suruhan leaves plant extract. Lastly, Group 5 was induced and treated with 80 mg/kg BW weight of Suruhan leaves extract. Before administering the treatment, the rats were measured to determine the amount of extract that could be given based on their dosage. The administration of the treatment was conducted orally using an oral probe and carried out daily for 14 days (Salma *et al*., 2013; Yuliani *et al*., 2016). During the treatment, there were variations in the dosages of the Suruhan leaf extract according to the predetermined groups. The Suruhan leaves extract was given orally to the rats at a maximum volume of 1% of their body weight. One day after the last administration of the extract, the rats' blood glucose levels were measured. The rats' body weights were recorded, and some rats were sacrificed to collect blood from the heart for the examination of their blood triglyceride levels. This was carried out because measuring blood triglyceride levels required a significant amount of blood, at least 3 ml, to be centrifuged and produce serum (Mahdi *et al*., 2020).

Statistical Analysis

The results are expressed as mean ± SD. Differences between groups were assessed by one-way ANOVA using the Statistical Package for Social Sciences (SPSS for Windows, version 22.0). Post hoc testing was performed for inter-group comparisons using the least significance difference (LSD) P-values < 0.05 were considered as significantly altered.

RESULTS AND DISCUSSION

Body weight changes

The body weight was analyzed during the 14 days of the experiment. The body weight changes in all diabetic and non-diabetic rats are represented in Table 1. Rats' body weight increased in the Normal Group and three group rats with diabetes with Suruhan extract treatment.

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Table 1. Body weight before and after being treated with suruhan extract

Group	Body Weight Before Treatment (gram)	Body Weight After Treatment (gram)	Percent BodyWweight Changes (%)
Normal	204.67 ± 2.31	277.67 ± 17.01	26.14 ± 3.81*
DM	189.67 ± 35.85	184.33 ± 37.29	-3.08 ± 2.26
DM+20	211.33 ± 53.20	223.67 ± 54.15	5.66 ± 0.91*
DM+40	176 ± 13.23	208.33 ± 24.66	15.21 ± 4.03*
DM+80	219.33 ± 81.46	249.33 ± 65.16	13.56 ± 8.82*

Note: The sign (-) in the number indicates the percent decrease in body weight. Values are expressed as the mean ± standard deviation (STD); *=P <0.05 vs Diabetic Group. Normal group (Rats without induced and treatment), DM (Rats Induced diabetes without treatment), DM+20 (Rats diabetes with treatment suruhan extract dose 20mg/kg BW), DM+40 (Rats diabetes with treatment suruhan extract dose 40mg/kg BW) and DM+80 (Rats diabetes with treatment suruhan extract dose 80mg/kg BW)

Table 2. Blood glucose levels of alloxan-induced diabetic rats treated with suruhan extract

Group	Blood Glucose levels before Treatment (mg/dL)	Blood Glucose levels after Treatment (mg/dL)	Percent decrease in blood glucose levels (%)
Normal	104 ± 14.73	143.67 ± 12.34	27.49 ± 9.18
DM	351 ± 215.65	525.67 ± 71.67	32.46 ± 41.81
DM+20	330.67 ± 103.37	288 ± 104.75	-12.91 ± 6.27*
DM+40	234.67 ± 34.43	142.67 ± 11.15	-64.54 ± 20.52*
DM+80	269 ± 18.36	136 ± 22.52	-102.55 ± 37.77*

Note: The sign (-) in the number indicates the percent decrease in blood glucose levels. Values are expressed as the mean ± STD; *=P <0.05 vs Diabetic Group. Normal group (Rats without induced and treatment), DM (Rats Induced diabetes without treatment), DM+20 (Rats diabetes with treatment suruhan extract dose 20mg/kg BW), DM+40 (Rats diabetes with treatment suruhan extract dose 40mg/kg BW) and DM+80 (Rats diabetes with treatment suruhan extract dose 80mg/kg BW)

According to the results, the untreated control group experienced a decrease in body weight after 14 days, with statistical significance observed at a *p*-value of 0,000. The changes in body weight gain were in three dosage variations of suruhan leaf extract. In this study, the most effective dose of extract was 80 mg/kg BW. Food and water intake were elevated whereas the body weight significantly decreased in diabetic rats compared with normal control rats. This possibly occurs in diabetes where the body is unable to use glucose as an energy source due to a lack of insulin and an increase in body weight implying that the anabolic effect has overridden the catabolic (Al-Attar & Alsalmi, 2019).

Blood glucose levels

Blood glucose level is the main factor in producing diabetes in animal models. This study found blood glucose level of the 12 test animals that were induced by alloxan 150 mg/kg BW was recorded at>200 mg/dL after 72 hours. Similarly, in alloxan-induced rats, a study showed that rats had fasting blood glucose (FBG) >200mg/dL (Yuliani *et al*., 2016). Rats having blood glucose levels of 220-250 mg/dL were considered diabetic (Rehman *et al*., 2023). Table 2 presents fasting blood glucose levels before and after treatment for 14 days. The group treatment with Suruhan leaves extract doses of 20 mg/BW, 40 mg/BW, and 80 mg/BW showed a significant decrease compared to the group DM control.

The study examined blood glucose levels within the control group and treatment group and observed which treatment with Suruhan extract significantly lowered blood glucose levels, resulting in a statistically significant result with a *p*-value = 0.007. The most effective dose to decrease blood glucose levels was found at an extract dose of 80 mg/kgBW. Several studies related to hypoglycemic activity have been carried out. Suruhan extract in three dose variations effectively reduces blood sugar levels (Salma *et al*., 2013). Blood Glucose in the control group increases because Alloxan is a diabetogenic drug (Sheriff *et al*., 2020). Treatment groups with Suruhan extract had been lowering blood glucose. It is probably phytochemicals, such as flavonoids that can improve insulin sensitivity (Bacanli *et al*., 2019). Phytochemicals in ethanol extract of Suruhan such as flavonoids and triterpenoids are thought to be related to the effect antioxidants can

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increase insulin expression and modulation of glucose transporter (Hidayati, 2021). On the other hand, suruhan extract has an α-amylase inhibitory activity that increases blood glucose levels (Men *et al*., 2022).

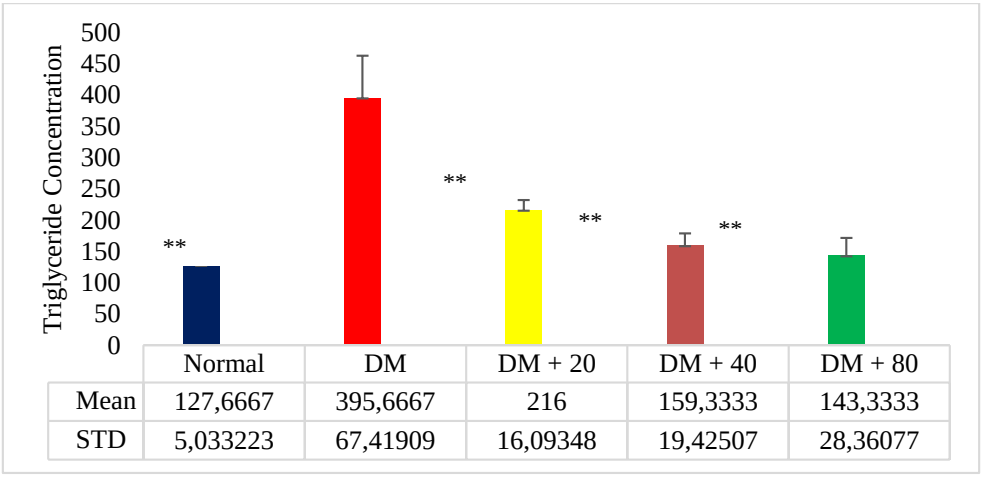
Blood triglyceride level

The blood triglyceride levels of the test animals were measured at the end of the treatment period, precisely one day after the completion of the 14-day treatment regimen. There were significant differences in the average blood triglyceride levels among the 5 groups (Figure 1). The group with the highest average blood triglyceride level was the DM control group, with a value of 395.67 mg/dl. On the other hand, the group with the lowest average blood triglyceride level was the normal group, with a value of 127.67 mg/dl. In the treatment groups, the blood triglyceride levels were closest to normal in the group receiving dose 3, with an average of 143.33 mg/dl. A study reported total triglyceride normal levels in rats is 26-145 mg/dL (Mahdi *et al*., 2020).

The graph depicts the blood triglyceride levels of the test animals in the 5 groups, where the highest blood triglyceride levels were found in the DM control group. Subsequently, the blood triglyceride levels of the test animals decrease sequentially in the groups of rats given doses of 20 mg/kg BW of the extract, 40 mg/kg BW of the extract, and 80 mg/kg BW of the extract. This indicates that as the dosage increases, the blood triglyceride levels in the test animals decrease. The most effective dose to decrease triglyceride levels was found at an extract dose of 80 mg/kg BW. Meanwhile, the lowest blood triglyceride levels were observed in the normal group.

The hypothesis testing results for the blood triglyceride levels of the test animals in the five groups p-value of 0.000 or < 0.05 was obtained. Therefore, this data suggests a significant difference in blood triglyceride levels among the five groups.

Hypertriglyceridemia is exceedingly common in diabetes (Simha, 2020). Diabetes mellitus frequently results in hypertriglyceridemia which increases the risk of arteriosclerotic disease (Kane *et al*., 2021). In Diabetic type 2 impaired glucose tolerance, the basic defect is postulated to be the loss of normal insulin sensitivity, leading to compensatory hyperinsulinemia and increased triglyceride secretion (Ye *et al*., 2019). However, Plant extract administration in the animal diabetic model resulted in a reduction in blood glucose and triglyceride levels (Bacanli *et al*., 2019; Khan *et al*., 2019). Phytochemical content in extracts such as flavonoids lowers triglyceride levels and modulates lipid metabolism through the inhibition of fatty acid synthase (Luna-Castillo *et al*., 2022). Other lipid metabolism-modulating effects of the extract are related to its ability to inhibit reactive oxygen species through the expression of antioxidants (Men *et al*., 2022). In this study identification of flavonoid compounds in the leaf extract of the suruhan plant (*Peperomia pellucida* L. Kunth) was carried out. The test results were positive for containing flavonoids with the formation of an orange-yellow color (Rao *et al*.,2016)



	Normal	DM	DM + 20	DM + 40	DM + 80
Mean	127,6667	395,6667	216	159,3333	143,3333
STD	5,033223	67,41909	16,09348	19,42507	28,36077

Figure 1. Blood triglyceride concentration of alloxan-induced diabetic rats treated with suruhan extract

Note: Each Value is presented as the mean ± standard deviation (STD); *Significant difference vs control group at P <0.05, **highly significant difference vs the diabetic group at P<0.01. Normal group (Rats without induced and treatment), DM (Rats Induced diabetes without treatment), DM+20 (Rats diabetes with treatment suruhan extract dose 20mg/kg BW), DM+40 (Rats diabetes with treatment suruhan extract dose 40mg/kg BW) and DM+80 (Rats diabetes with treatment suruhan extract dose 80mg/kg BW)

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CONCLUSION

According to the findings of the conducted study the administration of Suruhan leaves extract *(Peperomia pellucida* L. Kunth*)* at doses of 20 mg/kg BW, 40 mg/kg BW, and 80 mg/kg BW over 14 days to diabetic rats induced with intraperitoneal alloxan at a dose of 150 mg/kg BW, resulted in a significant impact. This was characterized by a reduction in blood glucose levels and the achievement of normal blood triglyceride levels. The optimum dose for reducing blood glucose levels and achieving normal blood triglyceride levels was found to be at the 80 mg/kg BW dose. The higher the dosage used, the greater the average decrease in blood glucose and blood triglyceride levels in the test animals subjected to treatment for 14 days.

ETHICS STATEMENT

This research has obtained ethical approval from the Ethics Committee of the Faculty of Medicine, University of Indonesia - Dr. Cipto Mangunkusumo National Hospital, with the reference number 21-07-0792.

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AUTHOR CONTRIBUTIONS

Conceptualization, H.N.; Methodology, H.N.; Validation, H.D.M., H.N.; Formal Analysis, H.D.M., H.N.; Investigation, H.D.M.; Resources, H.D.M., N.R.H.; Data Curation, H.D.M.; Writing - Original Draft, H.D.M., N.R.H.; Writing - Review & Editing, H.N.; Visualization, H.N.; Supervision, H.N.; Project Administration, N.R.H.; Funding Acquisition, H.D.M., H.N.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Jatropha curcas L. Leaf Extract Effects on Blood Pressure and Lipid Levels in Hypertensive Rats with High-Fat Diet

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Abstract

Background*: One of the main risk factors for cardiovascular diseases such as coronary atherosclerotic heart disease (CAHD) is dyslipidaemia or high levels of low-density lipoprotein (LDL) and triglycerides (TG) and low levels of high-density lipoprotein (HDL). Hypertension is also a cause of cardiovascular disease. One potential plant to lower LDL levels and blood pressure is Jatropha curcas, which is known to contain saponins, polyphenols, and flavonoids.* ***Objective****: The purpose of this study was to determine the effect of the ethanol extract of Jatropha curcas leaves (EEJCL) on blood pressure, LDL levels, and HDL levels in hypertensive rats given a high-fat diet.* ***Methods****: This study is an experimental study with a pretest-posttest control group design on male Wistar strain rats. Rats were divided into seven groups, namely the normal group, control group (induced with NaCl and given a high-fat diet), Captopril group, Simvastatin group, and EEJCL groups given doses of 1.8, 2.7, and 4.05 g/kg BW. The data obtained were analysed using the One-Sample Kolmogorov-Smirnov Test, Homogeneity of Variance, One-Way ANOVA, and Tukey Test.* ***Results****: The results showed that the administration of EEJCL could significantly lower LDL levels and blood pressure and increase HDL levels ($p < 0.05$) at doses of 1.8, 2.7, and 4.05 g/kg BW, and the dose of 4.05 g/KgBW was the most optimal dose.* ***Conclusion****: EEJCL has a potential for development in the treatment of hypertension and dyslipidaemia.*

Keywords*: blood pressure, cardiovascular, Jatropha curcas, HDL, LDL*

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INTRODUCTION

several other heart and blood vessel conditions, are the leading cause of global mortality and a major contributor to reduced quality of life. In 2017, CVDs caused around 17.8 million deaths worldwide, equivalent to 330 million years of life lost and 35.6 million more years lived with disability (Mensah *et al*., 2019).

Coronary atherosclerotic heart disease (CAHD) is characterized by dyslipidemia, manifesting as elevated levels of low-density lipoprotein (LDL) and triglycerides (TGs), alongside decreased levels of high-density lipoprotein (HDL). Elevated LDL levels can lead to plaque formation and inflammatory processes, resulting in the progression of atherosclerosis within arterial walls and thrombosis in CAHD. Meanwhile, HDL plays a protective role by reinforcing tissues surrounding arterial walls, inhibiting cholesterol deposition within arteries, and facilitating the repair of damaged endothelial membranes. Conversely, reduced levels of HDL impede the removal of cholesterol (Sun *et al*., 2022).

Evaluation of the lipid profile (triglycerides, LDL cholesterol, HDL cholesterol, and total cholesterol) in the blood is one way to identify the causes of hypertension, which is another cause of cardiovascular disease (Fuchs & Whelton, 2020). Research by Flint *et al*. (2019) on the influence of systolic and diastolic blood pressure on cardiovascular conditions explained that both systolic and diastolic blood pressure measuring ≥ 140/90 mm Hg and ≥130/80 mm Hg, respectively, significantly contribute to cardiovascular disease risk.

Hypertension, dyslipidemia, cardiovascular diseases (CVDs), and coronary atherosclerotic heart disease (CAHD) are interconnected conditions that can increase the risk of cardiovascular events. Dyslipidemia is a condition characterized by abnormal levels of lipids in the blood, and it is associated with an increased risk of hypertension. High levels of cholesterol can cause the blood vessels to become narrow and less elastic, leading to increased blood pressure (Hedayatnia *et al*., 2020).

Dyslipidemia is also a significant risk factor for CVDs, including CAHD. When dyslipidemia is present alongside hypertension, the risk of CVDs, including CAHD, increases. This is because both conditions contribute to the process of atherosclerosis, which is the buildup of plaque in the arteries. This plaque can lead to the narrowing and hardening of the blood vessels, reducing blood flow to the heart and increasing the risk of heart attack or stroke (Ariyanti and Besral, 2019). Futhermore, hypertension, dyslipidemia, CVDs, and CAHD are interconnected conditions that can increase the risk of cardiovascular events. Dyslipidemia is associated with an increased risk of hypertension and can exacerbate the risk of CVDs, including CAHD, when present alongside these conditions.

The prescriptions usually used for hypertension and dyslipidaemia are synthetic drugs such as Captopril and Simvastatin, but the use of herbal medicines is now developing and more preferred for long-term treatment due to their minimal side effects. One potential plant is *Jatropha curcas*, which is known to contain saponins, polyphenols, and flavonoids, that not only play a major role in treating various diseases, including bacterial and fungal infections, but also act as antioxidants (Ait Babahmad *et al*., 2018). According to Sadik *et al*. (2021), the administration of the ethanol extract of *Jatropha curcas* leaves can reduce blood pressure of hypertensive Wistar rats and can increase NO levels. In research conducted by Anita *et al*. (2023), it is reported that the administration of the ethanol extract of *Jatropha curcas* leaves can significantly reduce serum triglyceride levels at doses of 1.8, 2.7, and 4.05 g/kg BW. Research results on the effect of the ethanol extract of *Jatropha curcas* leaves on HDL and LDL levels have also been reported by Anigbogu (2015), revealing that the ethanol extract of *Jatropha curcas* leaves can increase HDL cholesterol concentration, thereby reducing LDL cholesterol concentration. This indicates that the ethanol extract of *Jatropha curcas* can be used for the treatment of cardiovascular diseases.

Due to the abundant presence of jatropha plants in Indonesia, numerous studies have examined the activity of the plants on blood pressure, LDL levels, and HDL levels. Therefore, the author intended to research the effect of giving the ethanol extract of *Jatropha curcas* leaves on blood pressure, LDL levels, and HDL levels in hypertensive rats given a high-fat diet.

MATERIALS AND METHODS

This study is an experimental study with a pretest-posttest control group design and has obtained ethical approval from Ahmad Dahlan University with the number 011804052. The test animals were grouped into seven groups, namely the normal group, negative control group, Captopril group, Simvastatin group, and EEJCL groups with doses of 1.8, 2.7, and 4.05 g/kg BW. The test animals in groups other than the normal group were induced with NaCl at 3.75 g/kg BW for 14 days to produce high blood pressure and a high-fat diet to produce hyperlipidaemia, while the normal group was only given standard feed.

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Materials

The materials used in this study were *Jatropha curcas* L. leaves obtained from the Gunung Kidul area of Yogyakarta and determined at the Biology Laboratory, Faculty of Applied Science and Technology, Ahmad Dahlan University, Yogyakarta, with the number 033/Lab.Bio/B/IV/2018, in addition to 96% ethanol, Captopril, Simvastatin, NaCl, Na-CMC, quercetin standard, gallic acid, Folin-Ciocalteau reagent, $AlCl_3$, Na_2CO_3, Silica gel 60 F_{254}, methanol, ethyl acetate, and chloroform. All the chemicals used were Merck chemicals of analytical grade. The test animals used were 35 male Wistar strain rats aged 2–3 months with weights of 200–250 grams.

Tools

The tools used included a drying cabinet, a blender, glassware, an analytical balance, a stirrer, a macerator, a vacuum, a rotary evaporator, water bath, a centrifuge, Eppendorf tubes, micropipettes, a vortex, and a UV-Vis spectrophotometer.

Methods

Preparation of the ethanol extract of *Jatropha curcas* Leaves (EEJCL)

As much as 1,700 grams of dried *Jatropha curcas* L. leaf powder was macerated using 96% ethanol as a solvent in a ratio of 1:4, stirred for 3 hours, and left to stand for 24 hours. Extraction was carried out 3 times. The extract was evaporated using a rotary evaporator at 70 °C and water bath until a thick extract was obtained (Anita & Bachri, 2023).

Compound identification using TLC

Thin layer chromatography (TLC) was carried out with silica gel F_{254} as the stationary phase, the mobile phase of hexane, ethyl acetate, and formic acid in the ratio of 6:4:0.2 for flavonoid analysis, and the mobile phase of HCl_3, MeOH, and H_2O in the ratio of 7:3.5:1. Sample spots were sprayed with $FeCl_3$ reagent for polyphenols and ammonia vapor for flavonoids and then compared to standard compound spots (quercetin serving as a flavonoid standard and gallic acid serving as a phenolic standard). The Rf value of each sample was determined (Susanto *et al*., 2023).

Total flavonoid test

The resulting sample with a concentration of 1% was pipetted at 2 mL and added with 2 mL of 2% $AlCl_3$. Absorbance was read with a spectrophotometer at a wavelength of 510 nm. The quercetin standard was prepared by dissolving quercetin in ethanol p.a at concentrations of 4, 6, 8, 10, and 12 µg/mL. The samples were examined with three replications. The flavonoid content was expressed as quercetin equivalent (Endah, 2016).

Total phenolic test

The resulting sample with a concentration of 1% was pipetted at 300 µL and added with 1.5 mL of Folin-Ciocalteu reagent. After being left for 3 minutes, 1.2 mL of 7.5% Na_2CO_3 solution was added and left again at room temperature. Absorbance was read with a spectrophotometer at a wavelength of 750 nm. Gallic acid standard solutions were made at concentrations of 15, 20, 25, 30, and 35 µg/mL, each being put into tubes and then added with 1.5 mL of Folin-Ciocalteu reagent (1:10). Afterwards, a calibration curve of the relationship between gallic acid concentration (µg/mL) and absorbance was made (Endah, 2016).

Antihypertensive activity test

The induced test animals in the control group were given a Na-CMC treatment, the Captopril group animals were given a Captopril suspension at a dose of 4.5 mg/kg BW, and the animals in the extract groups were given EEJCL at doses of 1.8, 2.7, and 4.05 g/kg BW, respectively. Treatments were applied orally from day 15 to day 21. Blood pressure measurements were carried out on day 14 for pre-treatment data. Blood pressure measurements were carried out on days 17, 20, and 22. The rats' systolic, diastolic, and mean arterial blood pressures were measured by the non-invasive blood pressure method using the CODA device. A tail cuff was placed on each rat's tail to monitor the rat's blood pressure. This CODA device has a VPR (Volume Pressure Recording) sensor, which uses a differential pressure transducer specifically designed to measure blood volume in the rat's tail non-invasively (Stanisavljevic *et al*., 2022).

LDL test

The induced test animals in the normal and negative groups were given an Na-CMC treatment, the positive group animals were given Simvastatin at 0.9 mg/kg BW, and the animals in the extract groups were given EEJCL at doses of 1.8, 2.7, and 4.05 mg/kg BW, respectively. Blood sampling was carried out twice, before and after treatment, with the rats fasting for ± 12 hours. Blood sampling of 3 mL was carried out through the retro-orbital sinus after the rats were anesthetized with ether (Nurmeilis, 2015). The blood was then centrifuged to obtain the serum. LDL cholesterol level data of the hypertensive Wistar rats given a high-fat diet were then analyzed. The enzymatic colorimetric test method was employed to directly measure LDL cholesterol levels.

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Table 1. TLC results on flavonoid content

Sample	Rf	Detection			Flavonoid
		UV 254	UV 366	Ammonia vapor	
Ethanol	1) 0.50	Yellow	Yellow	Yellow brownish	+
extract of	2) 0.62	Yellow	Yellow	Yellow brownish	+
Jatropha	3) 0.68	Yellow	Yellow	Yellow brownish	+
curcas leaves	4) 0.87	Yellow	Yellow	Yellow brownish	+
Quercetin	0.53	Greenish yellow	Greenish yellow	Yellow	+

HDL test

The induced test animals in the control group were given a CMC-Na treatment, the Simvastatin group animals were given a Simvastatin suspension at a dose of 0.9 mg/kg BW, and animals in the extract groups were given EEJCL at doses of 1.8, 2.7, and 4.05 g/kg BW, respectively. Blood sampling was carried out twice, before and after treatment, on day 15 and day 22. The obtained blood was separated between the serum and plasma. The serum was prepared with CHOD-PAP reagent and read on a UV-Vis spectrophotometer at a wavelength of 546 nm. Calculations were made on the obtained data to obtain HDL levels in blood.

Data analysis

Data analysis was conducted using SPSS with preliminary tests including the Kolmogorov-Smirnov test to determine if the data were normally distributed or not and the Levene test to determine if the variance was homogeneous or not. If the obtained data were normally distributed ($p > 0.05$) and homogeneous ($p > 0.05$), then it was followed by the parametric one-way ANOVA at a 95% confidence level. The analysis proceeded with a post-hoc test using Tukey test to show significant differences between treatment groups.

RESULTS AND DISCUSSION

Extraction of *Jatropha curcas* leaves

Extraction of dried powder from *Jatropha curcas* leaves resulted in 84.7 g of thick extract from a total of 1.7 kg of dried powder, with a yield of 4.98%.

Compounds contained in *Jatropha curcas* leaves based on TLC testing

The results of thin layer chromatography (TLC) testing of the extract after being passed under ammonia vapor in visible light indicated the presence of flavonoid compounds, with Rf 0.50. The ethanol extract of jatropha leaves was positive for flavonoids, as can be seen from the chromatogram profile in Figure 1. The TLC identification data of the ethanol extract of jatropha leaves can be seen in Table 1.

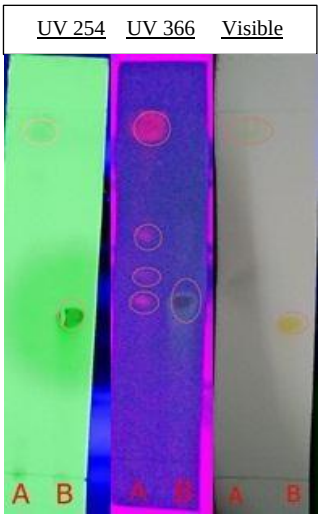

Figure 1. Flavonoid chromatogram profile of the ethanol extract of jatropha leaves: (A) *Jatropha curcas* L. sample; (B) quercetin standard

The results indicated that the ethanol extract of *Jatropha curcas* leaves contained phenolic compounds, with Rf 0.18. The ethanol extract of jatropha leaves was positive for phenolics, as can be seen from the chromatogram profile in Figure 2. The TLC identification data of the ethanol extract of jatropha leaves can be seen in Table 2.

