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Comparative Study of Densitometry and Videodensitometry for Quantitating the Active Pharmaceutical Ingredients Using Thin Layer Chromatography – Systematic Review

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Abstract

***Background**: Chromatography is one of the analytical techniques widely used for the quality control process in the pharmaceutical industry. One of the analytical methods used in drug analysis is Thin Layer Chromatography (TLC). The analysis process of TLC can be performed using densitometry (scanner) or videodensitometry (videoscan). The principal analysis of densitometry (scanner) is based on the density measured from each spot on the TLC plate using a specific wavelength range, and videodensitometry (videoscan) is performed by taking pictures of the plate using a Visualizer at a specific wavelength. **Objective**: This review article discusses the application of densitometry and videodensitometry methods for quantitative analysis of pharmaceutical products. **Methods**: This study was conducted using a systematic review method using the PRISMA statement from January to April 2023. Four databases were searched: PubMed, ScienceDirect, Scopus, and Google Scholar with inclusion criteria: studies on thin layer chromatography analysis using densitometry and videodensitometry. **Results**: Based on the ten articles in this study, it is known that the active ingredient concentrations in pharmaceutical products can be determined using densitometry and videodensitometry. The statistical analysis results show no significant difference between the two methods' chemical concentrations of active ingredients in pharmaceutical products. **Conclusion**: TLC densitometry and videodensitometry is a valid methods analysis that can be used for quantitating the active pharmaceutical ingredient concentration in finished pharmaceutical products.*

***Keywords**: densitometry, medicine, thin layer chromatography, videodensitometry*

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INTRODUCTION

Chemical compound analysis methods have been widely developed using various methods and analytical instruments. The analytical process aimed to identify the components (qualitative) or determine the concentration of the active ingredients (quantitative). Chemical compound analysis methods are mainly applied in several important sectors, such as quality control in industry, monitoring and control of contaminants, clinical and biological tests, and geological tests (Ege, 2021).

Quality control is important for guaranteeing a product's safety, efficacy, and effectiveness. For example, in the pharmaceutical, conventional, and herbal medicine industries, quality control is one of the critical points of the quality of a drug product produced. The selection of analytical techniques is essential for qualitative and quantitative analyses to ensure product quality. Qualitative analysis identifies certain compounds or groups of compounds, whereas quantitative analysis determines the concentration of compounds present in raw materials or products (Bandaranayake, 2006; Balekundri & Mannur, 2020)

Chromatography is an analytical technique that is widely used in quality control processes in the pharmaceutical industry. Thin-layer chromatography (TLC) is one of the analytical methods used in drug analysis. The analysis can be performed using densitometry (scanner) or videodensitometry (Videoscan). The principle of chromatography is the separation of chemical compounds based on their affinity for the stationary phase (solid or liquid) and mobile phase (liquid or gas) (Bittner *et al*., 2016). The choice of the analytical instrument used is relatively dependent on the type and characteristics of the sample to be analyzed. For example, TLC instruments are widely used to analyze chemical compounds in plants because they are simple, fast, and relatively inexpensive (Lucio-Gutiérrez, Coello & Maspoch, 2012).

TLC is the most frequently used method for qualitative and quantitative analysis of chemical compounds using densitometry (Liang *et al*., 2004). The analysis can be performed using densitometry (scanner) or videodensitometry (Videoscan). However, in compendial, the analysis of natural ingredients still uses TLC densitometry; however, video-densitometry is rarely used (Alaerts *et al.*, 2012). The videodensitometry method is a simple analytical method that uses images from the visualizer to be converted into a chromatogram profile. However, this method has a major weakness in that spectral analysis of each spot on the TLC plate cannot be performed. In addition, this method requires software that supports image analysis of the TLC plate to be converted into a chromatogram profile. Therefore, this method is rarely used for both qualitative and quantitative analysis. However, the videodensitometry method is advantageous because it can be used for the analysis of unstable samples on TLC plates, samples that require a reagent to detect the analyte, and through a derivatization process (Hahn, 2018).

Densitometric analysis was based on the density measured from each spot on the TLC plate using a specific wavelength range. Videodensitometry analysis was performed by taking pictures of the plate using a Visualizer at a specific wavelength, generally UV 254 or 366 nm, and then scanning it with videoscan software to obtain a chromatogram profile. The principle of videodensitometry is to group image pixels on each track on the TLC plate based on the value of the visual intensity of the color formed, consisting of *Red*, *Green*, and *Blue* (RGB) (Reich & Schibli, 2014). RGB values are one of the parameters used to describe colors precisely using mathematical models. This value was used to determine the intensity of the color formed from each spot on the TLC plate. The videodensitometry method utilizes images of visualization results from TLC plates, and the color intensity formed from each spot can be changed into RGB values. The conversion process from color to chromatogram profiles through a mathematical model approach requires particular software, such as videoscan from CAMAG. All image pixels from each spot on each track with the same Retardation Factor (Rf) were averaged and plotted as a distance function to produce an analog chromatogram curve. The principal analysis of both methods is explained in Figure 1. This analog chromatogram curve is the chromatogram profile of each track that has been analyzed; therefore, this technique can also be performed qualitatively and quantitatively (Srivastava, 2011; Fichou & Morlock, 2018).

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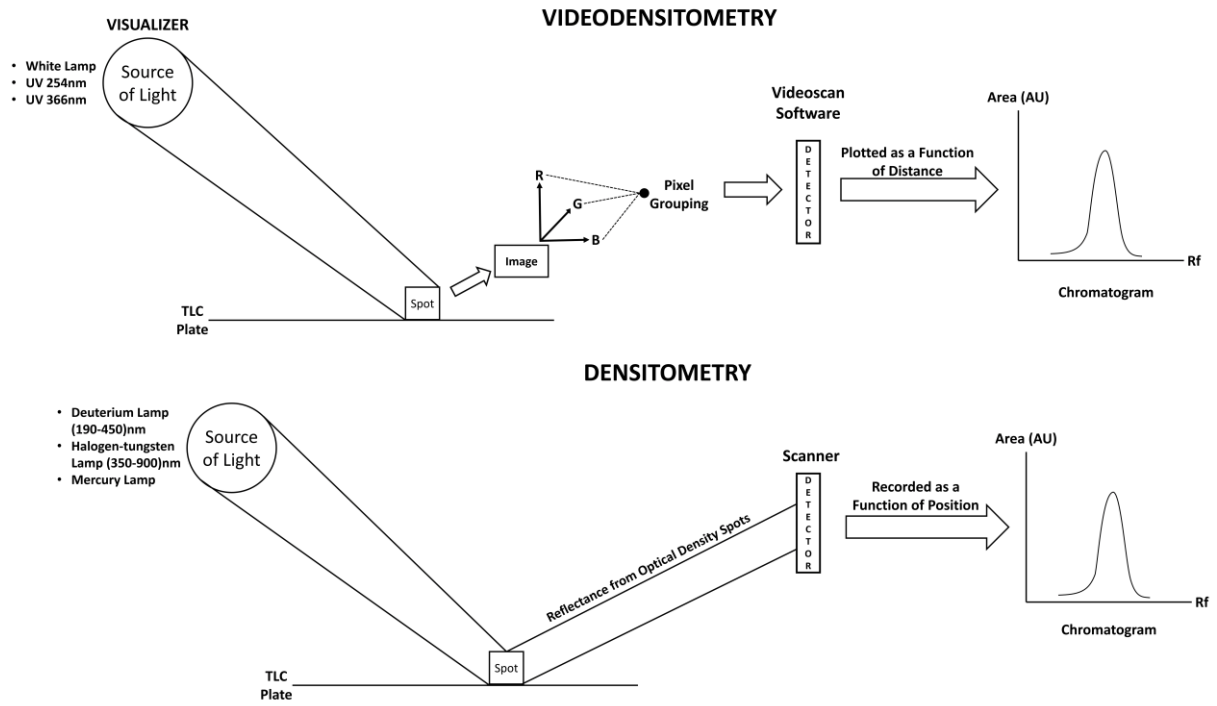

Figure 1. The different principle analysis of videodensitometry and videodensitometry

This study reviews articles related to TLC analysis using densitometry and videodensitometry instruments. Therefore, the development of an analytical process must be validated. According to the USP guidelines for method validation, reviewed articles show the results of several method validation parameters, including precision, accuracy, linearity, detection limit, and quantitation limit (USP, 2021). This study conducted a systematic review using the PRISMA statement to collect articles from several databases, including Google Scholar, PubMed, Science Direct, and Scopus (Page *et al.*, 2021). The review results are expected to provide new insights into the development of thin-layer chromatography for quantifying active ingredient concentrations using densitometry and videodensitometry.

METHODS

Eligibility criteria

The eligibility criteria in this systematic review were determined based on the research questions compiled in the following PICO (population, intervention, comparator, outcome) format.

- *Population*: Analysis using Thin Layer Chromatography
- *Intervention*: Densitometry and Videodensitometry
- *Comparison*: -
- *Outcome*: Quantitating Thin Layer Chromatographic Spots

In this systematic review, the PICO framework was used to develop literature search strategies to ensure comprehensive and bias-free searches. Generally, the PICO Framework is used in evidence-based practice, especially in evidence-based medicine, to provide solutions or formulate clinical or health care-related questions related to the research problem (Methley *et al.*, 2014). In this study, the PICO format was used to specify articles collecting data from several databases.

The inclusion criteria for this study were studies related to TLC analysis using densitometry and videodensitometry instruments, as well as articles containing comparative analysis and results from both methods from 2000 to 2023. The exclusion criteria were articles in languages other than English and systematic reviews, review articles, conference abstracts, case reports, editorials, proceedings, and letters to the editor.

Article selection and screening process

Searching and collecting article data were conducted online from January to April 2023 using the keywords "Thin Layer Chromatography AND densitometry AND videodensitometry" in several online databases, such as PubMed, Google Scholar, ScienceDirect, and Scopus. Furthermore, each article collected was screened using EndNote X9.3.3. The first

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screening stage was performed by checking for duplicates from the article search results, and then separating the duplicate articles found. After the article separation process, the sorting process continued, including the suitability of the title and abstract for this research topic, namely TLC analysis using densitometry and videodensitometry instruments. Furthermore, an eligibility test was carried out by reading the entire content of the article to determine its suitability with the inclusion criteria that had been previously set. The overall process of collecting and sorting articles was carried out by five people and double-checked by two others, and the risk of bias assessment of each article was determined using the PRISMA checklist. A research flow diagram is shown in Figure 2.

Data analysis

Data analysis of the articles obtained was carried out descriptively by comparing the data obtained from each article, including data on the validation of analytical methods and determination of concentrations from densitometry and videodensitometry methods. The data are presented in Table 1.

RESULTS AND DISCUSSION

A total of 319 articles were found in the four databases. Three hundred and nineteen articles were obtained using the search strategy:14 from PubMed, 120 from ScienceDirect, 164 from Scopus, and 21 from Google Scholar. After removing duplicate articles from the four databases, 107 articles were selected for systematic review. We excluded 81 articles because they listed the categories of book chapters, books, conference papers, and reviews that violated the eligibility criteria. Therefore, 26 full-text articles were reviewed according to the systematic review guidelines. After reading the full-text articles, 16 were excluded because they were irrelevant to the research question (Figure. 1).

TLC/HPTLC analysis methods using densitometry and videodensitometry for analyzing pharmaceutical products in various dosage forms have been widely developed (Srivastava, 2011). Generally, TLC analysis is performed using densitometry (scanner), but in several publications, TLC analysis methods were developed using videodensitometry. This systematic review aimed to compare the results of TLC analysis using densitometry and videodensitometry to determine active pharmaceutical ingredient concentrations, and to show that qualitative and quantitative TLC analysis can also be performed using videodensitometry. The results of this review are summarized in Table 1.

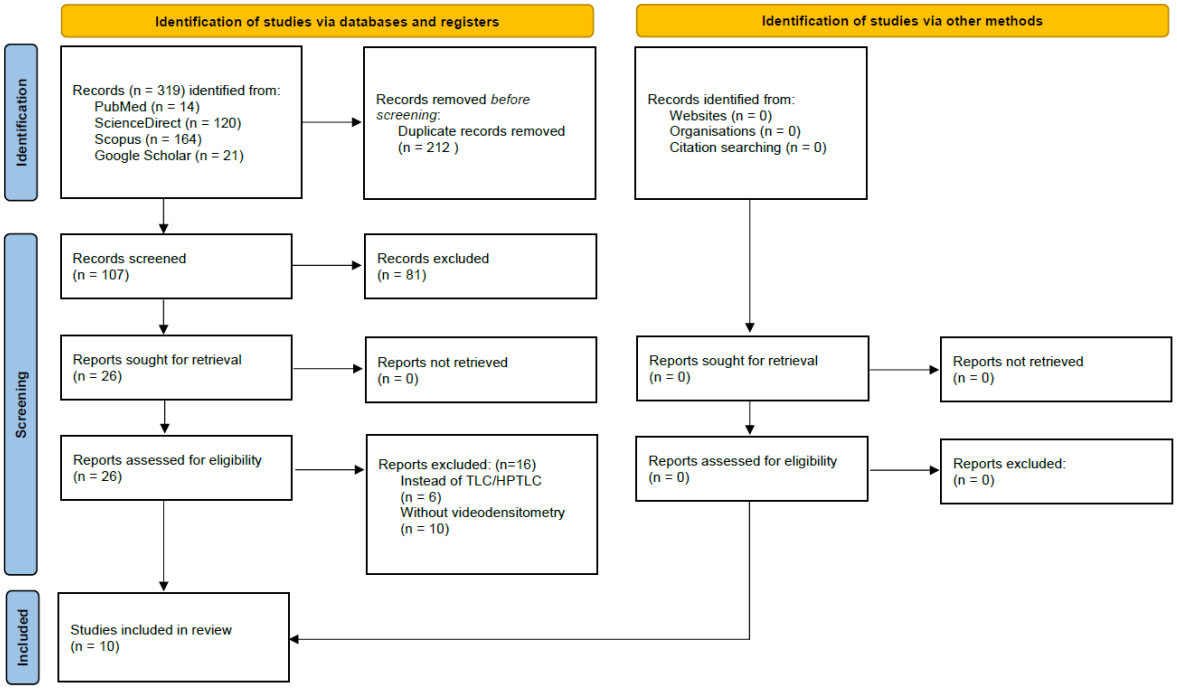

Figure 2. The research PRISMA 2020 flow diagram

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Table 1. Densitometry and videodensitometry applications in pharmaceutical products included in the review

Sample	Method Validation										Active Ingredient Concentration (mg)		References
	LOD (µg/spot)		LOQ (µg/spot)		Accuracy (%Recovery)		Precision (%RSD)		Linearity (r)				
	D	V	D	V	D	V	D	V	D	V	D	V	
Apo-Nadol® 80mg (Nadolol)	0.05	0.2	0.1	1	99.71 – 102.05	97.15 – 101.57	1.14	0.68	0.9972	0.9961	80.81 ± 0.86	80.28 ± 1.22	(Gumieniczek *et al*., 2002)
Apo-Pindol® 10mg (Pindolol)	0.05	0.05	0.1	0.1	98.41 – 101.57	97.37 – 101.14	0.74	0.79	0.9921	0.9960	9.93 ± 0.08	9.96 ± 0.06	
Betoptic 0.5% (Betaxolol)	2.0	1.9	2.3	2.2	95.36 – 96.36	94.02 – 98.21	1.51	1.82	0.9935	0.9857	5.32 ± 0.04	5.35 ± 0.05	(Hopkała *et al*., 2003)
Timohexal 0.1% (Timolol)	0.5	0.5	0.9	1	97.08 – 102.2	94.89 – 103.6	1.32	1.39	0.9966	0.9905	1.35 ± 0.04	1.31 ± 0.07	
Fluoxetine 20mg	0.05	0.15	N/A	N/A	101.00 – 106.66	96.25 – 100.75	1.10	1.59	0.9945	0.9997	21.02 ± 0.86	20.86 ± 0.64	(Skibiński, Misztal & Kudrzycki, 2003)
Paroxetine 20mg	0.02	0.15	N/A	N/A	103.00 – 106.50	98.50 – 107.00	1.04	1.78	0.9909	0.9996	19.83 ± 0.84	19.64 ± 0.96	
Fevarine 50mg (Fluvoxamine)	0.05	0.06	N/A	N/A	98.50 – 101.83	100.83 – 108.00	1.93	1.60	0.9983	0.9912	50.60 ± 1.26	49.48 ± 2.52	(Skibiński & Misztal, 2004)
Aurorix 150mg (Moclobemide)	0.02	0.07	N/A	N/A	98.30 – 107.67	96.50 – 108.50	1.25	1.87	0.9939	0.9843	151.72 ± 2.92	148.21 ± 5.91	
Cipamil® 20mg (Citalopram)	0.09	0.1	N/A	N/A	102.17 – 104.44	100.74 – 104.01	1.83	1.97	0.9946	0.9998	20.26 ± 0.37	19.89 ± 0.39	(Skibiński & Misztal, 2005)
Bezamidin 200mg (Bezafibrate)	9.39	9.81	24.21	24.85	97.17 – 103.61	91.88 – 100.45	0.50	1.06	0.9962	0.9934	200.78 ± 0.42	192.33 ± 1.17	(Misztal & Komsta, 2005)
Lipanor 100mg (Ciprofibrate)	11.65	11.89	23.55	23.15	95.41 – 100.93	95.03 – 100.57	1.91	1.91	0.9742	0.9678	98.01 ± 1.24	97.80 ± 1.13	
Liprox 20mg (Lovastatin)	N/A	N/A	N/A	N/A	97.90 – 104.2	95.80 – 104.6	1.88	1.49	0.9780	0.9650	20.82 ± 1.07	20.44 ± 1.32	(Komsta et al., 2007)
Simvahexal 20mg (Simvastatin)	N/A	N/A	N/A	N/A	97.20 – 102.2	96.80 – 106.2	1.43	1.49	0.9880	0.9671	19.72 ± 0.49	19.59 ± 0.87	
Seroquel 25mg (Quetiapine)	0.02	0.04	0.06	0.12	99.55 – 106.28	98.07 – 107.41	1.19	1.02	0.9862	0.9916	24.82 ± 1.26	24.84 ± 1.76	(Skibiński *et al*., 2008)
Fenoratio 100mg (Fenofibrate)	N/A	N/A	N/A	N/A	93.35 – 109.51	91.62 – 113.84	1.42	1.30	0.9770	0.9872	101.42 ± 0.87	102.73 ± 0.65	(Komsta & Misztal, 2005)
Gemfibral 450mg (Gemfibrozil)	N/A	N/A	N/A	N/A	93.80 – 107.13	95.29 – 102.35	1.95	1.41	0.9795	0.9790	452.12 ± 1.04	444.56 ± 1.21	
Atacand 16mg (Candesartan)	0.08	0.13	0.27	0.44	99.06 – 100.56	98.87 – 100.06	1.80	0.42	0.9997	0.9981	15.97 ± 0.79	15.91 ± 0.71	(Gumieniczek *et al*., 2011)
Xartan 50mg (Losartan)	0.12	0.11	0.39	0.37	99.80 – 101.12	99.00 – 100.04	1.38	0.81	0.9986	0.9982	50.23 ± 1.93	49.76 ± 1.59	

Notes: N/A Not evaluated; D: Densitometry; V: Videodensitometry

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Densitometry is a technique used to measure the density of a substance from each spot on a TLC plate. The principle of densitometry is based on the Kubelka-Munk theory, which provides a quantitative description of the absorption, reflectance, and scattering of light in a medium such as a TLC plate. In TLC analysis, the Kubelka-Munk theory can be used to quantitatively determine the concentration of components on a TLC plate by measuring their absorption, reflectance, and scattering properties. Analyzing absorption or reflectance data using the Kubelka-Munk equation makes it possible to obtain quantitative information (Reich & Schibli, 2014).

On the other hand, videodensitometry is a technique used in chromatography to quantitatively analyze and visualize the results of separated compounds from a TLC plate. The analysis process requires a video camera and image analysis software to capture and analyze the chromatograms. In videodensitometry, the chromatogram is illuminated with UV or visible light, and the video camera captures the image of the bands or spots on the TLC plate. The captured images were then analyzed using specialized software to identify and quantify the separate components in the plate based on their UV absorbance or color. The detection process for videodensitometry can be performed using several software, packagssuch as, ImageJ (U.S. National Institute of Health, Bethesda, MD, USA), Videoscan (CAMAG, Muttenz, Switzerland), Sorbfil TLC Videodensitometry (Jsc Sorbpolymer, Krasnodar, Russia), Macherey Nagel TLC scanner (Macherey Nagel, Düren, Germany), JustTLC (Sweday, Sodra Sandby, Sweden), TLC Analyzer (Amber, 2007), qtlc (cran.r-project), TLSee (AlfaTech, Genova, Italy), Matlab's imaging processing toolbox (MathWorks, Natick, MA, USA), and quanTLC (Fichou & Morlock, 2018).

Some of the above software, such as the TLC analyzer, qTLC, and MATLAB, are free and can be used for plate visualization, but data quantification requires additional software. qTLC has limited capabilities because data processing must be performed through a command prompt, which means that it is difficult for non-programmers. Other non-free software (VideoScan, Sorbfil TLC Videodensitometer, Macherey Nagel TLC scanner, JustTLC, TLSee, QuanTLC, and ImageJ) can be used for plate visualization and quantification of separated compound profiles on TLC plates. Hence, it is essential to select an analysis software for videodensitometry is essential to consider (Campus, 2011). Therefore, videodensitometry is a suitable method for analyzing the results of chromatographic separation and can provide important information regarding the quality and quantity of the TLC method. However, videodensitometry has some limitations, such as the need for high-quality video cameras and specialized software with the potential for analysis (Srivastava, 2011).

Based on the selected articles, we found that both densitometry and videodensitometry can be used to quantitatively analyze a sample's active pharmaceutical ingredients (API). The API concentrations obtained from these methods were not significantly different ($P>0.05$) when subjected to statistical analysis using *Student t-test*. Additionally, both methods met the requirements for method validation parameters, such as precision, accuracy, linearity, limit of quantification (LOQ), and limit of detection (LOD).

The linearity of the densitometry and videodensitometry methods was evaluated by analyzing a series of different concentrations of each standard. The linearity of the analytical method is the ability to provide results directly proportional to the concentration or amount of analyte measured over a specified range. The correlation coefficient 'r' was the acceptance criteria in linearity that indicate the linear relationship response between variables with the concentration or amount of the analyte. Based on this review article, the densitometry method has linearity over the correlation coefficient range from 0.9742 to 0.9972, while videodensitometry has a value of 0.9650–0.9998. A high 'r' value, close to 1, indicates good linearity (Harron, 2013). There was a linear correlation between the API concentration (µg/spot) and the peak area chromatogram. A comparison of the results obtained using the two methods using an independent sample t-test showed no statistical difference ($P>0.05$).

The limit of quantification (LOQ) and limit of detection (LOD) were also determined. LOD and LOQ are important parameters used to assess the sensitivity and reliability of analytical methods. Generally, the LOD and LOQ are often determined as the concentration or amount corresponding to a specified signal-to-noise ratio (S/N), but they can also be determined based on visual evaluation, standard deviation (SD), and slope response (Little, 2016). The LOD and LOQ were determined in the selected articles based on the standard deviation response and slope

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values in the linear regression. However, the LOD and LOQ values have not been determined in several articles. According to the USP guidelines for method validation, analytical procedures for determining the API concentration of major components in finished pharmaceutical products are classified in category I, where the detection limit and quantitation limit parameters are not necessarily required (USP, 2021). However, both of these parameters performed better during the validation process for the quantitative analysis. LOD was found to be 0.02 to 11.65 µg/spot for densitometry assay and 0.05 to11.89 µg/spot for videodensitometry assay, while the LOQ was found to be 0.06 to 24.21 µg/spot and 0.10 to 24.85 µg/spot for densitometry and videodensitometry assays, respectively. Moreover, no statistically significant differences were observed in the LOD and LOQ parameters (*P>0.05*). The sample matrix may affect the LOD and LOQ values, especially in complex matrices, as it may interfere with or alter the analytical signal of the target compounds (Yuwono & Indrayanto, 2005).

Accuracy is often assessed by calculating the percentage recovery, which compares the measured value obtained by the method to the known or reference value. Commonly used acceptance ranges for percent recovery include 80-120%, 90-110%, or tighter ranges defined by specific guidelines or compendials (European Medicines Agency, 2022). All selected studies used spiking concentrations of 80%, 100%, and 120% of the standard. The percent recovery achieved was 93.35 to 109.51 on densitometry and 91.62 to 109.51 on videodensitometry. This indicates that both methodologies accurately measure API concentrations in pharmaceutical products.

Precision refers to the degree of agreement or reproducibility between individual test results obtained from the analysis using a specific analytical method. Precision determination comprises three main categories: repeatability, intermediate precision, and reproducibility. Repeatability or intra-assay within-day precision methods from ten articles were analyzed using the lowest and highest standard concentrations of API in triplicate replications and determined as relative standard deviation (RSD). The densitometry results were 0.5 to 1.95%, and videodensitometry had a 0.42 to 1.91% RSD value. The results showed that the RSD did not exceed 2% for all API concentrations, indicating that the proposed densitometry and videodensitometry methods can be considered precise.

The densitometry and videodensitometry methods showed no statistical differences for all parameter validation. Both methods fulfilled the linearity parameters, precision, accuracy, LOD, and LOQ. The API concentrations obtained from the two methods were compared and then subjected to statistical analysis using the *Snedecor F test* and *Student t-test*. Through these statistical tests, it was found that there was no significant difference in the chemical content of API between the densitometry and videodensitometry methods (*P>0.05*).

Overall, when reviewed in more detail, it is known that the peak area from the videodensitometry method tends to be greater than that from the densitometry method. For example, according to Gumieniczek *et al*. (2011), the peak area from densitometry was 1376 AU, and videodensitometry was 3429 AU in Candesartan tablets (Gumieniczek *et al*., 2011). These conditions are caused by differences in the analytical principles of the two methods, in which the density of the substance in each spot is measured, whereas videodensitometry measures the intensity of the color formed in each spot on the TLC plate (Reich & Schibli, 2014). In addition, the results of the densitometry analysis depend on the wavelength of the analysis compound, which can be previously set on the application in the scanner instrument to measure the levels in the maximum wavelength region of the substance in each spot. In videodensitometry, the quality of the TLC plate image obtained determines the analysis results because the color intensity of the substance in each formed spot varies greatly depending on the type and quality of the image produced (Lucio-Gutiérrez *et al*., 2012).