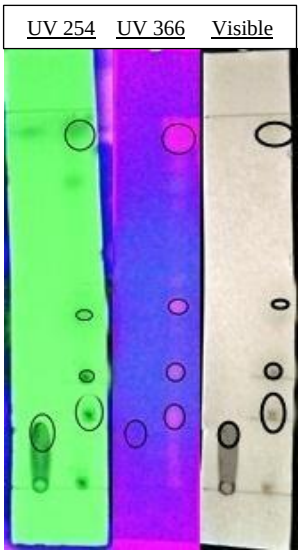

Figure 2. Phenolic chromatogram profile of the ethanol extract of jatropha leaves: (A) *Jatropha curcas* L. sample; (B) gallic acid standard

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Table 2. TLC results on phenolic content

Sample	Rf	Detection			Phenolic
		UV 254	UV 366	$FeCl_3$	
Ethanol extract of *Jatropha curcas* leaves	1) 0.18	blackout	Yellow	Black Grey	+
	2) 0.25	blackout	Yellow	Black Grey	+
	3) 0.43	blackout	Yellow	Black Grey	+
	4) 0.93	blackout	Yellow	Black Grey	+
Quercetin	0.12	blackout	Blue	Black Grey	+

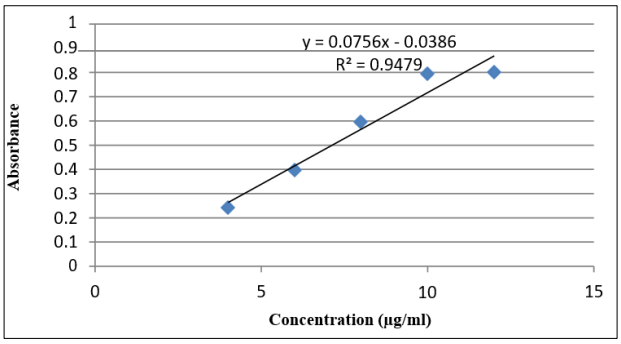

Figure 3. The concentration and absorbance graph of quercetin standard solution

Table 3. Total flavonoid content of the ethanol extract of jatropha leaves

Extract Weight (mg)	Absorbance	Total Flavonoid Content (%)
10.1	0.640	4.43
10.1	0.641	4.44
10.2	0.636	4.36
	Mean ± SD	4.41 ± 0.04

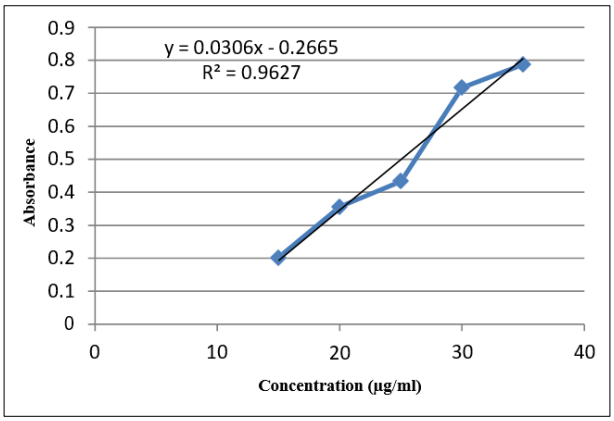

Figure 4. The concentration and absorbance graph of gallic acid standard solution

Table 4. Total phenolic content of the ethanol extract of jatropha leaves

Extract Weight (mg)	Absorbance	Total Phenolic Content (%)
10.1	0.422	11.1
10.3	0.466	11.6
10.5	0.404	10.4
	Mean ± SD	11.03 ± 0.60

Testing of total flavonoid and phenolic contents

Based on testing, the ethanol extract of jatropha leaves had a total flavonoid content of 4.41 ± 0.04%. A quercetin standard curve was developed based on this result, from which a linear regression equation as seen in Figure 3 was produced. The calculation results of flavonoid levels in the ethanol extract of jatropha leaves can be seen in Table 3.

Further testing showed that the ethanol extract of jatropha leaves had a total phenolic content of 11.03 ± 0.60%. A gallic acid standard curve was developed based on this result, from which a linear regression

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equation as seen in Figure 4 was produced. Calculation results on phenolic levels in the ethanol extract of jatropha leaves can be seen in Table 4.

The values obtained were above the total phenolic and flavonoid contents previously calculated by Sadik *et al*. (2017), who conducted extraction using the same method and solvent, where the total flavonoid and phenolic contents obtained were 1.48 ± 0.01% and 5.51 ± 0.01%, respectively. The differences in results were possibly due to differences in environmental conditions, such as temperature, soil, and plant cultivation processes.

Antihypertensive testing

The study was conducted on 7 groups of test animals, each consisting of 5 rats. Blood pressure measurements were conducted on days 14, 17, 20, and 22. The results obtained from each measurement can be seen in Tables 5, 6, and 7.

The data of systolic blood pressure measurement (Table 5) indicate that the administration of the ethanol extract of *Jatropha curcas* leaves (EEJCL) was able to lower systolic blood pressure. The most effective EEJCL dose according to these data was 2.7 g/kg BW, which could render a decrease greater than the comparative group (Captopril). A previous study by Sadik *et al.* (2021) also discovered that plants containing flavonoid compounds can lower blood pressure. Flavonoids can inhibit ACE by forming chelate complexes at the active centre of ACE, depending on their main structural features. The flavonoid content in the extract, as well as its antioxidant activity, supports the extract's ability as an antihypertensive agent (Guerrero *et al*., 2012). The one-way ANOVA results on systolic blood pressure showed a significant value ($p < 0.05$), meaning that there was an effect of decreasing systolic blood pressure after the application of the preparation. Therefore, it can be concluded that the ethanol extract of *Jatropha curcas* leaves (EEJCL) is effective as an antihypertensive on male Wistar strain rats.

The diastolic blood pressure measurement data (Table 6) indicate that the administration of the ethanol extract of *Jatropha curcas* leaves (EEJCL) was able to lower diastolic blood pressure. The most effective EEJCL dose according to the data was 2.7 g/kg BW, which could render a decrease greater than the comparative group (Captopril). The one-way ANOVA results on diastolic blood pressure showed a significant value ($p < 0.05$), meaning that there was an effect of decreasing diastolic blood pressure after the application of the preparations. This result supports the conclusion previously drawn.

Table 5. Systolic blood pressure measurement results on days 14, 17, 20, and 22

Group	Dose (g/kg BW)	Mean systolic blood pressure (mm Hg) ± SD			
		D-14	D-17	D-20	D-22
Normal	-	119.0 ± 6.51	123.0 ± 9.13	117.0 ± 5.56	120.0 ± 8.24*
Control	-	151.0 ± 7.74	145.8 ± 2.77	155.8 ± 6.91	137.4 ± 5.40
Captopril	0.0045	148.6 ± 12.4	122.8 ± 3.19*	118.0 ± 5.65*	121.4 ± 9.55*
EEJCL	1.8	143.2 ± 3.96	126.2 ± 10.98	126.4 ± 12.19*	114.8 ± 9.75*
	2.7	143.8 ± 11.88	141.0 ± 13.43	129.8 ± 18.55*	113.4 ± 8.26*
	4.05	139.6 ± 6.18	130.2 ± 17.15*	127.0 ± 16.53*	118.0 ± 8.71*

*) $p < 0.05$, indicating a significant difference from the control group

Table 6. Diastolic blood pressure measurement results on days 14, 17, 20, and 22

Group	Dose (g/kg BW)	Mean diastolic blood pressure (mm Hg) ± SD			
		D-14	D-17	D-20	D-22
Normal	-	84.8 ± 5.89*	76.6 ± 12.01*	76.8 ± 11.73*	73.0 ± 5.52*
Control	-	115.4 ± 7.19	116.2 ± 20.48	114.8 ± 16.33	92.8 ± 6.30
Captopril	0.0045	100.4 ± 26.85	99.0 ± 14.91	90.6 ± 12.30*	91.8 ± 20.50*
EEJCL	1.8	111.8 ± 6.05	99.6 ± 12.15	91.8 ± 15.62*	78.0 ± 7.71*
	2.7	109.0 ± 10.29	102.0 ± 12.58	95.0 ± 16.07*	77.6 ± 12.46*
	4.05	112.2 ± 9.36	94.4 ± 18.82*	90.0 ± 17.91*	81.8 ± 13.14*

*) $p < 0.05$, indicating a significant difference from the control group

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Table 7. Mean arterial blood pressure measurement results on days 14, 17, 20, and 22

Group	Dose (g/kg BW)	Mean arterial blood pressure (mm Hg) ± SD			
		D-14	D-17	D-20	D-22
Normal	-	98 ± 8.97	92.6 ± 7.40	94.4 ± 5.17	88.4 ± 6.02*
Control	-	127.8 ± 6.26	108.4 ± 12.3	128.2 ± 16.55	110.8 ± 14.75
Captopril	0.0045	123.4 ± 10.23	129 ± 19.27*	98.8 ± 8.40*	93.8 ± 6.64*
EEJCL	1.8	119 ± 5.47	115.6 ± 18.35	103.6 ± 15.37*	89.8 ± 7.56*
	2.7	118.6 ± 10.33	113.2 ± 10.35	106.4 ± 16.63*	90 ± 11.37*
	4.05	117.8 ± 10.32	98 ± 19.91*	105.2 ± 13.4*	90.6 ± 9.76*

*) $p < 0.05$, indicating a significant difference from the control group

Table 8. LDL level measurement results (mg/dL) after application of Simvastatin and the ethanol extract of *Jatropha curcas* leaves (EEJCL)

Group	Dose (g/kg BW)	Day-15[1)] (Mean ± SD)	Day-22[2)] (Mean ± SD)	Difference (Mean ± SD)	Decrease percentage (%)
Normal	-	24.25 ± 2.41	22.92 ± 4.64	1.33 ± 3.79*	5
Control	-	36.64 ± 1.58	48.56 ± 3.01	-11.91 ± 4.01	-32
Simvastatin	0.0009	42.74 ± 1.64	24.70 ± 1.89	18.04 ± 2.91*	42
EEJCL	1.8	37.82 ± 4.97	27.57 ± 3.90	10.24 ± 5.25*	27
	2.7	38.53 ± 2.87	26.16 ± 1.94	12.37 ± 4.02*	32
	4.05	38.47 ± 1.97	24.15 ± 1.22	14.31 ± 1.48*	37

*) $p < 0.05$, indicating a significant difference from the control group
1) Day 15, after being given high-fat feed and NaCl at 3.75 g/kg BW for 14 days
2) Day 22, after being given the ethanol extract of *Jatropha curcas* leaves (EEJCL) for 7 days

The mean arterial blood pressure measurement data (Table 7) show that the administration of the ethanol extract of *Jatropha curcas* leaves (EEJCL) was able to lower the mean arterial blood pressure, where the most effective EEJCL dose in this test was 1.8 g/kg BW, with a decrease greater than the comparative group (Captopril). Blood pressure measurements on days 20 and 22 had already shown significant decreases in blood pressure approaching normal. The statistical test results showed a significant difference between the dose groups and the induced groups, while the statistical results of the dose groups compared to the normal and Captopril groups showed no significant difference. The flavonoid compounds in the *Jatropha curcas* leaf ethanol extract exhibited ACE inhibitory activity, which was induced by the formation of chelate complexes at the ACE active centre; this activity depends on the main structural features of flavonoids. The flavonoid content in the extract, and its proven antioxidant activity, supports the extract's ability as an antihypertensive (Dhianawaty *et al*., 2018). As a result, the blood pressure of test rats in the EEJCL 1.8, EEJCL 2.7, and EEJCL 4.05 groups could be lowered approaching normal.

LDL testing

LDL level measurements were also conducted on all the test groups on days 15 and 22, the results of which can be seen in Table 8.

The statistical test results of the EEJCL 1.8, EEJCL 2.7, and EEJCL 4.05 groups showed a significant difference ($p < 0.05$) from the control group. The Simvastatin group was also significantly different ($p < 0.05$) from the control group. The EEJCL 1.8 and EEJCL 4.05 groups showed no significant difference ($p > 0.05$) from the Simvastatin group. Finally, the EEJCL 2.7 group showed a significant difference ($p < 0.05$) from the Simvastatin group. This shows that the administration of EEJCL could lower LDL levels, but not to the extent of normal levels. The data of the difference in rat LDL levels can be seen in Table 8.

The decreases in blood pressure and LDL levels are related to the presence of flavonoid compounds. Various studies have proven that flavonoids can lower blood pressure and LDL levels by inhibiting angiotensin-converting enzyme and binding free radicals and metal ion transitions in inhibiting lipid peroxidation (Loh *et al*., 2020). Flavonoids have the ability to stop oxidative damage and LDL oxidation. In addition, luteolin derivatives can trigger cholesterol barrier secretion, meaning cholesterol levels decrease. When cholesterol is transported from the intestine to the liver, flavonoids function as inhibitors of the HMGCoA reductase enzyme, the enzyme responsible for converting acetyl-CoA to mevalonate in cholesterol synthesis, thus reducing synthesis (Nuralifah *et al*., 2020). Thus, the administration of the ethanol extract of *Jatropha curcas* leaves (EEJCL) for 7 days can lower LDL levels in hypertensive rats given a high-fat diet.

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Table 9. HDL level measurement results (mg/dL) after application of Simvastatin and the ethanol extract of *Jatropha curcas* Leaves (EEJCL)

Groups	Dose (g/kg Bw)	Day15[1)] (Mean ± SD)	Day-22[2)] (Mean ± SD)	Difference (Mean ± SD)	Decrease percentage (%)
Normal	-	34.57 ± 1.17	36.67 ± 0.78	2.09 ± 1.63[*]	5
Control	-	23.39 ± 1.89	26.40 ± 2.38	3.01 ± 2.23	11
Simvastatin	0.0009	24.61 ± 1.21	33.82 ± 0.57	9.18 ± 1.12[*]	27
EEJCL	1.8	24.58 ± 2.35	27.45 ± 1.49	2.87 ± 1.56[*]	10
	2.7	24.58 ± 3.29	29.64 ± 1.49	5.05 ± 2.95[*]	17
	4.05	23.46 ± 2.86	35.60 ± 0.67	12.14 ± 2.32[*]	34

[*)] $p < 0.05$, indicating a significant difference from the control group
[1)] Day 15, after being given high-fat feed and NaCl at 3.75 g/kg BW for 14 days
[2)] Day 22, after being given the ethanol extract of *Jatropha curacas* leaves (EEJCL) for 7 days

HDL testing

At last, HDL level measurements were conducted on the test groups. The results of these HDL level measurements on day 15 and day 22 in each group can be seen in Table 9.

Before treatment, the control, Simvastatin, EEJCL 1.8, EEJCL 2.7, and EEJCL 4.05 groups had lower HDL levels compared to the normal group (Table 9). This was because groups other than the normal group were given a high-fat diet containing a lot of cholesterol. HDL is said to be low if the level is < 30 mg/dL (Hernáez *et al*., 2019). Then, the HDL levels in each group showed an increase after EEJCL administration. Table 4 shows that there was an increase in HDL levels after the application of the EEJCL treatment in each group. This shows that *Jatropha curcas* leaf ethanol extract is able to increase HDL levels. Previous research by Anigbogu *et al*. (2015) also discovered that *Jatropha curcas* leaf ethanol extract can increase HDL levels. The increase in HDL levels occurred following the administration of the ethanol extract of *Jatropha curcas* leaves, which is known to contain flavonoid compounds.

In this study, the highest HDL level increase occurred in the EEJCL 4.05 group. The effects resulted should go hand in hand with increasing doses. However, higher doses will have decreased effects. This is because the dose can no longer maximally provide effects. This case often occurs in traditional or herbal medicines, in which case these medicine no longer contain a single chemical compound, but several types of chemical compounds that work together to provide effects. It is not impossible that with increasing doses the amount of contained compounds also increases and unwanted reactions that can reduce effects occur (Siskayanti *et al*., 2017).

A similar study was conducted by Abdulmumin (2020), who reported that extracts of *Jatropha Curcas* leaves, peel, stems, and roots have hypolipidemic activity and may be useful in managing cardiovascular diseases. The acute toxicity (LD50) of *Jatropha curcas* leaf, peel, stem, and root extracts was found to be greater than 5,000 mg/kg, thus declared practically non-toxic to experimental animals (Mika'il *et al*., 2020). Administering treatments such as flavonoid-containing EEJCL is likely to increase endothelial nitric oxide (eNOS) synthesis, thus increasing NO bioavailability. Flavonoids can act as vasodilators with suitable signaling pathways and structural characteristics for strong vasorelaxant properties (Loh *et al*., 2020). In other words, the ethanol extract of *Jatropha curcas* leaves has a potential for development in the treatment of hypertension and dyslipidaemia. This study can be a reference for further research on similar topics, with the potential to lead to the development of more promising antihypertensive alternatives.

CONCLUSION

The ethanol extract of *Jatropha curcas* L. (EEJCL) leaves contains flavonoid and phenolic compounds. The administration of EEJCL can reduce blood pressure significantly in terms of systolic, diastolic, and average arterial blood pressure. In addition, it can increase HDL levels and reduce LDL levels in the blood of hypertensive rats given a high-fat diet. Therefore, it is concluded that the ethanol extract of *Jatropha curcas* L. (EEJCL) leaves has a potential for development in the treatment of hypertension and dyslipidaemia.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.S.B., W.Y.A., P.D.L., D.E.W., D.R.N., S.Y., W.W., L.H.N.; Methodology, M.S.B., S.Y., L.H.N., D.E., V.S.; Software, M.S.B.,

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Characterization and Stability Test of Hydrolyzed Collagen Glyserosomes

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Abstract

Background: *Hydrolyzed collagen is a protein obtained from enzymatic denaturation of collagen with a molecular weight of about 10 kDa, and it has been reported to produce anti-aging properties. Delivering hydrolyzed collagen into the dermis becomes a great challenge due to its large molecular weight, so glycerosome, a deformable vesicle containing glycerol as the edge activator, was developed to carry it into the dermis layer.* ***Objective****: The study aimed to determine the effect of increasing the concentration of glycerol and hydrolyzed collagen on the characteristics and stability of hydrolyzed collagen glycerosomes.* ***Methods****: Glycerosomes were composed of soy lecithin and prepared using a thin film lipid method. The lipid film was hydrated with phosphate-buffered saline pH 5 containing different glycerol concentrations (20% and 40%) and hydrolyzed collagen (2.5% and 5%). Then, characteristic tests and stability tests were carried out.* ***Results****: Hydrolyzed collagen glycerosomes had vesicle sizes of 170-180 nm, polydispersity index of 0.253-0.279, zeta potential values of -23.70 to -26.50 mV with deformability indexes of 2.25-3.49. The highest percentage of entrapment efficiency was 85.72%, achieved with a glycerol concentration of 40%. During the stability test at 25°C for 12 weeks, the hydrolyzed collagen glycerosomes did not experience pH and entrapment efficiency changes, but it increased the vesicle size.* ***Conclusion****: The use of 40% glycerol produced more deformable vesicles than 20% glycerol in hydrolyzed collagen glycerosomes; however, a formula improvement is required to improve the stability of glycerosomes.*

Keywords*: delivery system, glycerosome, glycerol, hydrolyzed collagen*

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INTRODUCTION

Human skin protects the body from the surrounding environment and maintains body temperature (Aguirre *et al.,* 2020). The skin consists of three layers; in the dermis layer, some fibroblasts produce collagen and other protein matrics so that the skin becomes elastic (Lukić *et al*., 2021). Getting older and exposure to ultraviolet light (UV) and air pollution can cause skin aging. In skin aging, fibroblasts decline function, reducing collagen production (Jhawar *et al*., 2019). The decreased amount of collagen generates skin inelasticity and wrinkles, aging lines appearances, and skin dryness (Wang, 2021). So, topical delivery of hydrolyzed collagen (Cao *et al*., 2020) is needed to increase skin collagen levels and overcome skin aging.

Hydrolyzed collagen can be isolated from cattle, sheep, pigs, and marine (León *et al*., 2019). Hydrolyzed collagen is obtained from an enzymatic denaturation of collagen, which breaks down the protein into smaller weight molecules (Larde *et al*., 2023). Hydrolyzed collagen has anti-aging benefits for the skin by increasing the amount of collagen to increase the skin's elasticity (Aguirre *et al*., 2020). It also works on the dermis layer by filling in broken collagen to form collagen fibers (Ferdinando *et al*., 2022). Previous research (de Miranda *et al*., 2021) shows that hydrolyzed collagen supplements can increase skin elasticity compared to placebo. However, hydrolyzed collagen has a large molecular weight (Schmidt *et al*., 2019), which limits its topical delivery. Therefore, a delivery system, i.e., a deformable vesicle, is needed to effectively carry hydrolyzed collagen to the dermis layer.

Glycerosomes are deformable nanovesicles with a hydrophilic core surrounded by a lipid bilayer membrane with glycerol as the edge activator. Glycerosomes can load hydrophilic and lipophilic drugs (Zaki *et al.,* 2022). The lipid bilayer of glycerosomes is deformable, which can improve the permeability of the active ingredients (Gupta et al., 2020). As an edge activator, glycerol will move to the curved area of the lipid bilayer when the vesicles are under mechanical pressure resulting from the transepidermal osmotic gradient (Opatha et al., 2020) so that glycerosomes can pass through the intercellular skin gaps and reform to their original shape when reaching the deeper skin layers (Touitou and Natsheh, 2021). It has been reported that a glycerol concentration of 10% to 50% can successfully increase the skin penetration of vesicles (Gupta et al., 2020). In addition, the higher the concentration of glycerol, the higher the entrapment efficiency (Rani et al., 2021).

On the other hand, hydrophilic hydrolyzed collagen will be entrapped within the intraliposomal phase of the vesicles, and the higher the entrapped hydrolyzed collagen level, the higher the anti-aging efficacy will be since the skin collagen level will be increased. However, this vesicle aqueous core has limited volume capacity. Thus, the variation level of hydrolyzed collagen can affect the entrapment efficiency. Therefore, both parameters need to be evaluated in this research.

This study aims to determine the effect of increasing the concentration of glycerol and hydrolyzed collagen on the characteristics and stability of hydrolyzed collagen glycerosomes. The physicochemical characteristics of hydrolyzed collagen glycerosomes were evaluated for organoleptic properties, pH, vesicle size, polydispersity Index (PDI), zeta potential, deformability index, and entrapment efficiency (EE). In addition, the stability test of hydrolyzed glycerosomes was further observed for 12 weeks at 25°C.

MATERIALS AND METHODS

Materials

Hydrolyzed collagen was purchased from Fenchem, Nanjing, China. Soy lecithin (Phospholipids content 30%) was purchased from Himedia, Mumbai, India. Glycerol was purchased from Smart Lab, Tangerang Selatan, Indonesia. Dipotassium hydrogen phosphate, Potassium dihydrogen phosphate, and Sodium hydroxide were purchased from SAP Chemicals, Bandung, Indonesia. The aquadest was used as the solvent in this study.

Method

Preparation of hydrolyzed collagen glycerosomes

The hydrolyzed collagen glycerosomes were prepared using the formula as presented in Table 1, in which F1 contained 2.5% hydrolyzed collagen and 20% glycerol, F2 contained 2.5% hydrolyzed collagen and 40% glycerol, F3 contained 5% hydrolyzed collagen and 20% glycerol, and F4 contained 5% hydrolyzed collagen and 40% glycerol. Firstly, glycerosomes were prepared by dissolving soy lecithin in chloroform (1:10) in a round bottom flask. Then, the chloroform was evaporated entirely using a rotary vacuum evaporator (Buchi, Indonesia) at 100 rpm and 45°C for 45 minutes until a thin lipid layer formed at the bottom of the flask. A hydrating solution containing hydrolyzed collagen, glycerol, and phosphate-buffered saline (pH 5) was added to hydrate the thin lipid film by rotating the flask at 100 rpm and 45°C for 60 minutes. The mixture was

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then sonicated for 20 minutes with a sonicator (Skymen, China) to obtain homogenous glycerosomes suspension (Gupta *et al*., 2020).

Characterization of hydrolyzed collagen glycerosomes

Organoleptic observation of hydrolyzed collagen glycerosomes

The physical appearance of the hydrolyzed collagen glycerosomes was organoleptically observed regarding their consistency, color, and odor (Table 2).

Morphology observation of hydrolyzed collagen glycerosomes

Morphological observations of hydrolyzed collagen glycerosomes were made using a scanning electron microscope (Thermo Fisher Scientific, US). Three drops of samples were placed in a holder and then dried for 24 hours. Then, the dried sample was coated with Au. The sample was then observed at 40.000x magnification (Gupta *et al*., 2020).

pH measurement of hydrolyzed collagen glycerosomes

The sample was diluted with distilled water (1:10), and the pH was measured using a pH meter (Horiba, Japan). The electrode was inserted into the sample solution until it showed a constant pH (Opatha *et al.*, 2020).

Vesicle size and polydispersity index (PDI) measurement of hydrolyzed collagen glycerosomes

About 100 µL of the sample was added with 3 mL distilled water, then placed in a cuvette and mixed until homogeneous by pipetting. Then, vesicle size and PDI were measured using the dynamic light scattering method with the Beckman Coulter Delsa Nano Particle Analyzer (US) (Santuso *et al*., 2023).

Zeta potential measurement of hydrolyzed collagen glycerosomes

About 100 µL of the sample was diluted with 3 mL distilled water, then put into a cuvette and mixed until homogeneous by the pipetting. Then, the zeta potential was measured using an electrophoretic scattering method with Horiba Nanoparticle Analyzer SZ-100 (Japan) (Santuso *et al*., 2023).

Deformability index measurement of hydrolyzed collagen glycerosomes

To measure the deformability index of the sample, about 1 ml of the sample was passed through a polycarbonate membrane (Millipore®, Merck) with 50 nm of pore diameter using an Avanti mini extruder (Avanti Polar Lipid, US) for 5 minutes. Then, the vesicle size was measured. The deformability index was calculated using the following formula (Opatha *et al*., 2020):

$$D = J\left(\frac{rv}{rp}\right)^2$$

D: Degree of deformability
A: volume of sample extruded in 5 minutes (ml)
Rv: vesicle size after extrusion (nm)
Rp: pore size (50 nm)

The determination of entrapment efficiency of hydrolyzed collagen in glycerosomes

The percentage of entrapment efficiency (EE) was determined by analyzing the free protein/hydrolyzed collagen content using the Bradford reagent (Himedia, Maharashtra, India) (Sari *et al.*, 2021). Firstly, about 1 ml of the sample was centrifuged at 10,000 rpm for 10 minutes. Then 0.1 ml of the filtrate was taken, put into a tube, and added with 5 ml of Bradford reagent. The mixture was then incubated for 10 minutes. The absorbance of free hydrolyzed collagen was then measured using UV-Vis Spectrophotometry at the wavelength of 595 nm. The percentage of entrapment efficiency was calculated using the following formula (Opathe *et al*., 2020):

$$\%\ EE = \frac{Total\ amount\ of\ the\ drug\ added - Amount\ of\ the\ free\ drug}{Total\ amount\ of\ the\ drug\ added} \times 100$$

Stability test of hydrolyzed collagen glycerosomes

The stability test of hydrolyzed collagen was observed by maintaining samples at room temperature (25±1°C) and relative humidity of 60±5% for 12 weeks. At the end of the study period, the sample was determined for pH, vesicle size, and %EE (Fayalil *et al*., 2020).

Table 1. Formulation of hydrolyzed collagen glycerosomes

Material	Function	concentration			
		F1	**F2**	**F3**	**F4**
Hydrolyzed collagen	Active ingredient	2,5%	2,5%	5%	5%
Soy lecithin	Phospholipid	2%	2%	2%	2%
Glycerol	Edge activator	20%	40%	20%	40%
Phosphate buffer saline (pH 5)	Hydrating solution	Up to 100%	Up to 100%	Up to 100%	Up to 100%

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Table 2. The organoleptic properties of hydrolyzed collagen glycerosomes

Formula	Organoleptic Parameter		
	Color	**Odor**	**Consistency**
F1	Yellow	Odorless	Liquid
F2	Yellow	Odorless	Liquid
F3	Yellow	Odorless	Liquid
F4	Yellow	Odorless	Liquid

Statistical data analysis

All experiments were determined in three replicates, and the values were presented as mean and SD. The physicochemical characteristics were analyzed using a One-Way Analysis of Variance (ANOVA), while the stability data were analyzed using the Paired T-test. The analysis was then followed by Tukey's post hoc test.

RESULTS AND DISCUSSION

The organoleptic properties of hydrolyzed collagen glycerosomes

The organoleptic properties of hydrolyzed collagen glycerosomes, such as color, odor, and consistency, were observed visually. The results showed that all hydrolyzed collagen glycerosomes had the same properties. All formulas produced odorless yellow color suspension and have a liquid consistency, as presented in Table 2. The yellow color occurs because of the natural color of the phospholipids and liquid hydrolyzed collagen used in the formula.

The morphological observations of hydrolyzed collagen glycerosomes

The morphology of hydrolyzed collagen glycerosomes was examined using SEM. Hydrolyzed collagen glycerosomes were observed as spherical vesicles with a size around 100 nm, as shown in Figure 1. In the aqueous environment, the phospholipids will spontaneously assemble into a bilayer membrane surrounding an inner water phase, forming spherical vesicles (Sun et al., 2022). The spontaneous drying process during sample preparation resulted in spherical vesicle formation. However,. However, further investigation is needed since the vesicles may be perturbed during this process.

The pH of hydrolyzed collagen glycerosomes

The pH measurement in (Figure 2A) shows that all glycerosomes have an average pH of 5.55. Varying the glycerol concentration and hydrolyzed collagen had no significant effects on pH parameters ($p>0.5$). In this study, phosphate-buffered saline with a pH of 5 was used as the hydrating solution, thus reflecting the pH of the hydrolyzed collagen glycerosomes. The pH of the sample is highly determined by considering the stability of hydrolyzed collagen, which is stable at pH 3.68-5.70 (León *et al.*, 2019). In addition, as a topical product, the pH of hydrolyzed collagen glycerosomes should correspond to the pH of human skin, 4.5-6.5 (Lukić *et al*., 2021).

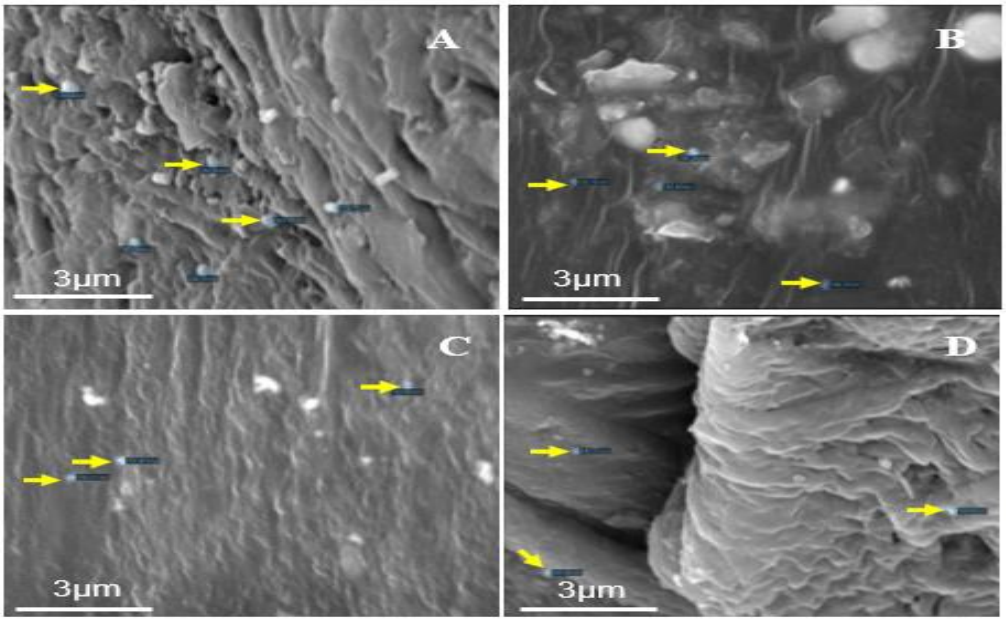

Figure 1. The morphological observations of hydrolyzed collagen glycerosomes (A) F1, (B) F2, (C) F3, (D) F4 by scanning electron microscopy. → = vesicle of glycerosomes

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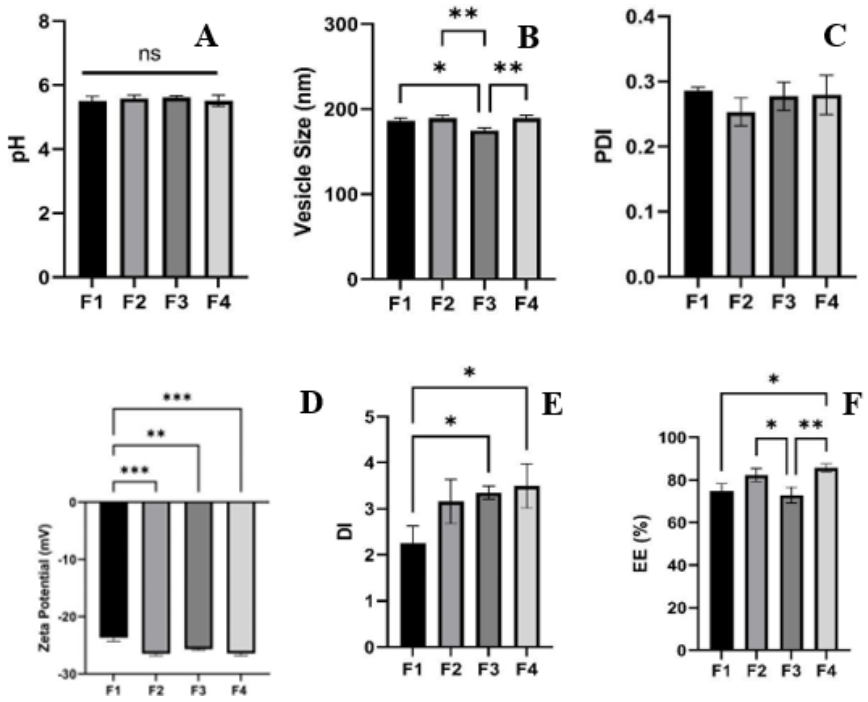

Figure 2. The evaluation of (A) pH, (B) vesicle size, (C) PDI, (D) zeta potential, (E) Deformability index, (F) %Entrapment Efficiency of hydrolyzed collagen glycerosomes

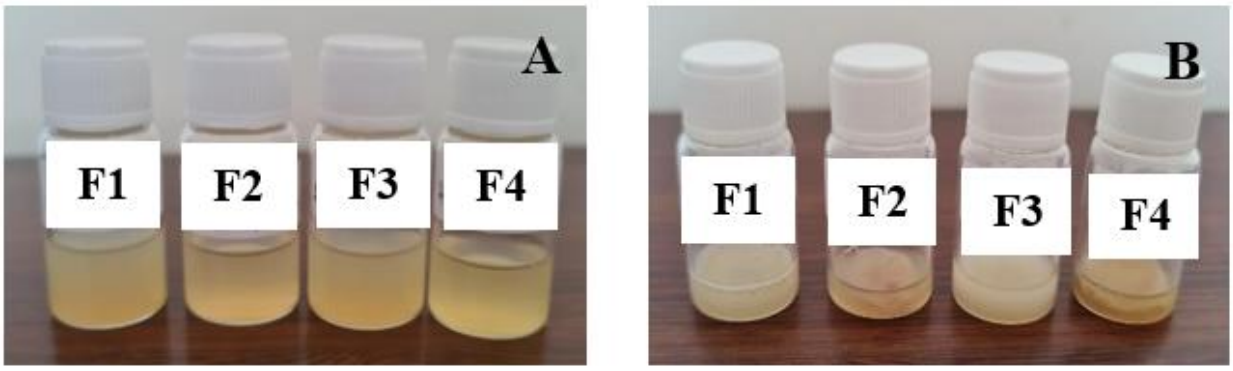

Figure 3. The organoleptic properties of hydrolyzed collagen glycerosomes (A) before and (B) after storage at a temperature of 25°C for 12 weeks

The vesicle size, Polydispersity Index (PDI), and zeta potential of hydrolyzed collagen glycerosomes

Designing a nanovesicle delivery system is essential to ensure penetration of the delivery system through deeper layers of the skin. The results show that the vesicle size of hydrolyzed collagen glycerosomes ranged from 170-180 nm, as presented in Figure 2B. Varying the concentration of glycerol and hydrolyzed collagen has a significant difference ($p<0.05$). It has been reported that increasing the edge activator level will increase the vesicles' size. Glycerol will damage the tight arrangement of phospholipids so that the phospholipids will stretch, causing the vesicles to become larger (Fatima et al., 2022).

In addition, the polydispersity Index (PDI) is a parameter of the size distribution homogeneity of glycerol vesicles, with a PDI value of 0.200 showing good homogeneity for the vesicles. The results showed that the PDI value of hydrolyzed collagen glycerosomes was 0.25-0.29, as shown in Figure 2C, which means all glycerosome formulas had homogeneous vesicle sizes. Varying the glycerol concentration and hydrolyzed collagen had no significant effects on the physical properties of the glycerosomes ($p>0.5$).

The zeta potential values of hydrolyzed collagen were around -20 mV, as presented in Figure 2D. Varying the concentration of glycerol and hydrolyzed collagen also had no significant difference ($p<0.5$). Zeta potential is related to glycerosome stability during storage. It has

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been reported that the zeta potential value of around ±30 mV indicates that the vesicles are stable during storage (Apriani., 2022).

The deformability index of hydrolyzed collagen glycerosomes

The deformability index is essential to determine the permeability of the delivery system (Rasheed *et al.*, 2022). Glycerol, as an edge activator, can make the lipid bilayer flexible by changing the orderly arrangement of phospholipids, thereby reducing the transition temperature of the bilayer membrane (Miatmoko *et al*., 2021). The results of the measured deformability index show that the deformability index value is 2.25 to 3.49 (Figure 3E). Varying the concentrations of glycerol and hydrolyzed collagen resulted in a significant difference in the deformability index ($p<0.5$). Hydrolyzed collagen glycerosomes containing 20% glycerol with deformability index values of 2.25-3.15 had a lower deformability index than hydrolyzed collagen glycerosomes containing 40% glycerol with deformability index values of 3.35-3.49. This result shows that the higher the glycerol level, the more flexible the glycerosomes produced (Leonyza and Surini., 2019). Variations in the concentration of hydrolyzed collagen influence the deformability index values.

The entrapment efficiency of hydrolyzed collagen in glycerosomes

Entrapment Efficiency (EE) is the value of the concentration of a drug trapped in a glycerosome vesicle (Opatha *et al*., 2020). As an edge activator, glycerol is important in increasing the EE value (Md *et al*., 2021). The EE results of hydrolyzed collagen glycerosomes differed significantly ($p<0.05$), as shown in Figure 3F. Hydrolyzed collagen glycerosomes containing 40% glycerol with EE values of 82.26-85.73% had higher EE than hydrolyzed collagen glycerosomes containing 20% glycerol with EE values of 72.90-74.90%. Glycerol, as an edge activator, can disrupt the stability of the lipid bilayer and increase its fluidity and elasticity. A high EE% indicates the successful formulation of a delivery system (Khan *et al*., 2022). Variations in hydrolyzed collagen concentration did not affect trapping efficiency. Hydrolyzed collagen is not all trapped in the vesicle because the compartments are limited, so the EE value has reached a maximum value.

The stability of hydrolyzed collagen glycerosomes

The stability test was evaluated by storing samples for 12 weeks at a temperature of 25±10°C with a humidity of 60±5% RH. After 12 weeks, the organoleptic properties of hydrolyzed collagen glycerosomes were evaluated. As shown in Figure 2, all formulas were still odorless; however, the visual appearance of several formulas has changed, and the F1 and F3 became cloudy, and F2 and F4 remained yellow (Figure 3B). Furthermore, lump-like sedimentation was observed in F2 and F4, as presented in Figure 3B. This sedimentation may occur because of the sticky nature of glycerol (Sharma *et al*., 2023). The pH of glycerosomes did not change ($p>0.05$) before and after the stability test, except for F4 (Figure 4A). The remaining pH can be maintained because phosphate buffered-saline pH 5 was used as a hydrating agent in the formula. This pH is related to the pH stability of hydrolyzed collagen, 3.68-5.70 (León *et al*., 2019), and the pH of the skin, 4.5-6.5 (Lukić *et al*., 2021).

In addition, the vesicle size of hydrolyzed collagen was significantly different before and after storage ($p<0.05$). The vesicle size became larger after 12 weeks of storage at room temperature (Figure 4B). This is probably due to the low zeta potential value (-26.50 mV until -23.70 mV), so the vesicles experience agglomeration (Apriani., 2022). There were negligible changes in %EE of hydrolyzed collagen glycerosomes before and after the stability test ($p>0.05$). In (Figure 5) the entrapment efficiency remains unchanged during 12 weeks of storage. If the phospholipid concentration is low, glycerosome leakage may occur (Zhu *et al*., 2022).

Based on this stability test, variations in glycerol concentration affected vesicle size and entrapment efficiency. The pH and entrapment efficiency remain unchanged in hydrolyzed collagen glycerosomes with hydrolyzed collagen concentrations of 2.5% and 5%. However, vesicle size changed with increased glycerol concentration.

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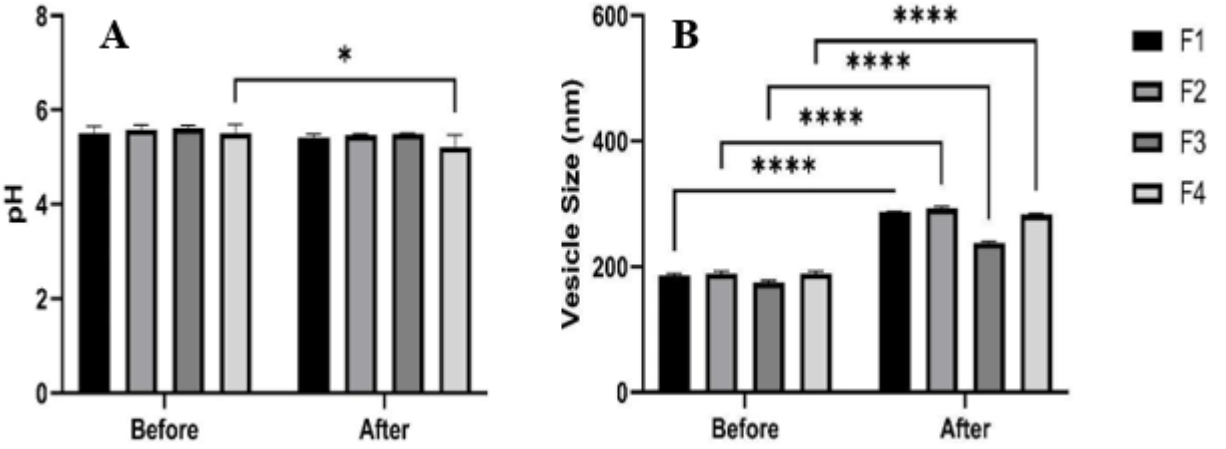

Figure 4. The pH (A) and vesicle size (B) hydrolyzed collagen glycerosomes before and after storage at a temperature of 25ºC for 12 weeks

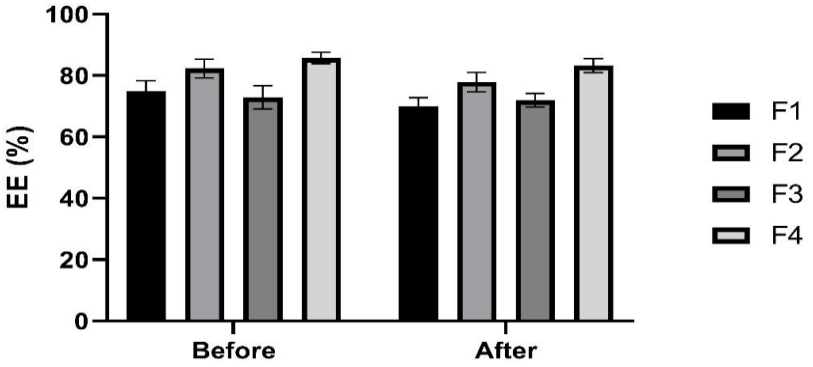

Figure 5. The results measurement of %EE of hydrolyzed collagen glycerosomes before and after storage for 12 weeks at a temperature of 25ºC

CONCLUSION

In conclusion, the use of glycerol affected the physical characteristics and stability of hydrolyzed collagen glycerosomes. Furthermore, this study also indicates that the concentration of hydrolyzed collagen had no effect on them, and a 40% glycerol concentration provided better physicochemical characteristics of hydrolyzed collagen glycerosomes than a 20% glycerol concentration at concentrations of hydrolyzed collagen of 2.5% and 5%.

AUTHOR CONTRIBUTIONS

Conceptualization, N. I. S. F., T. E., A. M., W. S.; Methodology, N. I. S. F., T. E., A. M.; Validation, N. I. S. F., T. E., A. M.; Formal Analysis, N. I. S. F., T. E., A. M.; Investigation, N. I. S. F., T. E., A. M.; Resources, N. I. S. F., T. E., A. M.; Data Curation, H.D.M N. I. S. F., T. E., A. M.; Writing - Original Draft, N. I. S. F., T. E., A. M.; Writing - Review & Editing, N. I. S. F., T. E., A. M.; Visualization, N. I. S. F., T. E., A. M.; Supervision, N. I. S. F., T. E., A. M.; Project Administration, N. I. S. F., T. E., A. M.; Funding Acquisition, N. I. S. F., T. E., A. M.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Molecular Docking and Pharmacokinetic Studies of *Moringa oleifera* As Angiotensin-Converting Enzyme Inhibitors

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Abstract

Background: *Hypertension in pregnancy is a vascular disorder that occurs before pregnancy or arises during pregnancy that there were 30% of cases of maternal death. Moringa oleifera's potential to lower blood pressure can be utilized as an alternative antihypertensive during pregnancy, minimizing the risk of preeclampsia.* ***Objective****: The purpose of this study was to determine the molecular target of Moringa oleifera is intended to optimize pharmacodynamic activity based on the interaction pattern of the compounds with the ACE inhibitor (PDB ID: 1O86).* ***Methods****: Molecular docking is carried out using Autodock 4.0 program (AutoDock Tools).* ***Results****: According to the binding energy value and ACE inhibitory interaction, α-Rhamnopyranosyl, β-Sitosterol, and Sinalbin are prospective Moringa oleifera compounds as alternative antihypertensive. These potential compounds can inhibit ACE with binding energy -8.23; -9.27; -9.14 kcal/mol. Pharmacokinetic predictions reported that the potential compounds are absorbed in the intestine and indicates as molecules are tightly bound to plasma proteins and, as well as CYP3A4 and CYP2C9 inhibitors. The prediction of toxicity indicates that the potential compounds are classified as drug-induced acute liver failure with low carcinogens.* ***Conclusion****: α-Rhamnopyranosyl, β-Sitosterol and Sinalbin can be suitable lead compounds for synthetic drugs for antihypertensive agents.*

Keywords*: angiotensin-converting enzyme, molecular docking, Moringa oleifera*

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INTRODUCTION

Maternal mortality ratio (MMR) is one of the health parameters in determining the degree of quality of women's health, especially pregnant women. MMR is one of the parameters in achieving development goals through indicators of improving maternal health with the main target being reducing the risk of maternal mortality during pregnancy. Maternal and Reproductive Health in 2013 stated that there were cases of death of 8000 mothers due to complications of pregnancy and childbirth. The main causes of death are bleeding, preeclampsia with hypertension, infectious diseases and indirect causes. The 2018 Basic Health Research stated that there were 30% of cases of maternal death caused by hypertension in pregnancy (Ministry of Health RI, 2018).

Hypertension in pregnancy is a vascular disorder is defined as blood pressure ≥140/90 mmHg on two or more measurements that occurs before pregnancy or arises during pregnancy or during the puerperium. Based on the International Society for the Study of Hypertension in Pregnancy (ISSHP) there are 4 categories of hypertension in pregnancy, namely preeclampsia, gestational hypertension, chronic hypertension and preeclampsia-chronic hypertension. Hypertension in pregnancy is the main cause of maternal death, as well as having other serious effects during childbirth. It was occurring in 5% of all pregnancies (Karthikeyan, 2015). In the United States the incidence was reaches 6 to 10%, where there are 4 million pregnant women and an estimated 240.000 are accompanied by hypertension each year. Hypertension is a risk factor for stroke and its incidence increases in pregnancy where 15% of maternal deaths are caused by intracerebral bleeding (Malha *et al*., 2018).

The angiotensin-converting enzyme (ACE) serves as essential for managing hypertension. The kidneys convert angiotensin I to angiotensin II through the renin-angiotensin-aldosterone pathway, which then activates bradykinin. Some synthetic ACE inhibitors, including captopril, lisinopril, ramipril, and enalapril, are frequently utilized to treat hypertension (Atlas, 2007). The number of ACE inhibitors regulated for medicinal usage highlights the value of rational drug design methodologies based on receptor structural understanding as well as molecular modeling methods. ACE inhibitors are used to control high blood pressure, prevent strokes, treat left ventricular dysfunction, congestive heart failure, and nephropathy; nevertheless, common side effects include persistent cough, headache, dizziness, weakness, increased uric acid levels, and so on (Nathisuwan and Talbert, 2002).

Methyldopa is recommended as a hypertension-lowering drug in pregnancy, even women of childbearing age with hypertension who want to become pregnant are advised to replace antihypertensive drugs with methyldopa or nifedipine, labetalol. It turns out that in research Calcium Canal Blockers are superior to methyldopa in the prevention of pre-eclampsia. Undesirable effects of methyldopa are sedation, drowsiness, dry mouth, depression, postural hypertension, rebound hypertension, withdrawal syndrome, and several autoimmune events (Kario et al., 2018). A prospective observational cohort study has been conducted on 261 first trimester pregnancies given methyldopa compared to 526 pregnancies without hypertension. The result was that there was no significant increase in adverse events between the two. It was concluded that methyldopa has no indication of a teratogenic effect, although caution is required in administering methyldopa in the first trimester of pregnancy (Hoeltzenbein *et al*., 2017).

The potential of Indonesia's natural ingredients has led to great public interest in using traditional medicine empirically. This data is supported by the many explorations of Indonesian traditional medicine in research. Moringa leaves (*Moringa oleifera*) was once a prima donna of traditional medicine with its various properties, ranging from treating allergies, rheumatic pain, rheumatism, wound infections, lowering blood sugar levels and uric acid levels to lowering blood pressure. Moringa leaf decoction can reduce systolic and diastolic blood pressure (Yanti and Nofia, 2020). Moringa leaves are rich in potassium so that sodium levels in the blood can be controlled, which has implications for reducing high blood pressure (Aminah, 2015).

The potential of Moringa leaves to lower blood pressure can be utilized as an alternative antihypertensive in pregnancy as a preventive step in suppressing the risk factors of preeclampsia. However, the identification of these active compounds against macromolecules or the molecular action targets for reducing blood pressure is not clearly and significantly known. Early identification of the target of molecular action and the mechanism of action of a chemical substance can facilitate the optimization of drug activity. Bioinformatics analysis is a method for identifying targets of molecular action by comparing the similarity of the chemical structure of a compound to various compounds whose targets are known in a database.

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Identification of the target of molecular action of an active compound is intended to optimize directed pharmacodynamic activity based on the interaction pattern of the drug with the target. The challenge faced in identifying the target of molecular action of an active compound and its interaction pattern is a long and costly testing process. One of the efforts to deal with this problem is a computational experiment through an in-silico method approach with molecular docking techniques. The rapid development and current advances in computational techniques allow for in silico tests to speed up the process of selecting compounds to be synthesized (Talele *et al*., 2010).

Molecular docking provides a scoring function through molecular mechanics in the form of repulsion, hydrogen bonding, electrostatics and desolvation, as well as scoring results that show the affinity of the ligand and the interaction model for the target protein as an indication regarding the mechanism of action of the tested compound (Forli *et al*., 2016). By utilizing the molecular docking technique, the target proteins of several chemical constituents of moringa which have pharmacological activity as lowering blood pressure can be predicted and identified precisely based on scores and models of ligand and protein interactions based on calculations using the AutoDock 4.0 program in AutoDock Tools. To predict the pharmacokinetic and toxicity profiles of various chemical constituents of moringa that can interact with antihypertensive therapy target proteins using the ADMETlab 2.0 webserver.

MATERIALS AND METHODS

Preparation of macromolecules and ligands

ACE Inhibitor (PDB ID: 1O86) were identified through SuperPred (https://prediction.charite.de/), which are webserver for target predictions of compounds. The macromolecule downloaded the structure from PDB (https://www.rcsb.org) in .pdb format (Muhammad and Fatima, 2015). Three-dimensional structure of compounds as the ligands that have been created with VegaZZ in .PDB format. The ligands were optimized with AutoDock Tools. The preparation is done through AutoDock Tools by separating the native ligand and water molecules, as well as adding hydrogen atoms (Wati *et al.*, 2020).

Validation method

Validation was carried out on the native ligand to find the right conformation. The previously prepared macromolecules were redocked with the native ligand. The docking conformation is subsequently aligned with the native ligand conformation on the crystallographic structure represented in root-mean-square deviation (RMSD). The RMSD value states that the conformational alignment of the structure is still acceptable with a value of less than 2.0 Å, if it is smaller or closer to the value 0 then the alignment value is getting better (Xuan *et al.*, 2011).

Molecular Docking

The docking is carried out using the AutoDock 4.0 program with AutoDock Tools (ADT). Setting docking with rigid macromolecular format as well as GA Runs (200) and Population Size (150). Then select the Output submenu for Lamarckian GA (4.2). The results docking all the test ligands resulted in $G_{binding}$ (kcal/mol) which was then analyzed and visualized using the Discovery Studio Visualizer Biovia to see the shape or model of the anchorage formed (Hasan *et al.*, 2023).