Recent research on developing TLC methods using videodensitometry can be performed using a smartphone (TLC-smartphone analysis). These data were excluded from the research criteria of the articles reviewed because they were only analyzed using videodensitometry, but this data is used as additional information about videodensitometry analysis, which is rarely known. Ibrahim *et al.* (2022) developed a qualitative and quantitative analysis method for determining loperamide (Immodium) and bisacodyl (Dulcolax) using a TLC smartphone based on the principle of videodensitometry analysis. The analysis was performed by taking pictures of TLC plates using a smartphone, and then analyzing using *Color Picker free software version 5.0.6*. Qualitative analysis is based on visual detection of the Rf values, whereas quantitative analysis is based on the color intensity of

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the compound spots. Methods evaluation supported by analytical method validation data included precision, accuracy, linearity, LOD, and LOQ. The intraday precision validation results have an RSD value of 0.76-1.78% and interday precision of 1.10-1.55% with triplicate replications. The method accuracy was calculated using the total recovery, with a 99.93-100.04% value. The linearity of the method from five different concentrations resulted in an r-value of 0.9996-0.9999, as well as LOD values of loperamide 0.57 µg/mL and bisacodyl 0.10 µg/mL and LOQ of loperamide 1.73 µg/mL and bisacodyl 0.30 µg/mL, calculated through the SD value and slope of the regression equation. Furthermore, determining loperamide concentration in immodium tablets using the TLC-smartphone method had a recovery value of 98.63 ± 1.68, and bisacodyl concentration in Dulcolax tablets was 100.23 ± 1.57. The results showed that the TLC-smartphone method can qualitatively and quantitatively determine the concentrations of loperamide and bisacodyl compounds (Ibrahim *et al.*, 2022).

However, densitometry and videodensitometry are challenging for analyzing natural products. Because raw materials contain many multicomponent components that work synergistically to cause pharmacological activity, this content will significantly affect the analysis process, starting from sample preparation techniques that must separate each component well. In addition, there is no reference standard for specific compounds that can be used for comparison (Xie *et al*., 2006; Renger *et al*., 2011). The variability factor in the composition of chemical compounds, influenced by environmental and plant genetic factors, also affects the analysis results (Kusumawati, 2021).

CONCLUSION

This systematic review shows that TLC densitometry and videodensitometry methods can be used for quantitative and qualitative analyses to determine the concentrations of active ingredients in pharmaceutical products. Through this review article, we intend to provide a new perspective that qualitative and quantitative TLC analyses can be performed using densitometry and videodensitometry.

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AUTHOR CONTRIBUTIONS

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

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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The Effect of Chitosan and Polyvinyl Alcohol Combination on Physical Characteristics and Mechanical Properties of Chitosan-PVA-*Aloe vera* Film

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Abstract

Background*: Wound is a condition where there is damage or disruption to the anatomical structure and function of the skin. Wounds that are not treated properly can lead to infection. As wound dressings, film dressings have many advantages such as being elastic, flexible, transparent, and can adapt to the wound shape. Film's characteristics of are affected by the plasticizer and the polymer. Combination of chitosan and polyvinyl alcohol (PVA) is able to improve the mechanical properties of the film such as its swelling capacity, tensile strength, and elongation at break.* ***Objective****: This study aims to determine the effects of chitosan and PVA in various concentrations on the physical characteristics and mechanical properties of the film.* ***Methods****: Film was prepared by solvent casting method, using chitosan and alginate in various concentrations of 0% to 1.5%, 1.5% Aloe vera, and 6% propylene glycol. Films' characteristics and mechanical properties were evaluated, such as swelling index, tensile strength, elongation at break, and Young's modulus.* ***Results****: The result showed that chitosan and PVA polymers had a significant effect on the swelling index, tensile strength, elongation at break, and Young's modulus. The effect of chitosan and PVA combination on the swelling index, tensile strength, and elongation at break is due to the hydrogen bonding between the hydroxyl group of PVA and the amine group of chitosan.* ***Conclusion****: The combination of chitosan and polyvinyl alcohol influenced the film's physical and mechanical properties. Film with chitosan and polyvinyl alcohol ratio of 1.5%:1.5% have best characteristics compared to others.*

Keywords*: aloe vera, chitosan, film dressing, mechanical properties, polyvinyl alcohol*

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INTRODUCTION

Skin wounds are the loss or damage to part of the body tissue caused by surgery, a blow, a cut, temperature changes, chemicals, pressure, or diseases such as carcinoma (Shankar *et al*., 2014). Skin wounds damage the protective function of the skin caused by a loss in the continuity of the epithelial tissue. This can occur with or without damage to other tissues such as muscles, bones, and nerves (Rodrigues *et al*., 2019). Wound care may include washing, administering ointments, and dressing the wounds. Wound washing often uses normal saline because it adjusts the pH of the skin and removes the dirt that sticks to the skin layer (Purnama *et al*., 2017; Gonzalez *et al*., 2016).

One wound-healing therapy is wound dressing (Oliveira *et al*., 2020). Modern wound dressing preparations and materials include plasters, film dressings, hydrogels, hydrocolloids, foams, medical collagen sponges, and gauze (Olivera *et al*., 2020; Han & Ceilley, 2017). The characteristics and properties of an ideal wound dressing include providing moisture to the wound area, preventing excess exudate production, preventing infection, providing low-cost treatment, providing comfort by relieving pain, using non-toxic materials, thermal insulation, and not causing pain (Matica *et al*., 2019; Tavakoli & Clark., 2020). Film dressing preparations have emerged as one of the most attractive topical preparations for effective drug delivery. They are defined as non-solid dosage forms that produce a film in situ after application to the skin or other body parts (Bornare *et al*., 2018). The advantages of film preparations include easy application, patient comfort owing to the non-invasive route of administration, and cost-effectiveness in formulation development (Patel *et al*., 2012). The type of wound dressing in the form of a film has many advantages: it is very elastic, flexible, can adapt to the shape of the wound and the body, and can be made transparent (Dhivya *et al*., 2015).

Chitosan is a natural polymer that forms films and acts as a coating material. This is because chitosan has a strong ionic bond between the negatively charged hydroxyl group and positively charged amine group, which can increase the moisture barrier (Liu *et al*., 2018; Arzate-Vazquez *et al*., 2012; Pratama *et al*., 2018). However, the use of pure chitosan films is very difficult because of their sensitivity to environmental conditions and poor mechanical properties (Peng & Li, 2014). Chitosan films also have high water vapor permeability (Liu *et al*., 2017). The flexibility of chitosan films can be improved by combining them with other polymers and plasticizers (Naqvi *et al*., 2021). Mixed polymer systems can be used to form films that are flexible, transparent, thin, and have good mechanical strength.

PVA is a hydrophilic synthetic polymer that is biocompatible, biodegradable, and can dissolve in water (Chandrakala *et al*., 2013). The polymer also has good elongation and surface tension properties (Rafieian *et al*., 2021) along with good tensile strength (TS). Therefore, PVA can be used for film formation (Birck *et al*., 2014; Srinivasa *et al*., 2003). PVA can form strong films but has several disadvantages such as poor adhesive properties, low gas diffusion, and low fluid absorption for wound dressings (Kamoun *et al*., 2017; Mok *et al*., 2020).

Aloe vera has anti-inflammatory, wound healing, antioxidant, and antimicrobial (Rahman *et al*., 2017). It is expected that *Aloe vera* could increase the activity of chitosan during wound healing. The use of mixed polymer combinations, such as chitosan and PVA, could help improve the flexibility properties of chitosan and the physical properties of PVA, thereby increasing its ability to absorb wound exudate.

This study aimed to investigate the effect of two variables in formulations, chitosan and PVA concentrations, and to explore the physical characteristics of new chitosan-PVA-*Aloe vera* films. The combination of chitosan and PVA can produce strong films with improved adhesion that can absorb wound exudates and maintain product biocompatibility. The ideal film should be elastic, not easily broken, suitable for skin pH, and have a high swelling ability.

MATERIALS AND METHODS

Materials

The chitosan polymer 107 cps was obtained from Biotech. Co.Ltd, Indonesia, Polyvinyl alcohol was obtained from Merck, Germany, *Aloe vera* powder was obtained from PT. Haldin Pacific Semesta, Indonesia, and propylene glycol were obtained from PT. Bratachem, Indonesia, and glacial acetate acid were obtained from Merck KGaA (Germany). All chemicals were of pharmaceutical grade.

Tools

Oven (MMM Medcenter Einrichtungen GmbH, Munchen), analytical scales (Ohaus PA-2102 C, USA), pH meter Trans Instrumen HP9010 PH-ORP-Temperature Meter (Walklab Series, Singapore), Desiccator, Digital thickness gauge (Syntex, China), tensile strength tester (MT, Moisture Analyzer HB43-S (Mettler Toledo, Switzerland), hotplate (Thermo

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Scientific, USA), and Fourier transform infrared spectroscopy (Alpha II, Bruker, USA).

Methods

Preparation of chitosan-PVA-*Aloe vera* films

The films were prepared by the solvent casting method using combinations of chitosan and PVA, as shown in Table 1. *Aloe vera* 1.5% was also added, along with propylene glycol 6% as a plasticizer. The chitosan solutions were prepared by dissolving chitosan in 1% (v/v) aqueous acetic acid, and the solution was stirred for 2 h with a magnetic stirrer. PVA solutions were prepared in distilled water at 90 ˚C under magnetic stirring at 250 rpm for 2 h, and *Aloe vera* powder was dissolved in water. PVA and *Aloe vera* solutions were poured into the chitosan solution and mixed with a magnetic stirrer at 250 rpm for 1 h. Propylene glycol was added to the mixed solution as a plasticizer. The solution was sonicated for 15 min to remove air bubbles. After the mixture was homogenized, the chitosan-PVA-*Aloe vera* film was prepared by pouring 5 ml of the solution into a Petri dish and drying at 40 ˚C in an oven for 5.5 hours. The film was weighed every hour until a constant weight was achieved. After drying, the chitosan-PVA-*Aloe vera* films were peeled off and stored in a desiccator until the evaluation tests.

Table 1. Formula Design of the Chitosan-PVA-*Aloe vera* Film

Polyvinyl Alcohol (PVA) (%w/v)	Chitosan (% w/v)			
	0	0.5	1	1.5
0	F1	F5	F9	F13
0.5	F2	F6	F10	F14
1	F3	F7	F11	F15
1.5	F4	F8	F12	F16

Organoleptic observations

The organoleptic evaluation of the film was carried out visually, including color, odor, elasticity, and surface texture.

Weight evaluation

The weight evaluation was carried out by taking three (3) films at random from each formula and replication for 3 times. Mean values were calculated. (Ali *et al*., 2016).

pH evaluation

The pH of the film was measured by immersing the chitosan-PVA-*Aloe vera* film in 20 ml distilled water for 1 h and calculating the change in the pH of the system using a digital pH meter (Walklab Series, Singapore).

Film thickness evaluation

This test was performed using the Digital Thickness tool (Syntex, China) by measuring ten parts of each film area. The film thickness was calculated as the average of ten individual measurements. The evaluation was performed in triplicate.

Moisture content (MC)

The films were weighed and stored for 24 h in a desiccator filled with silica gel. After storage, the film was weighed to determine the percentage moisture content using the following equation:

$$MC = \frac{\text{initial weight} - \text{final weight}}{\text{initial weight}} \times 100$$

Swelling index

The film sample ($1cm^2$) was weighed using an analytical balance, soaked in distilled water for 5, 10, 15, and 20 min, blotted with filter paper, and weighed accurately. The swelling index of the film was calculated using the following equation (Hajian *et al*., 2017).

$$\text{Swelling Ratio: } \frac{W_1 - W_o}{W_o} \times 100\%$$

W_1 is the weight of the wet film and W_o is the initial film weight.

Mechanical properties

Tensile strength was measured using a tensile tester (MTS bionix, China). The film was clamped between the two pull handles and pulled at a crosshead speed of 10 mm/min (Jantrawut *et al*., 2017). The tensile strength, elongation at break, and Young's modulus were calculated using the following formulas:

$$\text{Tensile strength (N/mm2)} = \frac{\text{Breaking Force (N)}}{\text{The cross} - \text{sectional area of the film (mm2)}}$$

$$\text{Elongation at break (\%)} = \frac{\text{Increasing in length at breaking point (mm)}}{\text{initial length (mm)}} \times 100$$

$$\text{Young's modulus} = \frac{F/A}{\Delta l/lo} = \frac{stress}{strain}$$

Folding endurance

Folding endurance is achieved by folding the film at the same point repeatedly until the film breaks or is folded up to 300 times without breaking.

Data analysis

The weight, thickness, pH value, moisture content, swelling index, tensile strength, elongation at break, and Young's modulus data were all statically examined using the two-way analysis of variance (ANOVA) method with a 95% confidence level (α=0.05) followed by a Post Hoc test. All experiments were performed in triplicate.

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Table 2. Organoleptic evaluation of chitosan-PVA-*Aloe vera* film

Formula	Organoleptic Observations		
	Color	Consistency	Odor
F1	Transparent	not forming a film, easy to break, can't peel off	*Aloe vera*
F2	Transparent	not forming a film, easy to break, can't peel off	*Aloe vera*
F3	Transparent	not forming a film, easy to break, can't peel off	*Aloe vera*
F4	Transparent	not forming a film, easy to break, can't peel off	*Aloe vera*
F5	Yellowish	not forming a film, easy to break, can't peel off	*Aloe vera*
F6	Yellowish	not forming a film, easy to break, can't peel off	*Aloe vera*
F7	Yellowish	a little can be peeled off, easy to break	*Aloe vera*
F8	Yellowish	a little can be peeled off, easy to break	*Aloe vera*
F9	Yellowish	a little can be peeled off, easy to break	*Aloe vera*
F10	Yellowish	Smooth texture, flat surface, and not easy to break	*Aloe vera*
F11	Yellowish	Smooth texture, flat surface, and not easy to break	*Aloe vera*
F12	Yellowish	Smooth texture, flat surface, and not easy to break	*Aloe vera*
F13	Yellowish	Smooth texture, flat surface, and not easy to break	*Aloe vera*
F14	Yellowish	Smooth texture, flat surface, and not easy to break	*Aloe vera*
F15	Yellowish	Smooth texture, flat surface, and not easy to break	*Aloe vera*
F16	Yellowish	Smooth texture, flat surface, and not easy to break	*Aloe vera*

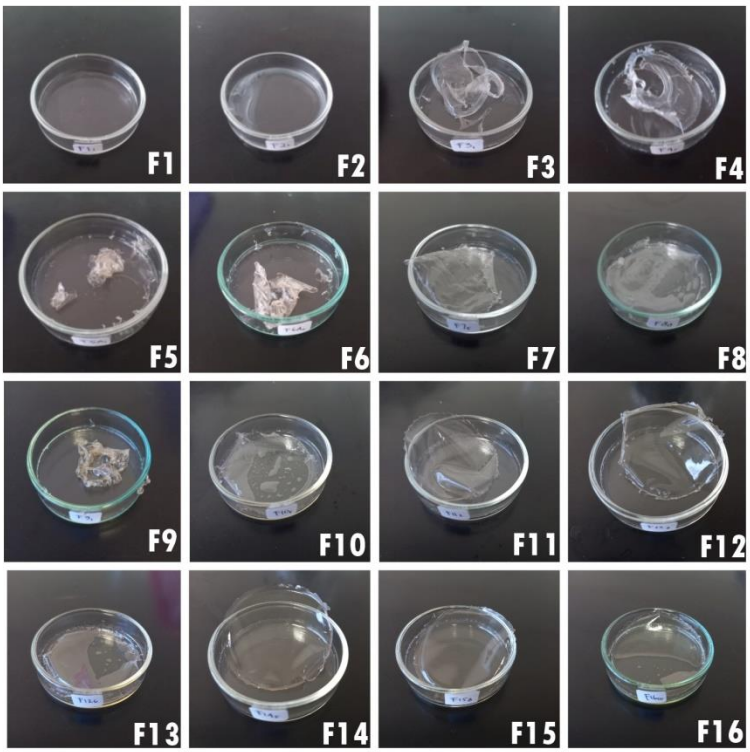

Figure 1. Photograph of chitosan-PVA-*Aloe vera* film with chitosan concentration 0.5%, 1%, 1.5% and PVA concentration 0.5%, 1%, and 1.5%

RESULTS AND DISCUSSION

The chitosan-PVA-*Aloe vera* film formulation is presented in Table 1. The results of the chitosan-PVA-*Aloe vera* film preparation are shown in Table 2 and Figure 1. From the sixteen formulas, six formulas could form films with good characteristics, such as easy to peel off, not easily broken, and having a smooth texture. The selected formulas (Table 3) were used for further evaluation.

Table 3. Selected Formulas of Chitosan-PVA-*Aloe vera* Film (% w/v)

Formula	Concentration (%)	
	Chitosan	Polyvinyl Alcohol (PVA)
F10	1	0.5
F11	1	1
F12	1	1.5
F14	1.5	0.5
F15	1.5	1
F16	1.5	1.5

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Organoleptic evaluation

The results of the organoleptic tests of the chitosan-PVA-*Aloe vera* films are shown in Table 2 and Figure 1. All formulas had the same odor and color. Chitosan-PVA-*Aloe vera* film has an *Aloe vera* odor with a light-yellow color. The texture of the chitosan-PVA-*Aloe vera* films was smooth and not easy to break.

Weight evaluation

Weight evaluation aims to determine the effect of varying the concentration of chitosan and PVA. The results of the weight evaluation of the chitosan-PVA-*Aloe vera* films are shown in Table 4. The two-way ANOVA statistical test for weight evaluation showed a significant difference between the other formulas (Table 6). The post-hoc Tukey HSD test showed that all formulas were significantly different (sig. value 0.000 < 0.05). Increasing the concentrations of chitosan and PVA affected the film weight. With increasing polymer concentration, the number of solids increased, affecting the weight of the film.

Thickness evaluation

The thicknesses obtained from the six formulae are listed in Table 4. The film thickness increased owing to the increase in chitosan and PVA concentrations. This is due to the large amount of water bound during the formulation process (El-Maghraby & Mona., 2015). The thickness of a film affects its flexibility, strength, and comfort. The acceptable film thickness is 0.05-1 mm (Karki., et al., 2016). The two-way ANOVA statistical test revealed a significant difference between the formulas (Table 6). Based on the post-hoc Tukey HSD test, all formulas were significantly different (sig. value 0.000 < 0.05). There was an interaction effect between chitosan and PVA on the film thickness.

Moisture content (MC)

The moisture content of the film can affect its mechanical strength, adhesive properties, and brittleness. The moisture content of the chitosan-PVA-*Aloe vera* films is shown in Table 4. The two-way ANOVA statistical test revealed a significant difference between the formulas (Table 6). The post hoc Tukey HSD test revealed that PVA 1.5% was significantly different (sig. value 0.000 < 0.05) with PVA 0.5% and 1.0%. Chitosan and PVA play a role in increasing the water content. The moisture content of the films increased with increasing chitosan concentration. The hydrophilic nature of chitosan and PVA caused an increase in the moisture content of the films. However, the significant effect of chitosan on increasing the water content was due to the higher number of amino and hydroxyl groups than PVA; thus, chitosan can absorb excess water molecules (Cazón *et al*., 2019; Chopra *et al*., 2022).

pH evaluation

As shown in Table 4, all chitosan-PVA-*Aloe vera* films had pH value of 5.24-5.67. Chitosan-PVA-*Aloe vera* film is acceptable for the skin because the skin has a pH of 4-6 (Prakash *et al*., 2017). pH plays a role in wound healing. Acidic pH provides an advantage in the wound healing process, which can increase the proliferation and migration of fibroblast cells (Jones *et al*., 2015). The two-way ANOVA statistical test for pH (Table 6) revealed a significant difference between the formulas. Based on the post-hoc Tukey HSD test, all formulas were significantly different (sig. value 0.000 < 0.05).

Swelling index

According to the observation, the films have good swelling ability and can maintain good structure (Figure 2). The swelling index values ranged from 272 % to 341 % (Table 4). The two-way ANOVA statistical test for swelling index showed a significant difference between the formulas (Table 6). The Tukey's HSD test showed that PVA 0.5% was significantly different (sig. value 0.000 < 0.05) from PVA 1% and 1.5%. Increasing the concentration of chitosan increased the swelling index of the film. Otherwise, as the PVA concentration increased, the film swelling ability decreased. This is due to the absorption of water in the film owing to the presence of hydroxyl groups (-OH) from PVA and amines ($-NH_2$) from chitosan bound to water molecules through hydrogen bonds and the presence of a porous network on the film. The swelling index value for wound dressings is 200-500% (Saarai *et al*., 2011). The chitosan-PVA-*Aloe vera* film compositions met the swelling index requirements.

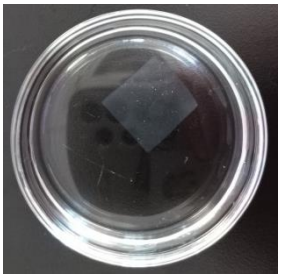

Figure 2. Photograph of swelling test on the chitosan-PVA-Aloe vera films

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Table 4. Physical Characteristics of the chitosan-PVA-*Aloe vera* film

Formula	Weight Evaluation (g)	Thickness (mm)	MC %	pH	Swelling Index %
F10	0.317 ± 0.002	0.131 ± 0.002	11.122 ± 0.164	5.67 ± 0.06	341.111 ± 8.389
F11	0.328 ± 0.007	0.154 ± 0.001	10.946 ± 0.066	5.51 ± 0.02	274.622 ± 7,337
F12	0.375 ± 0.005	0.209 ± 0.004	10.505 ± 0.273	5.33 ± 0.05	271.569 ± 6.560
F14	0.379 ± 0.002	0.161 ± 0.001	12.183 ± 0.099	5.53 ± 0.02	347.271 ± 2.779
F15	0.414 ± 0.006	0.189 ± 0.002	12.016 ± 0.091	5.45 ± 0.05	298.758 ± 4.411
F16	0.471 ± 0.005	0.234 ± 0.003	9.052 ± 0.289	5.24 ± 0.02	287.607 ± 9.255

Table 5. Mechanical Properties of Chitosan-PVA-*Aloe vera* Film

Formula	Tensile Strength (N/mm^2)	Elongation at Break (%)	Young's Modulus	Folding Endurance
F10	0.601 ± 0.019	27.63 ± 1,704	2.144 ± 0.135	> 300x
F11	0.654 ± 0.012	56.83 ± 3.512	1.154 ± 0.090	> 300x
F12	1.307 ± 0.065	64.33 ± 1.050	2.032 ± 0.095	> 300x
F14	0.615 ± 0.013	30.33 ± 3.855	1.850 ± 0.033	> 300x
F15	1.539 ± 0.050	43.10 ± 2.910	2.497 ± 0.089	> 300x
F16	2.194 ± 0.053	43.60 ± 1.803	2.123 ± 0.084	> 300x

Folding endurance

The folding endurance of the Chitosan-PVA-*Aloe vera* film is shown in Table 5. All formulas can be folded more than 300 times at the same point. This shows that the chitosan-PVA-*Aloe vera* film has good flexibility. Good film flexibility is > 300 times folded without breaking (Somepalli *et al.*, 2013). The polymer concentration affects folding power value (Nishigaki *et al.*, 2012). The combination of chitosan and polyvinyl alcohol as a film-forming polymer can provide strength to the film such that it is not brittle or easily broken. The interaction between the -OH and $-NH_2$ groups of the two polymers can increase the strength (Nugraheni *et al.*, 2018).

Tensile strength, elongation at break and young's modulus

The tensile strength test aims to determine the maximum strength that the film can withstand before the preparation is damaged by being pulled or stretched (Ma *et al.*, 2021). The tensile strengths of the chitosan-PVA-*Aloe vera* films are shown in Table 5 and Figure 3. The folding endurance and tensile strength are related to the strength of the film. Plasticizers affect tensile strength value (Irfan *et al.*, 2016). The two-way ANOVA statistical test for the tensile strength showed a significant difference between the formulas (Table 6). Based on the measurable results of ANOVA followed by the post-hoc Tukey HSD test, all formulas were significantly different (sig. value 0.000 < 0.05).

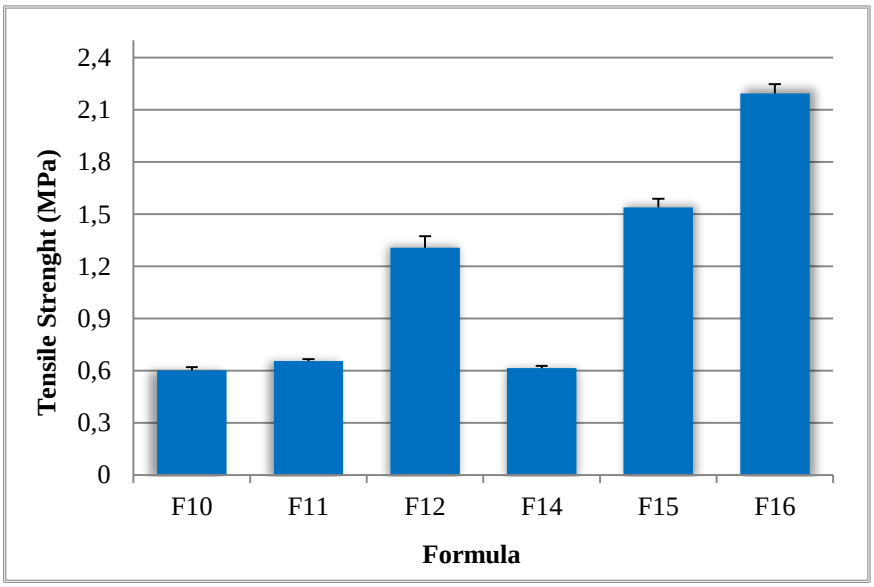

Figure 3. Tensile strength of the chitosan-PVA-*Aloe vera* film with different ratio of chitosan and PVA

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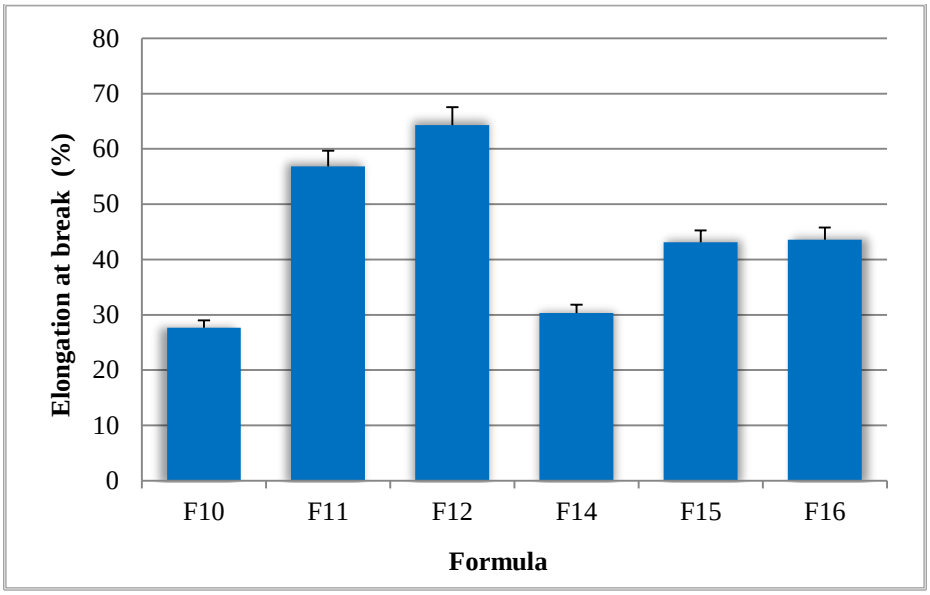

Figure 4. Elongation at break of the chitosan-PVA-*Aloe vera* film with different ratios of chitosan and PVA

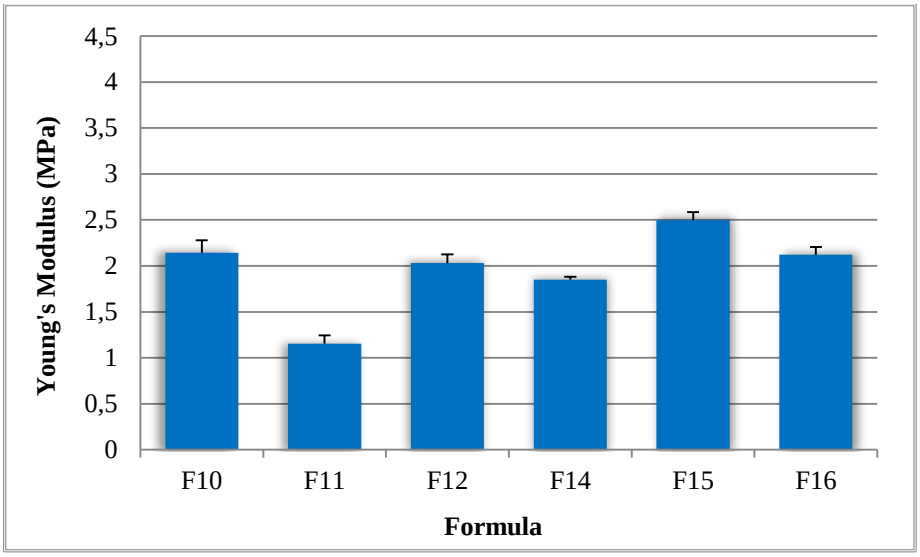

Figure 5. Young's modulus of the chitosan-PVA-*Aloe vera* film with different ratios of chitosan and PVA

Table 6. Results of the two-way ANOVA statistical test on the combination of chitosan and PVA

Film's characteristic	Statistical test results		
	Concentration of Chitosan	Concentration of Polyvinyl alcohol (PVA)	Interaction of the chitosan and PVA
Uniformity of weight	0.000[a]	0.000[a]	0.000[a]
Thickness	0.000[a]	0.000[a]	0.018[a]
pH value	0.000[a]	0.000[a]	0.008[a]
Moisture content (MC)	0.024[a]	0.000[a]	0.000[a]
Swelling degree	0.000[a]	0.000[a]	0.115[b]
Tensile Strength	0.000[a]	0.000[a]	0.000[a]
Elongation	0.000[a]	0.000[a]	0.000[a]
Young's modulus	0.000[a]	0.002[a]	0.000[a]

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The percentage elongation aims to determine the increase in length from the initial length to the point of breaking when the sample is pulled. Simultaneously, elongation-at-break refers to the point where the film can be stretched when it is torn or damaged. The elongation-at-break test was used to determine the flexibility of the film (Karki *et al*., 2016). The elongation-at-break value is below 15%, indicating that the material is easily brittle (El Hadi *et al*., 2017). Calculating of The elongation-at-break obtained using the six formulas were F10 (27.63%), F11 (56.83%), F12 (64.33), F14 (30.33%), F15 (43.10%), F16 (43.60%) (Table 5 and Figure 4). In the two-way ANOVA statistical test, sig = 0.000 < 0.05 means a significant difference (Table 6). From The post hoc Tukey HSD test, it was found that PVA 0.5% was significantly different (sig. value 0.000 < 0.05) from PVA 1% and 1.5%. PVA 1% was not significantly different from PVA 1.5%. The increase in elongation at break was due to the molecular interactions between chitosan and PVA through the formation of hydrogen bonds (Abraham *et al*., 2016). The maximum elongation-at-break was in the film with a combination formulation of chitosan 1% and PVA 1.5%. The Young's modulus indicates the stiffness or elasticity of the film. Films that have a high Young's modulus indicates that the film is not easily brittle (Karki *et al*., 2016). The Young's modulus of the Chitosan-PVA-*Aloe vera* film is shown in Table 5 and Figure 5. Based on the two-way ANOVA statistical test, the value of sig = 0.000 < 0.05 means a significant difference (Table 6). Tukey HSD test Young's modulus showed that PVA 1% was significantly different (sig. value 0.000 < 0.05) from PVA 0.5% and 1.5%. PVA 0.5% was not significantly different from PVA 1.5%.