Prediction of Pharmacokinetic and Toxicity Parameters

Prediction of pharmacokinetic and toxicity profiles was carried out online using webform ADMET Lab (https://admetmesh.scbdd.com). It has been widely recognized that absorption, distribution, metabolism, excretion and toxicity of potential compounds be evaluated for systematical as well as some physicochemical properties and medicinal chemistry friendliness. It is done by entering the SMILES code of the ligands retrieved from PubChem and clicking ADMET. The webserver will automatically standardize the submitted SMILES characters and calculate all of the endpoints. The prediction results are primarily displayed in tabular format in the browser, with a 2D molecular structure and a radar plot summarizing the compound's physicochemical properties. Concrete predictive values are provided for regression model-predicted endpoints such as Caco-2 permeability and plasma protein binding, among others Xiangya, 2021).

RESULTS AND DISCUSSION

Macromolecules preparation is the step in preparing the test protein in the molecular docking process by downloading the .pdb format in Protein Data Bank. The macromolecules were selected based on the identification of the ability of the potential compounds to target hypertension molecular action *Angiotensin-converting enzyme* (ACE) through *Super Prediction Anatomical Therapeutic Chemical (ATC).* As training data for the machine learning models, compounds with known ATC codes and target relations were used in the form of molecular fingerprints generated through their associated SMILES representation. For predicting targets, an artificial algorithm that learns was trained for

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each predictable target. The accuracy of the models was assessed using 10-fold cross-validation, with 82% of the target models obtaining at least 85% accuracy and only 5% scoring less than 70%. Due to the fact that the performance of the computational algorithms varies between targets, two different scores are reported, which produce the following outcomes: the probability that the input structure interacts with the target in question as determined by the corresponding machine learning hypothesis and the overall accuracy of the predictive algorithm (Gallo *et al.*, 2022).

The macromolecular requirements used are crystallographic structures (X-ray diffraction) with larger and more precise macromolecular structures. The conformational resolution value is also considered with a maximum RMSD value of 2.0 (Campbell, 2002). Downloaded macromolecules in .pdb format contain native ligand complexes and crystallized water molecules. The native ligand and water molecule must be removed from the macromolecule so as not to interfere with the molecular docking process. The native ligand is removed in order to obtain individual macromolecules that will be docked with the test ligand. Water molecules are removed because they can mediate the interaction between the test ligands and macromolecules which can affect the complexity of calculations in molecular docking. Additionally, the chemical environment is adjusted by removing non-residual amino acid molecules and adding charged atoms. Residues other than amino acids are removed because they can interfere with the interaction between the ligand and amino acid residues on the active site of the macromolecule. Removing residues other than amino acids needs to be reviewed if the macromolecule has a chromoprotein (Mg) or cofactor part that is retained in its main structure (Hypercube, 2002).

Macromolecule (ACE) with the identity PDB ID: 1O86 is the target protein chosen for first-line therapy in the treatment of hypertension, heart failure and myocardial infarction. This macromolecule with a crystallographic (X-ray diffraction) type of structure has formed a complex with lisinopril as the native ligand with a resolution of 2.0 Å (Natesh et al.,2003). Optimization of prepared macromolecules in Autodock Tools aims to determine parameters grid box. The grid box is analogous to the space where native ligands or active compounds can establish a conformation when anchored to macromolecule. The determination grid box was used to determine the coordinates of macromolecules' active sites. Arrangement grid box is done by setting the coordinates grid center and grid size (Rachmania et al., 2015). As for the coordinates grid center obtained are x= 40.935, y= 32.383 and z=47.285 with the setting grid size of 40x40x40.

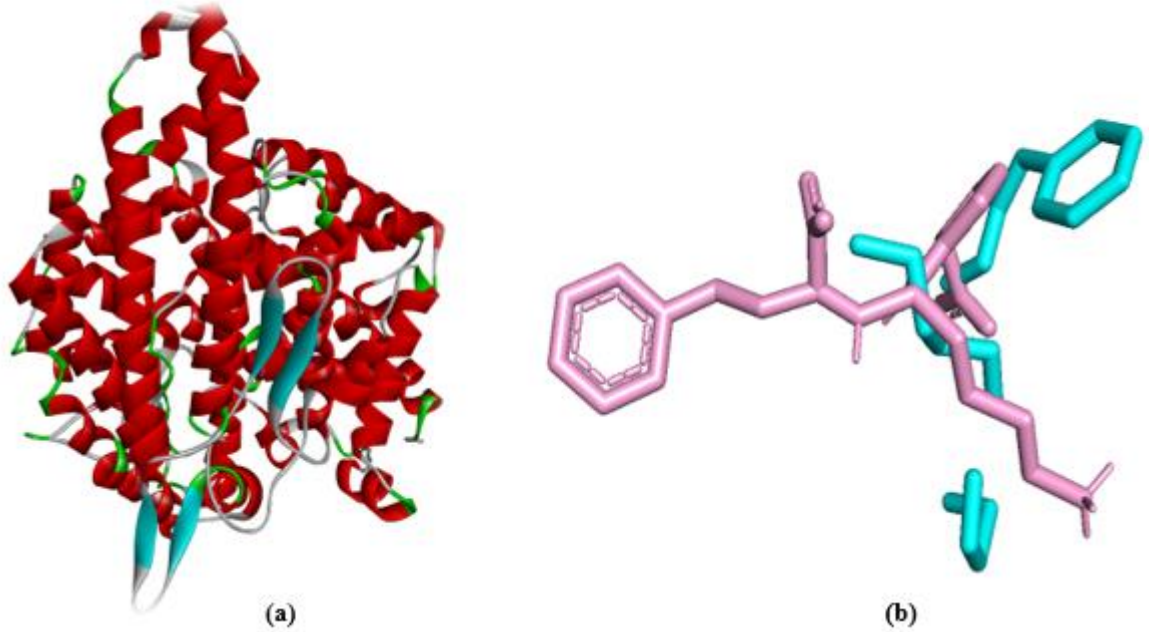

Figure 1. Macromolecule of ACE Inhibitor (PDB ID: 1O86) (a), Overlays of redocking ligand (pink) and reference ligand of crystallography data (blue) at 1O86 (b)

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Table 1. The results of molecular docking

Ligands	Bond energy (ΔG= kcal/mol)	Inhibitory constant (kI= uM)
Glucocochlearin	-6.48	17.93
Genistein	-6.65	13.44
Daidzein	-7.00	7.45
Niazirinin	-7.50	3.17
Niazidin	-7.38	3.88
Niazimicin	-7.10	6.24
Niazimin	-6.53	16.22
Niazine	-7.10	6.27
Niazim	-6.55	15.77
Niazicin	-6.68	12.70
α-Rhamnopyranosyl	-8.23	0.92452
Astragalin	-7.84	1.79
Vicenin	-3.76	1.75
Moringa	-5.67	69.70
β-Sitosterol	-9.27	0.15980
Hirsutrin	-7.55	2.91
Sinalbin	-9.14	0.20108
Amyrin	-5.65	72.43
Sitosteryl	-8.05	1.27
Chlorogenic	-7.68	2.33
Native ligand (Lisinopril)	-9.83	0.06216

The structure of ligands is studied in order to obtain a structure that is more convergent or able to concentrate on the binding pocket of the receptor. This is based on the amount of active torsion possessed by each tested ligand. Lisinopril as the native ligand has a number of bond rotations of 13 torsion. α-Rhamnopyranosyl, β-Sitosterol and Sinalbin as the best test ligands had a number of bond rotations of 16, 7 and 12 torsions respectively. The large number of active torsions can determine the search time for the best conformation and the results of molecular docking are longer and more difficult to obtain. Determination of the amount of active torque is intended to determine the active bonds that can rotate during the docking process (Rachmania *et al*., 2015).

The molecular docking parameter that determines the accuracy of the method is the suitability of the type of interaction of the tested ligand to the macromolecule aligned with the type of interaction of the native ligand. Table 2 describes the types of interactions that appear in the test ligands α-Rhamnopyranosyl, β-Sitosterol and Sinalbin as the test ligands with the lowest bond energy values. The mechanism of inhibition of the ACE enzyme by the inhibitor lisinopril is in the presence of a carboxy-alkyl carboxylic group bond with a zinc atom on the active site of ACE. Hydrogen bonds appear on the Glu384 residue and the oxygen atom is opposite the zinc atom on the active site of ACE. Van der Waals bonds appear in the phenylpropyl group with residues Val518 and hydrogen bonds Glu162, Lys511 and Tyr520 (Natesh, *et al*., 2003). α-Rhamnopyranosyl makes an H-bond interaction with Glu384 and Glu162. α-Rhamnopyranosyl and Sinalbin are well positioned to bind to the active-site zinc atom. Zinc is an important catalytic component of ACE and bound at the active site. Sinalbin makes an H-bond interaction with Lys511 and Tyr520. β-Sitosterol contains hydrogen bonds, but the amino acid residues are not identical to native ligand.

α-Rhamnopyranosyl has a molecular weight of more than 500 Da so that it has 1 deviating value for the Lipinski parameter as a condition for a good drug compound. Drugs with a molecular weight of more than 500 Da can make it difficult for the drug compound to penetrate the cell membrane of the target receptor. Sinalbin and β-Sitosterol complied with the Lipinski rules similar to Lisinopril (Lipinski, 2001). SA score is a medicinal chemical parameter that indicates the ease with which a compound can be synthesized. The lower the SA score (<6) indicates that the compound can be easily synthesized into drug products. α-Rhamnopyranosyl has the largest SA score so it has characteristics that tend to be quite difficult to synthesize compared to the native ligand and other tested ligands (Grinter and Zou, 2014).

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Table 2. The similarity of bonding interactions between potential ligands and native ligand

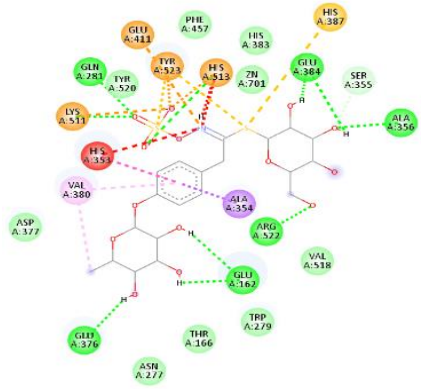
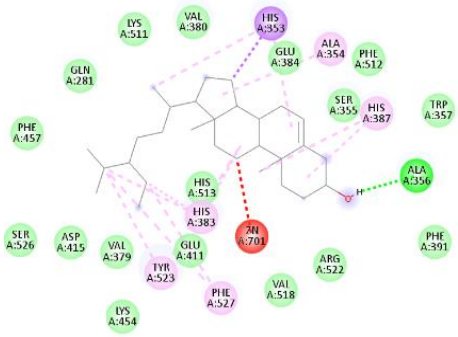
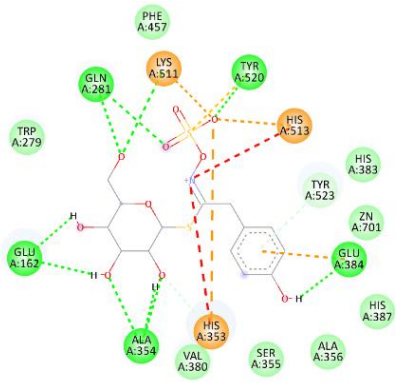
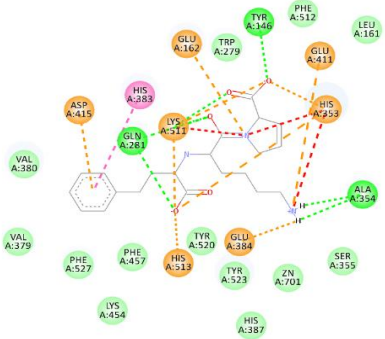
Ligands	Hydrogen bond Interactions	Non-Hydrogen bond Interactions	Visualization
α-Rhamnopyranosyl	Glu384, Glu162, Glu376, Ala356, Arg522, Gln281	Phe457, Tyr520, His383, Ser355, Trp279, Asn277, Asp377, Glu411, Lys511, His513, Zn701	
β-Sitosterol	Ala356	Lys511, Val380, Lys454, Ser526 Phe457, His513, His383, Glu384, Ser355, Phe512	
Sinalbin	Gln281, Tyr520, Lys511	Phe457, Ala356, His387, Ser355, Tyr523, His513, His353, Lys511, Glu384, Val380, Trp279, Zn701	
Native ligand (Lisinopril)	Ala354, Gln281, Tyr146, Glu384	Phe457, Phe527, His387, Ser355, Tyr523, Tyr520, His513, His353, Lys511, Val379, Val380, Trp279, Lys454, Leu161, Zn701	

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Table 3. Physicochemical parameters

Compounds	Physicochemical parameters						SA Score
	MW	nHA/nHD	nRot/nRing	Flex	TPSA	Log P	
Lisinopril	406.23	8/5	13/2	0.929	132.96	-1.535	3.172
α-Rhamnopyranosyl	571.10	15/8	9/3	0.429	245.26	-1.666	4.842
β-Sitosterol	414.39	1/1	6/4	0.300	20.230	7.663	4.388
Sinalbin	425.05	11/6	7/2	0.467	186.34	-1.130	4.225

Table 4. Pharmacokinetics and toxicity parameters

Compounds	Absorption		Distribution		Metabolism		Excrecy		Toxicity		
	Caco-2 Perm	HIA	PPB	VD	3A4 Inh	2C9 Inh	CL	$T_{1/2}$	DILI	ROAT	Carcino-genicity
Lisinopril	-6.194	+	19%	0.49	Yes	Yes	1.16	0.71	Yes	High	Low Carcinogen
α-Rhamnopyranosyl	-6.287	+	76%	0.65	Yes	Yes	0.78	0.23	Yes	High	Low Carcinogen
β-Sitosterol	-4.756	+	98%	1.96	Yes	Yes	16.6	0.01	Yes	Moderat	Low Carcinogen
Sinalbin	-6,163	+++	78%	0,32	Yes	Yes	0,74	0,61	Yes	Moderat	Low Carcinogen

Human Intestinal Absorption (HIA) is a parameter that describes the process of absorption in the intestine as a result of the bioavailability of absorption from the excretion ratio. An adequate HIA category is 20 – 70% (+) and good is 70 – 100% (++). The Caco2 parameter predicts the permeability of drug transport through intestinal epithelial cells derived from human colon adenocarcinoma with multiple transport pathways in vitro. The Caco2 cell parameter category is >70 nm/sec (high permeability); 4 – 70 nm/sec (medium permeability); <4 nm/sec (low permeability) (Cheng *et al*., 2013). Plasma Protein Binding (PPB) indicates that the drug molecule is tightly bound to plasma proteins. If the % PPB is below 90% it indicates that the molecule binds weakly to plasma proteins, and vice versa. The volume of distribution is a parameter that relates the drug concentration in blood plasma to the total amount of drug in the body. The optimal distribution volume parameter is 0.04 – 20 L/kg (Xiangya, 2021).

Drugs that are CYP2C19 and CYP2C9 inhibitors can increase plasma protein concentrations and sometimes cause side effects (Van Booven *et al*., 2010). CYP2D6 is responsible for the metabolism of most drugs and chemical compounds (Bertilsson *et al*., 2002). CYP2D6 is widely distributed in several tissues and is greatest in the liver (Ali et al., 2013). CYP3A4 is an enzyme that plays a major role in metabolism in the liver which is responsible for the oxidation process of small organic molecules, so they can be removed from the body (Dai *et al.*, 2001; Oyesakin *et al*., 2018).

Clearance (CL) is a parameter for determining the maintenance dose to achieve a desired plasma concentration or therapeutic concentration. High CL parameters with values >15 mL/min/kg and low CL values of <5 mL/min/kg. Half-life ($T_{1/2}$) is a parameter that predicts the time required for the drug level in the blood plasma to decrease to half the total level during the elimination phase. Short drug half-life is less than 3 hours and long drug half-life is more than 3 hours. All test ligands have short half-lives (Xiangya, 2021).

Drug induced liver injury (DILI) is a term that describes drug-induced acute liver failure. DILI is associated with direct dose-induced hepatotoxicity. There are two categories of hepatotoxicity, namely DILI with a high-risk category and no risk of hepatotoxicity. All potential compounds in Moringa leaves as antihypertensives are compounds with a risk of hepatotoxicity and are carcinogenic (Xiangya, 2021). Intravenous infusion of moringa extract at a dose of 40 mg/kg BW can reduce systolic pressure by 39.26 and diastolic by 20.79 mmHg in acetylcholine-induced rats (Mengistu, 2022). This is in line with research by Yanti and Nofia (2019), that Moringa leaf decoction can reduce systolic and diastolic blood pressure. Hypertension can be treated with Angiotensin Converting Enzyme Inhibitor (ACEI) drugs. ACE-Inhibitors inhibit the conversion of Angiotensin I to Angiotensin II resulting in vasodilation and decreased aldosterone secretion. In addition, the degradation of bradykinin is also inhibited so that blood levels of bradykinin increase and play a role in the vasodilatory

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effect of ACE inhibitors. Vasodilation will directly reduce blood pressure, while reduced aldosterone will cause water and sodium excretion and potassium retention (Nafrialdi, 2009). β-Sitosterol reduced serum creatinine levels in rat kidneys induced hypertension (Cadmium chloride) at a dose of 1.3 mg/kg/day ($p<0.05$) (Olaiya *et al*., 2014).

CONCLUSION

A molecular docking research suggests that potential compounds from Moringa oleifera, such as α-Rhamnopyranosyl, β-Sitosterol, and Sinalbin might be effective antihypertensive drugs due to their binding energy and amino acid interactions with ACE inhibitors. The results showed that the potential compounds to inhibit ACE with binding energy -8.23; -9.27; -9.14 kcal/mol. Pharmacokinetic predictions reported that the potential compounds are absorbed in the intestine and indicates as molecules are tightly bound to plasma proteins and, as well as CYP3A4 and CYP2C9 inhibitors. The prediction of toxicity indicates that the potential compounds are classified as drug-induced acute liver failure with low carcinogens.

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AUTHOR CONTRIBUTIONS

Conceptualization, RA.H.; Methodology, RI.H.; Software, RI.H.; Validation RA.H.; Formal Analysis, RA.H.; Investigation, RA.H.; Resources, RI.H.; Data Curation, RI.H.; Writing - Original Draft, RA.H.; Writing - Review & Editing, RI.H.; Visualization, RA.H.; Supervision, RA.H.; Project Administration, RA.H.; Funding Acquisition, RA.H.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Cost-Consequence Analysis of Levofloxacin Compared to Ceftriaxone in Community-Acquired Pneumonia of Adult Inpatients at X Hospital Surakarta

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Abstract

Background: *Community-acquired pneumonia is still a significant cost-burden disease in healthcare facilities. Pharmacoeconomic analysis using the cost-consequence analysis (CCA) method of ceftriaxone compared to levofloxacin as a first-line empirical antibiotic has never been carried out.* ***Objective****: to model the clinical and economic impact of administering ceftriaxone as a first-line empirical antibiotic compared to its comparator levofloxacin for community-acquired pneumonia therapy in hospitalized adult inpatients from the perspective of healthcare facilities.* ***Methods****: This research is a retrospective observational study that collects medical records and patient billing data in X Hospital Surakarta from January to December 2022 period. The study was conducted from June to July 2023. Subjects were adult inpatients aged ⩾18 years with community-acquired pneumonia and were given levofloxacin or ceftriaxone as first-line empiric antibiotics. The data taken included patient profile, antibiotic effectiveness and direct medical costs. Cost-consequence analysis (CCA) was used to compare levofloxacin to ceftriaxone to assess their impact on length of stay, antibiotic effectiveness, and direct medical costs based on a healthcare perspective.* ***Results****: The antibiotic effectiveness for levofloxacin was 75.00%, and ceftriaxone was 93.33%. The average length of stay for levofloxacin was 3.39 days, and ceftriaxone was 3.00 days. The total direct medical costs for levofloxacin were IDR 2,056,799, and ceftriaxone was IDR 1,969,627.* ***Conclusion****: The administration of ceftriaxone to levofloxacin as a first-line empirical antibiotic for community-acquired pneumonia in hospitalized adult patients had the consequence of increasing antibiotic effectiveness, reducing the length of stay and saving total direct medical costs by IDR 87,172.*

Keywords*: ceftriaxone, community-acquired pneumonia, cost-consequence, levofloxacin*

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INTRODUCTION

Health problems are still a concern of the world, especially in developing countries such as ASEAN countries, including Indonesia. One of the diseases that is still a concern for the World Health Agency is pneumonia. Pneumonia is a major cause of death and hospitalization worldwide, as well as being a large user of healthcare resources and costs. The burden of health costs due to community-acquired pneumonia worldwide is enormous, and most of it is contributed by patient hospitalization costs (Peyrani *et al*., 2019). Pneumonia is still one of the most significant health problems for children under five years of age (toddlers) (Oktaria & Mahendradhata, 2022). Pneumonia, as an infectious disease in humans, also contributes to relatively high morbidity and mortality rates (Farida *et al*., 2020).

It is possible that pneumonia will still overshadow Indonesia's health year after year in view of the bacterial resistance and mutation of viruses nowadays, which are still a problem. The problem of bacterial resistance is also an essential issue in the field of public health in Indonesia. These health problems are a burden for countries, including Indonesia, which is currently still developing. In general, bacterial pathogens that cause Community-Acquired Pneumonia (CAP) include *Streptococcus pneumoniae, Haemophilus influenzae, Mycoplasma pneumoniae, Staphylococcus aureus, Legionella species, Chlamydia pneumoniae, Moraxella catarrhalis* (Metlay *et al*., 2019). The emergence of *Methicillin-Resistant Staphylococcus Aureus* (MRSA) as a superbug (Nandhini *et al*., 2022) has drawn world attention to its handling and treatment costs, especially in pneumonia. The cost of treating antibiotic-resistant bacteria is undoubtedly increasing and becoming more expensive. Bacterial sensitivity test data at a Teaching Hospital in Surakarta City shows that the microorganisms that cause pneumonia in the hospital are *Streptococcus pneumoniae* (28%) followed by *Candida sp*. (21%), *Pseudomonas aeruginosa* (8.8%), *Klebsiella pneumoniae* (8.8%), and *Streptococcus viridans* (8.8%) where *Streptococcus pneumoniae* is sensitive to levofloxacin antibiotics (81.25%), ceftriaxone (62.5%), and ampicillin (75%) (Farida *et al*., 2019).

The cost of health care is the main concern of the Indonesian government through Badan Penyelenggara Jaminan Sosial (BPJS). The analysis of health costs has been paid attention to amidst the increasing cost of health in Indonesia. Pharmacoeconomics through cost-consequence analysis (CCA) can be a method for comparing the costs and consequences of applying a comparative drug to alternative drugs. Pharmacoeconomics can be a solution for health policymakers to determine the most rational drug therapy choice.

Pneumonia can attack anyone from young age to old age. WHO data from 2019 states that pneumonia was the cause of death in 14% of children under five. RISKESDAS 2018 data states that the prevalence of pneumonia in those aged 55 - 64 years is 2.5%, aged 65 - 74 years is 3.0%, and aged 75 years and over is 2.9% (Hatim, 2022). Data from the Central Statistics Agency of Surakarta (BPS) states that the number of pneumonia cases in Surakarta City in 2019 was 164 cases, in 2020 it was 178 cases, and in 2021 it was 262 cases (Dinas Kesehatan Kota Surakarta, 2022). The number of pneumonia cases has increased every year from 2019 to 2021. This is a concern on how to control the number of pneumonia cases so that they do not grow in number.

Pneumonia is a disease with a high burden in adults and is characterized by high morbidity (McLaughlin *et al*., 2020). The high cost of treatment and loss of productivity due to pneumonia is a significant economic burden for the government and the general public (Ekirapa-Kiracho *et al*., 2021). This makes it essential to do pharmacoeconomic evaluations for pneumonia to get the best therapy at an efficient cost.

Several recent economic evaluation studies regarding the use of antibiotics for pneumonia in hospitalized adult inpatients have been conducted by several researchers. Research at the Ajibarang Regional Hospital for the 2021 period concluded that giving ceftazidime antibiotic therapy was more cost-effective than ceftriaxone by comparing ACER values (Susanto *et al*., 2022). Research at RSU Karsa Husada, Batu City, from 2017 to 2018 found that the antibiotic levofloxacin was more cost-effective compared to ceftriaxone, cefotaxime and ciprofloxacin injections (Kolbiyah, 2019). Research at RST DD Hospital with adult inpatients found that the antibiotic ceftriaxone was more cost-effective than ceftizoxime for treating community-acquired pneumonia with indicators of the number of recovered patients and length of stay (Susanti *et al*., 2022). A Cost-Effectiveness Analysis study at the West Nusa Tenggara Provincial Hospital found that the antibiotic levofloxacin was more cost-effective than ceftriaxone for treating community-acquired pneumonia (CAP) (Rahmawati *et al*., 2023). The results of the study by Farida, Khoiry and Hanafi (2022) stated that the use of antibiotics for the treatment of pneumonia was ceftriaxone, levofloxacin, and the combination of ceftriaxone + azithromycin with the ACER value of the antibiotic levofloxacin was the most cost-effective

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compared to ceftriaxone and the combination of ceftriaxone + azithromycin.

Pharmacoeconomic studies using the cost-consequence analysis method for community-acquired pneumonia (CAP) have not been widely done. The study by Torres *et al*. (2020) has done an economic analysis of ceftaroline fosamil compared to other antibiotics for community-acquired pneumonia therapy in hospitalized adult patients with moderate/severe CAP using the cost-consequence analysis method. It is known that ceftaroline fosamil can be an alternative therapy compared to ceftriaxone, levofloxacin and the combination moxifloxacin – co-amoxiclav because the total cost of treatment is lower and clinical outcomes are relatively better than comparators (Torres *et al*., 2020).

Levofloxacin and ceftriaxone are broad-spectrum antibiotics widely used as first-line empiric antibiotics for community-acquired pneumonia (Farida *et al*., 2020; Kresnawati *et al*., 2021; Rahmawati *et al*., 2023; Sukriya *et al*., 2022). The big difference in drug unit prices between levofloxacin and ceftriaxone for antibiotic therapy causes the total cost of hospitalization with levofloxacin to be more expensive than ceftriaxone (Aoralia, 2022). Pharmacoeconomic evaluation through cost-consequence analysis for using the alternative drug (ceftriaxone) with its comparator (levofloxacin) for community-acquired pneumonia therapy has yet to be done nowadays. This study aimed to describe the clinical and economic impact of administering ceftriaxone as a first-line empirical antibiotic compared to its comparator levofloxacin for community-acquired pneumonia therapy in hospitalized adult inpatients from the perspective of healthcare facilities.

MATERIALS AND METHODS

Method

Study design

This research is a retrospective observational study with a cohort method that collects data retrospectively. The sampling technique is total sampling, where all data that meets inclusion during the period of January to December 2022 is taken. This research was carried out from June until July 2023 at a secondary-care hospital in Surakarta. This research received ethical clearance from the Health Research Ethics Committee of Kusuma Husada Surakarta University with the number 119/UKH.L.02/EC/IX/2022.

The inclusion criteria of this study were hospitalized adult inpatients in third-class rooms≥ 18 years old who were diagnosed with pneumonia with the ICD-10 code of J.12 - J.18 in the medical record (CAP cases confirmation in this study was that patients with a discharge date ⩽ 14 days before the index date were excluded from the study) (Konomura *et al*., 2017), patient was given levofloxacin or ceftriaxone injection as first-line empiric antibiotic therapy by the doctor at the time of initial hospitalization, self-paid and insurance (BPJS) category patients are all included because both of the direct medical costs are the same. The exclusion criteria for this study were patients with infections other than pneumonia (including COVID-19 positive), patients with severe comorbidities (cancer, immunocompromised, hematemesis melena), incomplete or missing medical record or patient billing, and patients who were forced to go home, died or referred to another hospital.

The data taken in this study were patient profiles, length of stay (LOS), antibiotic effectiveness and direct medical cost data. The patient profile includes age, gender and comorbidities. Length Of Stay (LOS) per antibiotic was determined by the patient being admitted to be hospitalized until they were allowed to go home by the doctor in charge. Antibiotic effectiveness was the antibiotic success rate of levofloxacin and ceftriaxone, which was defined as the ratio of the percentage of patients treated successfully (improved or cured) by levofloxacin or ceftriaxone antibiotic divided by the total number of patients given those antibiotics. The patient's treatment is stated to be successful (improved or cured) if the administration of levofloxacin or ceftriaxone as a first-line empiric antibiotic could improve the patient's clinical stability, marked by temperature ⩽ 37.8C, pulse rate ⩽ 100x/minute, respiratory rate ⩽ 24x/minute, systolic blood pressure ⩾ 90 mmHg, no requires oxygen supplements and can take oral medication and switching antibiotics from intravenous to oral. Besides, there is clinical improvement in one of four symptoms from baseline (cough, dyspnea, pleuritic chest pain, sputum production) with none worsening. The patient's treatment was stated to have failed if the patient did not achieve clinical stability and improvement and/or the antibiotic regimen was changed.

Direct costs were service costs, medication costs, and laboratory/diagnostic test costs. Service costs included room costs, medical procedures and doctor visits. Medication costs included costs of antibiotics, supporting drugs, pharmacy costs and consumables material. Laboratory/diagnostic test costs included costs for clinical laboratory tests (cost of electrolytes, blood counts, and blood chemistry test), diagnostic tests (chest

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x-ray) and microbiological sensitivity tests if needed. All fees were calculated in Indonesian Rupiahs (IDR) and based on the hospital's perspective.

Research data will be analyzed using the cost-consequence analysis (CCA) method by comparing the effectiveness of antibiotics, length of stay and direct medical costs. The comparison of costs and their consequences will be displayed in tabular form and analyzed descriptively.

Study population

The population of this study were adult inpatients with community-acquired pneumonia (CAP) that are given levofloxacin or ceftriaxone antibiotics and were hospitalized at X Hospital Surakarta in third-class room at least one night in the period of January to December 2022. The sampling method was total sampling, which took all samples from January to December 2022 as long as they met the inclusion criteria.

Materials

This research requires two groups of data: patient medical records (MR) and direct medical cost data, which met the inclusion criteria from January to December 2022 at X Hospital Surakarta. The patient's medical record was used to get patient profiles and clinical data. Patient direct medical cost data was taken from the billing print-out of patient care costs at the hospital cashier. Patient cost data was the direct medical costs of patient care during hospitalization.

Tools

This research requires a data collection form that was made as a tool to help in collecting patient data. This research also used Microsoft Office Excel 2019 and SPSS version 20 software to analyse the data.

Data analysis

The data obtained was analyzed descriptively and presented in tables. Patient profile data (gender, age, comorbidities) were analyzed using the Chi-Square test if it met the requirements and the Fisher' 's Exact or Kolmogorov Smirnov test if it did not meet the Chi-Square test requirements. Outcome data, which were length of stay (LOS) and antibiotic effectiveness, were analyzed using the Chi-Square test if it met the requirements and Fisher' 's Exact test if it did not meet the Chi-Square test requirements. Cost data was presented by the unit value of the Indonesian Rupiahs (IDR), and the significance value was analysed using a student-t-test if the data were normal and a Mann-Whitney test if they were not. Data analysis was continued with sensitivity analysis by calculating the base-case value at its lowest and highest cost value for each direct medical cost (service cost, medication cost, and laboratory/diagnostic test cost) of levofloxacin and ceftriaxone antibiotics.

RESULTS AND DISCUSSION

Subject selection

There were 146 data of adult inpatients diagnosed with pneumonia who were hospitalized from January to December 2022 at X Hospital Surakarta. Exclusions included missing medical records (n = 4), patient died (n = 21), missing patient print-out billings (n = 7), infections other than pneumonia (n = 12), patient's age was less than 18 years old (n = 1), there were severe comorbidities (n = 20), the patient was hospitalized other than third-class room (n = 11), and the patient who were given other empiric antibiotics for first-line empiric treatment besides levofloxacin and ceftriaxone (n = 33). The sample screening flow is shown in Figure 1.

The samples that met the inclusion criteria and were taken as subjects in this study were 37 patients divided into two groups (levofloxacin and ceftriaxone groups). The levofloxacin group consisted of 23 samples, and the ceftriaxone group consisted of 14 samples. Levofloxacin and ceftriaxone are the antibiotics most commonly prescribed in cases of pneumonia or lower respiratory tract infections (Farida *et al*., 2022; Rahmawati *et al*., 2023).

Samples were selected based on predetermined exclusion criteria. A total of 4 patients had missing medical records. Twenty-one patients were deceased and had their medical records recorded. Seven patients' billing data had not been found. Twenty-two patients had infections other than pneumonia, including patients who tested positive for COVID-19. There was one patient aged less than 18 years. Ten patients had comorbidities that met the exclusion criteria. There were 11 patients hospitalized in rooms other than the third-class room. Thirty-three patients were given other empiric antibiotics as first-line antibiotic therapy besides levofloxacin and ceftriaxone.

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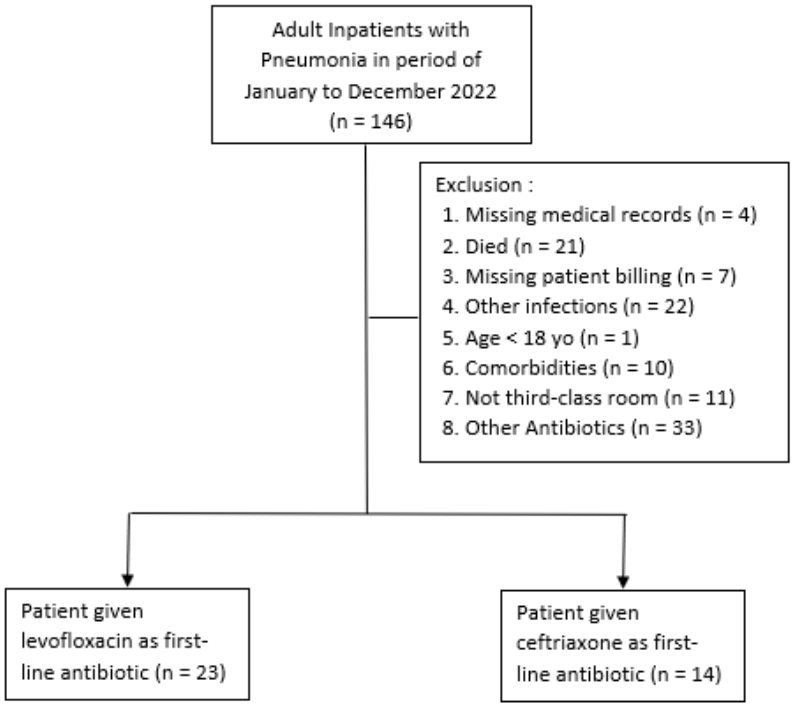

Figure 1. Sample screening flow

Patients with COVID-19 infections were not included in the study because COVID-19 is an infection by the SARS-CoV-2 virus that is different in general management from pneumonia (Bhimraj *et al*., 2022; COVID-19 Treatment Guidelines Panel, 2023; World Health Organization, 2021) and has an ICD-10 diagnosis code that is different from pneumonia, namely U07.1 (World Health Organization, 2019). COVID-19 disease has specific antiviral therapy treatment for the SARS-CoV-2 virus, including remdesivir, favipiravir or other antivirals with additional symptomatic therapy and administration of antibiotics if there are indications of bacterial infection (World Health Organization, 2021). Patients with comorbidities who were not included in this study were patients with comorbidity of cancer or tumours, liver disease and hematemesis melena. Cancer is a disease with high costs, many cases occurring, and high risk (Aisyah *et al*., 2018), so it was not included in this study. Meanwhile, liver diseases such as fatty liver, cirrhosis, which leads to variceal bleeding and hematemesis melena require expensive endoscopy procedures (Bakar, 2016) and, in some instances, require prophylactic antibiotics if the infection is indicated (Yoshiji *et al*., 2021). This study was limited to third-class room inpatients to limit variations in costs between inpatient room classes. The initial empirical antibiotic treatment included ampicillin-sulbactam, cefotaxime, ceftazidime, a combination of azithromycin and ceftriaxone, meropenem, a combination of cefuroxime and metronidazole, and a combination of azithromycin and levofloxacin. Cefuroxime was administered less frequently than levofloxacin and ceftriaxone.

Patient profiles

The descriptive data consisted of patient profiles that met the inclusion criteria in two groups, namely the levofloxacin group (23 patients) and the ceftriaxone group (14 patients), with a total of 37 patients. The patient profile was analyzed based on gender, patient age and comorbidities. Patient profiles can be seen in Table 1.

Patient gender profile data showed that there were 23 female patients more compared to 14 male patients. These results were in accordance with several previous studies where there were more women patients than men (Sukriya *et al*., 2022; Susanto *et al*., 2022). This result was different from other studies, which stated that the number of male patients dominated female patients (Farida *et al*., 2022; Susanti *et al*., 2022). The significance value (p-value) of gender data between the levofloxacin group and the ceftriaxone group was 0.365 (more than 0.05), so there was no significant difference between the two groups. The results of the gender data found that gender did not affect the prevalence of pneumonia because each region or hospital could have different results.

The result of age data showed that the total data for patients aged 51 - 60 years and 61 - 70 years were the

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most significant number of adult patients hospitalized during the study period, with 11 patients each. These results show that patients aged 51 to 70 years were vulnerable to pneumonia. This data was similar to the results of research by Susanto *et al*. (2022), in which the most significant number of adult inpatients were aged 56 - 65 years. In another study, it was found that patients aged more than 56 years were susceptible to pneumonia in, more significant numbers than young adults (less than 56 years) (Farida *et al*., 2020). The significance value of patient age data showed that there were no significant differences between the two groups, with a p-value of 0.625 (more than 0.05). The results of patient age data found that patients aged over 50 years were more susceptible to pneumonia than younger people.

The most common comorbidities are cardiovascular disorders, respiratory disorders, and diabetes mellitus, with the number of cases being 18 patients, 16 patients, and 13 patients, respectively. Comorbidities of cardiovascular disorders and diabetes mellitus were suffered mainly through the ceftriaxone group sample. Meanwhile, comorbidities of respiratory disorders were primarily suffered mainly by the levofloxacin group sample. This was in accordance with research at an academic hospital in Sukoharjo that found that cardiovascular disorders, respiratory disorders, and diabetes mellitus occupied the most cases in pneumonia patients (Farida *et al*., 2020). In another study, it was found that the most common comorbidities in hospitalized adult pneumonia cases were cardiovascular disorders and respiratory disorders (Susanti *et al*., 2022).

Table 1. Pneumonia patient profiles of adult inpatients at X Hospital Surakarta period of January – December 2022

No	Patient Profiles	Levofloxacin (n = 23, %)	Ceftriaxone (n = 14) (%)	Total (n = 37) (%)	p-value*
1	**Gender**				
	Male	10 (43)	4 (29)	14 (38)	0.365[A]
	Female	13 (57)	10 (71)	23 (62)	
2	**Age (years old)**				
	18-20	0 (0)	0 (0)	0 (0)	
	21-30	0 (0)	0 (0)	0 (0)	
	31-40	0 (0)	1 (7.1)	1 (2)	
	41-50	1 (4)	3 (21.4)	4 (11)	
	51-60	9 (39)	2 (14.3)	11 (30)	0.625[C]
	61-70	9 (39)	2 (14.3)	11 (30)	
	71-80	2 (9)	2 (14.3)	4 (11)	
	81-90	2 (9)	2 (14.3)	4 (11)	
	91-100	0 (0)	2 (14.3)	2 (5)	
3	**Comorbidities**				
	Other respiratory disorders	14 (32)	2 (6)	16 (21)	0.006[A]
	Diabetes mellitus	5 (12)	8 (23)	13 (17)	0.039[B]
	Hypoglicemia	0 (0)	1 (3)	1 (1)	0.378[B]
	Cardiovascular disorders	6 (14)	12 (34)	18 (24)	0.000[A]
	Stroke	0 (0)	1 (3)	1 (1)	0.378[B]
	Hypokalemia	5 (12)	3 (8)	8 (11)	1.000[B]
	Hyponatremia	2 (5)	2 (6)	4 (5)	0.625[B]
	Increased transaminase	1 (2)	0 (0)	1 (1)	1.000[B]
	Urology disorders	4 (9)	4 (11)	7 (9)	0.445[B]
	Dyslipidemia	0 (0)	1 (3)	1 (1)	0.378[B]
	Hematology disorders	2 (5)	0 (0)	2 (3)	0.517[B]
	Gastrointestinal disorders	2 (5)	0 (0)	2 (3)	0.517[B]
	Thyroid disorders	1 (2)	0 (0)	1 (1)	1.000[B]
	Skin disorders	1 (2)	0 (0)	1 (1)	1.000[B]
	Hypoalbuminemia	0 (0)	1 (3)	1 (1)	0.378[B]

***** Data from 37 patients who were hospitalized with a significance value (p-value) > 0,05 means there is no significant difference between the groups. A (Chi-Square Test), B (Fisher`s Exact Test), C (Kolmogorov-Smirnov Test)

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Table 2. Length of stay patients

Length of Stay (LOS)	Levofloxacin (n = 23) (%)	Ceftriaxone (n = 14) (%)	Total (n = 37) (%)	p-value*
	average = 3.39 days	**average = 3.00 days**	**average = 3.24 days**	
1 - 4 days	20 (87)	14 (100)	34 (92)	0.275
5 - 8 days	3 (13)	0 (0)	3 (8)	

*****Data from 37 patients who were hospitalized with a significance value (p-value) > 0,05 means there is no significant difference between the groups. P-value was determined using Fisher's Exact Test

Table 3. Antibiotic effectiveness of levofloxacin and ceftriaxone for the treatment of pneumonia

Antibiotic effectiveness	Failure (n = 7)	Success (n = 30)	Success percentage (%)	p-value*
Levofloxacin (n = 23)	6	17	75 %	0.217
Ceftriaxone (n = 14)	1	13	93.33 %	

*The significance value (p-value) was analyzed using Fisher's Exact test

The significance value of comorbidities was less than 0.05 for cardiovascular disorders, respiratory disorders, and diabetes mellitus, with p-values of 0.000, 0.006, and 0.039, respectively. This means that there was a significant difference (p-value less than 0.05) between the levofloxacin and ceftriaxone groups in terms of comorbid data on cardiovascular disorders, respiratory disorders and diabetes mellitus. Comorbid cardiovascular disorders and diabetes mellitus were most commonly suffered by patients in the ceftriaxone group. In another study, it was found that the highest usage of the ceftriaxone antibiotic (the highest DDD) was given to patients with cardiovascular disease along with diabetes mellitus and hypokalemia (Sukriya *et al*., 2022). Other research also states that ceftriaxone was often given to respiratory infection cases in South India (Sriram *et al*., 2013). Doctors' preferences in giving antibiotics to their patients influence the antibiotics used. Comorbid respiratory disorders occurred more frequently in patients in the levofloxacin group. This might be because levofloxacin is the fluoroquinolone antibiotic of choice for respiratory disorders *(Metlay et al*., 2019; Perhimpunan Dokter Paru Indonesia, 2014). Other research also found that the antibiotic levofloxacin was often given to adult pneumonia inpatients with comorbid respiratory disorders (Farida *et al*., 2020). It was found that there were no significant differences between other comorbidities between the two drug groups, with a significance value of more than 0.05.

The patient's length of stay (Table 2) was calculated using the length of stay (LOS) parameter obtained from medical record data with a length of stay of 1 to 4 days, totalling 34 patients and 5 to 8 days, totalling three patients. This data was similar to other similar studies in that the length of stay for most patients was five days or less in the levofloxacin and ceftriaxone groups (Farida *et al*., 2020; Putri; *et al*., 2018). The significance value (p-value) for the two antibiotic groups was 0.275, which means that there was no significant difference in the length of stay data for the levofloxacin group and the ceftriaxone group. However, if the average length of stay was calculated, it was found that the ceftriaxone group had the shortest average length of stay at 3.00 days compared to the levofloxacin group with 3.39 days. This means that patients in the ceftriaxone group went home sooner than those in the levofloxacin group.

Antibiotic effectiveness for pneumonia

Antibiotic effectiveness was determined using the antibiotic success rate parameters presented in Table 3. The success rate for antibiotics in the levofloxacin group with successful therapy was 17 patients, while failed therapy was six patients with a success rate percentage of 75%. The antibiotic success rate for the ceftriaxone group was 13 patients with successful therapy and one patient with failed therapy, with a success rate percentage of 93.33%. The significance value (p-value) was 0.217, which means that there was no significant difference between the two antibiotic groups. This followed the results of previous research that the effectiveness of levofloxacin and ceftriaxone as first-line empirical antibiotics was statistically the same (Rahmawati *et al*., 2023). The antibiotic success rate was different from the results obtained in other studies where the success rate of levofloxacin was higher than ceftriaxone (Farida *et al*., 2022; Sukriya *et al*., 2022). This might be caused by different comorbidities in each other group, which can affect the outcome of the success rate of both antibiotics (Sukriya *et al*., 2022). This difference also might be due to different germ resistance patterns in the hospitals where the research was carried out.

Table 4. Direct medical costs of pneumonia of adults inpatients in X Hospital Surakarta

	Levofloxacin (n = 23)		Ceftriaxone (n = 14)		p-value*
	Cost	Percentage (%)	Cost	Percentage (%)	
Average service cost	IDR485,609	23.6	IDR602,857	30.6	0.481
Average medication cost	IDR1,267,799	61.6	IDR945,199	48.0	0.008
Average laboratory/diagnostic test cost	IDR303,391	14.8	IDR421,571	21.4	0.012
Average total cost	IDR2,056,799	100	IDR1,969,627	100	0.079

* Significance value (p-value) was analyzed using the Mann-Whitney test

Table 5. Cost-consequence analysis (CCA) of levofloxacin and ceftriaxone group for pneumonia therapy

Antibiotic Groups	Therapeutic effectiveness	Effectiveness Difference	Length of stay (LOS)	LOS difference	Total direct medical cost	Cost difference
Levofloxacin (reference)	75.00%	-18,33%	3.39 days	0.39 days	IDR 2,056,799	IDR 87,172
Ceftriaxone (alternative)	93.33%		3.00 days		IDR 1,969,627	

Cost of pneumonia therapy

Direct medical costs (Table 4) are divided into three cost groups, which are service costs, medication costs, and laboratory/diagnostic test costs. Service cost data showed that the average cost for the levofloxacin group was IDR 485,609 with a percentage of 23.6% of the average total cost, and the ceftriaxone group was IDR 602,857 with a percentage of 30.6% of the average total cost. The significance value for the service cost group showed a p-value of 0.481, so there was no significant difference between the two groups. Service costs were the costs of services provided by the hospital while the patient is hospitalized.

Medication cost data showed that the average cost for the levofloxacin group is IDR 1,267,799 with a percentage of 61.6% of the total cost, and the ceftriaxone group is IDR 945,199 with a rate of 48.0% of the total cost. The significance value for the medication cost group showed a p-value of 0.008, which means there was a significant difference in medication costs between the two groups. Medication costs were all costs for administering medication to patients along with disposable materials during hospitalization, including the medications the patient takes home.

Laboratory/diagnostic test cost data showed that the average cost in the levofloxacin group was IDR 303,391 with a percentage of 14.8% of the total costs, and in the ceftriaxone group, was IDR 421,571 with a percentage of 21.4% of the total costs. The significance value in the medical cost group showed a p-value of 0.012, which means there was a significant difference in laboratory/diagnostic test costs between the two groups. Laboratory/diagnostic test costs were all laboratory/diagnostic test costs related to pneumonia for patients, such as chest x-ray costs, blood cell counts and electrolytes.

The average total cost was the summation of the average service costs, medication costs and laboratory/diagnostic test costs per antibiotic group. In the levofloxacin group, it was found that the average total cost was IDR 2,056,799. In the ceftriaxone group, it was found that the average total cost was IDR 1,969,627. The significance value was obtained with a p-value of 0.079, which means there was no significant difference in the average total cost between the two antibiotic groups.

The medication costs for the levofloxacin group were more expensive than those for the ceftriaxone group and were significantly different between both groups based on the p-value. The high cost of medication in the levofloxacin group was caused by the cost of levofloxacin antibiotics, which were much more expensive than the cost of ceftriaxone antibiotics. In addition, the high cost of medication in the levofloxacin group was supported by the cost of corticosteroid infusion drugs such as hydrocortisone infusion. In the ceftriaxone group, the high cost of medication was caused by the cost of other drugs than ceftriaxone itself, such as albumin infusion, insulin and complementary drugs cost. This was in accordance with other studies that found that the average total direct cost of levofloxacin is higher than ceftriaxone (Sriram *et al*., 2013).

Laboratory/diagnostic test costs in the ceftriaxone group were higher than those of the levofloxacin group and were significantly different between both groups based on the p-value. The higher laboratory/diagnostic test costs in the ceftriaxone group were due to the cost of blood chemistry tests being more frequent in the

ceftriaxone group compared to levofloxacin. Blood chemistry tests carried out on pneumonia patients include inflammatory biomarker tests and blood gas analysis (Julianti *et al*., 2023). Apart from that, blood chemistry tests also included checking blood glucose and lipids. The number of patients with cardiovascular comorbidities in the ceftriaxone group might have influenced the cost of blood chemistry tests.

It showed that laboratory/diagnostic test costs were more expensive in the ceftriaxone group than in the levofloxacin group (p-value 0.012). Meanwhile, medication costs were more expensive in the levofloxacin group compared to the ceftriaxone group (p-value 0.008).

Cost consequence analysis (CCA)

The cost-consequence analysis (CCA) of the levofloxacin and ceftriaxone groups for community-acquired pneumonia therapy can be seen in Table 5.

The therapeutic effectiveness of the ceftriaxone group was higher than that of the levofloxacin group, with a difference of 18.33%. This means that using levofloxacin as a first-line empirical antibiotic caused a reduction in the therapeutic effectiveness of community-acquired pneumonia therapy by 18.33% compared to ceftriaxone, but this was not statistically significantly different (p-value = 0.271).

Length of stay can be a consequence of administering antibiotics in an infection therapy. In the levofloxacin group, the average length of stay for inpatients in the hospital was 3.39 days. In the ceftriaxone group, the average length of stay for inpatients in the hospital was 3.39 days. In the levofloxacin group, it was shown that administration of levofloxacin had the consequence of increasing the length of stay for community-acquired pneumonia patients who were hospitalized by 0.39 days longer than the ceftriaxone group. However, this is not statistically significantly different (p-value 0.275).

The difference in total direct medical costs in the levofloxacin group and the ceftriaxone group was IDR 87,172. This shows that administering levofloxacin to adult community-acquired pneumonia patients hospitalized in hospitals had the consequence of increasing costs by IDR 87,172. Hospitals could save IDR 87,172 if they use ceftriaxone as the first-line empirical antibiotic for community-acquired pneumonia therapy in hospitalized adult patients instead of levofloxacin. This was different from the results of other studies that stated that levofloxacin is more cost-effective than ceftriaxone (Farida *et al*., 2022; Kolbiyah, 2019; Rahmawati *et al*., 2023). Differences in results occurred because, in those studies, the effectiveness of levofloxacin was higher than that of ceftriaxone, resulting in more cost-effectiveness.

Sensitivity analysis

Sensitivity analysis was done to determine the sensitivity of the direct medical costs to the cost difference between both groups so that the influence of uncertainty and robustness can be estimated. Sensitivity analysis is presented in Table 6. Sensitivity analysis was conducted by determining the lowest and highest costs for each service cost, medication cost, and laboratory/diagnostic test cost in the levofloxacin and ceftriaxone groups.

In the sensitivity analysis, it was found that changes in service costs, medication costs, and laboratory/diagnostic test costs in the levofloxacin and ceftriaxone groups could change the cost difference value. These results suggested that changes in the direct costs of either the levofloxacin or ceftriaxone groups can change the cost difference value. Changes in each direct medical cost of both antibiotics were sensitive to changes in the cost difference value.

Table 6. Sensitivity analysis

Sensitivity Analysis			Levofloxacin cost	Ceftriaxone cost	Cost difference
			IDR 2,056,799	IDR 1,969,627	IDR 87,172
Ceftriaxone	Service costs	lowest IDR 263.000	IDR 2,056,799	IDR 1,187,425	IDR 869,374
		highest IDR 1,501,000	IDR 2,056,799	IDR 3,844,974	IDR -1,788,175
	Medication costs	lowest IDR 442,461	IDR 2,056,799	IDR 1,076,461	IDR 980,338
		highest IDR 2,330,342	IDR 2,056,799	IDR 4,177,342	IDR -2,120,543
	Laboratory/Diagnostic test costs	lowest IDR 166,000	IDR 2,056,799	IDR 1,076,461	IDR 980,338
		highest IDR 814,000	IDR 2,056,799	IDR 4,177,342	IDR -2,120,543
Levofloxacin	Service costs	lowest IDR 308,000	IDR 1,470,335	IDR 1,969,627	IDR -499,292
		highest IDR 1,318,000	IDR 4,593,087	IDR 1,969,627	IDR 2,623,460
	Medication costs	lowest IDR 778,466	IDR 1,693,466	IDR 1,969,627	IDR -276,161
		highest IDR 2,708,087	IDR 4,593,087	IDR 1,969,627	IDR 2,623,460
	Laboratory/Diagnostic test costs	lowest IDR 65,000	IDR 1,366,155	IDR 1,969,627	IDR -603,472
		highest IDR 671,000	IDR 2,560,207	IDR 1,969,627	IDR 590,580

This study has several limitations. This study is a retrospective study with a small sample size and was conducted in one hospital. Therefore, there are limitations in measuring effectiveness and may not represent a representative sample. This study also only estimates direct medical costs. Indirect costs like loss of productivity and absence of family or caregivers are not analysed.