The combination of chitosan and PVA polymers increased the tensile strength, elongation at break, and Young's modulus values. It was noted that increasing the amounts of chitosan and PVA increased the mean tensile strength, and increasing the amount of PVA increased the elongation value. The combination of chitosan and PVA reflects the effect of the interaction between the two polymers. This is due to the interaction between the -OH and -NH_2 groups on the polymer (Bahrami *et al*., 2002; Bonilla *et al*., 2014). The positively charged polysaccharide chitosan moved towards the negative charge of the hydroxyl group of PVA, which improved the tensile strength and elongation at break of the plastic film due to the intermolecular interactions between chitosan and PVA through hydrogen bond formation (Abraham *et al*., 2016). Thus, it can improve the mechanical properties of the films. The mechanical properties are affected by the type and amount of polymer mixes. A concentration that is too low can result in intermittent film formation or poor mechanical resistance. In contrast, high concentrations produce a thick and rigid film on the skin, which is uncomfortable to use and may delay drug release (El Fray *et al*., 2012).

CONCLUSION

Based on this study, the physical characteristic and mechanical properties of the chitosan-PVA-*Aloe vera* films were affected by both polymers: chitosan (1%, 1.5%) and PVA (0.5%, 1%, 1.5%). The increased concentrations of chitosan and PVA caused an increase in the mechanical properties. However, there was a decrease in the swelling index. Based on the results, films with chitosan 1.5% and PVA 1.5% had the best characteristics and mechanical properties (swelling index, tensile strength, and elongation at break) compared to the other formulations. Therefore, this film has the potential to be developed as a wound dressing material.

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AUTHOR CONTRIBUTIONS

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

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Translation and Cultural Adaptation of Multi-Attribute Utility Instrument (MAUI) Indonesian Version of the 15D Questionnaire

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Abstract

***Background**: Diabetes mellitus is a chronic metabolic disorder that cannot be cured and progressively develop into a complication affecting the patient's health-related quality of life (HRQoL). The utility is a measure of preference-based HRQoL. Indirect utility measurements can be measured using the Multi-Attribute Utility Instrument (MAUI) instrument, one of which is the 15D questionnaire that has never been translated and adapted culturally in Indonesian. **Objective**: Translating and culturally adapting the 15D questionnaire to Indonesian diabetes mellitus patients. **Methods**: The translation process was carried out through the stages of forward translation, reconciliation, and backward translation involving two qualified translators and three experts in their field. The pilot test stage involved eight respondents consisting of 6 diabetes mellitus patients and two healthy individuals. **Results**: Problems in the linguistic validation process led to more conformity of word equivalents from the original to the target language. The agreement found was in the area of semantic equivalence, idiomatic equivalence, and experiential equivalence. The problem was resolved by reconciliation during the Focus Group Discussion, which translators and experts in their field attended and discussed with the original author to get equality of meaning in terms of language and culture. The Indonesian version of the 15D questionnaire tested on eight respondents showed results that were easy to understand and straightforward. **Conclusion**: The Indonesian version of the 15D questionnaire is valid from the linguistic and cultural adaptation stage. Further research is needed relating to the validation and reliability of the questionnaire.*

***Keywords**: 15D, backward translation, cultural adaptation, diabetes mellitus, forward translation*

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INTRODUCTION

Diabetes Mellitus is a chronic condition in which there is an increase in blood glucose levels caused by the inability of the body to produce sufficient insulin or insulin not working effectively. According to data from the International Diabetes Federation (IDF) in 2019, approximately 10% of the population has type 1 diabetes. In contrast to type 1 diabetes, type 2 diabetes is the most prevalent type of diabetes in the community, with a population of approximately 90%. Type 2 diabetes is caused by insulin resistance in muscles and the liver, and damage to pancreatic beta cells (Perkeni, 2019).

Diabetes mellitus is common in almost every part of the world. The countries with the highest number of diabetics, according to the International Diabetes Federation (IDF) in 2019 are China at 116.4 million, India at 77 million, the United States at 31 million, Brazil at 16.8 million, Mexico at 12.8 million, and Indonesia is in seventh place as many as 10.7 million. By region, the countries with the highest number of people with diabetes were North America (11%), Middle East and North Africa (10.8%), Southeast Asia (10.1%), Western Pacific (8.6%), and Central America (7.6%).

In Indonesia, the prevalence of diabetes mellitus diagnosed by a doctor by age > 15 years from to 2013-2018 was 2%. The prevalence of diabetes mellitus in East Java among residents aged > 15 years from to 2013-2018 was 2.6% (Silver *et al*., 2018)

Diabetes mellitus (DM) is an incurable chronic metabolic disorder that can progressively become a complication. High blood glucose levels can cause vascular damage, affecting the heart, eyes, kidneys, and nerves (Cho *et al*., 2017). Complications of diabetes can undoubtedly affect a patient's utility. Utility is a value that indicates a preference (chosen by a person) that reflects health conditions such as physical, mental, and social functions related to the weight of judgment. The best health condition was established with a value of 1.0, and mortality was characterized by a value of 0.0 (Rascati *et al*, 2013).

Direct and indirect measurements can be used to obtain utility values. Direct measurement using the time trade-off (TTO) method, standard gamble (SG) method, and Rating Scale *(*RS) method. Indirect utility can be measured using a Multi-Attribute Utility Instrument (MAUI). The instruments used were EQ-5D, SF-6D, HUI, AQOL, QWB, and 15D (Brazier *et al*., 2017). The Multi-Attribute Utility Instrument (MAUI) has been widely developed and adapted to various languages worldwide, including the 15D versions of the Turkish questionnaire, the 15D version of the Japanese questionnaire and the 15D version of the Greek questionnaire. Generic instruments translated into Indonesians and made cultural adaptations were EQ-5D, SF-6D, and QWB.

The 15D questionnaire has the most detailed dimensions among all types of MAUI (Richardson *et al.*, 2011). Key measurements include mobility, vision, hearing, breathing, sleep, eating, speech, elimination, daily activities, mental function, discomfort and symptoms, depression, anxiety, vitality, and sexual activity (Sintonen, 2001). The questionnaire also has better sensitivity for diabetic patients with complications of Chronic Kidney Disease and Diabetic Retinopathy than the EQ-5D and SF-6D questionnaires (Kontodimopoulos *et al*., 2012).

The 15D questionnaire, developed by Harri Sintonen in Finland, measures the perceived health condition of individuals developed by Harri Sintonen in Finland (Sintonen, 2001). In addition to the 15D questionnaire, there were 16D and 17D questionnaires. In contrast to the 15D questionnaire, the 16D questionnaire is aimed at adolescents aged 12-15 and covers 16 domains. The 17D questionnaire is aimed at children aged 8-11 years and covers 17 domains. The utility value generated by the 15D questionnaire can be helpful in pharmacoeconomic studies in determining the outcome of Cost-Utility Analysis, measuring and monitoring the patient's health status, and assisting medical decision-making by identifying the patient's health problems (as a diagnostic tool) and setting spending goals for hospitals or clinics. This 15D profile can be used for drug evaluation, surgical treatment, and rehabilitation. The assessment system is based on the application of multi-attribute utility theory. This instrument can convert health status into estimated utility values (1= perfectly healthy to 0=death) using a set of preferences based on the population or utility weight (Sintonen, 2021).

Questionnaire 15D has never been translated and culturally adapted for diabetic patients in Indonesia. The translation and cultural adaptation process is needed to reduce the possibility of bias due to cultural differences and to obtain the same understanding between the original questionnaire and the Indonesian version of the 15D questionnaire. The above description encourages translational research, cultural adaptation, and validation of the Indonesian version of the 15D questionnaire.

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MATERIALS AND METHODS

The linguistic validation of the questionnaire was conducted using international standards. There were two stages: The first stage was the translation process. The second stage was a pilot test or a respondent's test. The results of each stage are reported and discussed with the original authors. The 15D questionnaire used in this study received permission and license from the original author.

Phase I forward translation

This stage was completed by two translators from the Airlangga University Language Center who translated the original (English) questionnaire into the target (Indonesian) language. One of the translators was a professional translator who taught TOEFL/IELTS and Indonesian for Foreign Speakers (BIPA). The other is a professional translator and proofreader for Airlangga University's scientific literature on English. Both are Indonesian natives who live in Indonesia and speak both Indonesian and English fluently. The translator does not have a background in medicine, but has received explanations and guidance before beginning the forward translation process.

The goal was to produce a simple, clear, and understandable translation, without changing the meaning of the original language (English). Each translator had never heard of the 15D questionnaire and had worked independently or separately. Following the completion of each questionnaire, three experts in the field, one internal medicine specialist with expertise in endocrine, metabolic, and diabetes, two experts in questionnaire development and pharmacy practice, and two translators from the Airlangga University Language Center convened a meeting or Focus Group Discussion to reconcile or create an overview containing details of the translation reversals.

The report was initially emailed to the original author, along with the translator's name, qualifications, and/or experience, and the reconciliation results in English. Furthermore, the original author researched the report to step it up later. The original author of the questionnaire provided feedback to the researcher through comments or suggestions during the work process, and the researcher resubmitted the reconciliation results to obtain a final result.

The series of forward translation processes are shown in Figure 1.

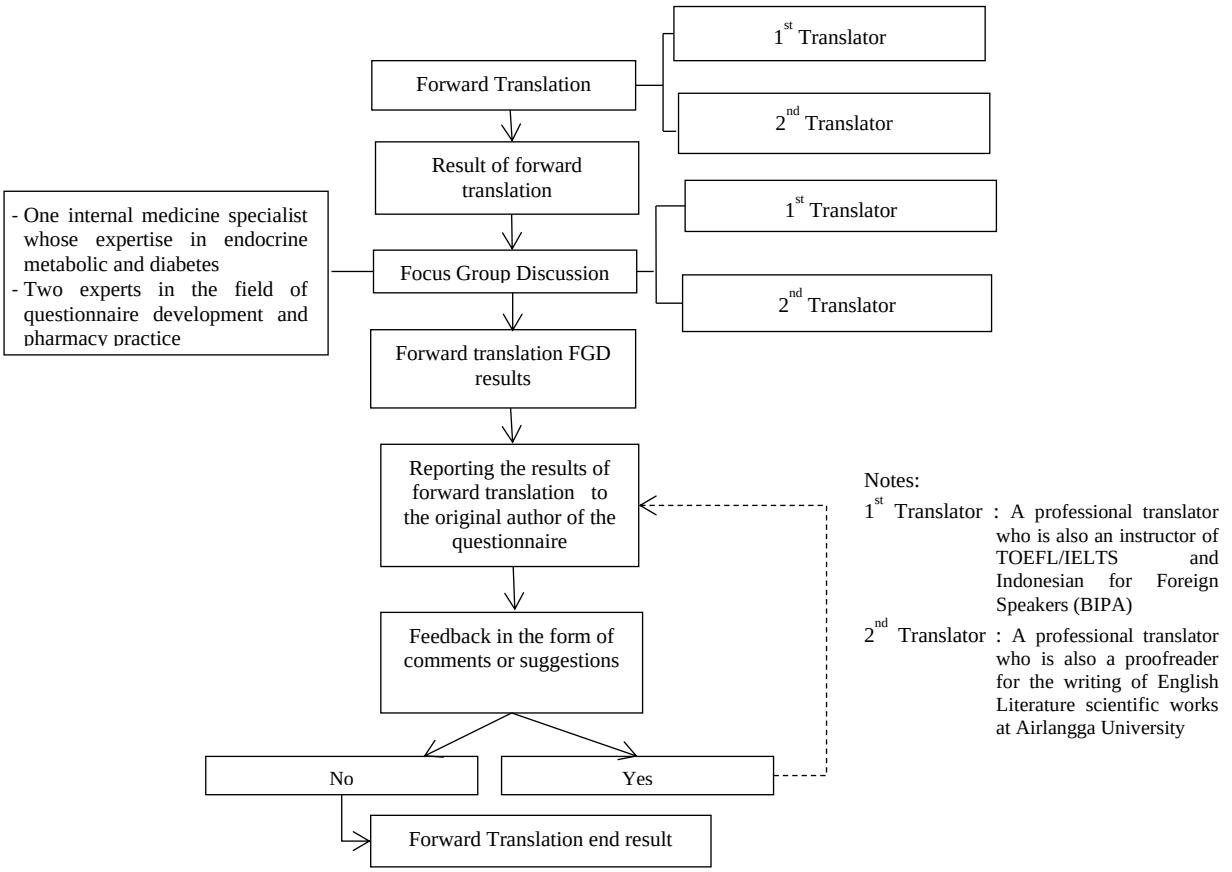

Figure 1. A series of forward translation processes

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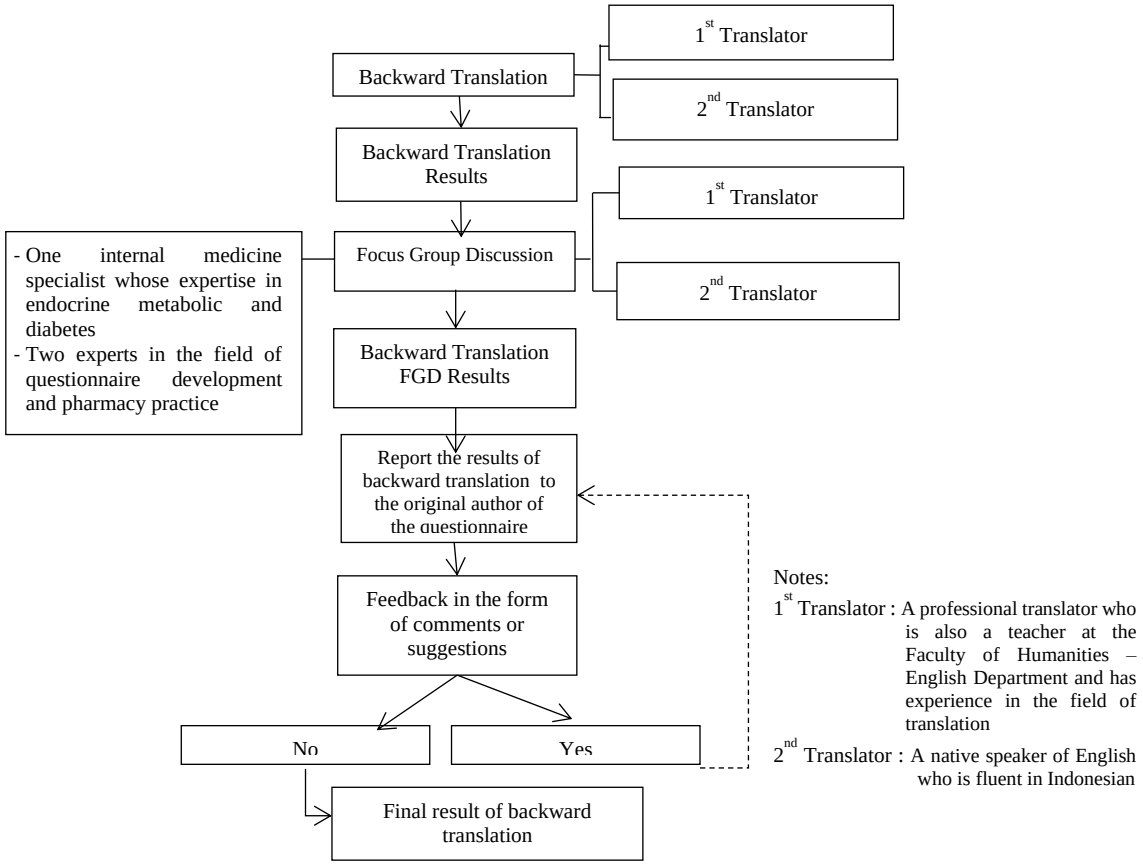

Figure 2. Backward Translation process sequence

Phase II backward translation

Two independent translators translated the Indonesian version of the 15D questionnaire, which was reconciled and then translated back into English. One translator is a professional translator who is also a teacher at the Faculty of Humanities – English Department–and has experience in translation. The other translator was a native English speaker fluent in Indonesian.

The translator then compared the backward translation results with the original version of the questionnaire. A meeting was held in the Focus Group Discussion, in which experts in the field attended to reconcile or create an overview containing details of the differences from the results of the translation, pay attention to the word structure, note the difficulties encountered, and seek the suitability of a simple language so that it has a conformity of meaning between Indonesian and English.

The reconciliation results in English were then emailed to the original author for review and permission to continue the pilot test phase. In the work process, the original author of the questionnaire provided feedback in the form of comments and suggestions. The reconciliation results were resubmitted for the final results. The sequence of the backward translation process is shown in Figure 2.

Phase III pilot test or respondent test

Following permission from the original authors of the 15D questionnaire, a pilot test or respondent test was conducted in which the questionnaire was tested on eight fluent Indonesian respondents. Among those who responded, were patients and healthy people who had never heard of the 15D questionnaire. This pilot test aimed to assess the clarity and comprehension of meaning between the native language and target language..

The trial involved eight participants who completed the questionnaire and interviews. The sample size was eight respondents because it referred to the original author's guidelines and the ISPOR guidelines. The respondents signed informed consent forms and completed persuasion and demographic data sheets. The interviews were recorded, and a summary of demographic information was sent to the original author of the questionnaire. Figure 3 shows the sequence of the pilot test process.

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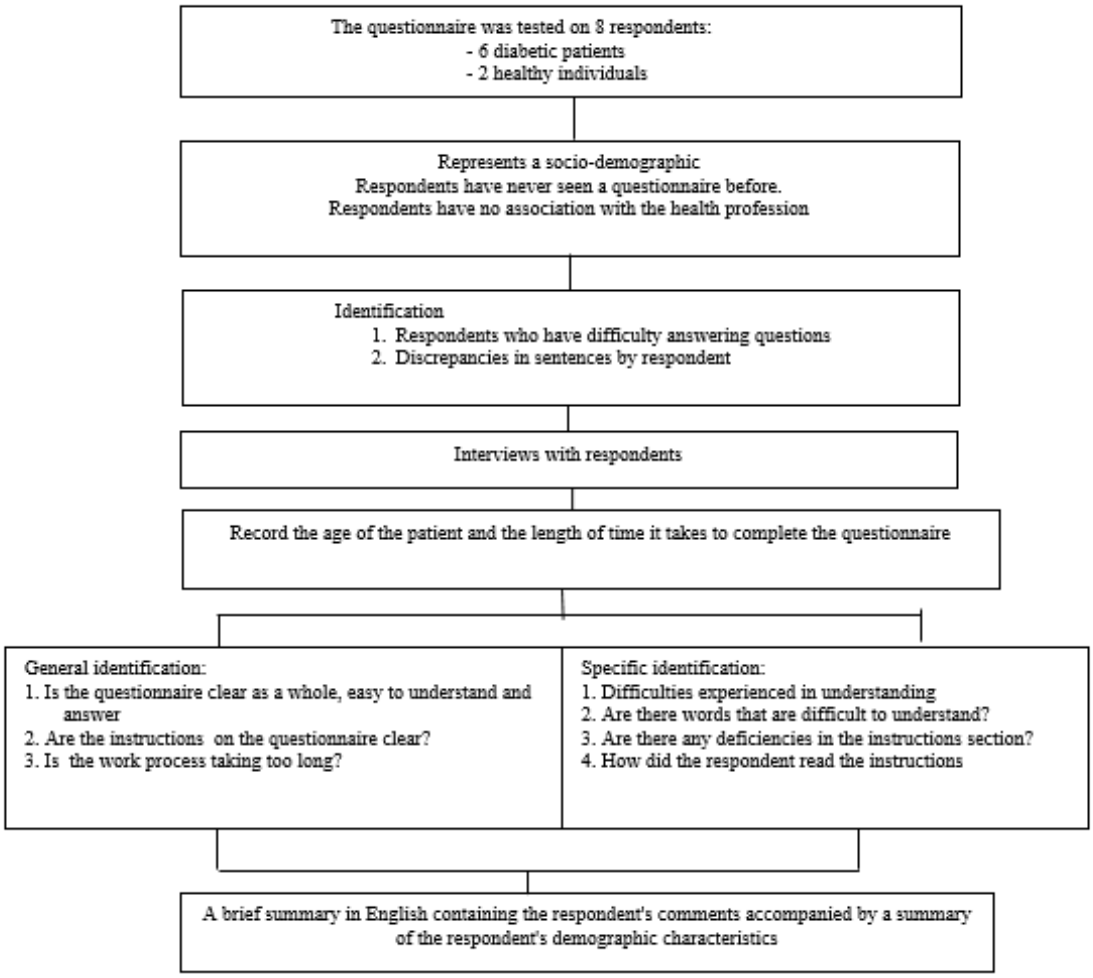

Figure 3. The pilot test process sequence

Ethics committee approval

This study was approved by the Health Research Ethics Committee, Faculty of Pharmacy, Universitas Airlangga (No.21/LE/2022).

RESULTS AND DISCUSSION

Forward translation

In translating health-related questionnaires into the target language, it is essential to ensure conformity of meaning between the words of the original language and the target language (Arnold *et al*., 2005). some problems and actions taken during the translation and cultural adaptation of the Indonesian version of the 15D questionnaire are described in Table 1.

The selection of translators and expert panels was essentialin, producing clear, simple translations of words and sentences that follow the original questionnaire's meaning. In the process of forward translation of the 15D questionnaire to Turkish, three native Turkish people fluent in Turkish and a panel of experts were selected from a 40 years old female doctor and a 40 years old male health service administrator (Akinci *et al*., 2005). One example of the result of changing the word *'I have moderate problems with sleeping (e.g., disturbed sleep or feeling I have not slept enough)*' was changed to '*I have moderate problems like I have felt that my sleep is disturbed and I have not slept enough*' (Akinci *et al*., 2005).

In the process of forward translation of the 15D questionnaire to Japanese, a panel of experts selected three men and one native Japanese person, each professing as an epidemiologist, health economist, and public health expert (Okamoto *et al*., 2013). However, this is not explained by the results of the forward translation.

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Table 1. Problems and solutions of the forward translation process and cultural adaptation to Indonesian

Item	Original Version	Issues	Actions	Area
Instruction	*Please read through all the alternative responses to each question before placing a cross (x) against the alternative which best describes your present health status*.	Based on the content of the questionnaire, it does not describe "*question*" so the word "*problem*" is preferred.	The word "*question*" is translated into Indonesian as "question" and the addition of the word "statement" or "statement" after the word *alternative.*	Semantic
Statement 1 part 1	*I am able to walk normally (without difficulty) indoors, outdoors, and on stairs.*	The use of the word "*stairs*" is more about the meaning of "stairs", while "*climb stairs*" is more about "climbing stairs".	The word "climbing stairs" or "climbing *stairs" is* chosen because it is more following the meaning of the word "*stairs*" in Indonesian.	Experiential
Statement 4 part 4	*I get shortness of breath even after light activity, e.g. washing or dressing myself.*	The word "*washing*" in Indonesia is not a light activity	In the opinion of the original author of the questionnaire, if the word "*washing*" is difficult to translate in Indonesian, it can be ignored as an example of light activity	Experiential
Statement 5 part 5	*I suffer severe sleeplessness, e.g. sleep is almost impossible even with full use of sleeping pills or staying awake most of the night.*	The word "*full use*" is defined by the translator as the frequency or routine of taking the drug.	The word "maximum dose" or "*full use*" was chosen according to the original author's explanation of the questionnaire.	Semantic
Statement 7 part 3	*I can make myself understood, but my speech is e.g. disjointed, faltering, stuttering or stammering.*	The words "*stuttering*" and "summering" have almost the same meaning; it is recommended to use one of them.	The words "stuttering" and "summering" are interpreted as "pausing."	Semantic
Statement 8 part 4	*I have serious problems with my bladder and/or bowel function, e.g. routine "accidents" or need of catheterization or enemas.*	The word *"catheterization"* or "enemas" may not be understood by ordinary people.	The word "*catheterization*" is interpreted as "kateterisasi" or "enemas" is interpreted as "enema" even though ordinary people do not understand it.	Semantic
Statement 10 part 2	*I have little difficulty in thinking clearly and logically, or my memory sometimes fails me.*	The sentence "*my memory sometimes fails me*" is a metaphor that is difficult to find matches for words in Indonesian	Used the word "sometimes interrupted" or "*sometimes impaired*" to replace "*sometimes fails me.*"	Idiomatic

Backward translation

The English version of the 15D translation and adaptation process was translated into Turkish through translation, cultural adaptation, validation, and reliability (Akinci *et al*., 2005). In the backward translation process, a research assistant was selected at the law faculty of a university who was also a *native speaker* because he was a native English speaker and a 36 years old male Turkish health service researcher.