CONCLUSION

In conclusion, there were different consequences in community-acquired pneumonia therapy for adult inpatients who used levofloxacin and ceftriaxone as first-line empirical antibiotics. We found that administering ceftriaxone than levofloxacin as a first-line empirical antibiotic therapy for community-acquired pneumonia in hospitalized adult patients had the consequences of increasing the therapeutic effectiveness by 18.33%, reducing the length of stay in the hospital by 0.39 days and saving total direct medical costs by IDR 87,172 per community-acquired pneumonia case in hospital. Although the results of several previous studies have differences, the use of first-line empirical antibiotics for community-acquired pneumonia in adult inpatients requires further pharmacoeconomic evaluations.

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AUTHOR CONTRIBUTIONS

Conceptualization, R.R., M.S.I., H.A.C.S.; Methodology, R.R., H.P.A., M.S.I., R.N.F., E.; Software, R.R., A.W.A., J.S.; Validation, H.P.A., M.S.I.; Formal Analysis, R.R., H.A.C.S.; Investigation, R.R., A.W.A., J.S., H.A.C.S.; Resources, R.R., J.S.; Data Curation, R.R., H.A.C.S.; Writing - Original Draft, R.R., A.W.A., H.A.C.S.; Writing - Review & Editing, J.S., E., R.N.F.; Visualization, R.R., J.S.; Supervision, H.P.A., M.S.I., R.N.F.; Project Administration, R.R., H.P.A., M.S.I., R.N.F.; Funding Acquisition, R.R., H.P.A., M.S.I., R.N.F.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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The Effect of Education on Writing Integrated Patient Progress Notes (IPPNs) at Several Government Hospitals in Bukittinggi, Indonesia

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Abstract

Background: *The writing of Integrated Patient Progress Notes (IPPNs) by pharmacists generally does not fulfil the correct writing standards.* ***Objective****: This study aimed to analyze the effect of education on the level of knowledge and writing profile of IPPN.* ***Methods****: A prospective analytic method research design was used with data collection techniques through questionnaires and total sampling for IPPN data. The researcher developed a valid and reliable questionnaire to measure pharmacists' level of knowledge. Education was conducted through "Focus Group Discussion" with PowerPoint slides of SOAP method writing material and SOAP framework leaflets. Quantitative analysis of IPPN data was performed using the Wilcoxon test on SPSS.* ***Results****: The results showed that the highest percentage of pharmacists' knowledge level before education was A.M Hospital (87%) and after education was B Hospital (95%). The profile of IPPN writing by pharmacists before being given education showed the highest percentage of IPPN writing suitability was at A.M Hospital (21.6%), and the completeness of IPPN writing was at M.H Hospital (99%). After education, the highest percentage of IPPNs writing suitability was in B Hospital (64.3%), and the completeness of IPPNs writing was in M.H Hospital (97.9%). Education has an effect on pharmacists' knowledge level (p-value 0.029) and the appropriateness profile of IPPN writing (p-value 0.013). However, education did not affect the completeness of writing Integrated Patient Progress Notes (IPPNs) (p-value 0.285).* ***Conclusion****: Education succeeded in improving pharmacists' knowledge of writing CPPT correctly.*

Keywords*: educational impact, integrated patient progress notes (IPPNs), IPPNs writing profil, SOAP notes*

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INTRODUCTION

Using the SOAP (Subjective, Objective, Assessment, Plan) technique, a healthcare practitioner writes an Integrated Patient Progress Note (IPPN) detailing how the patient's condition has developed and is integrated into the patient's medical record in a standardized format (Ministry of Health, 2022; Vijayakumar TM, 2016). An integrated medical record system is a means for professionals to make corrective and clinical decisions in analyzing and maintaining patient status (Lestari *et al*., 2020). The primary function of pharmacists in this activity is as an effort and intervention to solve the problem of irrational drug use. Efforts and interventions can be in the form of educational strategies, managerial strategies, and regulatory strategies (Kemenkes, 2011). Pharmaceutical care does not take over the work of doctors or other professions but complements the needs of the healthcare system that arise due to irrational therapy (Wydiati, 2016). In addition, clinical pharmacy practice contains information that reflects treatment actions between professionals and lay people. (Adejare *et al*., 2021).

Completion of IPPN using the SOAP method requires a framework that becomes a reference for pharmacists. Not all information obtained must be filled in the IPPN, depending on the type of visit and whether it is related to drug problems (Oregon State University, 2017). Correct documentation follows specific rules, including complete and readable documents, and the form and content of documents can be developed as needed. In the past, pharmacists did not have a common writing culture for evaluation and documentation of patient pharmacotherapy, which applies to all types of pharmaceutical practice (Ministry of Health, 2019). Further studies are needed to evaluate documentation by pharmacists in healthcare settings (Adam *et al.,* 2019). The daily involvement of critical care pharmacists in patient care most often results in the optimization of pharmacotherapy and avoidance of medication errors (Sledge *et al*., 2016). It was coupled with recent regulations that require documentation of patient clinical information to be organized electronically (Ministry of Health, 2022).

An important rule when creating SOAP is that it must be continuous and establish a connection between subjective and objective data. The data written should reflect what will be analyzed in the review. Plans are created sequentially according to the results of the evaluation (if there are multiple DRPs). Writing a SOAP must include the date and time of the letter and end with the pharmacist's initials, name, and job title (Ministry of Health, 2019). The publication of PMK No. 24 of 2022 on medical records is an issue under serious consideration, according to which all medical institutions must use electronic medical records in order to coordinate the writing activities of the IPPN. It is mandatory to create one. (Ministry of Health, 2022).

The results of Firza's research in RSUP Dr M. Djamil Padang showed that 32 pharmacist IPPNs were not written correctly (0%), and (78.12%) of pharmacist IPPNs were written completely (Firza, 2020). Likewise, the results of Hudria's research RSUP Dr M. Djamil Padang showed that the accuracy of pharmacIPPNs'IPPNs' writing was incorrect (0%) of 35 IPPNs, and 74.29% were incomplete (Hudria, 2020). The results of Kamil's research showed that health professionals see the importance of using IPPN but can only be implemented with educational and organizational support and that the use of electronic patient records may be more effective than paper records (Kamil *et al.*, 2020). The results indicate that health professionals see the importance of using IPPNs but only if implemented with educational and organizational support and that the use of an electronic patient record may be more effective than a paper record. Prioritize providing instructional and organizational support to facilitate the deployment of IPPNs. (Kamil *et al*., 2020). The results of Endri research showed that the analysis of the completeness of IPPNwriting (0%) and the suitability of IPPN writing (6.25%) of 31 IPPNat pharmacist one and the results of the analysis of the completeness of IPPN writing (0%) and the suitability of IPPN writing (9.67%) of 31 IPPNat pharmacist 2 (Endri *et al*., 2023).

MATERIALS AND METHODS

Research design

This research uses observational and analytical research methods with prospective data collection. Research data was taken from questionnaires and pharmacists' Integrated Patient Progress Notes (IPPN). Respondent data collection techniques for validity and reliability tests and pharmacist knowledge levels using questionnaires. The data collection technique for pharmacist respondents who will be given education uses purposive sampling. IPPNs data collection technique is in the form of total sampling.

Data collection

The data used was a questionnaire on pharmacists' level of knowledge regarding writing Pharmacist SOAPs and data on pharmacists' IPPNs writing at the research hospital. The questionnaire (Table 1) was made

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based on the literature and observations of researchers in several hospitals in Indonesia. The questionnaire to be used was first tested for construct validity and reliability on 30 respondents. Data collection was carried out by distributing questionnaires that had been made in the National HISFARSI group and the National LARSI Surveillance group via Google Form. The questionnaires collected were those filled out by pharmacist respondents who wrote IPPNs using the SOAP method in hospitals outside Bukittinggi, the province of West Sumatra.

Table 1. Research questionnaire

Put a check mark (√) on one of the most correct answers based on what you know

No.	Questionnaire Statement	True	False	hesitations
1.	*Subjective, Objective, Assessment, Plan* (SOAP) is one of the methods used in the Integrated Patient Progress Record (IPPNs) documentation.	√		
2.	In collecting *Subjective (*S) data, the pharmacist does not need to visit the patient's room.		√	
3.	Interviews can be conducted to complete subjective data not found in medical records required for *assessment* related to drug use.	√		
4.	History of disease is not Subjective (S) data.		√	
5.	Objective (O) data is sourced from observations, and measurements made by other health professions.	√		
6.	The doctor's diagnosis is Objective (O) data.	√		
7.	Information from patients related to drug use history is written on *Objective* (O) data.			√
8.	If more than one Drug Related Problem *(DRP) is found*, then the DRP writing should be numbered (with numbers 1, 2, and so on) sequentially in the *Objective* (O) data.			√
9.	The results of diagnostic tests such as culture tests do not need to be written on *Objective* (O) data.			√
10.	In writing SOAP, there must be continuity and relationship between *Subjective* data (S) and *Objective data* (O).	√		
11.	*Subjective* (S) and *Objective* (O) do not need to be written in the Integrated Patient Progress Notes (IPPNs).			√
12.	*Assessment* (A) is the pharmacist's assessment of the problems faced by patients related to drug use.	√		
13.	The doctor's diagnosis is Assessment data (A).			√
14.	*Assessment* (A) describes the indications of each therapy received by the patient.			√
15.	Writing *Drug Related Problem* (DRP) in *Assessment* (A) should not use sentences that justify certain professions.	√		
16.	Assessment of *Subjective (S) and* Objective (*O) data is carried out by referring to pharmacotherapy principles*, Evidence-Based Medicine (*EBM), and* guidelines *to determine the presence or absence of* Drug Related Problems *(DRPs).*	√		
17.	*Plan* (P) is a pharmaceutical service plan according to the *Drug Related Problem* (DRP) found.	√		
18.	*Plan (*P) does not need to be written if *the Drug Related Problem* (DRP) is not found.			√
19.	In recommending drug therapy for each d*rug-related problem* (DRP) found, it should be written in full the drug therapy recommendations along with the dosage and rules of use.	√		
20.	The counseling plan, recommendations and monitoring of drug therapy are written on *the Plan* (P) data.	√		

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Data on 'thepharmacist's level of knowledge was collected using a valid and reliable questionnaire. Data collection was carried out twice, using the same questionnaire for the pretest and posttest. The pretest was conducted at the beginning of the study at the research hospital using a questionnaire sheet, which was filled in by adding a checkmark in the column provided for the statement that was answered 'TRUE' or 'FALSE' or 'HESITATE'. One week after the pretest, the researcher educated respondents about filling out the IPPNs using the SOAP method. One week later, a posttest was conducted using the same questionnaire as the pretest.

The inclusion criteria for the IPPN data taken were those filled in last by the p pharmacist pharmacist on duty during hospitalization. IPPNs data collection was carried out during the first week before providing IPPNs writing education. The data collection method used was by reviewing documents using a checklist instrument. Then, the researchers provided IPPNs writing education using the FGD (Focus Group Discussion) method for one day. The educational media used are printed media in the form of SOAP writing leaflets and electrolyte media in the form of slides. After the FGD (Focus Group Discussion) activity, researchers also gave the SOAP writing framework leaflet to respondents. The SOAP writing framework leaflet is a summary of the SOAP writing leaflet prepared by researchers as a reference for pharmacists to familiarize themselves with changes in the previous IPPNs writing culture. Figure 1 displays the SOAP framework leaflet. One week following the educational session, another week was dedicated to collecting IPPN data.

Ethical clearance

All of the ethical clearance protocols of this study were approved by the Ethics Committee from the Faculty of Medicine, Andalas University. Number: 1056/UN.16.2/KEP-FK/2022.

Processing, analysis and interpretation of data

In this study, two steps were carried out, namely measuring the level of knowledge and analysis of IPPN writing before and after carrying out educational interventions on correct SOAP writing in accordance with the 2019 Ministry of Health regulations with appropriate variables. The first step is measuring the ppharmacist's level of knowledge using a questionnaire as the dependent variable, with the independent variable being the pharmacist who serves as a clinical pharmacist at the research hospital. The indicators used are questionnaires that have been validated and reliable. The assessment parameter is the suitability of the respondent's answer to the answer key that has been prepared. Respondents who answered correctly were given a value of 2, respondents who answered incorrectly were given a value of 1, while respondents who answered in doubtful were given a value of 0.

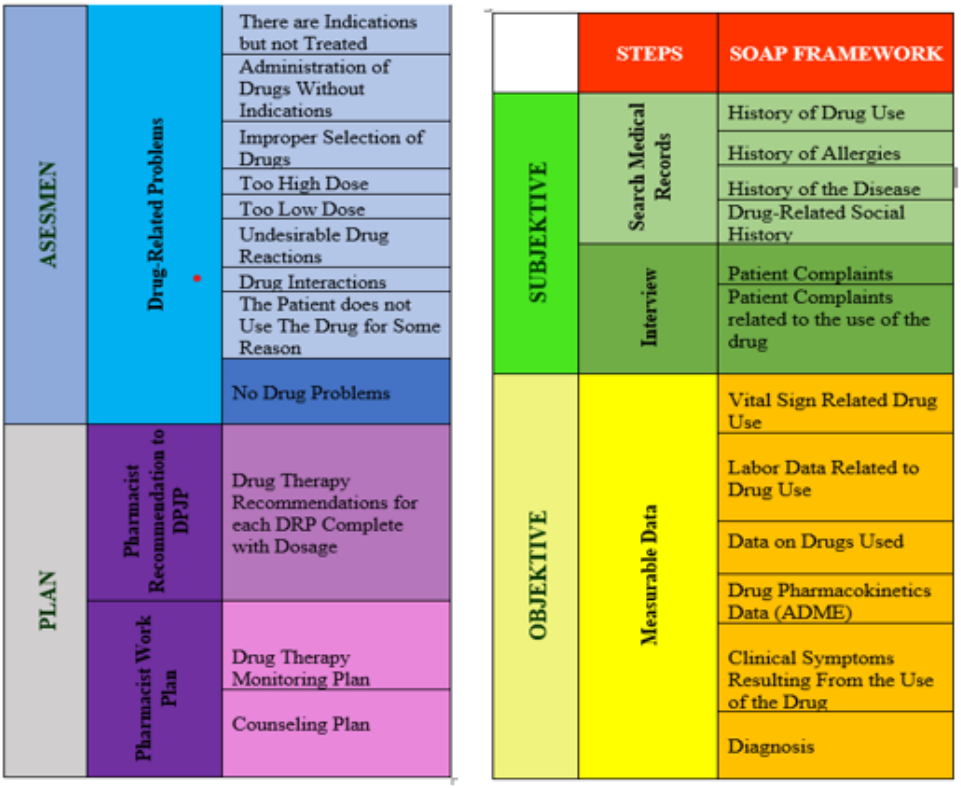

Figure 1. SOAP framework leaflet

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Second step: In the analysis of IPPN writing by pharmacists, the 2019 Ministry of Health regulatory policy is used as the independent variable, with IPPN data written by pharmacists as the dependent variable. The indicators used are the requirements for the completeness of the IPPN and the SOAP framework in accordance with the 2019 Ministry of Health Regulations. The assessment parameter is the suitability of the IPPN written by the pharmacist with the rules of the 2019 Ministry of Health Regulations. Writing a SOAP must include the date and time of the letter and end with the pharmacist's initials, name, and job title (Ministry of Health, 2019). The assessment parameter is the suitability of the IPPN written by the pharmacist with the rules of the 2019 Ministry of Health Regulations. The assessment of the IPPN writing is whether it is complete or incomplete according to the IPPN completeness requirements and whether the evaluation is appropriate/not in accordance with the rules of the SOAP writing framework. If one of the components is incomplete or inappropriate, it is given a score of 0, while those that are appropriate are given a score of 1.

The validity test of the questionnaire used Pearson's product moment with a significance <0.05. Responses from 30 participants on 20 questionnaire statements were scored as follows: 2 for correct answers, 1 for incorrect answers, and 0 for doubtful answers. Then the data was recapitulated and entered into the SPSS system. The questionnaire reliability test used the Spearman-Brown test with a Cronbach alfa value ⩾ 0.6.

The questionnaire responses from the hospital's participants were converted into percentages and then analyzed to compare the results before and after the educational intervention.. Arikunto (2008) claims that "scoring the level of knowledge" with the following formula (Arikunto, 2008).

$$P = \frac{F}{n} \times 100\%$$

P = The percentage value F = Accurate response
n = The total number of inquiries

As stated by Budiman & Riyanto (2013), in categorizing the level of knowledge can be grouped into two groups for the respondents studied, namely: Good category knowledge level if the value is> 75% and Poor category knowledge level if the value is ⩽ 75%" (Budiman & Riyanto, 2013).

Analysis of the authoring of Integrated Patient Progress Notes (IPPNs) using qualitative analysis techniques of data gathered from written document studies in Medical Records. Data from the IPPN was analyzed both before and after pharmacists received training. The Technical Guidelines for Pharmaceutical Service Standards in Hospitals, which were released by the Ministry of Health in 2019, were analyzed in order to determine the outcomes of the IPPN's writing profiles in a number of government hospitals located in Bukittinggi City. Completeness and appropriateness data were converted into percentages and compared between the pre- and post-education periods.

The effect of education on the level of pharmacist knowledge was carried out using quantitative analysis using SPSS software with the Wilcoxon hypothesis test. Data analysis of the pharmacist's knowledge level was obtained by filling out the questionnaire twice with the same questionnaire before (pretest) and after education (posttest). The questionnaire that pharmacist respondents filled in was presented and then entered into SPPS software to find the p-value.

The effect of education on the profile of IPPNs writing was carried out using quantitative analysis with software using the Wilcoxon hypothesis test. Data from the analysis of IPPNs writing on suitability before and after education and data on the completeness of IPPNs writing before and after education were processed using SPSS with the Wilcoxon hypothesis test to get the p-value. If the significance value (p-value) >0.05, then Ho is accepted, and if the significance value (p-value) <0.05, then Ho is rejected.

Conclusion drawing

The review of the suitability of writing IPPNs concludes that if IPPNs are written using the SOAP technique, they should refer to the 2019 Ministry of Health publication, Technical Guidelines for Pharmaceutical Service Standards in Hospitals. The IPPNs are classified as "not appropriate" if they are not written according to the writing guidelines. Additionally, the IPPNs' writing is deemed unsuitable if there are one or more writing inconsistencies in the data from the subjective, objective, assessment, or plan. An example of a mismatch in question is the placement of objective data written on subjective data such as diagnoses. Another example is a patient's complaint of pain in the subjective data but in the objective data, not pain scale data or pain medication used (there is a disconnection between subjective data and objective data).

The analysis of the completeness of IPPN's writing concludes that information is considered complete if it includes the date, time, and name of the visit, the ppharmacist's title, and their signature, in addition to recording subjective, objective assessment and plan data on the IPPNS sheet. The IPPNs data writing is classified

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as incomplete if one or more are not writing the completeness parameter data.

RESULTS AND DISCUSSION

The profile of writing (IPPNs) by pharmacists in several government hospitals in Bukittinggi City is as follows: The highest percentage of suitability of IPPNs writing before education was at A.M Hospital (21.6%). The highest percentage of completeness of IPPNS writing before education was at the M. H Hospital Bukittinggi (99%). The highest percentage of suitability of IPPNs writing after education was given at the B. Hospital (64.3%). The highest percentage of completeness of IPPNs writing after education was at the M. H Hospital (97.9%). The highest percentage of pharmacist knowledge level before education was at A. M Hospital (87%). The highest percentage of pharmacists' knowledge level after being given education is at the B Hospital (95%). Education affects pharmacists' knowledge level of IPPN writing in several government hospitals in Bukittinggi City (p-value: 0.029). There is an effect of education on pharmacists on the profile of the suitability of writing IPPNs in several government hospitals in Bukittinggi City with a value (p-value: 0.013). There is no effect of education on pharmacists on the completeness of IPPNs writing in several government hospitals in Bukittinggi City with a value (p-value: 0.285)

Questionnaire

There were two groups of respondents who participated in this research: group 1 was respondents for the validity of the questionnaire, and group 2 were respondents at the hospital where the study was conducted. Respondents for the validity of the questionnaire came from hospital pharmacists throughout Indonesia, apart from the province of West Sumatra, while respondents for the research data were in the city of Bukittinggi, West Sumatra. The total number of respondents who participated in this research was 30 people for the validity of the questionnaire, consisting of 12 men and 18 women. Meanwhile, the total number of respondents in several research hospitals was 15 people, consisting of 3 men and 12 women. The age range of respondents ranged from 26 - 55 years, with the majority being 35 years.

The results of the validity and reliability test of the questionnaire from the results of the SPSS calculation show that all statements are said to be valid because all r values (Pearson Correlation values) obtained are more significant than the r table (0.361). The significance value (p) or sig (2-tailed) found that the p-value on all items of the 20 questionnaire statements has a value <0.05 so it can be declared valid. The results of the questionnaire reliability test obtained a Cronbach's Alpha value $\geqslant$ 0.6, namely 0.764 so that it can be declared reliable (Sutriawan A, 2021).

Knowledge level

The ppharmacist's level of knowledge regarding CPPT writing will influence the IPPN writing pattern. This level of knowledge can be obtained from educational levels, seminars, workshops and training on writing IPPN using the SOAP method. The learning culture in each hospital also greatly influences the experience of IPPN pharmacists. Pharmacist staff graduates from the institution are also one of the contributors to the culture of IPPN writing profiles.

Analysis of the "Pharmacist Knowledge Level" questionnaire on IPPNs writing with the SOAP Method in the three research hospitals (Figure 2, 3, and 4) shows that at M.H Hospital and A.M Hospital, it was in a good category before education was given. However, it is still in the category of weight I because, in theory, it is included in the group that already knows and understands. The B Hospital is in the poor category because it has a percentage value of $\leqslant$75% (Budiman and Riyanto, 2013).

Researchers realize that one of the weaknesses of questionnaires is that it is difficult to get good responses from respondents. Even though the time and atmosphere are conducive, strong motivation from respondents is still needed for good data. According to researchers, even a level of knowledge that has been assessed as good does not guarantee good IPPN writing. Other factors can influence pharmacists in writing IPPN, including a. Pharmacist's desire to continue to develop personal competence. b. Hospital systems that support the implementation of filling out IPPN include job descriptions and DCA (Details of Clinical Authority), adequate number of pharmacists with supporting educational levels, availability of supporting data to help assess drug use, and easy access in collecting supporting data for writing IPPN. c. A supportive work environment such as facilities and infrastructure (electronic drug information system and updates) and conducive collaboration between the Pharmacist team and Caregiving Professional. d. A good pharmacist work culture includes a uniform understanding of cases, routine FGD activities, continuing to look for up-to-date references and supervision by the appointed pharmacist regarding the consistency of the IPPN filling profile.

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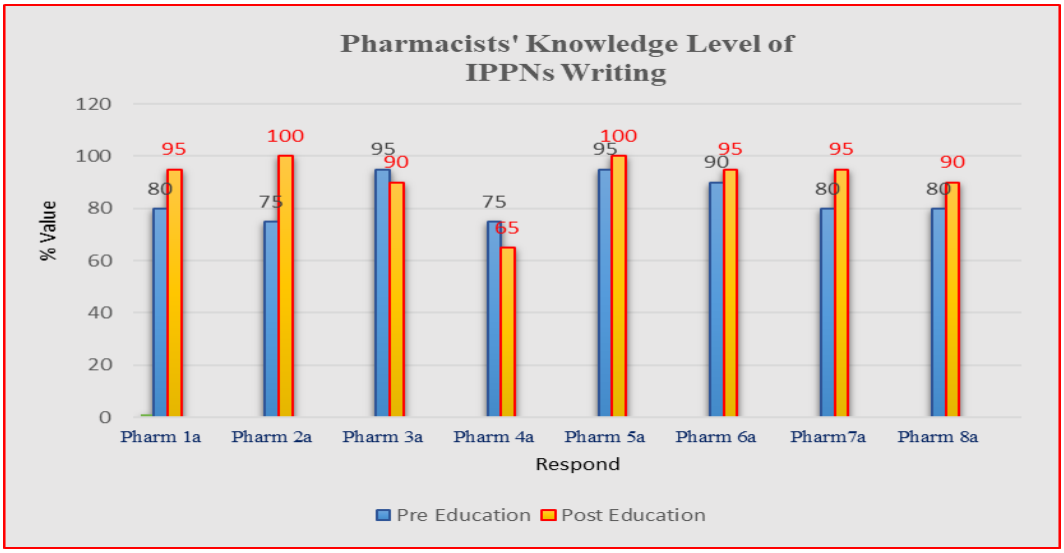

Figure 2. Histogram of the results of the pharmacist knowledge level questionnaire on CPPT writing at M. H Hospital Bukittinggi

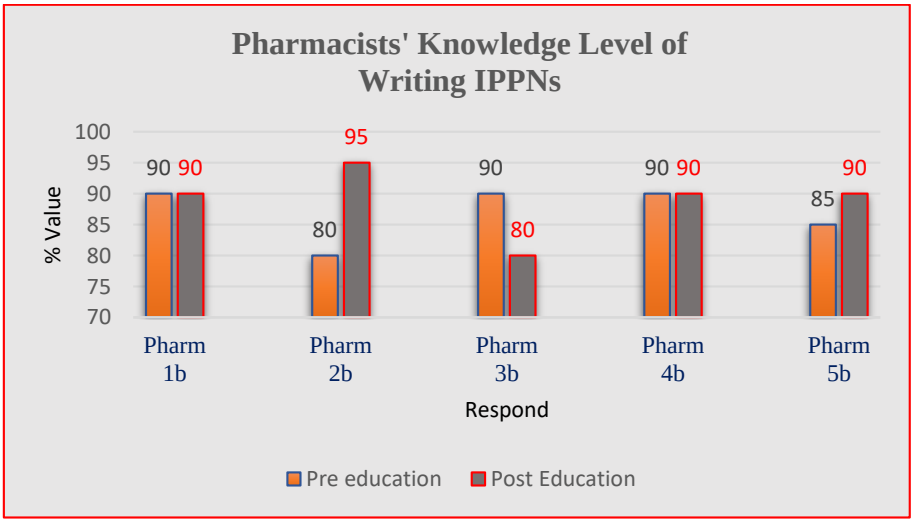

Figure 3. Histogram of the Results of the Pharmacist Knowledge Level Questionnaire on IPPNs Writing at A. M Hospital Bukittinggi

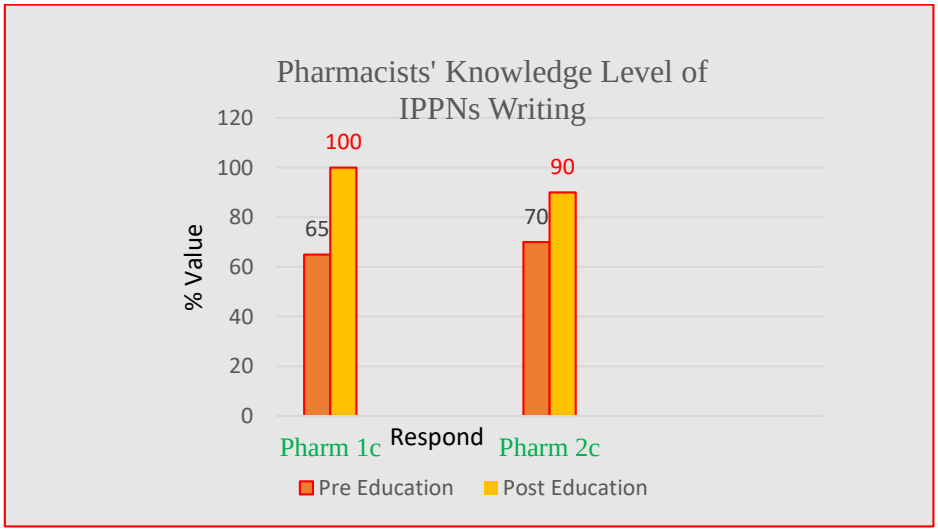

Figure 4. Histogram of the Results of the Pharmacist Knowledge Level Questionnaire on IPPNs Writing at B Hospital

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Analysis of the suitability and completeness of writing Integrated Patient Progress Notes (IPPNs)

This research was conducted at several government hospitals as research sites. However, they still have different conditions and systems. The total IPPN data was obtained from the three hospitals where the study was conducted, and there were 313 Integrated Patient Progress Notes (182 IPPNs before education and 128 IPPNs after education). The profile of the results of the analysis of the suitability and completeness of the Pharmacist's IPPNs data filling (Tables 2, 3 and 4) shows that all SOAP elements have been filled in, but there is still a lot of subjective and objective information that has not been explored so that the assessment of drug use assessment is not optimal. Plan writing has also been done, but some of them are still not in writing.