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Similar to the Indonesian version of the 15D questionnaire, several changes were made to resolve these problems. Some sentence arrangements have been changed from the original language to produce meaningful and easy sentences for Turks to understand (Akinci *et al*., 2005). Some of these problems are listed in Table 2.

Pilot test

The questionnaire was tested on a small sample to see if there were still some things missing from each item, and if respondents could provide responses and suggestions after reading the questionnaire. A 15D pilot test of the Indonesian version of the questionnaire was conducted on eight respondents. Respondent characteristics were obtained as described in the table. The following are the reasons for selecting diabetes patients and healthy people. First, refer to the original author's guidelines and the ISPOR guidelines for translation and cultural adaptation, where the goal of the pilot test is to ensure comprehension of each sentence regarding the perceived health condition. Second, because the 15D questionnaire is a comprehensive generic instrument, the questions were not limited to diabetes mellitus. Third, subject selection seeks to determine the utility range. The best health condition is assigned a value of one, whereas death is assigned a value of zero (Rascati *et al*., 2013). The utility value of healthy people is greater than that of patients (Pea-Longobardo et al., 2017). The respondents' data obtained were grouped according to the type of respondent's demographic data, as described in Table 4.

Table 2. Problems and completion of the backward translation process and cultural adaptation of the 15D questionnaire to Indonesian

Item	Original Version	Issues	Actions	Area
Statement 2 part 3	I am able to walk without help indoors (with or without an appliance), but outdoors and/or on stairs only with considerable difficulty or with help from others.	After being translated back to English, the word "sufficient" or "considerable" changed to "*moderate difficulties*", while according to the original author of the questionnaire, the word "*moderate difficulties*" had a lower severity than "considerable.″	Revised the Indonesian translation so that it is in accordance with what the original version means to be "quite a lot."	Semantic
Statement 2 part 5	I can't see enough to walk about without a guide, which is I am almost or completely blind.	The word "I can't see clearly" "*I can't see enough*" has a different meaning than "*I cant see clearly.*"	Revised the Indonesian translation to match what the original version meant "I can't see well" or "*I can't see well enough.*"	Experiential
Statement 7 part 3	I can make myself understood, but my speech is, e.g. disjointed, faltering, stuttering or stammering.	The words"stuttering" and "stammering" have almost the same meaning as "pause."	The word"stammering" is deleted.	Semantic
Statement 15 part 1	My state of health has no adverse effect on my sexual activity.	The word "*adverse effect*" is changed to "*negative effect.*"	Revised Indonesian translation "My health level does not have a negative effect on my sexual activity."	Semantic

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Table 3. Characteristics of respondents on pilot test

Respondent	Age (years)	Gender	Patient	Work	Time to fill in 15D
P1	66	L	DM with Cardiovascular Disease	Teacher	4 minutes
P2	72	P	DM with Neuropathy	Housewife	5 minutes
P3	45	P	DM with Cardiovascular Disease	Housewife	6 minutes
P4	59	L	DM with Cardiovascular Disease	Civil servant	4 minutes
P5	70	P	DM with Retinopathy	Housewife	6 minutes
P6	38	L	Healthy individuals	SOE Officer	5 minutes
P7	63	P	DM with Nephropathy	Housewife	4 minutes
P8	31	L	Healthy individuals	SOE Officer	4 minutes
Mean age: 55.5 years					Mean: 4 minutes
Median age: 61					Median: 4 minutes

Note: P = female, L= male, DM= Diabetes Mellitus, SOE= State Owned Enterprise

Table 4. Demographic characteristics of respondents

No.	Description	n (%) [#]
1	**Gender**	
	Male	4 (50)
	Female	4 (50)
2	**Age**	
	26-35	1 (12.5)
	36-45	2 (25)
	46-55	0 (0)
	56-65	2 (25)
	>65	3 (37.5)
3	**Work**	
	Employed	4 (50)
	Unemployed	4 (50)
4	**Highest education**	
	Primary school	0 (0)
	Junior High School	1 (12.5)
	High School	3 (37.5)
	Bachelors degree	4 (50)
5	**Health Conditions**	
	Diabetes Mellitus	6 (75)
	Healthy Individuals	2 (25)

Notes:
*) Age grouping divided by the Ministry of Health of the Republic of Indonesia in 2009
[#]) Percentage (%): represents the number of patients divided by the total number of subjects (5) times 100%.

In the process of translating and culturally adapting the Japanese version of the 15D questionnaire, a pilot or *respondent test* was conducted on eight respondents with an age range of 44 to 75 years, consisting of four men and four women (not from the health profession) (Okamoto *et al*., 2013). The same number of respondents were also shown in translating and cultural adaptation of the Turkish version of the 15D questionnaire, pilot *test or respondent test* conducted by eight respondents consisting of four men and four women with an age range of 26 to 75 years. The female professionals included retired high school teachers, economists, homemakers, and housekeepers. Men work as porters, drivers, engineers, and university employees (Akinci *et al*., 2005).

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The problem encountered in the Turkish version of the 15D translation process is that cultural differences cause researchers to modify or change the sentence from the original language 'I have moderate problems with sleeping (for example, disturbed sleep or feeling I have not slept enough)' to 'I have moderate problems like I have feelings that my sleep is disturbed and I have not slept enough. '. In addition, the word '*depressed*' does not have a suitable word equivalent in Turkish; the term '*depressed*' indicates a state of bad mood and morale.

The 15D questionnaire was also translated into Romanian (Adina & Mihaela, 2021) through *forward-backwards translation* stages and pilot tests conducted on 25 respondents, comprising 15 cancer patients and ten healthy individuals. The problem is the difference in meaning when translated into the target language, as in Domain 1 point 4, which is '*I am able to walk indoors only with help from others*. Translated differently by the first and second translators. Thus, the action taken by combining the results of the translation into a simpler sentence but still in accordance with its original meaning is' *Pot să merg în casă doar cu ajutorul unei alte personae*'. The same problem in the translation process between the Indonesian version of the 15D questionnaire and the 15 Romanian version is in the 7 point 3 domain, the word 'stuttering or stammering' has the same meaning. Because the translation results from the second translator are more complete, the action taken in the Romanian version of the 15D translation process was to use them. In the Indonesian version, the word "stammering" was deleted and only used the word "stuttering" was used on the recommendation of the original author. Data related to word clarity in the questionnaire are presented in Table 5.

Table 5 shows the respondents' identification of word clarity, while Table 6 shows an overview of the problems encountered and the solutions in a simple language, according to the meaning of the original version of the 15D questionnaire.

The Indonesian version of the 15D questionnaire can be helpful in pharmacoeconomic studies and can be used to determine a patient's health condition. This study is the first to translate 15D, and a cultural adaptation was made to Indonesian using international standards, involving several experts in the field. The limitations of this study were that the selected patients had diabetes with complications only and healthy people. In the 15D Turkish version of the study, the respondents had diabetes without complications. In addition, the 15D questionnaire has not been validated and its reliability has not been verified. Therefore, further studies are warranted.

Table 5. Identification of the word clarity in the Indonesian version of 15 D questionnaire

No	Question	Yes n (%)
	General Impression	
1	Is it globally clear, easy to understand, easy to answer?	8 (100)
2	Is it too long	0 (0)
3	Are the instructions clear?	8 (100)
	Instruction in the 15D	
1	Did you have difficulty understanding the instructions?	8 (100)
2	Are there words that you find difficult to understand?	8 (100)
3	How would you have worded the instructions?	8 (100)
4	is there anything missing from the instructions?	8 (100)
	Question	
1	Did you have difficulty understanding this question?	6 (75)
2	What does it mean for you?	6 (75)
3	How would you have worded the question?	4 (50)
4	Are the response choices clear and consistent with the question?	8 (100)

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Table 6. Problems and solutions to the Indonesian version of the 15D questionnaire derived from the pilot test

No	Item	Original version	Issues	Suggestion	Actions
1	Statement 12 part 1	I do not feel at all sad, melancholic or depressed.	The word "melankolis" translate into easily carried away in the situation	No suggestion given	No changes, "melankolis" is still chosen because the meaning matches the English version
2	Statement 8 part 1	My bladder and bowel work normally and without problems	The word " normal tanpa masalah" have a similar meaning	Choose one	Revised into "kandung kemih dan usus saya bekerja tanpa masalah"
3	Statement 4 part 4	I get shortness of breath even after light activity, e.g. washing or dressing myself.	The word "Napas Pendek" was not a daily term used daily and felt uncommon	Change to "Sesak Napas"	No changes, "napas pendek" or "*shortness of breath*" is still chosen because the meaning matches the English version
4	Statement 10 part 5	I am permanently confused and disoriented in place and time	The word "kehilangan orientasi" was difficult to understand	Word " kehilangan pandangan" is more easily understood	No changes, "kehilangan orientasi" or "*disoriented*" is still chosen because the meaning matches the English version
5	Statement 5 part 2	I have slight problems with sleeping, e.g. difficulty in falling asleep, or sometimes at night	The word "saya sulit jatuh tertidur" was difficult to understand	Word "saya sulit tertidur" is more easily understood	No changes, "Saya sulit jatuh tertidur" or "*difficulty in falling asleep*" " is still chosen because the meaning matches the English version
6	Statement 6 part 5	I am unable to eat at all, so I am fed either by tube or intravenously.	The word "Intravena" can not be understood by laypeople	No suggestion given	No changes, "intravena" or "*intravenously*" " is still chosen because the meaning matches the English version

CONCLUSION

The Indonesian version of the 15D questionnaire has been validated linguistically, and cultural adaptation has been performed using international standard methods with the participation of experts in the field using predetermined criteria.

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AUTHOR CONTRIBUTIONS

Conceptualization, Y. N., L., D. P.; Software, Y. N., L., D. P.; Validation, Y. N., L., D. P.; Formal Analysis, Y. N., L., D. P.; Investigation, Y. N., L., D. P.; Resources, Y. N., L., D. P.; Data Curation, Y. N., L., D. P.; Writing - Original Draft, Y. N., D. P.; Writing - Review & Editing, Y. N., L., G. N.; Visualization, D. P.; Supervision, Y. N., L.; Project Administration, Y. N., L., D. P.; Funding Acquisition, Y. N., D. P.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Antihyperglycemic Activity of Red Fruit Oil (*Pandanus conoideus* Lam) on Improving Kidney Function in STZ- NA-Induced Nephropathy Rats

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Abstract

Background*: Type 2 diabetes mellitus can cause complications, one of which is diabetic nephropathy. Parameters that indicate damage to the kidneys are the increase in creatinine and albumin levels. One of the traditional medicines used in the treatment of DM is red fruit (Pandanus conoideus Lam).* ***Objective****: The purpose of this study was to determine the antihyperglycemic activity and the effect of red fruit oil administration on creatinine levels, microalbumin, and renal histopathology in STZ-NA-induced rats.* ***Methods****: This study used 30 male Wistar rats conditioned with type 2 DM with STZ-NA induction. The rats have grouped into 6 groups: group I, the normal control, group II, the negative control, group III, the positive control (pioglitazone 15 mg/kg BW), and groups IV, V, and VI, the red fruit oil respectively 1.35 mL/kg BW,2.7 mL/kg BW, and 5.4 mL/kg BW. Red fruit oil is made in traditional way and prepared for 2 days. Parameters tested in the study include blood glucose levels, creatinine, microalbumin, and kidney histopathology. Data analysis used the ANOVA method followed by Tukey's post hoc test.* ***Results****: The results showed that a red fruit oil dose of 5.4 mL/kg BW was an effective dose in reducing blood glucose levels, microalbuminuria, and serum creatinine, and repairing damage to the kidneys of rats. The percent activity of a red fruit oil dose of 5.4 mL/kgBW for blood glucose levels, microalbuminuria and serum creatinine were 84.69%, 76.30%, and 92.20% respectively.* ***Conclusion****: Red fruit oil can reduce blood glucose levels, creatinine levels, microalbumin and can repair kidney damage.*

Keywords*: creatinine, diabetic nephropathy, microalbumin, Pandanus conoideus Lam.*

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INTRODUCTION

Diabetes mellitus has been recognized by the WHO as one of the four non-communicable diseases and is the third highest risk factor for death worldwide. Data from the IDF in 2019 showed that approximately 463 million people were living with diabetes worldwide, and it is predicted that this number will continue to increase to 578 million by 2030 and 700 million by 2045 (Infodatin, 2020). Diabetes nephropathy is a microvascular complication caused by impaired renal function in patients with type 2 diabetes mellitus. Impaired renal function begins with a progressive hyperglycemic state that triggers changes in renal cell hypertrophy, capillary permeability, and extracellular matrix synthesis (Brownlee *et al*., 2010).

Red fruit (*Pandanus conoideus* Lam.) is a traditional plant that acts as a local biological resource for the people of the Central Highlands of Papua and has been known by the local population for generations as a natural food supplement with medicinal qualities. In addition, local people also process red fruit in the form of oil to treat degenerative diseases such as diabetes mellitus (Wawo *et al*., 2019; Keim & Sujarwo, 2020). The antioxidant role of tocopherol and carotene in red fruit is higher than that in other fruits or vegetables, such as papaya, bean sprouts, tomatoes, and carrots (Ayomi, 2015). According to previous studies, red fruit has been proven to be antidiabetic (Astuti & Dewi, 2007; Febriyanti, 2011).

According to Agnesa *et al.* (2014), red fruit extract can reduce blood glucose levels by 31.51%. Diah *et al.* (2016) showed that red fruit extract is effective in reducing blood sugar levels by 68%. Alkatiry *et al.* (2014) reported that red fruit oil could reduce blood glucose levels by 63%. Another study also found that red fruit oil has a decreasing effect on serum creatinine and urea levels in rats with maximum physical activity (Sinaga *et al*., 2019) and a reducing effect on the level of kidney cell degeneration in test animals (mice) exposed to plumbum (Pb) (Aprilianti *et al*., 2020).

Based on previous research, research was conducted on the antihyperglycemic effect of red fruit oil on rats with type 2 diabetes mellitus induced by streptozotocin-nicotinamide and the histopathological picture of the kidneys of rats conditioned with nephropathy.

MATERIALS AND METHODS

Materials

The material used in this study was red fruit obtained from the North Sorong District, West Papua. The other ingredients were STZ, nicotinamide, pioglitazone, 95% alcohol, 1% phenolphthalein (PP), NaOH, acetic acid, chloroform, potassium iodide, distilled water, sodium thiosulfate, 1% starch solution, beta carotene, butylated hydroxytoluene (BHT) 0, 1% ethyl acetate, toluene, diethyl amine, methanol, water, n-hexane, diethyl ether, acetic acid 10%, Ragendorff reagent, Lieberman Burchard, KOH, phosphate buffered saline (PBS), ether, 70%, 80%, and 90% ethanol, xylene solvent, warm water, and hematoxylin and eosin (HE).

Tools

The tools used included a pycnometer, oven, cup, desiccator, water bath, and sterile Bidwell. TLC plate, capillary tube, ruler, scissors, blood glucose meter, probe, Eppendorf tube, ice bath, centrifuge, freezer, spectrophotometer, vortex, tissue cassette, microtome, refrigerator, glass object, and light microscope Olympus CH20.

Method

Manufacture of red fruit oil

Red fruit was collected from the North Sorong District, West Papua, and was analyzed at the Natural Materials Laboratory, Department of Pharmacy, Gorontalo State University. Red fruit oil is traditionally produced. Red fruit seeds are boiled continuously until the oil produced tends to be dark red and concentrated. The first boiling step was conducted for 2 h to facilitate pounding to obtain red fruit starch. The second boil took a full day to separate the oil from the dregs. The third purpose of boiling was to make the red fruit oil last longer and free of bacteria. Red fruit oil was produced in amounts as high as 1 liter.

Characterization and analysis of red fruit oil

Oil quality tests included specific gravity, moisture content, free fatty acids, peroxide value, and total carotenoid content. Specific gravity was measured using a pycnometer with three replicates. Water content was determined using the Sterling Bidwell tool. Free fatty acid and peroxide numbers were tested using the titration method, whereas the determination of total carotenoid levels was performed using a slightly modified method from Knockaert *et al*. (2012).

Preparation and treatment of test animals

The test animals used in this study were 30 Wistar male white rats, 8 weeks old, weighing 150–200 g. The rats were acclimatized for one week before treatment. Rats were grouped into 6 treatment groups, where all treatment groups consisted of 5 rats, including K1 (normal control), K2 (negative control), K3 (positive control: pioglitazone, 1.35 mg/kg BW), K4 (MBM, 1.35 mL/kg BW), K5 (MBM, 2.7 mL/kg BW), and K6

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(MBM, 5.4 mL/kg BW). All groups, except the normal group, were induced by STZ at a dose of 45 mg/kg BW rats and NA at a dose of 110 mg/kg BW rats intraperitoneally. The induced rats were then checked for their blood glucose levels. A person with a blood glucose level ≥200 mg/dL is declared to have diabetes and left for two months to reach a state of nephropathy. The parameters of diabetic nephropathy include serum creatinine and urine albumin levels.

Measurement of rat blood glucose levels

Blood glucose levels were measured using the GOD-PAP method. This method uses enzymatic serum or plasma samples to identify the red color formed in proportion to the level of glucose formed in the sample. The serum was mixed with glucose liquid chromatography reagent and incubated for 10 min at 20−25ºC or 37ºC ℃ for 5 min. Standard and sample absorbance measurements were performed using a spectrophotometer (Rias & Sutikno, 2017).

Examination of microalbumin

Microalbumin examination using fresh urine and immunoturbidimetry Rats were orally administered 8 mL of warm water. The rats were then placed in the metabolic cage, and 20 µL of urine was collected. Subsequently, 350 µL of reagent 1 (TRIS pH 7.5 + NaCl) and 70 µL of reagent 2 (TRIS pH 8.0 + NaCl) were added, and the absorbance was read using a spectrophotometer. Then calculate the albumin ratio. The reference value for the ratio of urine albuminuria was normal (<30 mg/L) and was expressed as microalbuminuria (≥30 mg/L).

Creatinine check

The Jaffe method was used to examine creatinine levels. A normal creatinine level was 1.2 mg/dL. The serum creatinine levels were measured using a spectrophotometer. To determine the creatinine level, a mono-reagent was first prepared by mixing four parts of reagent 1 (sodium hydroxide) with one part of reagent 2 (picric acid), and 10 µL of the test serum was reacted with 1000 µL of the test reagent (creatinine reagent) and then homogenized using a vortex. The absorbance was measured with a spectrophotometer (490-510 nm) at 37ºC ℃ for 60 s (A1), and then the absorbance was measured again after 120 s (A2). The difference between A2-A1 was used to calculate creatinine levels for blanks (reagent and distilled water) and standards (reactant and creatinine standards).

Histopathological test

Experimental animals were anesthetized first with ether, then sacrificed, the kidney organs were taken, and histopathological preparations were made. Kidney organs were fixed in formalin in phosphate-buffered saline (PBS) at pH 7.4. The next step is embedding. The embedding procedure consists of several stages, including dehydration, clearing, and paraffin block preparation. The tissue pieces were then removed, placed on a glass slide, and stained with hematoxylin and eosin or HE. Kidney tissue preparations were observed under a microscope at 400x magnification. An analysis was carried out on the changes that occurred, and the average of these changes was calculated.

Data analysis

The data obtained in this study were statistically analyzed. If the data were normally distributed (p> 0.05), then the parametric test was continued with the post hoc test, namely Tukey, to determine whether there was a difference between each treatment group.

RESULTS AND DISCUSSION

Characterization and oil quality test

The characterization and quality testing of red fruit oil were carried out on the parameters of specific gravity, moisture content, free fatty acids (FFA), peroxide number, and total carotenoids. The characterization and quality test results for the red fruit oil are shown in Table 1.

The specific gravity obtained in this study is 0.93. This result is similar to that of a previous study by Widowati *et al.* (2009), who reported that the specific gravity of red fruit oil was 0.92. Specific gravity describes the number of components contained in a substance. The more chemical components in the oil, the higher the specific gravity (Kristian *et al.*, 2016).

Table 1. Characterization and oil quality test results

No.	Parameters	Result	Requirements	Literature
1.	Specific gravity	0.93±0.002	-	-
2.	Moisture content	4.98%±0.008	0.5%	(CPO SNI, 2006)
3.	Free fatty acids	0.62%	0.5%	(CPO SNI, 2006)
4.	Peroxide number	7.95 meq O2/kg	10 meq O2/kg	(CPO SNI, 2013)
5.	Total carotenoids	4889.32 mg/kg	-	-

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The obtained red fruit oil had an average water content of 4.98% on average. The standard for red fruit oil has not yet been established, but according to a previous study by Pratiwi *et al.* (2020), the moisture content obtained was 0.92%. Meanwhile, when compared to the SNI for CPO, the maximum moisture content was 0.5%. The high water content of the oil causes hydrolysis reactions to occur. The very high water content in red fruit oil can be caused by the extraction process of red fruit oil using a wet extraction method, where it is suspected that the water separation process does not take place properly, so that the amount of water included in the oil is still quite high (Pratiwi *et al*., 2020). More attention will be given to the process of separating water and oil, which can be done so that the water contained in the oil is not too high.

Free fatty acids are an important parameter for determining the quality standards of oils and fats because they can indicate the rancidity of oil during storage. The obtained result was 0.62%. The FFA level of CPO according to the SNI 2006 was 0.5%, which means that the FFA level of the research results is still unqualified when referring to the standard value for CPO. The results of this study are in line with those of previous studies that reported FFA values higher than SNI. Pratiwi *et al.* (2020) reported that the FFA content produced was higher than the specified standard of 0.66% for crude oil. The high FFA content of red fruit oil can be caused by the extraction process of red fruit oil, hydrolysis reactions that are influenced by temperature, air, heating time, and the container used (packaging) (Pratiwi *et al.*, 2020; Sarungallo *et al.*, 2011). Previous research also reported FFA levels of red fruit oil ranging from 7.24-8.5%. FFA values depend on the extraction method used. The wet extraction method had a higher FFA value than the dry extraction method because water was added to the extraction process to allow hydrolysis. Red fruit oil with a high FFA value requires a refining process with four general stages: degumming, neutralization, bleaching, and deodorization (Sarungallo *et al*., 2014). The peroxide number is an important parameter that can indicate damage to oil owing to the rancidity of fat due to oxidation. The peroxide number measured in this study was 7.95 meq O2/kg. This peroxide value meets the peroxide number required for cooking oil, which is 10 meq O2/kg (SNI 2013). According to Pratiwi *et al.* (2020), the low peroxide number value can be due to the presence of active components, such as carotenoids and tocopherols, which are quite high in red fruit oil. The peroxide number of red fruit oil in this study was lower than that reported by Sarungallo *et al.* (2011), who reported the traditional extraction of red fruit oil (17.6 mg O2/100 g) and commercial oil (16.62 mg/kg). The difference in the peroxide number was mainly due to the length of the cooking process. Merdey's traditional extraction, with a total extraction process time of more than 30 h, causes the peroxide number of the oil to be very high. Their findings revealed that by shortening the extraction time, both dry and wet extraction could reduce the level of oxidative damage manifested by lower peroxide values (Sarungallo *et al*., 2011).

Red fruit oil has been reported to contain carotenoids as one of its most important active components (Pratiwi *et al*., 2020). Carotenoids are natural pigments that give red color to red fruit oil, and are antioxidants that are needed by humans (Santoso *et al.*, 2018). This study produced carotenoid levels of 4889.32 mg/kg. This result is still lower than those reported in previous studies. According to Pratiwi *et al.* (2020), the total carotenoid content in crude red fruit oil is 6299 ppm. Santoso *et al.* (2018) reported a total carotenoid content of 6398 ppm in crude red fruit oil. Sarungallo *et al.* (2018) reported a total carotenoids of 7857 ppm. The difference in carotenoid content can be attributed to the differences in the extraction process. This study used a wet extraction method (boiling) in which a large amount of water was used. Excess water during the extraction process is thought to cause oxidation and hydrolysis reactions in oil. These reactions can trigger damage to the carotenoid components. In addition, the processing process using a heating process is also a factor in the damage to carotenoid components (Sarungallo *et al*., 2014). Wilska and Jeszka (2002) stated that carotenoids are very sensitive to oxygen, light, and temperature because they have conjugated double bonds that contain many reactive electrons and are easily oxidized (Pratiwi *et al.*, 2020; Sarungallo *et al.*, 2018).

Blood glucose levels of rats

Blood glucose levels were measured six times at different times on days 0, 3, 24, 31, 38, and 45. Day 0 (T0) was the day the rats were acclimatized for 7 days. Blood glucose levels measured in this study ranged from 60 to 70 mg/dL. This is because T0 has not been given any treatment, so it can be ascertained that: the Test animals were healthy. All groups except the normal group were then given STZ-NA intraperitoneally to condition the test animals to experience diabetes mellitus (DM). The results of measuring Blood glucose levels in rats are shown in Figure 1.

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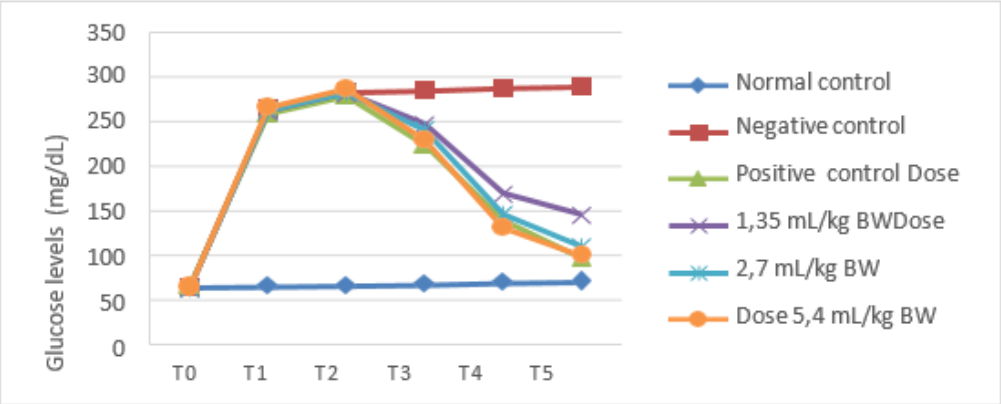

Figure 1. Average blood glucose levels of rats

Blood glucose levels were measured on days three (T1) and 24 (T2). At this time, the test animals had already experienced DM and were conditioned for 21 days to induce nephropathy. The measurement of blood glucose levels showed an increase in blood glucose levels due to STZ-NA induction. This is due to the mechanism of STZ, which can damage pancreatic beta cells via nitric oxide (NO) production, the formation of reactive oxygen species (ROS), and DNA alkylation, which can cause cell death or total cell damage. Therefore, NA induction was also performed to partially protect pancreatic beta cells from STZ exposure (Ghasemi *et al.*, 2014; Kishore *et al.*, 2017; Szkudelski, 2012).

Blood glucose levels were measured at T3, the first week (day 31); T4, the second week (day 38); and T5, the third week (day 45). As shown in Figure 1, the measurement of blood glucose levels at T3, T4, and T5 showed a decrease in blood glucose levels in the group administered pioglitazone (the positive control) and the test preparation. The decrease in blood glucose levels indicated that there was an effect on the blood glucose levels after treatment. The 5.4 mL/kgBW red fruit oil group was not significantly different from that in the positive control group. This proves that the administration of red fruit oil group at 5.4 mL/kg BW decreased blood glucose, which continued to increase. This proves that the longer the administration time, the higher is the lowering effect shown by the test preparation.

Statistical analysis showed significant differences in the measurement of blood glucose levels in each group ($P<0.05$). Blood glucose levels at T5 in the positive group (pioglitazone) were closest to normal when compared to the test preparation group, while in the test preparation group, the dose of K6 (red fruit oil, 5.4 mL/kg BW) was closest to the positive and normal control groups.

The positive control (pioglitazone) reduces blood glucose levels by acting on peroxisome proliferator-activated receptor agonist (PPAR) to increase insulin stimulation, thereby increasing glucose uptake in peripheral tissues. Pioglitazone can reduce blood sugar levels due to its mechanism of increasing insulin sensitivity in the liver and adipose tissue and with a lower risk of hypoglycemia-related side effects (Ulfa & Arfiana, 2020).

The test preparation can reduce blood glucose levels because of its chemical content, which acts as an antihyperglycaemic agent. According to previous research, red fruit oil contains flavonoid compounds, which are antioxidants that can prevent the formation of AGE chains that cause pathological changes under hyperglycemic conditions. Flavonoids reduce blood glucose levels through direct and indirect protection of pancreatic beta cells from damage and oxidative stress, which can increase insulin secretion (Ghasemi *et al.*, 2014). Red fruit oil contains carotenoids. Carotenoids can improve pancreatic function so that insulin secretion by the beta islets of Langerhans can increase (Heriyanto *et al.*, 2021; Stahl & Sies, 2005).

Microalbuminuria levels in rats

Microalbuminuria measurement is the initial examination to detect the occurrence of diabetes nephropathy. Microalbuminuria is characterized by higher than normal albumin excretion of more than 30 mg/day (Natesan & Kim, 2021; Rivandi & Yonata, 2015; Verdiansah, 2016). Figure 2 shows the results of measuring microalbumin levels in rats.

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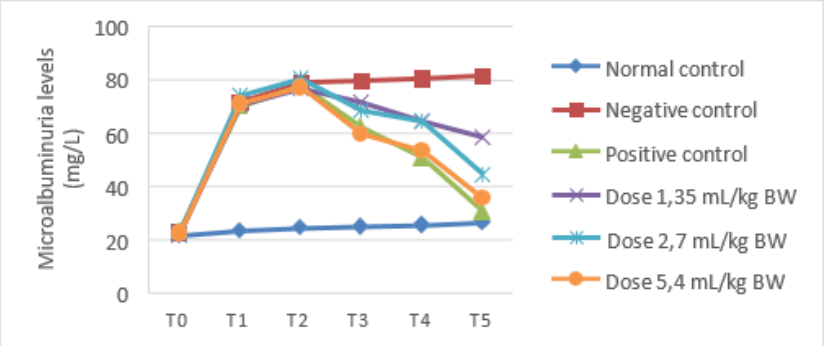

Figure 2. Average microalbuminuria level of rats

The measurement of microalbuminuria levels in the test animals began on day 0 (T0). The average albumin level in the urine of test animals at T0 was <30 mg/L. In contrast to the urine albumin levels measured at T1 (day 3) and T2 (day 24), which revealed that they had experienced microalbuminuria, with urine albumin levels exceeding the normal value of >30 mg/L. The increase in urinary albumin levels is due to STZ-NA induction, which causes blood glucose levels to exceed normal so that diabetic nephropathy occurs, indicating disruption of kidney function.

Examination for microalbuminuria is very important for detecting impaired renal function. The initial stage begins with renal hypertrophy, hyperfunction, and thickening of glomerular and tubular membranes. The process of glomerulosclerosis that continues to occur results in increased glomerular permeability, which causes albumin to escape glomerular filtration and be found in the urine (Verdiansah, 2016).

Urine albumin levels were measured on days 31, 38, and 45. Based on the average graph in Figure 2, urine albumin levels at the three measurement times showed a decrease compared to the negative control. The decrease in urine albumin levels was because, on day 24, each group was given treatment, except for the normal group and the negative control. As shown in the graph, the decrease from T3 to T4 and from T4 to T5 was different for each treatment group. Data on urine albumin levels at T5 were subjected to statistical analysis. Statistical analysis from T5, namely the 45th day, showed a significant value (P <0.05) in the one-way ANOVA test. Testing was continued using a post-hoc Tukey test. The results showed that the positive control group was closest to the normal group. Then the red fruit oil group 5.4 mL/kg BW is not different from the positive control so the positive control group (pioglitazone) has proven its effectiveness in reducing urinary albumin levels, and the red fruit oil group 5.4 mL/kg BW is the dose closest to the positive group, so it can be said to have the effect of reducing urinary albumin levels best compared to the red fruit oil groups 1.35 mL/kg BW and 2.7 mL/kg BW. However, when compared to normal urine albumin levels, normal urine albumin levels were not reached.

The development of microvascular complications in renal mesangial cell growth that occur during diabetic nephropathy results in increased levels of AGEs in the blood. AGEs bind to the AGEs receptor (RAGE), which then triggers the generation of ROS and activates NF-κB in target cells, mesangial cells, the endothelium, and macrophages, resulting in increased vascular permeability. This results in transvascular albumin leakage that causes microalbuminuria. The decrease observed in the test preparation treatment group could be due to the presence of antioxidant compounds from flavonoids and carotenoids. Antioxidants reduce the production of ROS-modified proteins in mitochondria to prevent the progression of diabetic nephropathy through the effect of ROS scavenging on mesangial cell mitochondria (Murnah & Indranila, 2014).

Serum creatinine levels of rats

High creatinine levels in blood indicate weak kidney function (Aditya *et al.*, 2018; Aji *et al.*, 2019; Alfarisi *et al.*, 2012; Martono & Satino, 2014; Verdiansah, 2016). Creatinine levels above normal (<1.2 mg/dL) indicate impaired renal function (Alfarisi *et al.*, 2012). The following is a graph of the results of creatinine level measurements in rats.

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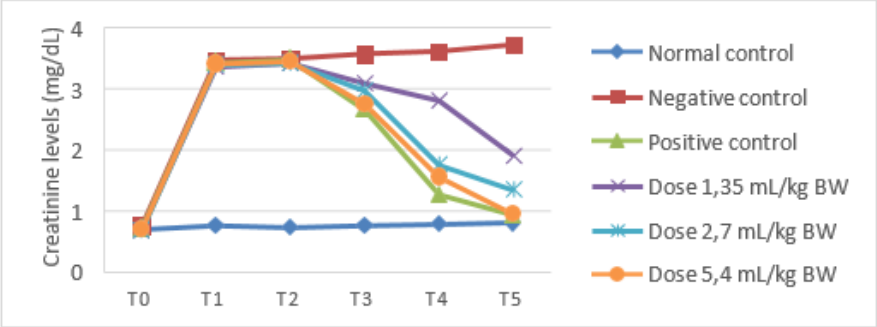

Figure 3. Average rat creatinine levels

Creatinine levels increased on days 3 (T1) and 24 (T2). According to Aditya *et al.* (2018), creatinine levels in the blood that increase above 1.5 mg/dL indicate weak kidney function. Increased creatinine levels are caused by damage to glomerular filtration. The damage that occurs can be caused by exposure to toxic substances, such as STZ-NA. STZ is a diabetic agent that causes pancreatic beta cell death and increases blood glucose levels through an increase in ROS. High blood glucose levels can cause oxidative stress, which triggers the production of NO to increase, resulting in the release of vasoconstrictive mediators that can then affect kidney function, namely, a decrease in glomerular filtration in rats (Aji *et al*., 2019). Glomerular damage can be determined by measuring creatinine levels because one of the factors that affects kidney function is determined by serum creatinine levels (Martono & Satino, 2014).

For 21 days, measurements of creatinine levels in test animals were carried out three times, namely, on day 31 (T3), day 38 (T4), and day 45 (T5), to observe the effect of decreasing creatinine levels in each treated group. The measurement results at T3, T4, and T5 based on the graph in Figure 3 show that there was a decrease in creatinine levels in the positive control and treatment groups compared to before the administration of the test preparation. The measurement data of creatinine levels at the three time points were then subjected to statistical analysis. Statistical test results using one-way ANOVA showed a significance value of 0.000 ($P<0.05$). Furthermore, to determine the difference in the decrease in creatinine levels in each group, a post-hoc Tukey test was conducted on the 45th day. The test results showed that the administration of pioglitazone as a comparison drug provided the best reduction in creatinine levels because it was closest to the normal control. The results also showed that there was no difference between the positive control and 5.4 mL/kgBW red fruit oil groups. Therefore, the 5.4 mL/kgBW red fruit oil group had reduced creatinine levels and improved kidney function in test animals experiencing diabetic nephropathy.

The effect of decreasing creatinine levels that occurred in the 5.4 mL/kg BW red fruit oil group could be due to red fruit oil containing antioxidants, such as flavonoids and carotenoids, which indirectly reduce creatinine levels in test animals with impaired kidney function. Flavonoids can capture free radicals by releasing hydrogen atoms from their hydroxyl groups. Carotenoids can quench singlet oxidation and their antioxidant content binds to free radicals. The bond does not eliminate the electrons but reduces the energy possessed so that it is not able to induce other cells (Palupi & Martosupono, 2009).

Rat kidney pathology

The histopathology of kidney organs aims to determine the damage that occurs in the tubules and glomeruli of the kidneys. Renal histopathological observations were performed using hematoxylin-eosin (HE) staining. Necrosis was also observed. Each group was administered three preparations to read the histopathological picture of the kidneys. Observations were made using a light microscope at 400X magnification.

Histopathological changes were evaluated according to the Arshad system by classifying histopathological changes in rat kidneys as no change (0), mild damage (1), moderate damage (2), and severe damage (3). Percentage of mild damage (changes <30%), moderate damage (changes <50%), and severe damage (changes >50%) (Jannah & Budijastuti, 2022). The results of the histopathological observations and percentage of kidney damage are shown in Figure 4 and Table 2.

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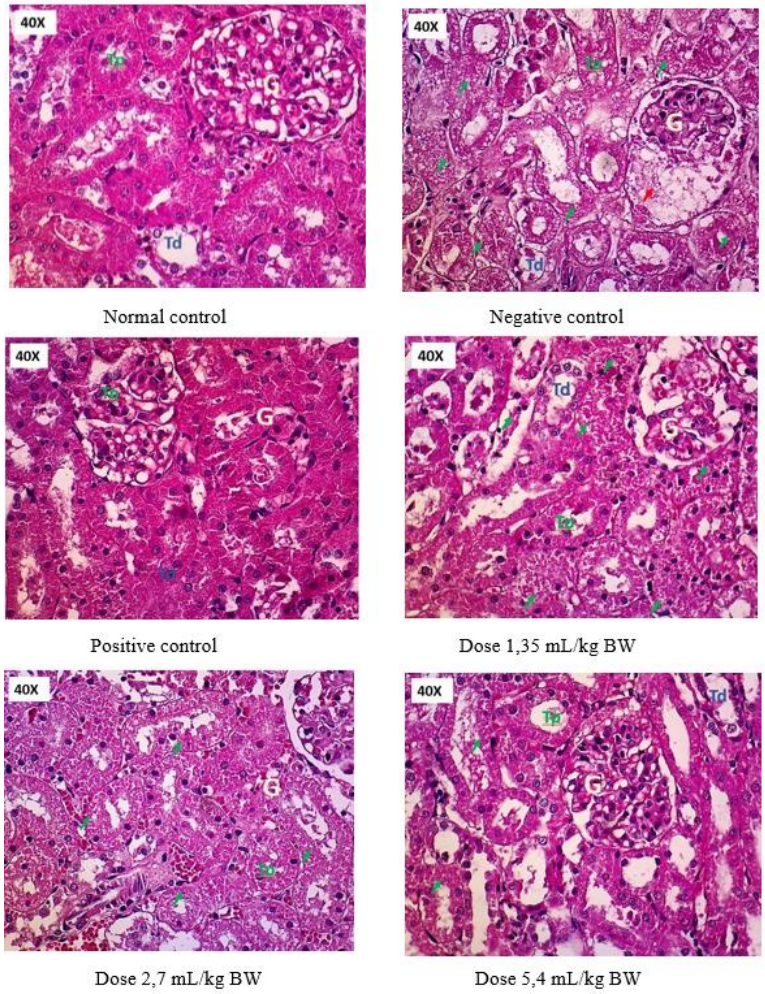

Figure 4. Histopathology of rat kidney

Description:
Tp: Proximal tubule
Td: Distal tubule
G: Glomerulus
Green arrow: Tubular necrosis
Red arrows: Glomerular necrosis

Table 2. Average and scoring of rat kidney damage

Group	Mean percentage of tubule necrosis ± SD (%)	Scoring
I	0	0
II	41.7 ± 14.34	2
III	5 ± 0	1
IV	35 ± 8.50	2
V	16.7 ± 4.71	1
VI	6.7 ± 2.36	1

Description:

I: Normal control
II: Negative control
III: Positive control (pioglitazone)
IV: Red fruit oil 1.35 mL/kg BW
V: Red fruit oil 2.7 mL/kg BW
VI: Red fruit oil 5.4 mL/kg BW

(0) No changes occurred
(1) Mild cell damage with less than a 30% change
(2) Moderate cell damage with less than a 50% change
(3) Severe cell damage with changes > 50%

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Damage to the kidneys can be caused by several factors, including toxic substances that enter the body and interfere with kidney function. Damage to the kidneys due to toxic substances can be identified through structural changes in histology, including cell necrosis, which is morphologically characterized by deconstruction of proximal tubule epithelial cells. Proximal tubule epithelial cells are sensitive to anoxia and are easily destroyed in cases of poisoning owing to metabolic waste excreted by the kidneys. Therefore, histological changes that occur in the kidneys can be ascertained from the number of compounds that enter the body. Necrosis is a cell tissue that undergoes death due to injury while the individual is still alive. Changes occur in the nucleus in the form of loss of chromatin images: the nucleus appears denser and becomes wrinkled, no longer vascular, the color becomes dark (picnosis), the nucleus is divided into torn fragments (cariorexis), and the nucleus appears pale or unreal because it no longer takes up much color (karyolysis). no longer takes up much color (karyolysis) (Jannah & Budijastuti, 2022).

The improvement in kidney damage closest to that of the normal control was a positive control. Histopathological examination of the kidneys in the positive control group showed that the glomeruli and tubules were relatively normal. Likewise, the scoring was 1, with mild cell damage. This is because the positive control was administered with a comparator drug, pioglitazone. This drug acts on PPAR and reduces the amount of glucose in the blood stream. Pioglitazone has strong antihyperglycemic action by reducing insulin resistance (Schernthaner *et al.*, 2013).

The test preparation group showed the best dose VI group in repairing kidney damage, and approaching the positive control was the 5.4 mL/kg BW red fruit oil group. The damage that occurred in dose group VI was less than that in the other dose groups, with an average damage score of 1 for mild damage. The histopathological picture of the kidneys in red fruit oil (5.4 mL/Kg BW) also shows that necrosis was reduced and observed better than in other dose groups.

The ability of red fruit oil to reduce or repair cell damage through the improvement of cell metabolism and cell division means that cells that experience necrosis can be replaced with new cells. This ability can be attributed to the content of red fruit oil, which is rich in antioxidants, proteins, and unsaturated fatty acids, such as oleic acid and linoleic acid. Antioxidants, such as carotenoids, counteract free radicals, which are highly reactive and harmful to cell life (Suastika, 2011).

CONCLUSION

Based on this study, the physical characteristic and mechanical properties of the chitosan-PVA-*Aloe vera* films were affected by both polymers: chitosan (1%, 1.5%) and PVA (0.5%, 1%, 1.5%). The increased concentrations of chitosan and PVA caused an increase in the mechanical properties. However, there was a decrease in the swelling index. Based on the results, films with chitosan 1.5% and PVA 1.5% had the best characteristics and mechanical properties (swelling index, tensile strength, and elongation at break) compared to the other formulations. Therefore, this film has the potential to be developed as a wound dressing material.

AUTHOR CONTRIBUTIONS

Conceptualization, A. C. K., T. W., G. P. W.; Methodology, A. C. K., T. W., G. P. W.; Software, A. C. K.; Validation, A. C. K.; Formal Analysis, A. C. K., T. W., G. P. W.; Investigation, A. C. K.; Data Curation, A. C. K., T. W., G. P. W.; Writing - Original Draft, A. C. K.; Writing - Review & Editing, T. W., G. P. W.; Visualization, A. C. K.; Supervision, T. W., G. P. W.; Project Administration, A. C. K.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Antimalarial Potential of Fraction 5 from Ethanolic Leaves Extract of *Artocarpus Altilis*

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Abstract

Background*: Artocarpus altilis leaf extract (AAL.E) was separated by VLC, and six fractions were obtained. Fraction 5 (AAL.E.5) showed antimalarial activity with an IC_{50} value of 3.71 µg/mL.* ***Objective****: This study aimed to determine the antimalarial activity of AAL.E.5 subfractions against P. falciparum, the mechanism of action against Plasmodium Falciparum Malate quinone oxidoreductase (PfMQO), and the active substances.* ***Methods****: The AAL.E.5 was separated by open-column chromatography and eluted with chloroform-methanol gradient elution in order of increasing polarity. The antimalarial activity of all subfractions was assessed using a lactate dehydrogenase (LDH) assay against P. falciparum and the mechanism of action of the PfMQO enzyme. The profiles of the most active subfractions were analyzed using High-Performance Liquid Chromatography (HPLC).* ***Results****: The separation of fraction 5 (AAL.E.5) yielded 11 subfractions (AAL.E.5.1–AAL.E.5.11). Screening antimalarial activity at 10 µg/mL in this subfraction showed that only five subfractions (AAL.E.5.6-AAL. E.5.10) inhibited P. falciparum and two subfractions (AAL.E.5.6 and AAL.E.5.10) inhibited the PfMQO enzyme. Only subfraction 6 (AAL.E.5.6) inhibited both, with IC_{50} values of 6.609 µg/mL and 20.34 µg/mL. The thin layer chromatography profile of AAL.E.5.6 revealed reddish-orange spots, indicating the presence of flavonoid compounds, and was also presumed from the UV-visible to HPLC chromatogram for band I in the 300 – 400 nm range and band II in the 240–285 nm range.* ***Conclusion****: Subfraction 6 has antimalarial activity against P. falciparum and is thought to have a mechanism of action in PfMQO. Based on the TLC, HPLC, and UV-Vis spectra, subfraction 6 was assumed to be a flavonoid.*

Keywords*: antimalaria, Artocarpus altilis, flavonoid, lactate dehydrogenase (LDH), PfMQO*

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INTRODUCTION

Malaria is an infectious disease caused by the protozoan parasite *Plasmodium*, which is transmitted by the bite of female Anopheles mosquitoes. Malaria has the world's most extensive and endemic distribution, primarily in the tropical and subtropical climates. Malaria affected an estimated 229 million individuals in 2019, with an increase of 228 million in 2018 (WHO, 2020). An estimated 219 million malaria cases were reported in 2017 compared to 217 million (WHO, 2018). Globally, malaria deaths have decreased steadily from 451,000 in 2016 to 409,000 (WHO, 2020). Indonesia is malaria endemic, with approximately 180,000 confirmed cases of malaria from 26 malaria-endemic areas nationally (Sitohang *et al*., 2018). The province with the highest number of malaria cases in Indonesia was Papua (12.07%). West Papua (8.64%), East Nusa Tenggara (1.99%), North Maluku (1.36%), and Maluku (1.21%) (RISKESDAS, 2019).

Malaria is difficult to control owing to its widespread treatment resistance. Malaria control in Indonesia remains restricted in terms of treatment due to discrepancies in malaria case management at all levels of health care, as well as the rise of malaria parasite resistance to available commercial antimalarial drugs, such as chloroquine and pyrimethamine sulfadoxine (Cui *et al*., 2015). However, this therapeutic modification is considered ineffective. The lack of Artemisinin Combination Therapy (ACT) coverage is caused by numerous non-malaria-endemic locations, such as Jakarta, which are still relatively weak in dealing with malaria cases from endemic areas (Kinansi *et al*., 2021). As existing antimalarials are ineffective, a novel technique to prevent malaria transmission is required (Noronha *et al*., 2019). As malaria therapies have become increasingly resistant, new drugs derived from various plants are being explored.

The Mulberry family (Moraceae) is found worldwide, with the majority of species found in Asia, while the Indo-Pacific Islands have 60 genera and 1,400 species (Berg, 2001). *Artocarpus* is the largest genus of Moraceae, is high in phenolic compounds, and is widely used in traditional medicine. Widyawaruyanti (2007) revealed that heteroflavone C, a prenylflavonoid compound isolated from the stembark of *Artocarpus champeden*, had an IC_{50} antimalarial activity of 0.001 – 1 µmol/L. A prenyl chalcone, morachalcone A, was isolated as an active antimalarial agent from the ethanol extract of *A. champeden* stem bark by Hafid (2012) and had an IC_{50} of 0.18 µg/mL. Bourjot (2010) examined an ethyl acetate extract from the bark of *Artocarpus styracifolius*, which has antimalarial activity against *P. falciparum* with an IC_{50} of 0.5 µg/mL - 6.9 µg/mL and succeeded in isolating prenylated flavonoids. From a dichloromethane extract of *Artocarpus altilis* root, Boonphong (2007) isolated nine prenylated flavones with antimalarial activity ranging from 1.9 - 4.3 µg/mL. Ethanol extracts from *A. altilis* leave suppressed the development of *P. falciparum in vitro* with an IC_{50} value of 1.32µg/mL and *P. berghei in vivo* with an ED_{50} of 0.82 mg/kg body weight, according to Hafid (2016). Lactate dehydrogenase (LDH) assay revealed that fraction-6 of *Artocarpus sericicarpus* dichloromethane exhibited an IC_{50} value of 1.53 ± 0.04 µg/mL against *Plasmodium falciparum* (Tumewu *et al*., 2020).

Syah (2006) isolated two acylated flavonoid derivatives from *Artocarpus altilis* leaves: 2-geranyl-2, ’ 4, ’ 3,4-tetrahydroxidihidroxcone and 8-geranyl-4', 5,7-trihydroxy flavanone. According to Fajriah *et al.* (2013), the ethyl acetate fraction of *A. altilis* leaves produces prenylated flavonoids, specifically the 1-prenylated flavonoid (2,4-dihydroxy phenyl) -3- (8-hydroxy-2-methyl-2-ethane-2-ethane-2-ethane-2-e (4-methyl-3-pentenyl) -2H-1benzopyranyl] -1-propanone. Nguyen *et al.* (2013) obtained the auron flavonoid altilisin H-J from a methanolic extract. Nguyen *et al.* (2013) isolated eight geranylated dihydrochalcones from the leaves of the methanolic extract of *A. altilis*, designated sakenins A-H.

Based on a previous study, fractionation of ethanol extract of *A. altilis* leaves obtains six active fractions (AAL.E.1-AAL. E.6). The microscopic antimalarial results showed that AL. E.2, AAL.E.4, and AAL.E.5 were active as antimalarials (Hidayati, 2020). AAL.E.2 isolated 1-(2,4-dihydroxy phenyl)-3-[8-hydroxy-2-methyl-2-(4-methyl-3- pentenyl)-2H-1-benzopyran-5-yl]-1-propanone, and AAL.E.4 isolated geranyl-2′, 4 ′, 3, 4-tetrahydroxy-dihydrochalcone. Both inhibit *P. falciparum* and *Pf*MQO enzymes (Hidayati *et al*., 2023). Further studies are needed on fraction 5 (AAL.E.5) to determine the active substance that acts as an antimalarial agent. This study aimed to identify the active substance that functions as an antimalarial agent in AAL.E.5 of *A. altilis*.

MATERIALS AND METHODS

Materials

Plant material

Fresh *Artocarpus altilis* leaves were obtained from the Purwodadi Botanical Garden, East Java, Indonesia. A licensed botanist identified this plant in the Purwodadi Botanical Garden in East Java (No: B-

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107/IPH.06/AP.01/II/2020). The specimens were stored in the herbarium of the Natural Product Medicine Research and Development (NPMRD) at the Institute of Tropical Diseases, Universitas Airlangga, Indonesia.

Parasite

P. falciparum was obtained and cultivated at the Natural Product Medicine Research and Development (NPMRD), Institute of Tropical Diseases, Universitas Airlangga, Surabaya, East Java, Indonesia.

Methods

Fractionation and HPLC

A total of 800 mg of AAL.E.5 was separated using silica gel in open-column chromatography and eluted with a chloroform-methanol gradient elution in order of increasing polarities (9.8:0.2 v/v) and obtained eleven subfractions (AAL.E.5.1- AAL.E.5.11). In future studies, the profile of the most active subfractions will be analyzed using High-Performance Liquid Chromatography (HPLC). The subfraction was eluted in the mobile phase acetonitrile-water (3:7 v/v) at a flow rate of 0.5 ml/min, a PDA detector, and an injection volume of 40 µL.

In vitro* cultivation of *Plasmodium falciparum

The chloroquine-sensitive *P. falciparum* culture was maintained using numerous modification approaches described by Trager and Jansen (1997) at the Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia. Fresh human erythrocytes of type O were suspended at 2% hematocrit in RPMI 1640 (Gibco) containing 25 mM HEPES buffer, 50 g/mL hypoxanthine, 2 mg/mL Natrium Bicarbonate, and 10 g/mL gentamycin to culture the parasites. The Incubation was carried out at 37°C in a gas mixture of 5% O_2, 5% CO_2, and 90% N_2.

Antimalarial LDH assay

The antimalarial assay was conducted using the LDH (Lactate Dehydrogenase) method. Samples were screened at a dose of 10 µg/mL. Briefly, a stock solution was prepared by dissolving 10 mg of the sample in 1000 µL DMSO. Several concentrations of the sample were prepared from 0.05 to 100 µg/mL. 0.4 µL sample from each concentration and 100 µL of parasites (ring stage) were added to the well plate. Adding (0.4 µL) were added to the well as a positive control (chloroquine diphosphate). The microplate was incubated for 72 h with a gas mixture of 5% CO_2, 5% O_2, and 90% N_2. After incubation, 200 µL PBS was added to the well plate and centrifuged at 1300 rpm for 10 min at room temperature. The supernatant (240 µL) was removed and the well plate was kept at -30°C for 24 h. Anti-malarial activity was measured using the LDH method. Ten milliliters of LDH-buffer (Tris-HCL, Sodium L-Lactate, Triton X-100, deionized water) was added to 2 mg NBT (10 mg/ml, Sigma), 50 µL APAD stock (10 mg/ml, Oriental Yeast Co., Ltd), and 200 µL diaphorase stock (50 units/mL, Sigma). The substrate was mixed gently and kept in the dark. Add 90 µL of substrate per well plate and shaker at 400 rpm for 30 min. The absorbance was then measured using an ELISA Reader at a wavelength of 650 nm, and the results were analyzed using the GraphPad Prism program.