Typically, subjective data entries include (in descending order) patient complaints, medical history, drug use history, and allergy history. Data regarding drug-related social history or patient complaints related to drug use were not collected before or after education.

Table 2. Profile of the results of the analysis of the suitability and completeness of IPPNS writing at M. H. Hospital Bukittinggi

Respond	Pre-Education					Post-Education				
	N1	AIW	%	CIW	%	N2	AIW	%	CIW	%
Pharm 1a	13	0	0	12	92.3	6	0	0	5	83.3
Pharm 2a	12	0	0	12	100.0	9	0	0	9	100.0
Pharm 3a	24	0	0	24	100.0	15	12	80.0	15	100.0
Pharm 4a	6	0	0	6	100.0	15	8	53.3	15	100.0
Pharm 5a	13	0	0	13	100.0	7	4	57.1	7	100.0
Pharm 6a	13	4	30.8	13	100.0	7	0	0	7	100.0
Pharm 7a	6	0	0	6	100.0	14	0	0	4	100.0
Pharm 8a	3	0	0	3	100.0	12	0	0	12	100.0
IPPNs Total	90	4		89	100.0	75	24		74	

Description:
N 1: Number of IPPNs before education AIW: Appropriateness of IPPNs Writing
N 2: Number of IPPNs after education CIW: Completeness of IPPNs Writing

Table 3. Profile of the results of the suitability analysis and completeness of IPPNs writing at A. M Hospital Bukittinggi

Respond	Pre-Education					Post-Education				
	N1	AIW	%	CIW	%	N2	AIW	%	CIW	%
Pharm 1b	15	5	33.3	0	0	6	6	100.0	0	0
Pharm 2b	28	11	39.3	0	0	6	3	50.0	0	0
Pharm 3b	13	4	30.8	13	100.0	7	4	57.1	7	100.0
Pharm 4b	16	0	0	16	100.0	13	0	0	13	100.0
Pharm 5b	8	1	12.5	8	100.0	7	1	14.3	7	100.0
IPPNs Total	80	21		37		39	14		27	

Description:
N 1: Number of IPPNs before education AIW: Appropriateness of IPPNs Writing
N 2: Number of IPPNs after education CIW: Completeness of IPPNs Writing

Table 4. Profile of the results of the suitability analysis and completeness of IPPNs writing at B Hospital

Respond	Pre-Education					Post-Education				
	N1	AIW	%	CIW	%	N2	AIW	%	CIW	%
Pharm 1c	8	0	0	0	0	7	5	71.4	6	85.7
Pharm 2c	4	1	25	0	0	7	4	57.1	7	100.0
IPPNs Total	12	1		0		14	9		13	

Description:
N 1: Number of IPPNs before education AIW: Appropriateness of IPPNs Writing
N 2: Number of IPPNs after education CIW: Completeness of IPPNs Writing

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According to the Ministry of Health 2019, what needs to be studied from a social history perspective is the social (lifestyle) and economic situation of the patient in relation to the disease. Patient complaints related to drug use were not written by pharmacists but by researchers' observations. Pharmacists asked patients about their complaints, and some patients submitted their complaints that were not written in the IPPN. Researchers could not find them at the Ministry of Health and Welfare. Explanations and examples can be found in the 2019 Health Literature. Disease diagnosis data that should be included in the O data may also be entered in the S data (Ministry of Health, 2019). There is also subjective data with embedded striped symbols (S: ~). As a result of interviews with concerned parties, it appears that there have been no complaints. Researchers say data marked "S-" is acceptable for newborns and newborns with no medical history or disease since babies cannot communicate yet. However, for adult ICU patients or patients who lose consciousness during their visit, information should be obtained from the patient's family or other Caregiving Professional.

Typically, writing objective data includes Vital signs, medication taken data, work data (in order from highest to lowest) and disease diagnosis. Pharmacokinetic data (ADME) and clinical symptoms due to drug use were not collected before and after informed consent. Writing objective data unrelated to subjective data, not writing pain scales, and not calculating ClCr from creatinine data were improved through awareness campaigns. The diagnosis is written in O (objective) data. Disease diagnostic data includes data discussed at the study hospital. Among the survey data (validity test participants and sample participants), some diagnostic data were answered correctly, and others were answered incorrectly. In the 2019 Ministry of Health literature, the diagnosis is included in other PPAs, in this case, objective data, one of the doctor's observations, but not explicitly or in detail. The policy of the director of M. Hatta Tertiary Brain Hospital is that "diagnosis is not the pharmacist's SOAP data," which is consistent with his 1992 ASHP Literature Module 2 (American Society of Health-System Pharmacists, 1992). It is understood, according to. Whereas the diagnosis data at A M Hospital and B Hospital, some pharmacists write in subjective data and some in objective data. After being given education, the diagnoses were written by pharmacists, and pharmacists wrote diagnoses using objective data.

Review data typically includes drug-related problems, drug interactions, untreated indications, administration of drugs without indication, overdose, inappropriate drug selection, and underdosing (in order from highest to lowest) not included. Data regarding side effects or patients not taking the drug for any reason were not written before or after informed consent.

Side effect data is data that does not occur frequently. At the time of the study, there may not have been any cases in the three hospitals where the study was conducted. Data about patients who are not taking their medication for a specific reason should include information collected from the patient. When interviewing patients, pharmacists should search more thoroughly for information about the patient's medications, whether they were obtained from a health service or self-administered, including herbs. Noncompliance by patients may be due to patient discomfort with the prepared drug, perhaps a bitter taste or large amounts of the drug. It may also be due to the patient's noncompliance with medication. It may also be due to inadvertent patient non-adherence, with the result that drug evaluation is influenced by such information.

Planning data generation includes (from highest to lowest) drug therapy monitoring plans and drug therapy recommendations for each DRP, including medication regimens and counselling plans. Although advanced therapy phrases are common before training and change after training, some pharmacists still write them. Evaluation data were written into the plan before and after training. No errors were found when writing review data. Data regarding counselling plans are not included in his P data but are related to the Education and Drug Information Center (DIC). Some assessment data in the form of a DRP does not include treatment recommendations from pharmacists but is completed through Adverse Drug Reaction Monitoring (ADR) or Drug Use Evaluation (DUE). Drug counselling plans are rarely developed by pharmacists at research hospitals. Researchers' observation, experience, and consulting activities will work better if pharmacists can communicate extensively with patients and patients' families. This requires special skills but can be honed with daily practice.

The IPPN writing at M. H Hospital Bukittinggi is already using the E-IPPN system, so the implementation of filling out the IPPN can be verified through the application. IPPN integrity data is supported by a system with a password for each PPA, so visit date and time, full name and electronic signature are automatically recorded. The same subjective and objective Caregiving

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professional data (vital signs) filled in by the doctor will automatically be present in the other pharmacist/Caregiving Professional's IPPN. The hospital system has allowed each Caregiving Professional to adapt to its own needs. Data can be edited, added, and even deleted if it is not set through the SOAP method of each Caregiving Professional. Data can be accessed from anywhere with the registered pass I.D., making it very convenient for pharmacists to understand medication status smoothly. SOAP data can be completed even after patient discharge. This is the benefit of an electronic system that can fulfil the elements of accreditation assessment and guarantee legal immunity (Ministry of Health,2022).

The IPPNs filling system at the A. M Hospital is still manual. Currently, pharmacists are divided by room according to hospital policy. The pharmacists on duty stay in their respective rooms. If one pharmacist holds more than 1 room, the activities in the room follow the doctor's visit hours in each room. A. M Hospital has implemented a joint visit system so that some patient problems can be directly discussed during the visit. For DRP, especially drug interactions cannot be resolved during the visit because pharmacists need time to study literature.

Founded just a year ago, B Hospital is a Type C facility. The pharmacists' writing of IPPNs is still done manually. Not only does the pharmacist team lack a certificate of clinical pharmacy training or seminar, but the pharmacist in charge of the Ranap division is a recent graduate. During pharmacist lectures, the Head of the Pharmacy Installation and Field Practice experience guided filling out IPPNs. There isn't currently a staff pharmacist with a master's degree in clinical pharmacy at B Hospital.

The influence of education on pharmacist IPPN writing profiles

Education is one of the non-formal education methods used by pharmacists for learning. Education can be direct or indirect. Education is the independent variable that consists of attitude, knowledge, motivation, experience, and work conditionsThis research uses direct education methods through the FGD system, accompanied by SOAP writing leaflets, PowerPoint presentations, and IPPN writing framework leaflets, using the SOAP method according to the Ministry of Health 2019. The education provided requires several case examples so that there is a clear picture of the intent and purpose. Individualized education is also needed because people's ability to receive information is different.

In the three hospitals, the education was conducted in the same pattern, and the good condition of the FGD activities can affect the purpose of the FGD itself. A good presentation of information is also essential when conducting education, including the ability of the educator himself to understand what will be conveyed and the way of delivery. In the FGD activities that have been carried out, the improvement is more focused on the discussion because the IPPNs filling has taken place and is even carried out by staff who are competent in their fields. For Type A and Type B hospitals, the discussion was generally on equalizing perceptions of the SOAP framework in the 2019 Ministry of Health literature. For type C hospitals, the emphasis was on information and questions and answers.

Study limitations: This research refers to the literature on Technical Guidelines for Standards of Pharmaceutical Services in Hospitals issued by the Ministry of Health in 2019. Writing rules that are in accordance with the SOAP framework according to the literature is acceptable even though the depth of content is not yet appropriate. If the writing is correct, this research can be continued with the suitability of the content of drug therapy assessment and the usefulness of Pharmacist IPPN for other caregiving professionals.

CONCLUSION

Education succeeded in improving pharmacists' knowledge in writing IPPN correctly.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.; Methodology, H.N.; Software, S.; Validation, Y.O.S.; Formal Analysis, S.; Investigation, S.; Resources, S.; Data Curation, H.N.; Writing - Original Draft, Y.O.S.; Writing - Review & Editing, H.N.; Visualization, H.N., Y.O.S.; Supervision, H.N.; Project Administration, H.N.; Funding Acquisition, S.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Identification of Drug Related Problems (DRPs) in Rheumatoid Arthritis Patients at Palembang City Hospital

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Abstract

Background: *Rheumatoid arthritis is a chronic systemic inflammatory arthritis disease that affects mainly synovial joints. The incident of RA can lead to the emergence of complications or comorbidities, which then allows patients to receive a variety of therapies that can trigger the incidence of DRPs during the treatment. The prevalence of RA in Indonesia itself in 2018 has reached 7.30%, with the highest percentage occurring in the elderly age group and more prevalent in women.* ***Objective****: This study aimed to determine the incidence of DRPs in RA patients in Palembang city hospitals based on the category of DRPs identified as related to drug selection and dose selection problems, as the relationship between demographic factors and the incidence of DRPs.* ***Methods****: This research is non-experimental study conducted with a retrospective cross-sectional survey. Data collection was carried out by looking at patient medical record data at X and Y Hospital in Palembang from January 2021 to March 2023.* ***Results****: The results showed that the most frequent drps in the drug selection category were drug interactions (72.03%), while in the dose selection category were insufficient dosage regimens (60.74%). The results of bivariate analysis between the incidence of DRPs and gender (p=0.809), age (p=0.879), the number of drugs used (p=0.001), and comorbidities (p=0.089).* ***Conclusion****: There is no relationship between demographic factors and comorbidities with the incidence of DRPs, and there is a relationship between the number of drugs and the incidence of DRPs.*

Keywords*: drugs, drug-related problems, drug selection, dose selection, Rheumatoid arthritis*

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INTRODUCTION

The immune system is the body's ability to fight organisms or toxicants that tend to damage tissues or organs. When the immune response attacks antigens in the body's own tissues, which are found inside or on the surface of cells, autoimmunity will occur [1]. Apart from osteoarthritis, one of the most common autoimmune diseases in Indonesia is rheumatoid arthritis (RA), osteoarthritis, is rheumatoid arthritis. Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease that can damage the bones and cartilage of the joints (Prihanto *et al.*, 2022).

The prevalence of RA in Indonesia itself in 2018 has reached 7.30% especially in South Sumatra at 6.48%, with the highest percentage occurring in the elderly age group and more prevalent in women than men (Kemenkes RI, 2018). The increasing incidence of RA can cause various complications, such as osteoporosis, infection, coronary artery disease, atherosclerosis, and other diseases. One study in Korea mentioned that RA patients are more at risk of having comorbidities, such as hypertension, dyslipidemia, and myocardial infarction or angina (Jeong *et al.*, 2017). This allows patients with a diagnosis of RA to get a variety of drug therapies that can trigger drug-related problems (DRPs) during their treatment.

Research conducted by Hasan and Mumtazah (2016) showed that RA patients had the most comorbidities, namely osteoarthritis, and the most patients experienced DRPs in the category of inappropriate drug combinations at 54.2%. Ma *et al*. (2019) in their research found that 78.5% of 289 patients had DRP problems, namely side effects, drug interactions, and drug selection problems. The research of Sah *et al*. (2022) explained that 88.4% of patients experienced DRPs in the form of medication safety, advanced age, polypharmacy, and other related factors.

This study aims to determine the incidence of DRPs in RA patients in Palembang city hospitals based on drug and dose selection categories. It also aims to determine the correlation between demographic factors, the number of drugs, and comorbidities and the incidence of DRPs in RA patients in Palembang city hospitals.

MATERIALS AND METHODS

Study design and setting

This study was a non-experimental study conducted with a retrospective cross-sectional survey. The study was conducted at X Hospital and Y Hospital in Palembang City from May to June 2023.

Sampling technique

The population of this study included patients diagnosed with rheumatoid arthritis at X Hospital and Y Hospital in Palembang City. The sampling technique was carried out in taking research subjects using purposive sampling. The inclusion criteria included patients with or without comorbidities and patients aged 20 to 65 years. The exclusion criteria included patients with incomplete and/or illegible medical records and patients who were pregnant and/or breastfeeding. The study subjects who met the inclusion criteria were 112 patients.

Data collection

Data collection was carried out retrospectively in the form of secondary data taken from medical record data and the status of patients with a diagnosis of rheumatoid arthritis who met the inclusion and exclusion criteria.

Data analysis

The obtained data were then grouped and analyzed using the Statistical Package for the Social Sciences (SPSS) Version 25.0 for Windows with the Chi-Square test. If the expectation value results in the Chi-Square test were more than 20%, Fisher's exact test was continued.

Ethical approval

This study was approved by the Health Research Ethics Committee of the Health Polytechnic of the Ministry of Health Palembang (Number 0697/KEPK/Adm2/VII/2023).

RESULTS AND DISCUSSION

Demographic data

Based on the results of the study, it is known that the female sex dominates rheumatoid arthritis patients in Palembang city hospitals by 74%, which is in line with research conducted by Wahid et al., (2021) which states that the majority of rheumatoid arthritis patients are female by 70%. This happens because women have the hormone estrogen, which can affect the immune system. In addition, the hormone estrogen also functions to help maintain bone density, but if the levels are excessive, it will cause autoimmune diseases (Susarti and Romadhon, 2019).

The age of rheumatoid arthritis patients in Palembang city hospitals is mainly in the 46-55 year age group at 32.14%. This is almost similar to research conducted by Sah *et al*., (2022) which states that the majority of rheumatoid arthritis patients are aged 41-60 years and research by Wahid *et al*., (2021) that the majority of rheumatoid arthritis patients range from 45-

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55 years. This is due to increasing age, which causes decreased body function, so that the protective layer of the joint, which functions as a barrier to bone friction, will begin to thin out, and the bone fluid that functions as a lubricant will begin to thicken, so that it will cause pain when moved (Elsi, 2018).

The number of drugs used by rheumatoid arthritis patients in Palembang city hospitals is not only given for indications of rheumatoid arthritis disease but also given to treat comorbidities. Based on the results of the study, rheumatoid arthritis patients in Palembang city hospitals are known to consume the majority of the number of drugs <5 by 58.93%, so it can be said that the majority of patients do not experience polypharmacy. This indicates that this study is not in line with research conducted by Sah et al. (2022) and Ma et al. (2019), which state that rheumatoid arthritis patients experience polypharmacy.

The results showed that the majority of rheumatoid arthritis patients in Palembang City hospitals had comorbidities, and the most common comorbidity was osteoarthritis. This is in line with research conducted by Hasan and Mumtazah (2016) , which states that osteoarthritis is the most common comorbidity in rheumatoid arthritis patients.

Table 1. Demographic data of rheumatoid arthritis patients

Characteristics	Frequency	Percentage
Aged		
≤ 25	11	10
26-25	8	7
36-45	26	23
46-55	36	32
56-65	31	28
Gender		
Male	29	26
Female	83	74
Number of drugs used		
< 5	66	59
≥5	46	41
Comorbidities		
Comorbidities present	67	60
No comorbidities	45	40

In this study, it was also known that rheumatoid arthritis patients in Palembang City Hospital were given csDMARD monotherapy, corticosteroids, NSAIDs, and vitamins or supplements. The administration of csDMARD monotherapy is carried out by giving Methotrexate drugs, which dihydrofolate reductase inhibitors will inhibit chemotaxis, thus providing anti-inflammatory effects through induction of adenosine release. Research conducted Savitri *et al.*, (2019) showed the use of Methotrexate monotherapy by 3%. While the most corticosteroid administration is Methylprednisolone, where this drug will affect the decrease in Disease Activity Score-28 (DAS28) value compared to other drugs because of its role as an anti-inflammatory and pain reliever. Furthermore, the administration of NSAIDs in patients is most common in diclofenac sodium, which works as a cyclooxygenase enzyme inhibitor that reduces the production of prostaglandins that cause inflammation, fever, and pain.

DRPs in the drug selection category

The identification of drug-related problems was carried out based on PCNE V9.01. The results showed that DRPs in the drug selection category mostly occurred in drug interactions at 72.03%. This is in line with previous research conducted , which showed that the largest percentage of DRPs occurred in drug interactions at 54.2%. Potential drug interactions in patients mostly happen in the use of Diclofenac and Meloxicam, where the use of Diclofenac will increase anticoagulants and serum potassium. In this co-use, Diclofenac will increase Meloxicam levels through anionic drug competition for renal tubular clearance. The increase in anticoagulant will result in an increased risk of bleeding, while the rise in serum potassium will increase the risk of hyperkalemia.

The percentage of drugs without indications of 16.09% occurred in the administration of diclofenac sodium together with other NSAID drugs. Based on the literature, diclofenac sodium has contraindications when used together with other NSAIDs and will increase side effects in the form of stomach ulcers. Meanwhile, the percentage of too many drugs for the same indication was 6.33%, the majority of which occurred in the use of diclofenac sodium and Meloxicam, both of which are NSAIDs to treat pain and inflammation. Based on the literature, the use of NSAIDs should be given in low doses, even if the dose will be reduced or stopped if the DMARD is effective. Too many drugs for the same indication can lead to drug interactions and several side effects.

Drug administration according to guidelines but with contraindications occurred at 2.90% in rheumatoid arthritis patients who were given diclofenac sodium together with other NSAIDs. The literature states that diclofenac sodium has contraindications when used together with other NSAIDs, which Diclofenac will

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increase side effects in the form of stomach ulcers in patients.

Indications without drugs in patients amounted to 2.11%, the majority of which occurred in patients with indications of Hypothyroid. Hypothyroidism occurs when thyroid hormone levels are less than optimal in the body due to congenital abnormalities of inflammation or inflammation in the thyroid gland or iodine deficiency (Ningsih, 2023). Based on research conducted Yi-jing *et al.,* (2022) concluded that rheumatoid arthritis patients have a high risk of experiencing thyroid dysfunction, especially Hypothyroid. Cross-sectional research conducted [illegible] mentioned that some of the things that are mentioned as potential links between rheumatoid arthritis and hypothyroidism are autoimmuneidism are autoimmune, syndromes and genetic factors, treatment factors, environmental triggers, and inflammation.

Drug administration not according to guidelines of 0.26% occurred in the administration of xanthine-oxidase in rheumatoid arthritis patients. According to the literature, patients with rheumatoid arthritis are given pharmacological therapy in the form of DMARDs, folic acid supplementation, corticosteroids, and NSAIDs. Meanwhile, inappropriate duplication of active ingredients occurred at 0.26% in the administration of diclofenac sodium. Duplication of treatment occurs when the use of two or more drugs that have the same active substance and for the same indication. According to Cipolle *et al.,* (1998), duplication of drug therapy can provide potential toxic effects of drugs and have little or no positive impact on patient outcomes.

DRPs in the dose selection category

DRPs in the dose selection category occurred most in the insufficient dose regimen of 60.74%, which is not in line with research conducted by Ma *et al.,* (2019) which states that dosing problems in rheumatoid arthritis patients are mostly caused by drug doses that are too high or dosing regimens that are too frequent. Drug administration with an insufficient dosage regimen occurred in the administration of Aspirin, Furosemide, Glucosamine, Ibuprofen, Mecobalamine, and Diclofenac sodium.

Too frequent dosing regimen occurred at 18.52% in the administration of Meloxicam. Meloxicam administration in rheumatoid arthritis patients was given at a frequency of three times a day, while in the literature, rheumatoid arthritis patients were given Meloxicam at a frequency of once a day at a dose of 15 mg. The administration of too-low drug doses of 17.04% also happened in the administration of Meloxicam, which aims to treat pain and inflammation. The literature shows that the use of Meloxicam for rheumatoid arthritis is 15 mg once a day, but rheumatoid arthritis patients are given at a dose of 7.5 mg once a day.

Incorrect/unclear / non-existent dosing time instructions occurred in 2.22% of rheumatoid arthritis patients, which in the administration of Ceftazidime, Diclofenac Sodium, and Eperisone, did not explain the dose of drugs to be used by patients. Meanwhile, too high drug doses of 1.48% occurred in the administration of Simvastatin, which is a Statin class drug that reduces LDL and triglyceride levels and increases HDL levels in the blood. Based on the literature, Simvastatin has a dosage of 5-10 mg once a day for patients with dyslipidemia, while it is known that patients with comorbid dyslipidemia are given the drug at a dose of 20 mg once a day.

Table 2. DRPs in the drug selection category

DRPs Category	Number of patients	Percentage	Frequency	Percentage
Drug selection				
-Drugs not in accordance with guidelines	1	0.57	1	0.26
- Medication as per guidelines, but there are contraindications	11	6.29	11	2.90
-Drug without indication	50	28.57	61	16.09
-Drug interactions	80	45.71	273	72.03
-Inappropriate duplication of active ingredients	1	0.57	1	0.26
-Indication without drug	8	4.57	8	2.11
-Too many drugs for the same indication	24	13.71	24	6.33

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Table 3. Distribution of DRPs on drug selection category

DRPs Category	Frequency
Drugs not in accordance with guidelines	
Allopurinol	1
Total	**1**
Medication as per guidelines, but there are contraindications	
Diclofenac sodium	11
Total	**11**
Drug without indication	
Amlodipine	7
Atorvastatin	5
Azithromycin	1
Candesartan	2
Cefadril	1
Cetirizine	2
Eperisone	1
Fenofibrate	2
Flunarizine HCl	1
Furosemide	2
Gabapentin	1
N(2)-L-Alanyl-L-Glutamine	1
Lansoprazole	11
N-Acetylcysteine	1
Omeprazole	20
Simvastatin	1
Doripenem	1
Betahistine mesylate	1
Total	**61**
Inappropriate duplication of active ingredients	
Diclofenac sodium	1
Total	**1**
Indication without drug	
Amyotrophic Lateral Sclerosis (ALS)	1
Diabetes mellitus	1
Dsypnea	1
Hypothyroidism	2
Hypertensive Heart Disease (HHD)	1
Osteoarthritis	1
Vertigo	1

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DRPs Category	Frequency
Total	**8**
Too many drugs for the same indication	
Amlodipine and Candesartan	1
Amlodipine and Ramipril	1
Aspirin and Meloxicam	2
Aspirin and Spironolactone	1
Glucosamine and Acetaminophen	1
Lansoprazole and Antacids	1
Lansoprazole and Sucralfate	1
Meloxicam and Mefenamic acid	1
Metformin, Glimepiride, and Vildagliptin	1
Methylprednisolone and Dexamethasone	2
Diclofenac sodium and Aspirin	1
Diclofenac sodium and Meloxicam	9
Omeprazole and Lansoprazole	2
Total	**24**

Table 4. DRPs in the dose selection category

DRPs Category	Number of patients	Percentage	Frequency	Percentage
Dose selection				
-Too low drug doses	21	19.44	23	17.04
-Drug doses too high	2	1.85	2	1.48
-Insufficient dose regimen	59	54.63	82	60.74
-Dosing regimen are too frequent	23	21.30	25	18.52
-Incorrect / unclear / non-existent dosing time instructions	3	2.78	3	2.22

Table 5. Distribution of DRPs on dose selection category

DRPs Category	Frequency	Information
Too low drug doses		
Meloxicam	23	The patient was given a dose of 7.5mg/day; literature 15 mg/day
Total	**23**	
Drug doses too high		
Simvastatin	2	The patient was given a dose of 20mg/day; literature 5-10mg/day
Total	**2**	
Insufficient dose regimen		
Aspirin	6	Patients are given once a day, whereas in literature, every 3-4 hours
Furosemide	5	Patients are given two times a day; whereas in literature every 6-8 hours
Glucosamine	17	Patients are given once a day, while in literature three times a day
Ibuprofen	4	Patients are given once a day, whereas in literature, every 4-6 hours
Mecobalamin	16	Patients are given once a day, while in literature three times a day

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Diclofenac sodium	34	Patients are given two times a day, whereas in literature, every 8 hours
Total	**82**	
Dosing regiment are too frequent		
Meloxicam	25	Patients are given three times a day; while in literature, once a day
Total	**25**	
Incorrect/unclear/non-existent dosing time instructions		
Ceftazidime	1	No instructions
Eperisone	1	
Diclofenac sodium	1	
Total	**3**	

Table 6. Results of bivariate analysis between factors and the incidence of DRPs in RA patients

No	Factor	P value		Interpretation
		Drug selection	Dose selection	
1	Gender	0.809	0.901	No relationship
2	Aged	0.879	0.832	No relationship
3	The number of drugs used	0.001	0.000	There is a relationship
4	Comorbidities	0.089	0.086	No relationship

Bivariate analysis

Based on the results of bivariate analysis between demographic factors and the incidence of DRPs, it can be seen that there is no relationship between gender and age with the incidence of DRPs in both drug selection and dose selection categories. In addition, there was also no relationship between comorbidities and the incidence of DRPs in rheumatoid arthritis patients in Palembang city hospitals, both in the drug selection category and the dose selection category. Meanwhile, this study found that there was a relationship between the number of drugs and the incidence of DRPs in the drug selection category (P = 0.001) and the dose selection category (P = 0.000). Which, this result is in line with the findings of Hasan and Mumtazah (2016) that there is a relationship between the number of drugs and the incidence of DRPs.