***Plasmodium falciparum* malate quinone oxidoreductase (*Pf*MQO) enzyme assay**

The stock solution of the sample was prepared by dissolving 10 mg in 1000 µL DMSO to produce a final concentration of 10,000 µg/mL as stock solution. The *Pf*MQO enzyme test mixture solution consisted of 20 mL of Hepes 50 mM (pH 7.0), 200 µL of 12 mM dichlorophenolindophenol (DCIP), 12 µL of 100 mM decilubiquinone (duQ), and 25 µL of *Pf*MQO 13.2 mg/mL. The assay solution in the microwell plate was then supplemented with 1 µL sample, and mixing of the solution in the microwell plate was carried out in a well plate mixer at 650 rpm for 1 min. then, while maintaining the temperature at 250 °C for 3 minutes, read the absorbance at a 600 nm wavelength. Next, 5 µL of L-malate (enzyme) was added to each well (apart from well number 12) and shaken with a plate mixer for 20 s. The absorbance at a wavelength of 600 nm was then measured after the incubation at 25°C for 10 min. Samples exhibiting more than 50% resistance were analyzed using GraphPad Prism to determine their IC_{50} values.

RESULTS AND DISCUSSION

Separation of fraction 5 (AAL.E.5)

The ethanolic extract and fraction of *Artocarpus altilis* exhibited antimalarial activity. This study focused on *A. altilis*'s the antimalarial activity of A. altilis (AAL.E.5). Open column chromatography was used to isolate ethanol 5 from *A. altilis* (AAL.E.5) using a gradient elution of chloroform: methanol (98:2). Subsequently, 11 subfractions were generated (subfraction 1-11). All of–1-11 (AAL.E.5.1 - AAL.E.11) were examined using thin layer chromatography (TLC) with silica gel 60 RP-18 $_{F254}$ as the stationary phase and acetonitrile: water (7:3 v/v) as the mobile phase. The flavonoids were observed more clearly after spraying with H_2SO_4 reagent on the TLC plate. TLC spots indicate orange-yellow spots. Flavonol glycosides are indicated by orange-yellow hues (Gwatidzo *et al*., 2018). Subfractions (7 – 9) also

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performed well, as evidenced by their R_f values of 0.7 (AAL.E.5.7), 0.7 (AAL.E.5.8), and 0.68. (AAL.E.5.9) (Figure 1).

Antimalarial activity of subfraction *Artocarpus altilis*

Lactate dehydrogenase assay (LDH)

Antimalarial activity testing was performed to investigate the possible inhibition of *P. falciparum* growth by the samples. All subfractions (11 fractions) were screened for antimalarials at a concentration of 10 ug/ml, and only five subfractions (AAL.E.5.6 – AAL.E.5.10) showed inhibition of more than 50% (Figure 2). Therefore, IC_{50} calculations were performed on these subfractions.

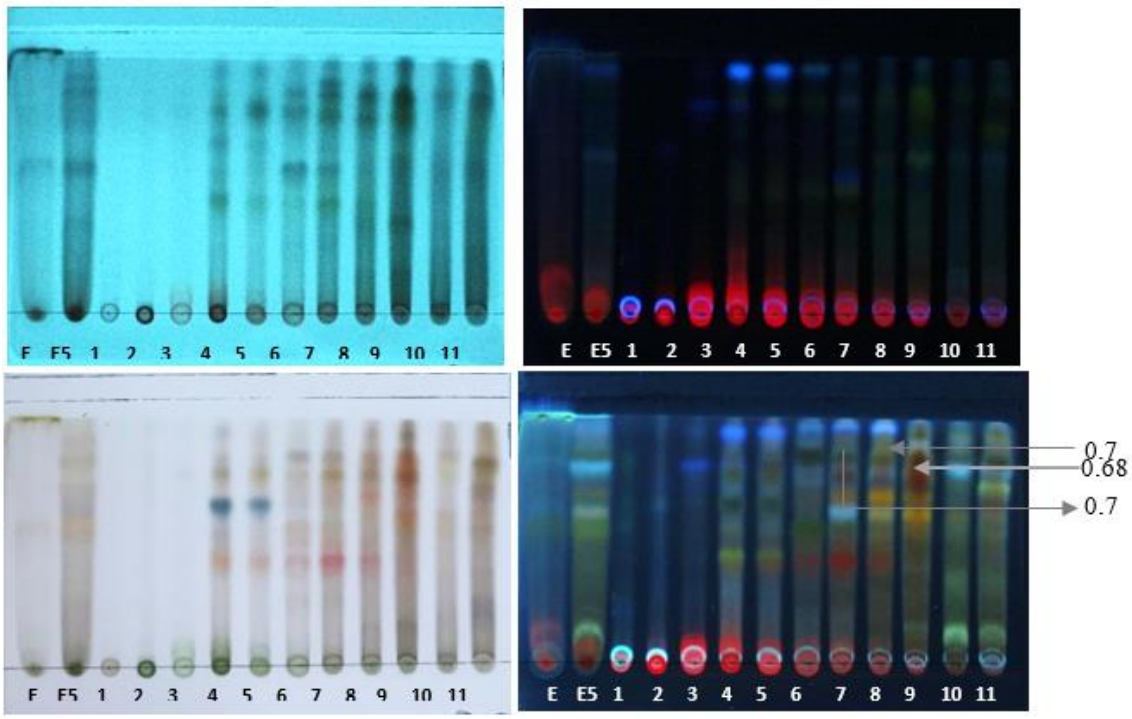

Figure 1. TLC profiles for detecting flavonoids in ethanol leaves of *Artocarpus altilis* using silica gel 60 RP-18 $_{F254}$ as the stationary phase and acetonitrile: water (7:3 v/v) as the mobile phase. The TLC spots were seen in the following conditions: UV 254 nm (a), UV 366 nm (b), white light after being sprayed with 10% H_2SO_4 and heated to 105° C for 5 min (c), and UV 366 nm after being sprayed with 10% H_2SO_4 and heated to 105° C for 5 (d)

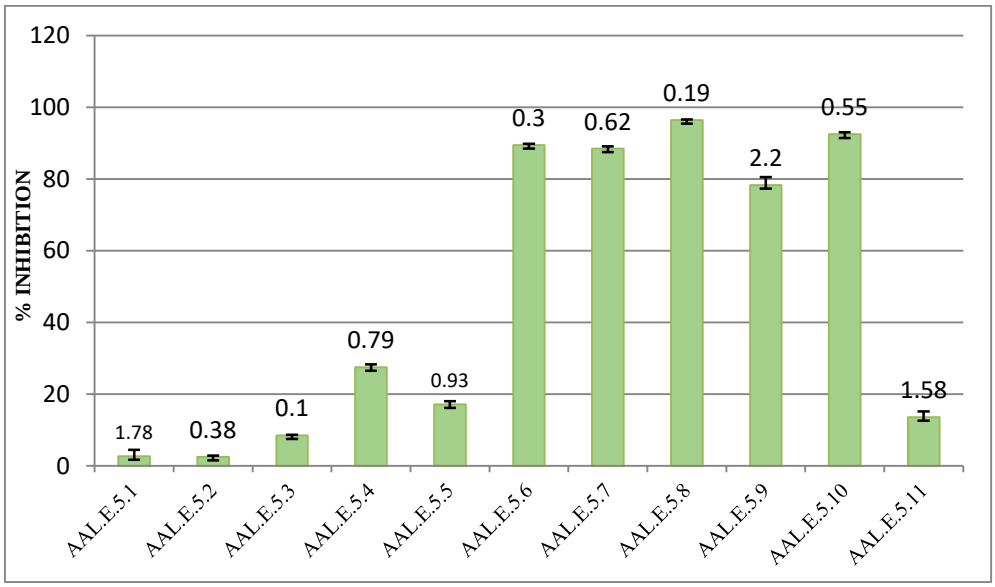

Figure 2. Inhibition of parasite growth by LDH assay at 10 µg/mL from AAL.E.5.1 – AAL.E.5.11 with duplicate measurement

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GraphPad Prism was used to calculate the IC_{50} values for the subfractions from 6 to 10 (AAL.E.5.6 to AAL.E.5.10), which had shown *P. falciparum* inhibitory activities of more than 50% (Table 1). The results showed that five subfractions (AAL.E.5.6 – AAL.E.5.10) were active as antimalarials. The criteria for the antimalarial activity of the extract, according to Basco *et al*. (1994) and Dolabela *et al*. (2008), were as follows: IC_{50} <10 µg/mL was the active extract, IC_{50} 10-50 µg/mL was included in the moderate criteria, IC50 50-100 µg /mL extract had low activity, and IC_{50} >100 µg/mL indicated that the extract was inactive. Subfraction 8 (AAL.E.5.8) has the highest antimalarial activity compared to the other subfractions, with the IC_{50} value of 6.01±0.03 µg/mL. Subfraction 6 (AAL.E.5.6) had an IC_{50} of 6.61 ± 0.03 µg/mL.

Table 1. The IC_{50} Values of subfractions from AAL.E.5.6 – AAL.E.5.10.

Sample	IC_{50} (µg/mL)
AAL.E.5.6	6.61 ± 0.03
AAL.E.5.7	6.97 ± 0.03
AAL.E.5.8	6.01 ± 0.03
AAL.E.5.9	9.39 ± 0.01
AAL.E.5.10	6.80 ± 0.02

Malate quinone oxidoreductase (*Pf*MQO)

All subfractions (AAL.E.5.1 – AAL.E.5.11) were screened for inhibitory potential against *P.falciparum* malate quinone oxidoreductase (*Pf*MQO) at 10 µg/mL (Figure 3).

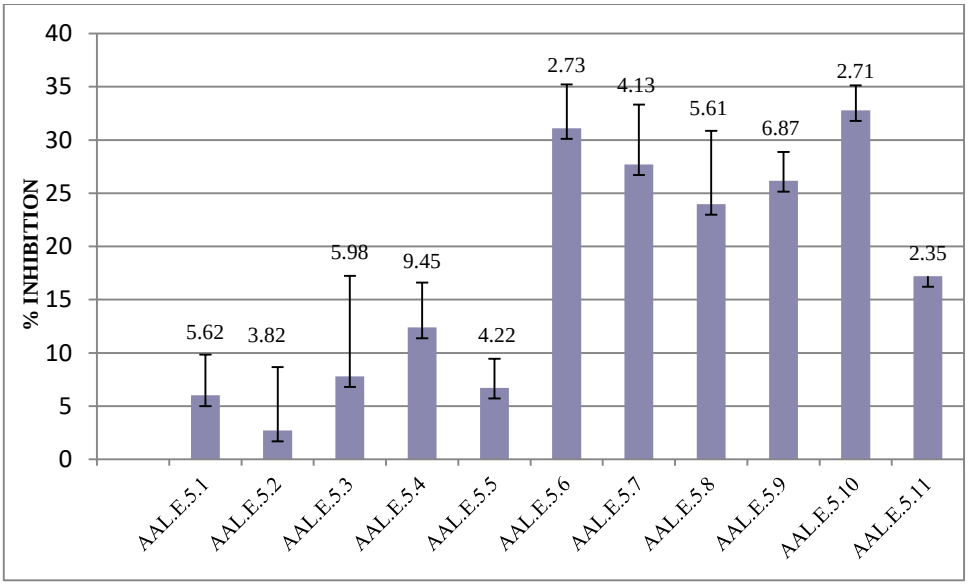

Figure 3. Inhibition of Parasite Growth by *Pf*MQO assay at 10 µg/mL from AAL.E.5.1 – AAL.E.5.11 with duplicate measurement

Therefore, the subfractions with the best inhibition were taken further to determine their IC_{50}. According to the findings, subfractions 6 (AAL.E.5.6) and 10 (AAL.E.5.10) exhibited medium IC_{50} values of 20.34 and 16.35 (g/mL), respectively. (Table 2).

Table 2. The IC_{50} value of AAL.E.5.6 – AAL.E.5.10

Sample	IC_{50} (µg/mL)
AAL.E.5.6	20.34 ± 0.99
AAL.E.5.10	16.35 ± 0.98

It can be claimed that the antimalarial activity was moderate when the IC_{50} was between 10 and 50 µg/mL. Subfraction 6 (AAL.E.5.6) and subfraction 10 (AAL.E.5.10) showed decreased MQO enzyme activity. Subfraction 10 displayed the highest inhibitory activity (AAL.E.5.10). These findings demonstrate that subfractions 6 (AAL.E.5.6) and 10 (AAL.E.5.10) displayed the best *Pf*MQO enzyme inhibition activity compared to the other subfractions because higher inhibition percentages resulted in lower enzyme activity, whereas lower inhibition percentages resulted in higher enzyme activity. The inhibitory process was performed by blocking the activity of *Pf*MQO. Hartuti *et al*. (2018) assumed that chemical ubiquinone binds to succinate dehydrogenase in mammals by inhibiting *Pf*MQO.

Identification of subfraction 6 (AAL.E.5.6)

The UV spectra of AAL.E.5.6 were identified using high-performance liquid chromatography (HPLC). The

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maximum absorption was observed in the UV spectra for AAL.E.5.6, at wavelengths of 215 nm and 272 nm (Picture 4). The U.V. spectra of the majority of flavonoids exhibit two substantial absorption bands: band I in the range of 300 – 400 nm and band II at 240-285 nm (Markham & Mabry, 1975)

The typical absorption peaks of the flavan-3-ols, proanthocyanidins, and dihydrochalcone classes of flavonoid compounds were visible in the UV spectra of the primary peak. While flavanones and dihydroflavonols also have modest shoulders in band I at approximately 320 nm, proanthocyanidins, dihydrochalcones, and flavan-3-ols generally exhibit an absorption maximum in band II between 270 and 290 nm. This is because there is less conjugation between the B ring and other molecules, which results in decreased absorption of the I band (no double bonds other than the B ring). Except for anthocyanins and some aurons, the maximal absorption bands of most flavonoids I and II were below 400 nm. It can be inferred that the UV-VIS spectrum is frequently substituted when the term "UV flavonoids" is used (Santos-Buelga *et al*., 2003; Marston and Hostettman, 2006) (Figure 4).

Identification of subfraction 10 (AAL.E.5.10)

Then, using an HPLC Shimadzu LC-6AD, an analysis was carried out to determine the profile of the chromatogram compounds in subfraction 10 (AAL.E.5.10) using a stationary phase LiChrospher® 100 RP-18 (5 m) analytical column with a concentration of 1 mg/ml, flow rate of 0.5 mL/min, and isocratic solvent mixture of acetonitrile: water (3:7 v/v) (Figure 5). The HPLC chromatogram revealed a strong peak in subfraction 10 at a wavelength of 210 nm. *Pf*MQO is thought to be actively inhibited by this peak, which has a retention duration of 2.4 minutes. Flavonoids, especially dihydrochalcone compound as mentioned above, are said to have a UV spectrum with an absorption maximum at 280 nm (Figure 6 and 7).

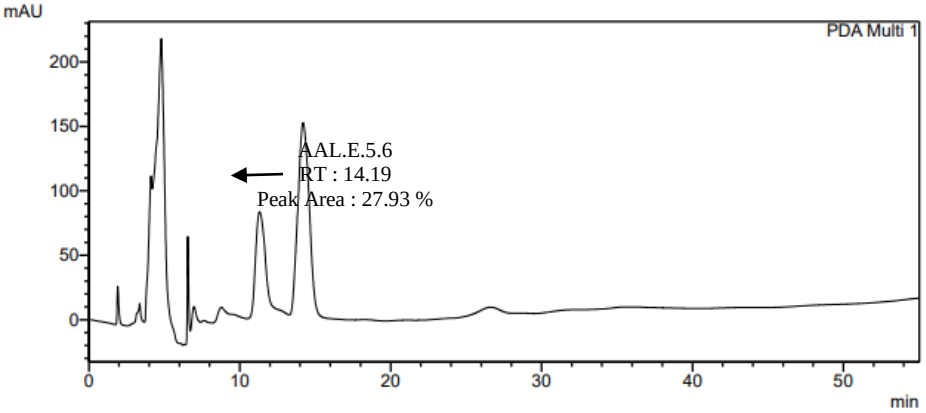

Figure 4. HPLC profile of AAL.E.5.6. The with acetonitrile: water (3:7 v/v) as mobile phase at a flow rate of 0.5 mL/min, injection volume 40 µL and the major peak was observed

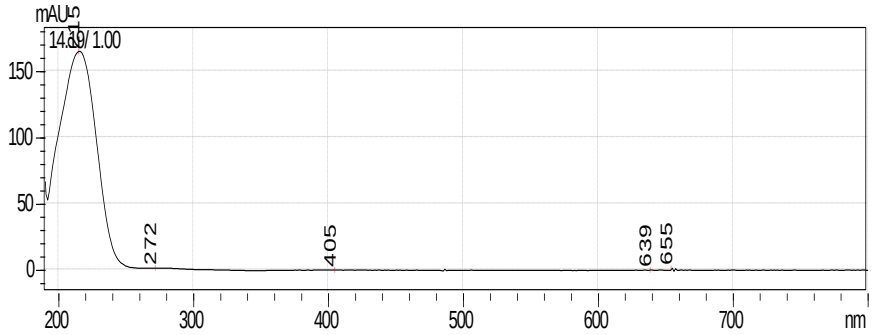

Figure 5. UV-Visible spectra of major peak of AAL.E.5.6

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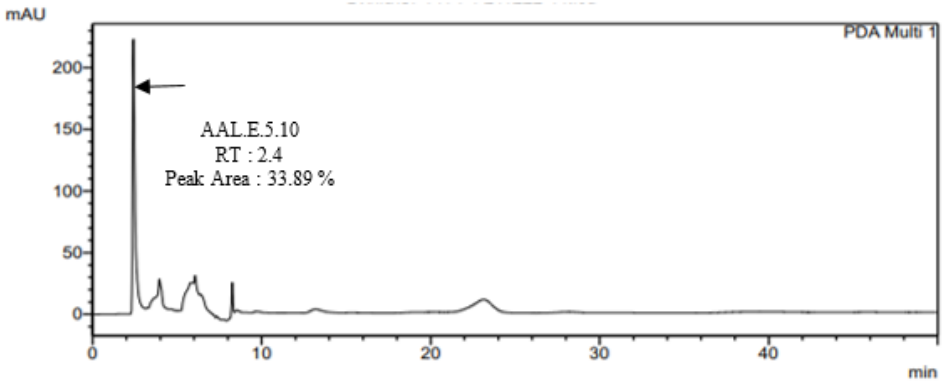

Figure 6. HPLC profile of AAL.E.5.10. The with acetonitrile: water (3:7 v/v) as mobile phase at a flow rate of 0.5 mL/min, injection volume 40 µL and the major peak was observed

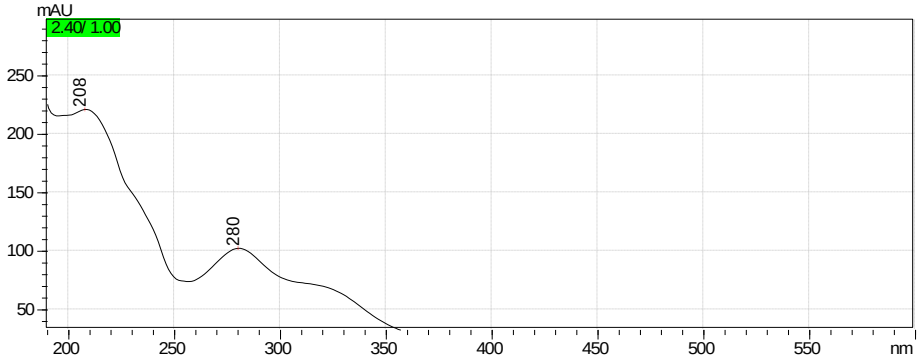

Figure 7. UV-VIS chromatogram of AAL. E.5.10

CONCLUSION

Artocarpus altilis subfraction 8 (AAL.E.5.8) had the most potent antimalarial activity among all the subfractions. However, subfraction 6 (AAL.E.5.6) showed antimalarial efficacy and *Pf*MQO enzyme inhibition. It may be assumed that the antimalarial activity against *P. falciparum* and *Pf*MQO enzyme inhibition may be due to the presence of important plant secondary metabolites, flavonoids. However, further research is needed to confirm this hypothesis. Flavonoid compounds in AAL.E.5.6 that were detected by yellow spots on TLC and UV profiles at a wavelength of 272 nm on HPLC are encouraged to proceed further for isolation to develop pure compounds that can subsequently serve as antimalarials.

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AUTHOR CONTRIBUTIONS

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

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Using Simulated Patients to Understand Non-Prescription Antibiotic Dispensing in Indonesia: A Systematic Review

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Abstract

***Background**: Dispensing antibiotics without a prescription at community pharmacies is a significant contributor to the ongoing global public health issue of antibiotic resistance. **Objectives**: To estimate the proportion of antibiotics that are dispensed without a prescription in community pharmacies in various Indonesian cities. **Methods**: A literature review was conducted via PubMed, Science Direct, Google Scholar, Garuda, and Neliti for articles published between January 2007 and December 2022 combined with Boolean operators. The literature search keywords were (simulated patientsOR mystery shopper OR sample patients OR dummy patients) AND ("antibiotics without prescription OR non-prescription antibiotics OR self-medication of antibiotics). The keywords are also used in Indonesian language (Bahasa), including "simulasi pasien" OR "sampel pasien" AND "antibiotik tanpa resep" OR "swamedikasi antibiotik". **Results**: Seven studies from various cities have complied with the inclusion criteria and were considered when reviewing 199 articles. The findings of our studies were consistent with the extensive use of non-prescription antibiotics throughout the review. A simulation patient study design was used in all seven studies in this review. Amoxicillin recorded the highest percentage of dispensing without a prescription, while other drugs often purchased include chloramphenicol, ciprofloxacin, and cefadroxil. Among the studies reviewed, one study utilized the pre-test and post-test methods, while the others did not. **Conclusion**: The lack of prescriptions for antibiotics dispensing has often occurred in community pharmacies throughout Indonesia. The community pharmacist's role is needed as the final gate of pharmaceutical services in providing rational treatment and controlling the dispense of antibiotics without a prescription.*

***Keywords**: antibiotics, mystery shopper, self-medication, simulated patients*

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INTRODUCTION

The effects of antibiotic resistance on morbidity, mortality, and healthcare expenditure illustrate that it is one of the world's most pressing public health issues. Low- and middle-income countries (LMICs) are particularly at risk due to the increasing use of antibiotics in their populations (Klein *et al*., 2018). Community pharmacies and drug stores, which are part of the private drug retail sector, are often the first point of care for minor illnesses in many LMICs because they are close by, trustworthy, easily accessible, and provide essential medicines, such as antibiotics, often without a prescription (Miller & Goodman, 2016; Suy *et al*., 2019). Antibiotics are readily accessible in urban areas, and people can obtain them from pharmacies and drugstores without the need for prescriptions, even though this practice is against national regulations. This stems from the term """""""""""""""antibiotic therapy" """" "" """", “" where ' 'it's believed that antibiotics could cure many common illnesses such as diarrhea, flu, and many others (Wulandari *et al*., 2021).

The World Health Organization (WHO) has warned that LMICs misuse between 20% and 50% of antibiotics. According to previous studies, more than two-thirds of the antibiotic sales in the pharmaceutical industry in LMICs are attributed to self-medication (Torres *et al*., 2019). Indonesia and the rest of Southeast Asia are at the epicenter of a global crisis regarding the emergence and spread of antimicrobial resistance (AMR) due to their large populations, rapid but unequal economic development, weak health systems with widely varying access to quality healthcare, high rates of infectious diseases, and lax enforcement of antibiotic policies (Limato *et al*., 2022).

There are 3,042 hospitals in Indonesia in 2021, 10,292 primary care clinics, 30,199 pharmacies, and an estimated 9,752 retail pharmacy stores in the community providing OTC medications. In Indonesia, community pharmacies may stand as independent institutions, be part of a larger chain, be connected to a clinic, or be part of both. Antibiotics require prescriptions and may only be supplied by a registered pharmacist. Pharmacies are not allowed to sell antibiotics without prescriptions. In addition, a licensed pharmacist or pharmacy technician must always be present in retail drug stores to monitor the medication distribution. Despite these regulations, research has shown that antibiotics can be purchased through community pharmacies without a prescription or with only a few initial assessments (Hadi *et al*., 2010; Limato *et al*., 2022; Puspitasari *et al*., 2011).

Extrapolating data from various nations may prove difficult as factors leading to antibiotic misbehavior may vary spatially, owing to human behavior, health literacy, economy, and legislation. Several initiatives have been implemented by governments and nonprofit organizations worldwide to decrease the improper distribution of antibiotics (Langford *et al*., 2021). Controlling antimicrobial resistance (AMR) in public hospitals, including monitoring antibiotic prescriptions, is one of the measures implemented by the Indonesian government to reduce inappropriate antibiotic use. Additionally, it is crucial to monitor policies to ensure that they have the desired effect (Godman *et al*., 2021).

The simulated patient technique has been used to increase the conformity of policy applications with real-world societal conditions (Soltani *et al*., 2020). This procedure is widely used to evaluate several aspects of pharmaceutical practice. Simulated patients are used to assess the quality of service, discover how customers react to cognitive benefits, and determine if pharmacy employees adopt training programs in an era when these services are increasingly being established alongside traditional dispensing and sales (Björnsdottir *et al*., 2020). This is comparable to self-report methods that allow participants to provide socially desirable responses (Green & Norris, 2015).

Several studies have described the use of simulated patients to understand the dispensing of non-prescription antibiotics in Indonesia. There is a scarcity of systematic reviews on this topic. To address this gap, this study proposes the use of a systematic review to provide a complementary perspective to conclusions from non-prescribed antibiotics.

METHODS

Search strategy

Guidelines for reporting systematic reviews and meta-analyses (PRISMA) 2020 were followed for this systematic review (Page *et al*., 2021). Articles were sourced from three international databases (PubMed, ScienceDirect, and Google Scholar). In addition, two Indonesian databases (Garuda [Garba Rujukan Digital] and Neliti) were used to find the original articles. The use of Boolean operators (AND/OR) in the literature search yielded the following combinations of terms """""""""""""("simulated patients" "" "" "" "" "" OR """""""""""""""mystery shopper" " " " " " " " " "" OR """""""""""""""sample patients" """" """" """"" OR """""""""""""""dummy patients"" """" "" "" "") AND """""""""""""("antibiotics without prescription" " " " " " " " " "" OR """""""""""""""non-prescription antibiotics" """" """"

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"""" OR """""""""""""""self-medication of antibiotics" " " " " " " " """"). The keywords are also used in Indonesian language (Bahasa), including "simulasi pasien" OR "sampel pasien" AND "antibiotik tanpa resep" OR "swamedikasi antibiotik."

Selection criteria

This study evaluated whether each included article was appropriate to the selected criteria for the main research question, """""""""""""""How is non-prescribed use of antibiotics in Indonesia"""""""""""""""?". The study implemented the following inclusion criteria: i) research published over the last 15 years (2007-2022); ii) research studies that presented findings from primary research; iii) full-text articles that were published in the five databases mentioned above; iv) research locations in the article were carried out in Indonesia; and v) focus on using simulated patients to illustrate the dispensing of antibiotics without a prescription. The decision to focus the literature search more narrowly on several regions in Indonesia to concentrate the search more specifically on one country. Moreover, each nation may have different policies; hence, drawing comparisons between regions in several countries will bias the findings of the review. The authors crosscheck each target database search and then confirm one another to reduce bias in selecting articles in one area.

Screening and eligibility assessment

In this study, steps were taken under the established criteria. After identifying all articles that met the criteria, duplicates were removed. Subsequently, the abstracts and keywords relevant to the study were chosen. Article eligibility was determined using inclusion criteria. Afterwards, it is not only the abstracts and keywords that are read, but also all parts of the article (Patton, 2014). A content analysis of the most relevant data was performed to provide a more in-depth view of the review process and address research questions on dispensing antibiotics without prescriptions in Indonesia (Table 1).

Data extraction

The studies included 199 research articles from five databases. After removing 15 duplicate articles, 184 were screened based on their relevance to the titles and abstracts. Finally, 159 articles were excluded. Consequently, only the 25 remaining full-text articles were assessed for eligibility. Therefore, of the remaining 25 studies, 18 were excluded because they did not meet the inclusion criteria, and seven studies were included in the final systematic review. This process is illustrated in the PRISMA flowchart in Figure 1.

Figure 1. PRISMA diagram outlining the screening and selection process in literature search

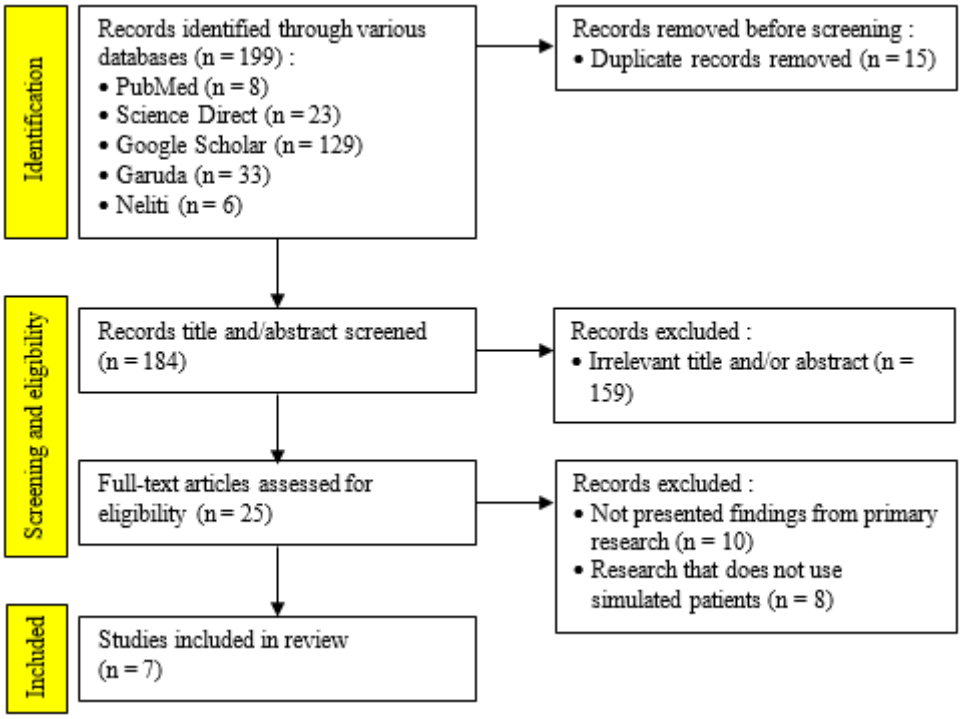

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Table 1. Details of eligible studies

Authors (years)	Study purpose	Study design and location	Number of pharmacies involved	Number of pharmacies dispensed without prescription (%)	Symptoms of antibiotics dispensed without a prescription
Hadi *et al*., 2010	To assess the potential connection between resistance and substandard antibiotics sold in the area	Simulated patients visited pharmacies in Surabaya to request specific antibiotics product	104	79 (76%)	• Amoxicillin (n = 15) • Chloramphenicol (n = 18) • Ciprofloxacin (n = 14) • Cotrimoxazole (n = 14) • Tetracycline 250 mg (n = 15) • Tetracycline 500 mg (n = 2)
Puspitasari *et al*., 2011	To assess antibiotic sales without prescription and provision of drug information services	Simulated patients visited pharmacies in Surabaya by the three scenarios for each pharmacy	88	80 (91%)	• Ciprofloxacin (n = 80, 91%) • Tetracycline (n = 80, 91%) • Amoxicillin (n = 74, 84%)
Brata et al., 2019	To ascertain the appropriate advice given in response to the antibiotic self-medication	Simulated patients test was conducted at community pharmacies in an eastern Indonesian provincial capital	80	36 (45%)	Any antibiotics for diarrhoea
Saibi et al., 2020	To assess the sale of antibiotics without a prescription and drug information services	Simulated patients collected data randomly in pharmacies located in South Tangerang	100	49 (49%)	Cefadroxil for diarrhea
Simamora et al., 2020	To assess antibiotic sales without a prescription	Simulated patients visited pharmacies in the district of Plaju, Kertapati, Jakabaring, and Sungai Musi (South Sumatera)	17	17 (100%)	Random types of antibiotics • Amoxicillin (n = 9) • Cefadroxil (n = 2) • Chloramphenicol (n = 1) • Ciprofloxacin (n = 2) • Cotrimoxazole (n = 3) • Erythromycin (n = 3)
Listyorini et al., 2021	To investigate the actions taken by pharmacists	Simulated patients visited pharmacies in 3 districts located in Tangerang	55	49 (89%)	Random types of antibiotics by showing the old empty strip

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	on requests for antibiotics without prescription and drug information services				
Wulandari *et al*., 2021	To determine the magnitude of inappropriate dispensing of antibiotics by drug outlets in urban and rural settings	Simulated patients portrayed three different clinical case types to bought antibiotics without a prescription from pharmacies and drug outlets located in Bekasi (an urban area in West Java Province) and Tabalong district (a rural area in South Kalimantan Province)	362	275 (76%)	The clinical cases included diarrhea, tuberculosis, and upper respiratory tract infection. • Amoxicillin (n = 152) • Trimethoprim (n = 45) • Fradiomycin/Gramicidin (n = 39) • Cefadroxil (n = 35) • Ciprofloxacin (n = 17) • Cefixime (n = 15) • Nifuroxazide (n = 10) • Ampicillin (n = 5) • Others (n = 29)

RESULTS AND DISCUSSION

A total of 199 studies were identified by searching various databases, including PubMed, ScienceDirect, Google Scholar, Garuda, and Neliti. Among these, seven were included in the in-depth content analysis (Table 1). This in-depth review helped enhance the accuracy and reliability of the findings, providing valuable insights into the trends and patterns of non-prescribing antibiotic usage in Indonesia through simulated patients.

Study characteristics

All seven studies included in the review on dispensing antibiotics without a prescription from Indonesian community pharmacies used a simulated patient study design. Two studies were reported from Surabaya (Hadi *et al*., 2010; Puspitasari *et al*., 2011), one study from Tangerang (Listyorini *et al*., 2021), South Tangerang (Saibi *et al*., 2020), and eastern Indonesian provincial capital (Brata *et al*., 2019). Moreover, one study was conducted in four districts in Palembang (Simamora *et al*., 2020), and one study was conducted in urban and rural areas, namely Bekasi City and Tabalong District (Wulandari *et al*., 2021). The purpose of these studies was to investigate the actions taken by community pharmacists on requests for antibiotics without prescriptions. In addition, several studies have sought to assess pharmacists' provision of drug information services for dispensing antibiotics without a prescription (Listyorini *et al*., 2021; Puspitasari *et al*., 2011; Saibi *et al*., 2020).

The sample size in each study differed based on the number of pharmacies in the study area. Most samples were obtained from research conducted in two cities (Bekasi and Tabalong) with 362 pharmacies, whereas the least sample was obtained from a study in Palembang, with only 17 pharmacies involved. In six studies, ethical approval from the relevant institutions was documented (Brata *et al*., 2019; Hadi *et al*., 2010; Listyorini *et al*., 2021; Puspitasari *et al*., 2011; Saibi *et al*., 2020; Wulandari *et al*., 2021), while one did not include a letter of ethical approval (Simamora *et al*., 2020). One study applied interaction with audio recordings to minimize bias in the study *(Wulandari et al*., 2021), and the remaining studies utilized a data sheet (check-list) to record information such as the purchase date, name and address of the pharmacies, the antibiotic's name (and whether it was obtained with or without a prescription), the antibiotic's storage conditions, the packaging type, information correctness during antibiotic dispensing, and price.

Types of Simulated patients scenarios

Multiple types of antibiotic requests, including those based on symptoms and specific products, were employed in these analyses. The simulated patients were well prepared to present their cases to pharmacists

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before their in-person visits. Five studies relied solely on direct requests using a product name (Hadi *et al*., 2010; Listyorini *et al*., 2021; Puspitasari *et al*., 2011; Saibi *et al*., 2020; Simamora *et al*., 2020), one study used an old empty package of antibiotics to be purchased (Listyorini *et al*., 2021), and one study reported that family members entrusted the antibiotics purchased (Saibi *et al*., 2020). Two other studies involved symptom-based requests, specifically employing instances of diarrhea scenarios (Brata et al., 2019), and diverse medical cases, including tuberculosis, diarrhea, and upper respiratory tract infection (Wulandari *et al*., 2021).

Six studies reported training for simulated patients before data collection, while one did not mention training for simulated patients (Simamora *et al*., 2020). Most studies have reported that each pharmacy was visited once by simulated patients. However, there are also studies in which pharmacies were visited more than once. For instance, to lessen the likelihood that the same staff member would attend to all three simulated patients, they spread their visits to the pharmacy for one–three days at different times of the day. They presented them with three clinical scenarios (diarrhea, tuberculosis, and upper respiratory tract infections) (Wulandari *et al*., 2021). Another study was designed with three scenarios for each pharmacy that were visited by three simulated patients during business hours for each of the three scenarios (ciprofloxacin, tetracycline, and amoxicillin) at different times (Puspitasari *et al*., 2011).

Significant findings: inappropriate dispensing practices

Table 1 displays the names and percentages of antibiotics prescribed in response to patient requests. Amoxicillin had the highest dispensing rates without prescription across all seven studies (Hadi *et al*., 2010; Simamora *et al*., 2020; Wulandari *et al*., 2021). Chloramphenicol, ciprofloxacin, and cefadroxil were also purchased without a prescription. Another study found that amoxicillin, the active ingredient in most over-the-counter antibiotics (86.9%), was analyzed in 61 studies (Batista et al., 2020).

Our research supports the hypothesis that non-prescription antibiotics are widely used in Indonesia. Research conducted in the districts of Plaju, Kertapati, Jakabaring, and Sungai Musi (South Sumatra Province) indicates that among the 17 pharmacies visited, each dispensed antibiotics without requiring a prescription (Simamora *et al*., 2020). Two studies conducted in Surabaya within a year reported similar results, with 79 and 80 pharmacies dispensing antibiotics without prescriptions, respectively (Hadi *et al*., 2010; Puspitasari *et al*., 2011). Another study performed at pharmacies in the provincial capital of eastern Indonesia found that 36 of 80 pharmacies surveyed provided antibiotics without a prescription (Brata *et al*., 2019). Moreover, research in Tangerang showed that as many as 89% of pharmacy staff dispensed antibiotics without a prescription to simulated patients. However, simulated patients did not identify in more detail whether the staff were pharmacy technicians or others (Listyorini *et al*., 2021). Interestingly, one of the simulated patients received an antibiotic that was not properly labelled and was packaged in a plastic bag (Wulandari *et al*., 2021).

Diarrhea and respiratory issues, including tuberculosis, flu, cold, and upper respiratory tract infection, were the most prevalent reasons why antibiotics were administered without a prescription. Similarly, another study found that sore throat, fever, and respiratory disorders, including cold/flu and cough, were the most common conditions in which people used antibiotics without a doctor's prescription *(Alhomoud et al*., 2017).

Among the seven studies reviewed, one study used pre- and post-test methods. After observing the pre-test with the simulated patients, the pharmacist was educated. The impact of this education can be seen when conducting post-test observations: some pharmacies no longer sell antibiotics without a prescription (Simamora *et al*., 2020). This approach is more suitable for future research if similar research is conducted. Although it requires a longer time, it could yield a more positive impact in regulating the dispensing of antibiotics without a doctor's prescription.

There are several reasons that may contribute to the dispensing of antibiotics without prescriptions. The most probable cause is the lax approach to law enforcement (Zawahir *et al*., 2019). Additionally, insufficient or inefficient supervision by the Food and Drug Authority in Indonesia (BPOM) conducts inspections of pharmacies to guarantee proper pharmacy practices. Another factor is that non-licensed pharmacy personnel lack knowledge of antibiotic dispensing regulations. These field studies showed that, despite existing regulations, pharmacies in several regions of Indonesia still dispense antibiotics without a prescription. These antibiotics are available in all community pharmacies and are easy to purchase. A common concern among community pharmacists is that refusing a customer's demand for antibiotics could

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adversely affect their pharmacy business. They also think that if they do not give antibiotics, the customer will seek them elsewhere (Wulandari *et al*., 2021). This is among the reasons why pharmacists ultimately dispensed antibiotics without prescription.

Many prior studies have analyzed this issue. Most of those who used antibiotics for self-treatment lived in Asia (51 of 89 articles) (Batista et al., 2020). Another review found that poor national medicine regulations, shortage of qualified pharmacists, commercial pressure on pharmacy staff, consumer demand, inappropriate prescribing practices, and lack of awareness of antimicrobial resistance all play a role in the sale of antimicrobials that do not require a prescription (Sakeena *et al*., 2018). A review of 59 articles from LMICs showed that 83.3% dispense antibiotics without a prescription (Batista et al., 2020). Another study found that antibiotics dispensed without prescription had a reasonably high proportion in South America (almost 78%) (Auta *et al*., 2019).

Research impacts

This systematic review revealed that the number of dispenses of antibiotics without a prescription in Indonesia remains high, particularly in regions with low levels of supervision from related government authorities. Hopefully, this review will lead the government to take stricter actions to ensure that antibiotics are not dispensed without prescription throughout Indonesia. In line with this, studies in China show that the government has striven to curtail non-prescription antibiotic dispensing (NPAD) in the past decades through stricter policies (Wang *et al*., 2020). Moreover, this research would also impact community pharmacists, who are required to inform the public about the risk of antibiotic resistance. There is an urgent need for community pharmacists and health workers to enhance public awareness of microbial resistance resulting from antibiotic misuse of antibiotics (Halboup *et al*., 2020).

This research is the initial step to provide a glimpse into some actual conditions that exist in Indonesia with regard to unprescribed antibiotics. To become a concern for policymakers in the formulation of effective policies, research needs to be more extensive and cover a wide range of areas in Indonesia. Other systematic reviews conducted in one country did not use the patient simulation method. For example, a review in Saudi Arabia, which was only concerned with the prevalence of antibiotic misuse in several cities, revealed a considerably high prevalence among the Saudi population (Alnemri *et al*., 2016). Alternatively, a review conducted in China aimed to estimate the prevalence of the general population's irrational use of antibiotics, including incorrect antibiotic recognition, inappropriate usage, and ignorance of potential adverse outcomes (Duan et al., 2021).

Strengths, limitations, and recommendations

This is the first systematic study to illustrate the availability of antibiotics in Indonesian community pharmacies without a doctor prescription. Owing to variations in scenario types, pharmacy counts, and environmental conditions, a unified statistical analysis was not possible. Therefore, this evaluation can only provide a descriptive analysis of the dispensing of antibiotics for self-medication in different locations. We suggest that researchers conduct studies on a broader scale in Indonesia. Thus, it can describe the conditions of the community and serve as a reference for policymakers to determine the next steps.

CONCLUSION

Based on a literature review, this study concludes that antibiotics are available without a prescription in community pharmacies in many Indonesian cities. No one can do more than community pharmacists to provide appropriate therapy and regulate over-the-counter dispensing of antibiotics. Furthermore, the government's active role in strengthening regulations and supervisory measures and driving the obligation for pharmacists to guarantee the safety of drug services according to their authority and carry out audits to ensure that these services are urgently needed to overcome this.

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AUTHOR CONTRIBUTIONS

Conceptualization, F. M., A. W. W.; Methodology, F. M., A. W. W.; Validation, A. W. W.; Formal Analysis, F. M.; Investigation, A. W. W.; Resources, F. M.,; Data Curation, F. M.,; Writing - Original Draft, F. M.,; Writing - Review & Editing, A. W. W.; Visualization, F. M., A. W. W.; Supervision, A. W. W.; Project Administration, F. M.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Risk Factor Analysis of Adverse Effects of Kanamycin and Capreomycin on Kidney Function in Multidrug-Resistant TB Patients

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Abstract

***Background**: Multidrug-Resistant TB treatment's high side effects and long duration are barriers to successful TB therapy. Various side effects such as age, gender, body weight, comorbidities, and drug dose can cause severe side effects, including impaired renal function (nephrotoxic).* ***Objectives**: This study aimed to analyze the risk factors of side effects of the failure of kanamycin and capreomycin therapy that can cause impaired renal function in Multidrug-Resistant TB patients.* ***Methods**: Data were collected retrospectively by searching and recording the medical records of Multidrug-Resistant TB patients at the Multidrug-Resistant TB Polyclinic. There were 183 patients at Dr Soetomo Hospital who met the inclusion criteria.* ***Results**: There was a significant relationship between gender in the kanamycin group and the appearance of side effects of renal impairment (p= 0.035). There was no effect of age, comorbid diseases, body weight, and dose of drug administration on the side effects of kanamycin and capreomycin in treating Multidrug-Resistant TB on impaired renal function (nephrotoxic). However, nephrotoxic side effects in elderly patients were more common in the kanamycin group (p=0.001).* ***Conclusion**: Gender affects the side effects of kanamycin and capreomycin in treating Multidrug-Resistant TB in nephrotoxic patients. In addition, stricter supervision of the use of kanamycin in elderly patients (>40 years) to minimize the incidence of side effects of impaired renal function in the treatment of Multidrug-Resistant TB.*

***Keywords**: capreomycin, kanamycin, multidrug-resistant TB, nephrotoxic*

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INTRODUCTION

The high incidence of tuberculosis (TB) and multidrug-resistant Tuberculosis (MDR-TB) in Indonesia deserves special attention. The high incidence of side effects and long duration of Multidrug-Resistant TB treatment are significant obstacles to successful Multidrug-Resistant TB therapy. Drug side effects are among the leading causes of treatment failure; therefore, special attention must be paid to the occurrence of side effects in TB treatment. The World Health Organization (WHO) recommends second-line injectable drugs for the management of Multidrug-Resistant TB cases, including the aminoglycoside class consisting of streptomycin, kanamycin, amikacin, and capreomycin polypeptide class (Ratnawati *et al*., 2018). However, some drugs can cause severe side effects such as ototoxicity, electrolyte disturbances (hypokalemia, hypomagnesemia, hypocalcemia), and impaired renal function (nephrotoxicity) (Heysell *et al*., 2018). Some problems associated with the high incidence of adverse drug events can be influenced by factors such as age, sex, weight, comorbidities, and the dose of drugs administered (Tamirat *et al*., 2020).

Drug-resistant TB continues to be a public health concern. Globally, Multidrug-Resistant or Rifampicin Resistant TB (MDR/RR TB) accounts for 3.4% of new TB cases and 18% of previously treated cases, with the highest proportion (>50% of previously treated TB cases) in the Soviet Union (WHO, 2019). In Indonesia, in 2018, there were an estimated 845,000 total TB cases with MDR/RR TB cases in Indonesia, totalling 24,000 cases. An estimated 2.4% of resistant TB cases were new cases, and as many as 13% were previously treated resistant TB cases (WHO, 2019). Several researchers have reported a success rate of Multidrug-Resistant TB treatment of only 52%, consisting of 37.2% cured and 14.5% complete treatment. This can lead to high treatment failure rates. Data from the East Java Provincial Health Office in 2018 showed that the number of TB cases reached 229,961. While the data on the most TB cases in East Java Province is Surabaya City, which is 3990 cases, and the TB mortality rate in Surabaya City is estimated to reach 10,108 BTA-positive patients, namely 3990 cases (Dinkes Jatim, 2018).

Kanamycin is an aminoglycoside antibiotic with side effects on the kidneys because it contains a cationic group (Mamlouk *et al*., 2019). Capreomycin is a polypeptide antibiotic with a structure similar to that of the aminoglycoside group; therefore, it can be used as a second choice if the patient experiences adverse drug effects on the use of kanamycin or if there are contraindications to kanamycin. Both drugs are second-line or group B injectable drugs for the classification of Multidrug-resistant TB drugs (WHO, 2016). Neither drug can be absorbed orally, making it injectable. Side effects of kanamycin are common. The occurrence of nephrotoxicity is an effect of the administration of kanamycin caused by toxicity to the renal tubules, decreased glomerular filtration, and decreased blood flow to the kidneys, leading to impairment of renal function characterized by non-oliguria, hypoosmotic polyuria, increased serum urea and creatinine, and as many as 13% of cases of resistant TB that have been treated before (Kwiatkowska *et al*., 2021).

Previous studies have shown that kanamycin and capreomycin have side effects on kidney function and electrolytes. Research conducted by Arto *et al* (2017) through collecting patient medical record data from 2012-2017 revealed that after the first month of Multidrug-Resistant TB treatment, there was a significant decrease in mean serum potassium (4.0 + 0.4 mEq/L to 3.7 + 0.5 mEq/L) in patients using kanamycin and (4.1 + 0.5 mEq/L to 3.2 + 0.6 mEq/L) in patients receiving capreomycin therapy (Soeroto *et al*., 2019). Based on research by Ratnawati *et al.* (2018), using kanamycin in patients with MDR-TB caused functional disorders in 147 patients (61.8%). In addition, nephrotoxicity also occurred in 9.8% of patients at 4.8 months of MDR-TB treatment (Mwansasu *et al*., 2017). Kanamycin and capreomycin are used in Multidrug-Resistant TB treatment at Dr. Soetomo Hospital. The long-term use of these drugs requires regular monitoring of renal function and serum electrolyte levels. The number of possible risk factors that can affect kidney function during treatment will also be a separate consideration. Therefore, researchers are interested in examining the risk factors for the side effects of kanamycin and capreomycin on kidney function in patients with multidrug resistance at Dr. Soetomo Hospital.

MATERIALS AND METHODS

Study design

This was a retrospective observational research, specifically tracking and recording data on patients with Multidrug-Resistant Tuberculosis who received treatment at Dr. Soetomo Hospital between January 2018 and June 2020. The inclusion criteria in this study were adult Multidrug-Resistant TB patients (aged >18 years), including those who were pregnant, dropped out of treatment, died, transferred treatment to Public

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Health, received short-term and individualized regimens, and had complete laboratory examination data. The exclusion criteria included patients with a history of kidney disease and thyroid disorders, undergoing hemodialysis, NSAIDs, ACEIs, and ARBs, and interactions with other drugs that affected serum electrolytes.

Method of collecting data

Data were collected by searching and recording the medical records of Multidrug-Resistant TB patients at the Multidrug-Resistant TB Polyclinic Dr. Soetomo Hospital from January 2018 to June 2018, who met the inclusion criteria for data analysis. The instruments used in this study included patient medical records containing patient observational record sheets that recorded the patient's clinical condition, laboratory examination result sheets, and data collection sheets. Data recorded in the data collection sheet included medical record number, demographic data, doctor's diagnosis, disease history and drug history, laboratory data, and supporting clinical data such as regimens obtained, gene expert results, and side effects of drugs.

Data analysis

Analysis of the influence of risk factors of sex, age, weight, presence or absence of comorbidities (Diabetes Mellitus, Hypertension, and Hepatitis B), and drug dose on the adverse effects of renal impairment was performed using binary logistic regression statistical test. If the data were normally distributed, the chi-square statistical test was used to compare the effects of kanamycin and capreomycin on adverse effects, such as impaired renal function and electrolyte disturbances. If the data were not normally distributed, an independent t-test was used.

Ethical approval

This study was approved by the Health Research Ethics Committee of Dr. Soetomo Hospital (number 0105/LOE/301.4.2/VIII/2020).

RESULTS AND DISCUSSION

Demographic data

Many factors can influence adverse drug events, including patient-derived factors, such as sex, age, comorbidities, race, and genetic polymorphism (Tamirat *et al*., 2020). Clinical examination data and supporting examinations written in the patients' medical records were used to determine drug side effects. The only drug side effect observed was renal function (nephrotoxicity). The term nephrotoxic refers to a condition in which serum creatinine levels rise by more than 0.3 mg/dL (1.5-2 times) from baseline (Kemenkes RI, 2019).

The number of patients obtained in the medical record was 183 and included as many as 112 patients in the study inclusion criteria: 63 received kanamycin, and 43 received capreomycin in their therapy regimen. The results of the demographic status assessment are presented in Table 1. Most patients were male (56.25%, n=63), while 43.75% (n=49) were female. Males are more susceptible to Multidrug-Resistant TB due to their heavy workload, irregular rest, and unhealthy lifestyles, such as smoking and drinking alcohol (Nugroho *et al*., 2018). Most patients were 41-60 years old, which is included in the elderly age category. In this age range, a person tends to have high mobility, so the risk of exposure to TB germs is high. The high comorbidity of diabetes mellitus in patients with Multidrug-Resistant TB is because diabetes mellitus is a risk factor for tuberculosis. Patients with diabetes mellitus have impaired immune responses that facilitate the multiplication of Mycobacterium tuberculosis and cause pulmonary tuberculosis (TB). Patients with diabetes mellitus have a 2-3 times risk of developing pulmonary TB disease than people without diabetes mellitus and are mostly found at the age of over 40 years (Wijaya, 2015). Most patients received short-term regimens based on therapy regimens. Regarding the initial status of patients, most Multidrug-Resistant TB patients are new relapse patients; this occurs due to non-compliance with treatment.

Adverse Effects of Kanamycin and Capreomycin in Multidrug-Resistant TB

Kanamycin causes the loss of potassium and magnesium by a mechanism similar to that of diuretic drugs, blocking the chloride pathway associated with sodium reabsorption and inhibiting transport in protein membranes. This increases the sodium in the collecting tubules, which is exchanged for potassium (Suparyatmo *et al*., 2014). Capreomycin causes hypokalemia through a mechanism similar to that of kanamycin. Kanamycin and capreomycin can induce electrolyte balance through the stimulation of calcium-sensing receptors (CaSR) on the thick ascending branch of the arch of Henle (Penn-Nicholson *et al*., 2022).