CONCLUSION

Based on the results of the research that has been done, it can be concluded that there are drug-related problems in rheumatoid arthritis patients in Palembang City hospitals. Drug related problems in the drug selection category occurred in drug interactions (72.03%), drugs without indications (16.09%), too many drugs for the same indication (6.33%), drugs according to guidelines but there are contraindications (2.90%), indications without drugs (2.11%), drugs not according to guidelines (0.26%), and inappropriate duplication of active ingredients (0.26%). Drug-related problems in the category of dose selection occurred in insufficient dose regimen (60.74%), too frequent dose regimen (18.52%), too low drug dose (17.04%), wrong/unclear/no dosing time instruction (2.22%) and too high drug dose (1.48%). There was no relationship between demographic factors and the incidence of DRPs. There was a relationship between the number of drugs and the incidence of DRPs. There was no relationship between comorbidities and the incident of DRPs. Future research is expected to explore more deeply and in detail related to the incidence of DRPs itself, both in rheumatoid arthritis patients and in patients with other diseases. It is suggested that related professionals implement strategies that can minimise the possibility of DRPs in patients, such as making SOPs on how to manage RA therapy or other diseases.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.M., H.N.; Methodology, S.M., H.N.; Software, S.M.; Validation, S.M., H.N., S.H.A.; Formal Analysis, S.M.; Investigation, S.M.; Resources, S.M.; Data Curation, S.M.; Writing - Original Draft, S.M.; Writing - Review & Editing, S.M., H.N., S.H.A.; Visualization, S.M.; Supervision, H.N., S.H.A.; Project Administration, S.M., H.N., S.H.A.; Funding Acquisition, S.M.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Molecular Docking and QSAR Study of 5-*O*-acylpinostrobin Derivatives as Topoisomerase IIα Inhibitors

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Abstract

Background: *Cancer is one of the top causes of death worldwide. A wide range of illnesses known as cancer can start in almost any organ or tissue in the body when abnormal cells multiply uncontrollably. Cancer patients have higher levels of the Topo IIα protein in their cells, this protein has been proposed as a relevant target for anticancer treatment development.* ***Objective****: This study aims to predict the anticancer activity of pinostrobin and 5-O-acylpinostrobin derivatives against topoisomerase IIα by docking molecular and QSAR study.* ***Methods****: In silico analysis was performed using the structure of the topoisomerase IIα (PDB: 5GWK)) as templates. Molecular docking analysis was performed with AutoDock Vina.* ***Result****: All 5-O-acyl pinostrobin derivatives, showed lower ΔG values than the parent pinostrobin. The 5-O-acetyl pinostrobin compound showed the highest score, namely -9.14 kcal/mol. 5-O-acetyl pinostrobin is predicted as the most powerful inhibitor that can cause inhibition of topoisomerase IIα.* ***Conclution****: The results of the best QSAR equation obtained can be used as a reference for predicting the activity of the new pinostrobin derivatives to be synthesized by inserting the electronic (E_{tot}) parameter values of the compounds into the equation.*

Keywords*: modification, pinostrobin, QSAR, topoisomerase IIα*

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INTRODUCTION

Cancer is a broad category of disorders that can originate in nearly any organ or tissue in the body when aberrant cells proliferate out of control, cross normal boundaries to infect other body parts, or spread to other organs. Roughly 10 million deaths, or roughly one in six deaths, globally will be attributable to cancer in 2020 (Martel *et al.,* 2020). The Global Burden of Cancer Study (Globocan) from the World Health Organization (WHO) recorded that total cancer cases in Indonesia in 2020 reached 396,914 cases and 234,511 deaths (Sung *et al.,* 2021).

Cancer treatment can currently be done in several ways, such as chemotherapy, surgery and radiation therapy, which depends on the stage of the cervical cancer. In chemotherapy treatment therapy, most cancer drugs are designed to destroy and prevent the growth of cancer cells by triggering apoptosis (Wong, 2011). The selective initiation of apoptosis is a process used in cancer treatment, as it stimulates cancer cells to undergo self-destruction in a precise manner, without affecting healthy cells or creating inflammation (Nagata, 1997). Apoptosis is initiated when topoisomerase IIα (Topo IIα) inhibition induces DNA damage. The occurrence of DNA damage triggers the activation of p53, which controls the progression of the cell cycle and initiates programmed cell death (Sheng *et al.*, 2006). Topo IIα is a crucial regulator of DNA topology since it controls the wrapping and unwinding of DNA strands during transcription or DNA replication. Topo IIα is more prevalent in cancer cells compared to normal cells, making it a prime target for the development of anticancer drugs (Delgado *et al.,* 2018).

For the treatment of cancer, doxorubicin is one of the chemotherapy treatments. Doxorubicin is an antineoplastic drug that functions as a Topo IIα inhibitor (Mastrangelo *et al.,* 2022). This medicine has been established as the primary choice for chemotherapy in treating different forms of cancer. Recent studies have shown that doxorubicin can cause cardiotoxicity in humans (Zhao *et al.,* 2018). The ideal cancer drug is a drug that has a specific target, high selectivity and is abundant in nature (Pal *et al.,* 2020; Sher *et al.,* 2018).

Research on natural ingredients has been proposed as a safer alternative therapy for cancer. The rhizome of *Boesenbergia pandurata* (Roxb.) Schlecht is reported to contain active anti-cancer compounds. Ethanol extract of *B. pandurata* had more significant cytotoxic activity against HeLa cells (IC_{50} of 60 µg/mL) than Vero cells (IC_{50} of 125 µg/mL) (Listyawati *et al.,* 2016). So it was concluded that the ethanolic extract of *B. pandurata* has selective toxicity against cancer cells. According to Pratama *et al.,* (2022), the flavonoids found in *B. pandurata* have demonstrated potential as an anti-cancer agent by effectively suppressing ER-α and HER2 in silico. One of the compounds containing flavonoids and is a marker compound from these plants is pinostrobin. Pinostrobin has cytotoxic activity against carcinogen-induced fibrosarcoma in mice (Sukardiman *et al.,* 2014). Pinostrobin has cytotoxic activity in vitro against cervical cancer cells HeLa with IC_{50} of 50 µM, breast cancer cell T47D with IC_{50} of 2,93 mM, and no cytotoxic effect on normal HEK293 cells (Jaudan *et al.,* 2018; Widiandani *et al.,* 2023). Even though it has good activity against cancer cells, anti-cancer activity of pinostrobin is still lower than currently available drugs.

One effort to increase the anticancer activity of pinostrobin is by adding a group to its basic structure or known as a structural modification. Structure modification on the hydroxyl group of pinostrobin was synthesized, and five 5-*O*-acylpinostrobin derivatives were obtained as analgesics (Siswandono *et al*., 2020; Suryadi *et al*., 2021). Another study on the breast anticancer activity of 5-*O*-acylpinostrobin derivatives *in silico* indicated that the compound had an affinity for the ErbB4 protein in breast cancer cells (Praditapuspa *et al*., 2021). Pinostrobin structure modification by acyl groups in producing 5-*O*-acylpinostrobin derivatives is highly rational to be developed. Acyl group addition can be determined through the Topliss model for aliphatic substances. The addition of acyl groups improves lipophilic (π), electronic (δ*), and steric (Es) properties, hence increasing pharmacological activity (Siswandono, 2016). Computational approaches are currently crucial in expediting the drug development process and are vital in uncovering novel pharmaceuticals. One of the computational strategies for drug discovery is simulation with molecular docking and quantitative structure-activity relationships (QSAR) (Meng *et al*., 2012).

Molecular docking aims to understand and predict molecular recognition, study drug-receptor interactions, and predict candidate ligands' binding affinity or energy match to target proteins (Meng *et al.,* 2012). The QSAR was used to determine the quantitative relationship between descriptors and inhibitory activity (Daoui *et al.,* 2021). In this study, descriptors represent the most important physical and chemical properties of the molecular structure of pinostrobin and its derivatives. In silico anticancer activity was obtained from the molecular docking of pinostrobin compounds and their derivatives against inhibition at Topo IIα.

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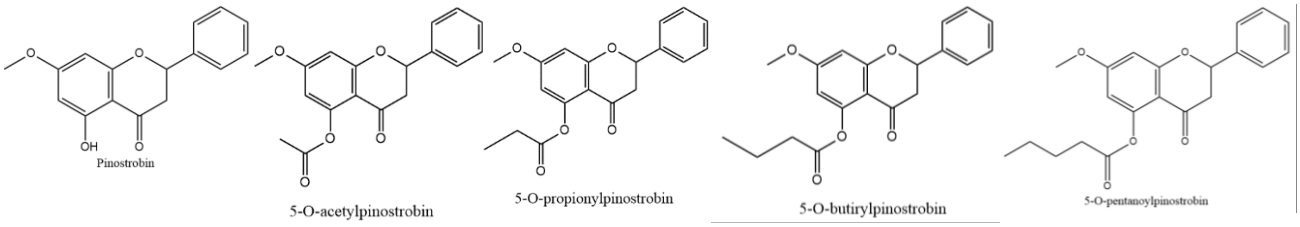

Figure 1. Structure of pinostrobin and four 5-O-acylpinostrobin derivatives

MATERIALS AND METHODS

Materials

The material used in this research were pinostrobin and four 5-O-acylpinostrobin derivative compounds. The structure of the compound was drawn using the ChemDraw 20.0 application (PerkinElmer). The results can be seen in Figure 1. The three-dimensional structure of Topo IIα which forms a complex with DNA and antagonist ligands (PDB ID: 5GWK) was downloaded in .pdb format from the RCSB PDB site https://www.rcsb.org/structure/5GWK has a resolution of 3.15 Å. The 5GWK protein code that binds to the etoposide ligand (with the code EVP).

Tools

The hardware used is the ASUS X409FJ computer with Intel® Core™ i5 8265U @ 1.80 GHz Processor specifications and 4 GB Random Access Memory (RAM). The software used is ChemDraw 20.0 and Chem3D 20.0 to create the structure of the test ligand compounds, AutoDockTools 4.2.6 for the preparation of target macromolecules, AutodockVina in the PyRx 0.8 system and Discovery Studio Visualizer v.19.1.0.18287 for in silico testing, and SPSS Ver. 21 to determine QSAR equation.

Method

Ligan preparation

The structure of the tested compounds were pinostrobin, 5-*O*-acetyl pinostrobin, 5-*O*-propionylpinostrobin, 5-*O*-butiryl pinostrobin, 5-*O*-pentanoylpinostrobin. The native ligand of this receptor is etoposide. The structures were designed using the ChemDraw 20.0 program and then converted into a three-dimensional structure using the Chem3D 20.0 program and optimized to minimize energy. The most stable form is saved in *.mol2 format, then open the compound in PyRx, click Load Molecule, and create a ligand to dock with the target receptor.

Protein Preparation

Protein structure preparation was using AutoDock Tools. Topo IIα used chain D from the crystal model of the Human Topoisomerase IIα in complex with DNA and etoposide. Water molecules and all non-standard residues from the initial structure were removed. Then, all the missing hydrogen and Kollman charges were added to the system, and the prepared protein receptors were saved in *.pdbqt format and immediately placed into the PyRx workspace folder.

Molecular docking

The prepared macromolecules are subjected to preliminary tests and method validation to ensure that the method used is valid in the molecular docking process for the test ligand. Validation of the docking method for the acyl ligand was carried out to look for the conformation of the original ligand. This is done by determining a grid box to determine the coordinates of the active site of the target receptor macromolecule. Validation of the docking method was carried out to look for the 3D conformation of the cocrystal ligand and copy ligand for the target protein using PyMOL software which was expressed in a Root Mean Square Deviation (RMSD) value $\leqslant$ 2Å. The molecular docking test was performed with five repetitions using the same grid box settings.

Quantitative structure-activity relationship (QSAR) of pinostrobin derivatives

The parameters observed in this study were physicochemical parameters, including (1) Lipophilic Parameters (LogP and LogS), (2) Electronic Parameters (E_{tot}, E_{LUMO} and E_{HOMO}), (3) Steric Parameters (WM and MR), and *in silico* activity parameters, namely value of binding affinity (ΔG). The QSAR equation is made to see the relationship between physicochemical properties parameters and activity parameters using IBM SPSS Ver 21.

RESULTS AND DISCUSSION

Moleculer docking validation

Table 1 shows the gridbox settings and RMSD values of the target macromolecules. The RMSD calculation result between the original ligand and the docking results was 1.083 Á, which shows that the method used is valid and can be used for the test ligand.

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Table 1. Gridbox settings and RMSD values of target macromolecules

Makromolecul	Grid Center			Grid Size (Å)	RMSD	Condition
	X	Y	Z			
Topoisomerase IIα (5GWK)	31.3837	-22.6558	-58.1512	25	1.083 Å	< 2 Å

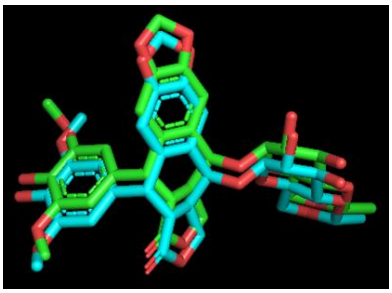

Figure 2. Topo IIα overlay results validate the docking method with PyMOL. Green (crystallography results) and blue (re-docking results)

Table 2. Results of $\Delta G_{binding}$ values and results of interaction of amino acid residues between pinostrobin and 5-O-acylpinostrobin with Topoisomerase IIα

Compound	$\Delta G_{binding}$ (kkal/mol)*	Residues	
		Hydrogen Bond	Non-Hydrogen Bond
Native Ligand	-12.82 ± 0.04	**Arg487**, **Gly462**, **Asp463**, **DT9**	Gly488, **DG13**, **Met766**, Pro803
Doxorubicin	-11.78 ± 0.04	Gly488, **Asp463**, **Gly462**, DC8	Glu461, **Arg487**, DA12, **DG13**
Pinostrobin	-8.5 ± 0.00	DC8	**DG13**, **Arg487**, Met762
5-O-acetyl pinostrobin	-9.14 ± 0.05	DC8, **Arg487**, **DT9**	**DG13**, Met762
5-O-propionylpinostrobin	-8.94 ± 0.05	**Arg487**, **DT9**	DC8, Met762, **DG13**
5-O-butiryl pinostrobin	-8.86 ± 0.05	**Arg487**, **DT9**	**DC13**, Met762, DC8
5-O-pentanoylpinostrobin	-8.88 ± 0.04	DC8, **Arg478**	**DG13**, Tyr805

Note: *average results of 5 replications. Bold print represents the similarity of amino acids to the original ligand.

In addition to the RMSD value, the similarity of the interaction of the redocked ligand and the original ligand is crystallographic to the amino acid residues in the active site of the target protein. The overlay results between the original ligand and the crystallographic ligand can be seen in Figure 2.

Moleculer docking

Molecular docking of the test ligand is carried out in the same way as the validation process using the same size and position of the grid box. The parameters observed to determine the affinity of a ligand for a receptor are binding free energy (ΔG), amino acid residues, and the number of hydrogen bonds (Kontoyianni *et al*., 2004). The binding affinity score (ΔG) is a parameter of conformational stability between ligand and macromolecule. Molecular docking results are ranked based on the value of binding affinity score from the smallest to the largest. The lowest binding affinity score indicates that the conformation formed is stable. So the lowest binding affinity, more potent the macromolecular-ligand complex (Daoui *et al*., 2021).

There are 2 types of topoisomerase, namely topoisomerase I and topoisomerase II (Topo IIα and Topo IIβ) (Vejpongsa and Yeh, 2014). Topoisomerase IIα was chosen as the inhibitory target because Topo IIα is overexpressed in cancer cells while Topo IIβ is expressed in normal cells (Florkemeier *et al*., 2021). So suppressing Topo IIα activity using potential inhibitors is very important to inhibit DNA replication. Therefore, this research was carried out to predict the Topo IIα inhibitory activity of pinostrobin derivative compounds using the molecular docking method. The selected Topo IIα macromolecule has a cocrystal ligand and X-Ray crystallography results with the PDB ID code 5GWK. The parameters observed are the affinity of the ligand for the receptor which is expressed in the form of binding free energy values (ΔG), residue interactions and hydrogen bonds of 5-*O*-acylpinostrobin derivative compounds. The results of molecular docking studies on macromolecular targets with Autodock Vina. Analysis of the ΔG value and amino acid residues of molecular docking results on Topo IIα can be seen in table 2. The results of native ligand redocking obtained a ΔG value of -12.82 kcal/mol interacting with the 5GWK binding site. Doxorubicin as a positive control obtained a ΔG value of -11.78 kcal/mol. The 5-*O*-acylpinostrobin

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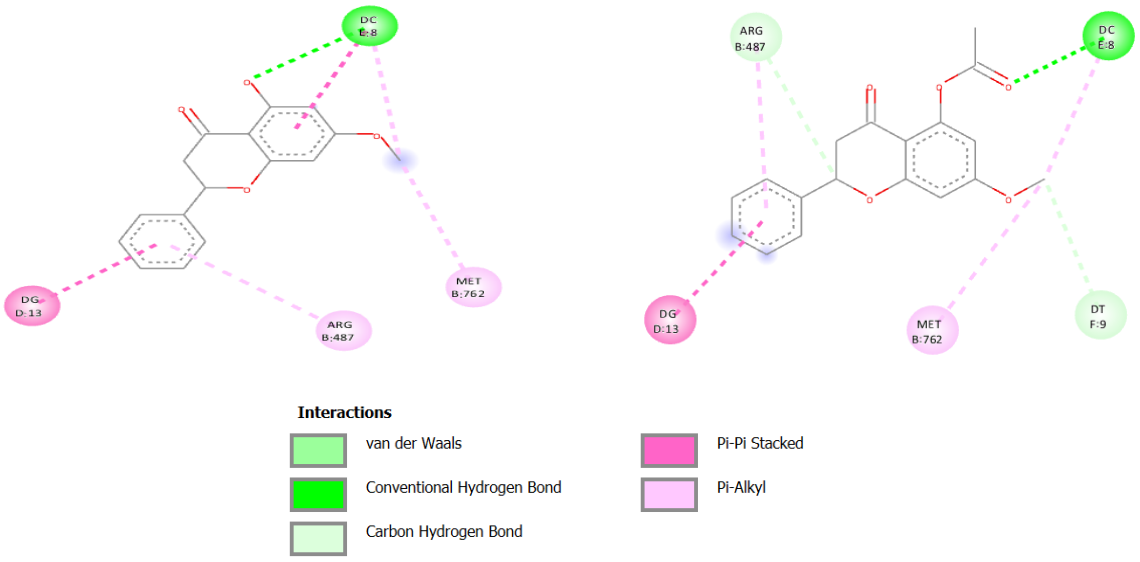
derivative obtained a ΔG value of -8.88 to -9.14 kcal/mol, while pinostrobin obtained a ΔG value of -8.5 kcal/mol. The results of this study showed that 5-*O*-acylpinostrobin derivatives obtained lower ΔG values than pinostrobin.

The best 5-*O*-acylpinostrobin derivative is 5-*O*-acetylpinostrobin where this compound shows stronger inhibitory activity than other 5-*O*-acylpinostrobin derivatives, because the ΔG value of 5-*O*-acetylpinostrobin is the most negative, namely -9.14 kcal/mol. The increase in the ΔG value in the 5-*O*-pentanoylpinostrobin compound occurs due to the formation of hydrogen bonds with residues Arg487, DT9 and DG13. Interactions at the same residue as the positive control are predicted to inhibit DNA strand unwinding so that replication does not occur, which then causes anticancer activity. According to a study conducted by Agustin *et al.*, (2022) and Widiyana *et al.*, (2023), pinostrobin derivatives exhibited stronger anti-cancer effects on the HER-2 and Erα receptor in silico compared to pinostrobin. According to Praditapuspa *et al.*, (2021), acetyl pinostrobin derivatives can be used as possible anticancer drugs because they have stronger apoptosis activator mechanisms than pinostrobin. Pinostrobin derivative compounds exhibit enhanced efficacy and selectivity in vitro compared to pinostrobin, making them highly promising for advancing as potential anticancer agents (Widiandani *et al.*, 2023). Based on research conducted by Sukardiman *et al.* (2000), pinostrobin isolate from B. pandurata has inhibitory activity against topoisomerase in vitro. Pinostrobin can interfere with the DNA strand unwinding reaction mediated by topoisomerase, thereby inhibiting cancer cell replication and transcription and inducing apoptosis in T47D breast cancer cells. This indicates that the 5-*O*-pentanoylpinostrobin derivative compound has the potential to be better than pinostrobin for development as an anticancer drug.

QSAR is an important stage of drug development so as to obtain equations that can later be used for further drug development. The QSAR technique has the concept that similar structures have the same properties and the more differences there are between molecules, the more difficult it is to relate their physical and chemical properties to biological activity. The physicochemical properties that provide the greatest contribution to activity are lipophilic, electronic and steric properties (Siwandono, 2016). In this stage, we study the quantitative relationship between physicochemical property parameters and the inhibitory activity of the Topo IIα anti-cancer receptor in silico. The physicochemical parameter values (Log P, Log S, E_{tot}, E_{HOMO}, E_{LUMO}, MW and MR) and the results of in silico of binding free energy values (ΔG) of the inhibitory activity of the Topo IIα for pinostrobin and four 5-*O*-acylpinostrobin derivative compounds can be seen in Table 3.



Pinostrobin **5-acetylpinostrobin**

Figure 3. Interaction of pinostrobin and 5-Oacetylpinostrobin compounds with Topo IIα

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Table 3. Physicochemical parameters and activity of pinostrobin and four pinostrobin acyl derivatives

Compound	LogP	LogS	E_{tot}	E_{HOMO}	E_{LUMO}	MW	MR	Log (1/ΔG)
Pinostrobin	2,75	-3,84	46,19	-11,21	-3,45	270	74,02	-0.9294
5-*O*-acetil pinostrobin	3,00	-3,64	56,72	-11,31	-3,53	312	83,49	-0.9609
5-*O*-propionyl pinostrobin	3,27	-3,94	55,52	-11,31	-3,58	326	88,30	-0.9513
5-*O*-butiryl pinostrobin	3,43	-4,17	55,31	-11,31	-3,57	340	93,11	-0.9474
5-*O*-pentanoyl pinostrobin	3,72	-4,52	55,11	-11,31	-3,57	354	97,91	-0.9484

Note: Log P = Logarithm of octanol/water partition; Log S = Logarithm of solubility (in water); Etot = Total energy (kcal/mol); EHOMO = Energy Highest Occupied Molecular Orbital (eV); E_{LUMO} = Energy Lowest Unoccupied Molecular Orbital (eV); MR= Molar Refraction (cm3/mol); MW= Molecular Weight (Da)

Table 4. QSAR equation

No.	QSAR Equation	n	r	SE	F	Sig
1	Log (1/ ΔG) = - 0.011 Log P – 0.912	5	0.360	0.199	0.446	0.552
2	Log (1/ ΔG) = - 0.004 Log S – 0.963	5	0.110	0.095	0,037	0.860
3	Log (1/ ΔG) = - 0.003 E_{tot} – 0.812	5	0.942	0.016	23.589	0.017
4	Log (1/ ΔG) = 0.226E_{HOMO} + 1.604	5	0.884	0.041	10.770	0.046
5	Log (1/ ΔG) = 0.144 E_{LUMO} – 0.438	5	0.679	0.141	2.563	0.208
6	Log (1/ ΔG) = 0.000 MW – 0.882	5	0.576	0.178	1.493	0.309
7	Log (1/ ΔG) = - 0.001 MR – 0.895	5	0.481	0.198	0.902	0.412
8	Log (1/ ΔG) = - 0.419 Log P + 0.063 Log P^2 – 0.260	5	0.521	0.250	1.088	0.479
9	Log (1/ ΔG) = - 0.453 Log S - 0.055 Log S^2 – 1.876	5	0.207	0.164	0.260	0.793

CONCLUSION

Based on research, 5-*O*-acetylpinostrobin has a high affinity for topo IIα inhibitors with ΔG -9.14 kcal/mol so it can be a cancer drug candidate. The best QSAR equation results (Log (1/ΔG) = - 0.003 Etot – 0.812) obtained can be used as a reference for predicting the activity of new pinostrobin derivatives that will be synthesized by entering the electronic value (Etot) of the compound into the equation.

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AUTHOR CONTRIBUTIONS

Conceptualization, T.W.; Methodology, T.W., J.E., S.R.; Software, T.W., J.E., S.R., P.A.P..; Validation, T.W., J.E., S.R.; Formal Analysis, T.W., J.E., S.R.; Investigation, S.R., P.A.P.; Resources, T.W.; Data Curation, T.W., J.E., S.R.; Writing - Original Draft, S.R., P.A.P.; Writing - Review & Editing, T.W., J.E.; Visualization, S.R.; Supervision, T.W.; Project Administration, T.W., S.R.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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