Kanamycin is an aminoglycoside antibiotic with nephrotoxic side effects of which is nephrotoxic (Penn-Nicholson *et al*., 2022). This antibiotic causes nephrotoxicity because it has a cationic amino group, which causes the drug to accumulate in the proximal tubules (Purnasari *et al*., 2019). Nephrotoxic side effects were defined as elevated creatinine levels, classified as grade 1 if an increase of >0.3 mg/ml or 1.5-2x above baseline (Kemenkes RI, 2019). Capreomycin has a

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longer t1/2 than kanamycin, and the interval of drug administration in patients with both drugs was the same; therefore, the blood levels of capreomycin were higher than the blood levels of kanamycin. This leads to saturation of drug levels in the proximal tubules, which is an important factor in nephrotoxicity. The nephrotoxic properties of aminoglycosides are determined by the number of amine groups in their structure. Kanamycin has 4 amine groups, while capreomycin has five protonated amine groups and nine unprotonated amine groups; therefore, there are differences in the affinity of kanamycin and capreomycin to cell membrane phospholipids (Molitoris, 2017).

Table 1. Demographic data of multidrug-resistant TB patients at Dr Soetomo Hospital.

Patient Characteristics	Frequency	Percentage
Gender		
Male	63	56.25
Female	49	43.75
Aged		
< 20 years	9	8.04
21-40 years	38	33.93
41-60 years	56	50
> 60 years	9	8.04
Comorbid		
DM	42	37.5
DM + HT	6	5.36
HT	4	3.57
Hepatitis B chronic	1	0.89
No comorbid	59	52.68
Smoking and alcoholic status		
Smoking	40	35.71
alcoholic	6	5.36
Characteristic Patient		
Regimen obtained		
Short term regimen	82	73.21
Regimen Individual	10	8.93
STR moved to individual	20	17.86
Reasons for switching regimens		
Resistant	7	
Drug Adverse Effects	10	
No conversion after three months	1	
Unknown	2	
Gene Expert results		
Very Low	4	3.6
Low	20	17.86
Medium	60	53.57
High	28	25
Initial State		
New	31	27.68
Relapsed	39	34.82
Drop Out / neglect	9	8.04
Fail Category 1	31	27.68
Failed long regimen	2	1.79
Drugs used		
Kanamycin	69	61.61
Capreomycin	43	38.39
Adverse Effects of Nephrotoxic Drugs		
Kanamycin	14	20.29
Capreomycin	13	30.23

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Table 2. Risk factors that influence drug side effects for hypokalemia in multidrug-resistant TB patients in Dr. Soetomo Hospital

No	Risk factor	P value		OR (odds ratio)	
		Kanamycin	Capreomycin	Kanamycin	Capreomycin
1	Aged	0.792	0.770	1.239	1.010
2	Gender	0.443	0.075	0.680	0.221
3	Weight	0.524	0.101	0.987	0.951
4	Yes/no Comorbid	0.790	0.428	1.143	0.586
5	Dosage/Kg BW	0.157	0.108	5.348	1.229

Table 3. Risk factors that influence drug side effects on kidney function in Multidrug-Resistant TB patients in Dr. Soetomo Hospital

No	Risk factor	P value		OR (odds ratio)	
		Kanamycin	Capreomycin	Kanamycin	Capreomycin
1	Aged	0.571	0.905	0.090	1.187
2	Gender	0.035	0.581	1.016	1.039
3	Weight	0.420	0.788	0.537	0.934
4	Yes/no Comorbid	0.468	0.170	0.992	0.085
5	Dosage/Kg BW	0.373	0.220	1.959	1.466

This was based on the results of the study of the risk factors that affect the side effects of drugs on kidney function (Table 2). Table 3 shows that only the risk factor of gender in the kanamycin patient group had a statistically significant effect on the occurrence of nephrotoxic side effects because the variable had a significant value smaller than 5% (p=0.035).

Sex influences the appearance of side effects in kidney disorders. Men generally have a heavier workload and often have more contact with a larger environment outside the home than women, in addition to lifestyle factors such as smoking habits in men (Longe & Bindukkinasih, 2022; Nugroho *et al*., 2018). Results of the research conducted by (Reviono *et al*., 2014). We found that male patients had more impaired renal function than female patients did. Based on the data obtained from the patients' medical records in this study, 40 patients had a smoking history, of which 39 (97.5%) were male, and only one (2.5%) was female.

In this study, there was no effect between body weight and the incidence of side effects on the kidneys and hypokalemia, which was indicated by the results of p-value> 0.05, namely the value of p = 0.420 in the kanamycin group and p = 788 in the capreomycin group. Differences in outcomes can be attributed to differences in the definition of impaired renal function, different populations, and the use of various drugs (Ratnawati *et al*., 2018).

This study found no influence of comorbid factors on impaired renal function. However, most MDR-TB patients in this study had comorbid diabetes mellitus. Diabetes mellitus can increase the risk of side effects and serum creatinine with an RR value of 2.049 (95% CI:1.242–3.379) (Soedarsono *et al*., 2021). Unregulated DM is correlated with acute ketoacidosis and chronic complications, including diabetic nephropathy (Onuka *et al*., 2017). Diabetes causes glomerular hyperfiltration, which is hypothesized to predispose patients to irreversible nephron damage, thus contributing to the initiation and progression of renal disease in diabetes (Tonneijck *et al*., 2017). A study in Mexico showed that patients with diabetes mellitus had a higher risk of experiencing serious side effects of Multidrug-Resistant TB treatment, including renal impairment with an OR of 6.5 (95% CI:1.9 - 21.8) (Muñoz-Torrico *et al*., 2017).

Dosing accuracy is very important for TB treatment therapy because the treatment obtained is maximized with the appropriate dose and patient therapy is guaranteed. If the dose given is lower than the standard dose, it can cause the desired therapeutic effect of the drug not to be achieved, so that the treatment objectives are effective in patients, which can prolong treatment, and patient recovery will take longer (Shibeshi *et al*., 2019). Excessive doses can also cause various side effects in patients with Multidrug-Resistant TB. Less than 1% of the gut absorbs aminoglycosides. All aminoglycosides were rapidly absorbed in injectable form. The highest concentrations were found in the renal cortex and vestibular regions. Aminoglycosides cause ototoxic, nephrotoxic, and electrolyte disturbances (hypokalemia, hypomagnesemia, and hypocalcemia) (Suparyatmo *et al*., 2014). Patients experiencing nephrotoxic side effects have a significantly longer duration of aminoglycoside treatment and a larger total dose (Shibeshi *et al*., 2019). However, in this study, no

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dose effect was observed on the occurrence of side effects in the kidneys.

The demographic characteristics of the patients using kanamycin and capreomycin in Table 4 show significant differences in age between those using kanamycin and those using capreomycin. While other characteristics, namely sex, body weight, presence or absence of comorbidities, dose per kg of body weight, and initial potassium levels of patients, showed no significant differences between the two groups of patients.

Previous studies have reported that old age is a risk factor for impaired renal function when administering aminoglycosides (Ahmad *et al*., 2018; Alene *et al*., 2019; Ratnawati *et al*., 2018). In this study, the results of statistical tests using binary logistics showed that age did not significantly affect the occurrence of the side effects of renal impairment. However, the age characteristics of the patients in the capreomycin group were significantly different from those in the kanamycin group. Nephrotoxic side effects in older adults were more common in the kanamycin group. In line with the results of research from (Ratnawati *et al*., 2018), who stated that the relationship between risk factors and side effects that occur in Multidrug-Resistant TB patients who receive kanamycin drug therapy, they found that those aged> 59 years were 3.8 times more likely to have impaired renal function than those aged < 40 years. Aged 40-59 years were 1.89 times more likely to have renal impairment than patients aged < 40 years.

This study has limitations, namely that most Multidrug-Resistant TB patients have a history of diabetes mellitus, so there is an influence of the use of insulin by patients on potassium levels, which can affect kidney function disorders. Some of these factors may have caused the bias in this study. The comparison of the number of patients in the characteristics of age and BMI in this study is less balanced and does not mention the influence of genetic factors that might have affected the investigation results.

Table 4. Baseline characteristics of patients taking kanamycin and capreomycin in multidrug-resistant TB patients in Dr. Soetomo Hospital

No	Characteristic	Statistic Test	P value	Interpretation
1	Aged	Independent t-test	0.001	Significant
2	Gender	Pearson Chi-square	0.750	Not significant
3	Weight	Independent t-test	0.561	Not significant
4	Yes/no Comorbid	Pearson Chi-square	0.262	Not significant
5	Dosage/Kg BW	Independent t-test	0.708	Not significant
6	Initial potassium levels	Independent t-test	0.700	Not significant

CONCLUSION

Male sex affects the occurrence of side effects of kanamycin and capreomycin in the treatment of Multidrug-Resistant TB with impaired renal function (nephrotoxic). Body weight in Multidrug-Resistant TB patients did not affect the occurrence of side effects of renal impairment, and there was no influence of comorbid factors or the dose administered on renal impairment. Stricter supervision of kanamycin use in elderly patients (>40 years) is needed to minimize the side effects of renal impairment in the treatment of Multidrug-Resistant TB.

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AUTHOR CONTRIBUTIONS

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

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Optimization and Prevalidation of TLC-Densitometry Method for Fucoidan Analysis in *Sargassum sp*. Aqueous Extract

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Abstract

Background*: Fucoidan is sulfated polysaccharide that has gastroprotector activity, and it is distributed in brown algae cell walls. Currently, there is no method for fucoidan analysis in compendia. Furthermore, analysis of Fucoidan is proven to be challenging due to the lack of chromophores and its high polarity.* ***Objective****: To develop the optimal condition of TLC-Densitometry method for fucoidan analysis in Sargassum sp. aqueous extract and to evaluate the stability of Fucoidan as a preliminary study.* ***Methods****: Chromatography was performed on Silica gel 60F_{254} TLC-plate as a stationary phase. The developed plate was stained with H_2SO_4 10% in absolute ethanol and heated in oven at 105°C for 15 minutes. Optimization is carried out by determining composition of the mobile phase, analytical wavelength, and spotting volume. Stability test of Fucoidan in standard and extract solution at 0, 4, 8, and 24 hours also 0 and 60 minutes after derivatization.* ***Results****: The optimal condition which produces a good separation of Fucoidan was achieved by using n-butanol:methanol: water (10:6:10 v/v/v) as a mobile phase, 400 nm as an analytical wavelength, and 1 µl as a spotting volume. Fucoidan was stable after storage until 24 hours. The stained spots were stable until 60 minutes after derivatization.* ***Conclusion****: Optimal condition of the TLC-Densitometry method for Fucoidan analysis was selective and can be applied to stability tests in preliminary study. Fucoidan was stable in standard solution and extracted solution until 24 hours after storage at 4°C, and the stained spots were stable until 60 minutes after derivatization.*

Keywords*: fucoidan, prevalidation, Sargassum sp., TLC-densitometry*

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INTRODUCTION

As a maritime country, Indonesia has high marine potential. One of these is the existence of *Sargassum sp.*, which consists of approximately 400 species spread across the Java Sea to the Banda Sea. *Sargassum sp.* contain bioactive compounds that can be utilized in the health sector (Rohim *et al*., 2019). Fucoidan is a bioactive compound in *Sargassaum sp*. that has gastroprotective activity (Hu *et al*., 2020). Fucoidan (Table 1) is a type of sulfate polysaccharide found in the cell wall of *Sargassum sp.*, which contains fucose as its main component (Li *et al*., 2017).

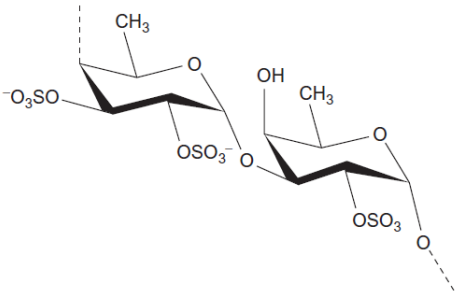

Figure 1. Chemical Structure of Fucoidan

Sargassum sp. extract is used as an industrial raw material and source of fucoidan. Therefore, finding a reliable, fast, and inexpensive method for fucoidan analysis is very important. However, there is no analytical method for fucoidan analysis in the compendia. Therefore, an analytical method for fucoidan analysis must be developed. Several analytical methods for fucoidan analysis have been developed, including fluorometric assays and UV spectrophotometry (Venkatesan *et al*., 2018; Yamazaki *et al*., 2016). However, because herbal extracts are composed of several substances, they are not likely to be simple to apply, and the process requires the isolation of fucoidan from the other components. Chromatography is a method used to separate multiple compounds. High-performance liquid chromatography (HPLC) methods for fucoidan analysis use photodiode array (PDA), refractive index (RI), and mass spectrometry (MS/MS) detectors (Isnansetyo *et al*., 2017; Zhao *et al*., 2021; Zhu *et al*., 2018).

In addition to HPLC, thin-layer chromatography (TLC) can also be used for fucoidan analysis. The TLC method was used to separate compounds, such as fucoidan, from the crude extract of *Sargassum turbinarioides* and *Sargassum ilicifolium* for screening crude extracts (Artemisia *et al*., 2019). In that study, a densitometer was not used, whereas the detector contained in the densitometer allowed the TLC method to achieve accurate qualitative and quantitative results (Ekasari *et al*., 2020). Therefore, a TLC densitometry method for fucoidan analysis was developed. TLC densitometry is a relatively simpler and less time-consuming chromatography method than HPLC (Ahmad *et al*., 2020; Ekasari *et al*., 2020; Satpathy *et al*., 2017; Sherma & Rabel, 2018). Fucoidan analysis with TLC densitometry was challenging because of the lack of a chromophore group and its high polarity. In this study, the development of a TLC-densitometry method for fucoidan analysis was carried out at an optimization stage to determine the optimal condition of the TLC-densitometry method for fucoidan analysis in *Sargassum sp.* aqueous extract and to evaluate the stability of fucoidan as a preliminary study.

MATERIALS AND METHODS

Materials

Fucoidan standard (Sigma Aldrich) and *Sargassum sp.* extract powder were obtained from the pharmaceutical industry in Indonesia (PT. Natura Laboratoria Prima), methanol (Merck), and water (PT. Ikapharmindo Putramas), n-butanol (Merck), n-hexane (Merck), acetone (Merck), ethyl acetate (Merck), isopropanol (Merck), acetonitrile (Merck), ethanol (Merck), sulfuric acid (Merck), and silica gel $60F_{254}$ TLC) aluminum plates (20 × 20 cm (Merck).

Tools

Analytical balance (Mettler Toledo), Twin Trough Chamber 20 × 20 cm (Camag), Linomat 5 (Camag), TLC Scanner 4 with a UV detector (Camag), VisionCATS software (Camag), oven (Medcenter), and reagent sprayer.

Method

Preparation of standard solution

A standard solution (2000 µg/ml) was prepared by dissolving 10 mg of the fucoidan standard in 5 ml of water: methanol 1:9 (v/v). The mixture was then vortexed until homogenous.

Preparation of sample solution

Sargassum sp. extract powder (10 mg) was dissolved in 5 ml of water: methanol 1:9 (v/v). The mixture was then vortexed until homogenous.

Preparation of mobile phase

The mobile phase was freshly mixed with various compositions for the optimization stage, as shown in Table 1. The development chamber was then left to saturate with mobile phase vapor for 2 h before each eluation.

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Table 2. Optimization parameters

No.	Optimization	Parameter	Criteria
1.	Composition of the mobile phase		
	(1) n-hexane : acetone (4:6, v/v)		
	(2) Acetone : water (7:3, v/v)	Rf	0.2-0.8
	(3) Isopropanol : ethyl acetate : water (7:2:1, v/v/v)	Rs	≥ 1.5-2.0
	(4) Acetonitrile : n-butanol : water (6:3:1, v/v/v)		
	(5) n-butanol : methanol : water (10:6:10, v/v/v)		
2.	Analytical wavelength		
	fucoidan spot was tested for its UV spectrum profile at wavelengths of 200-700 nm	Maximum wavelength	Maximum wavelength (based on UV spectrum)
3.	Spotting volume		
	(1) 1 µl	Area and Peak Shape (Symmetry factor (As))	Largest area with symmetry peak shape (As : 0.9-1.2)
	(2) 2 µl		
	(3) 3 µl		

Table 1. Variation of mobile phase composition

No.	Mobile phase composition
1.	n-hexane : acetone (4:6, v/v)
2.	Acetone : water (7:3, v/v)
3.	Isopropanol : ethyl acetate : water (7:2:1, v/v/v)
4.	Acetonitrile : n-butanol : water (6:3:1, v/v/v)
5.	n-butanol : methanol : water (10:6:10, v/v/v)

Optimization of analytical conditions

Optimization was performed by determining the composition of the mobile phase, analytical wavelength, and spotting volume. The parameters observed in this optimization stage are listed in Table 2. The chamber was saturated with the mobile phase. Standard and sample solutions were added to the plates. The plate was then developed in a saturated chamber until a migration distance of 70 mm from the origin was reached. After development, the plate was dried with a hair dryer for 20 min at room temperature and then dried in an oven for 15 min at 40°C. To visualize the spots, the plate was sprayed with H_2SO_4 10% in absolute ethanol and then heated in an oven at 105°C for 15 min. The absorbance of fucoidan from yellow to dark spots was measured using a densitometer.

A mobile phase that could separate fucoidan from other compounds with a resolution (Rs) value ≥ 1.5-2.0 and had a retardation factor (Rf) range of 0.2-0.8 was chosen. The analytical wavelength and spotting volume that produced the largest area and sharper peaks were selected. The symmetry factor (As) is a parameter that can characterize the shape of the peaks. The optimal values for the symmetry factor of the peaks were 0.9–1.2 (Czyrski & Sznura, 2019).

Stability test for standard and extract solution

The stability test is a pre-validation stage that aims to determine the stability of fucoidan in standard and extract solutions. Standard and extract solutions were spotted, developed, and analyzed at 0, 4, 8, and 24 h after storage at 4°C. The stability of the resulting spots after derivatization was also tested 0 and 60 min after derivatization.

RESULTS AND DISCUSSION

Optimization was performed by determining the composition of the mobile phase, analytical wavelength, and spotting volume. In this optimization stage H_2SO_4 10% in absolute ethanol was used as the visualizer reagent because of the lack of a chromophore group in fucoidan. Mobile phase optimization is a crucial step in TLC method development, because it ultimately affects the quality of separation (Chaudhari & Shirkhedkar, 2020). The results of the mobile phase optimization are listed in Table 3. Based on mobile phase optimization, only the n-butanol: methanol: water (10:6:10, v/v/v) mobile phase could separate fucoidan from other compounds. The resolution (Rs) value and the retardation factor (Rf) value of the fucoidan peak, were 0.58 and 3.60, respectively. It means that this mobile phase can produce an optimal separation of fucoidan spot with another compound (Rs ≥ 1.5-2.0), and the Rf value met the requirement range of 0.2-0.8 (AOAC International, 2019; Indrayanto *et al*., 2009; Yuwono & Indrayanto, 2005). Meanwhile, the results of fucoidan analysis using various other mobile phases showed that fucoidan was still at the starting point of the spotting area, so the resulting Rf value of fucoidan spots was around 0 (out of the requirement range), and the separation process could not occur. This could be due to

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the high polarity of fucoidan, and the polarity of the stationary phase is also polar, so the fucoidan is strongly bonded with the stationary phase (Zayed *et al*., 2020). In this case, a mobile phase with high polarity is required, resulting in the optimal Rf value for fucoidan. Therefore, n-butanol: methanol: water (10:6:10, v/v/v) was selected as the mobile phase because it can migrate the fucoidan spot until it meets the requirement for the Rf value and can optimally separate fucoidan with good Rs values. Different compositions of the mobile phase also differ in polarity, where the separation process depends on the polarity of the mobile phase, which affects the Rf and Rs values.

Table 3. The results of fucoidan analysis using a variation of mobile phase composition

Mobile Phase	Rf	Rs
1	0.01	25.21
2	0.01	3.00
3	0.01	5.85
4	0.01	8.24
5	0.58	3.15

Subsequently, the fucoidan spot was tested for its UV spectral profile. Spectral measurements were performed at wavelengths of 200-700 nm. The Fucoidan spectrum is shown in Figure 2. The fucoidan spectrum shows that the maximum wavelength of fucoidan is 400 nm, which provides the maximum absorption for fucoidan. Therefore, 400 nm was selected as the analytical wavelength of fucoidan. TLC densitograms of fucoidan in the standard and extract solutions at a wavelength of 400 nm are shown in Figure 3.

Spotting volume optimization was conducted by spotting fucoidan standard with various spotting volumes such as 1 µL, 2 µL, and 4 µL on the stationary phase, and then analyzed according to a previous procedure, including scanning with a densitometer at 400 nm wavelength. The observed response areas and peak shapes of fucoidan with various spotting volumes are shown in Table 4. Based on the results in Table 4, the largest response area was produced by 3 µL, but the peak shape was asymmetric (fronting). A spotting volume of 2 µL also produces asymmetry (fronting). A spotting volume of 1 µL was chosen for each analysis because it can produce a symmetrical peak shape, although with the lowest response area. The amount of sample to be applied in a spot is sometimes difficult to determine because it depends on several variables, such as the sample matrix itself, sorbent thickness, and sample solvent. If the applied sample is overloaded, poor chromatographic separation on thin layers will occur (Wall, 2005). This is the reason why the spotting volume needs to be optimized.

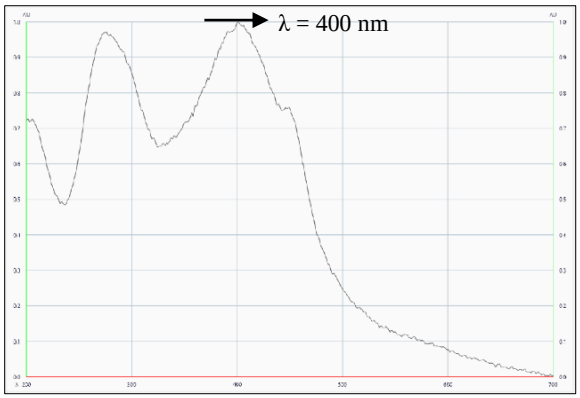

Figure 2. The fucoidan spectrum at a wavelength 200-700 nm

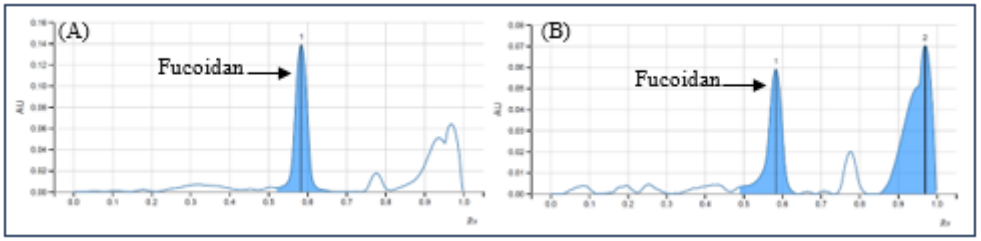

Figure 3. TLC densitograms of Fucoidan in standard (A) and extract (B) solution at a wavelength of 400 nm

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Table 4. The observed response area and peak shape of Fucoidan with various spotting volumes

Concentration	Spotting Volume	Area	As	Peak Shape
2 µg/µl	1 µl	0.00446	0.90	Symmetry
2 µg/µl	2 µl	0.00687	0.71	Asymmetry
2 µg/µl	3 µl	0.00904	0.77	Asymmetry

Table 5. Stability test results for fucoidan standard and extract solution

Spotting time (hours)	0 min after derivatization		60 min after derivatization	
	Average Area of Standard Solution	Average Area of Extract Solution	Average Area of Standard Solution	Average Area of Extract Solution
0	0.00676	0.00390	0.00637	0.00374
4	0.00572	0.00317	0.00584	0.00327
8	0.00719	0.00427	0.00768	0.00448
24	0.00649	0.00497	0.00664	0.00535

The results of the stability test of fucoidan in a preliminary study are shown in Table 5. One-way ANOVA statistical analysis was performed with a 95% confidence interval ($\alpha = 0.05$) on the fucoidan area from each spotting time. The one-way ANOVA results showed a significance value of 0.068 for the standard solution and 0.050 for the extract solution. The significance value was more than α (0.05). This indicates that there was no significant difference in the fucoidan area obtained from each spotting time in the standard and extract solutions. It can be concluded that fucoidan is stable until 24 h of storage at 4°C. A paired t-test statistical analysis was carried out with a 95% confidence interval ($\alpha = 0.05$) on the results of the fucoidan standard and extract solution area at 0 and 60 min after derivatization. The paired t-test statistical analysis results showed that the significance values of the standard solution and extract solution areas between 0 and 60 min after derivatization were 0.488 and 0.253, respectively. This indicates that there was no significant difference between the areas observed at 0 and 60 min after derivatization. Therefore, it can be assumed that the derivatized stain is stable up to 60 min after derivatization.

CONCLUSION

The optimal conditions for Fucoidan analysis were n-butanol: methanol: water (10:6:10, v/v/v) as the mobile phase, 400 nm as the analytical wavelength, and 1 µL as the spotting volume. This method was selective and can be applied to stability tests in a preliminary study. Fucoidan was stable in the standard solution and extracted solution until 24 h after storage at 4°C, and the stability of stained spots was stable until 60 min after derivatization. This optimal condition can be validated in future research.

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AUTHOR CONTRIBUTIONS

Conceptualization, L. I., M. Y., R. P.; Methodology, L. I., M. Y., R. P.; Software, L. I.; Validation, L. I.; Formal Analysis, L. I.; Investigation, L. I.; Resources, L. I., M. Y., R. P.; Data Curation, L. I., M. Y., R. P.; Writing - Original Draft, L. I., M. Y., R. P.; Writing - Review & Editing, L. I., M. Y., R. P.; Visualization, L. I., M. Y., R. P.; Supervision, L. I., M. Y., R. P.; Project Administration, L. I., M. Y., R. P.; Funding Acquisition, L. I., M. Y., R. P.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Comparative Analysis of Real Costs and INA CBG's Rates in BPJS Kesehatan Patients with Schizophrenia

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Abstract

Background*: Schizophrenia is the most severe mental disorder because its risk of death is to 2-3 times higher.* ***Objective****: To determine the average real costs, the difference between real costs and INA-CBG rates of inpatients with schizophrenia, and the factors that affect real costs.* ***Methods****: The study was conducted retrospectively from the hospital perspective using the total sampling method from January 2020-December to 2021. The study sample included inpatients patients and the real costs of the hospital. The data obtained were analyzed using the Mann-Whitney U-test and multiple linear regression tests.* ***Results****: About 112 patients met the inclusion criteria. The average real cost of inpatients with schizophrenia at Tombulilato General Hospital from January 2020 to December 2021 is Rp. 9,895,102 and the average INA-CBG rate of inpatients with schizophrenia was Rp. 14,820,778. There was a difference between the real costs and INA-CBG rates (p = 0.002), with the highest average hospital real cost component in the inpatient room (Rp. 3,397,723 (34.34%). The factor that affected the real costs of inpatients with schizophrenia patients is the length of stay (p = 0.000).* ***Conclusion****: The real costs of the hospital were lower (p = 0.002) than those of the INA-CBG. The highest real cost of the hospital was the inpatient room (34.34%), and the factor that affected the real costs was the length of stay (p = 0.000).*

Keywords*: cost analysis, hospital real costs, INA-CBG's rates, schizophrenia*

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INTRODUCTION

Mental disorder is a syndrome of individual behavioral patterns associated with a symptom of suffering (*distress*) or *impairment (impairment*) in one or more essential functions of humans, namely psychological, behavioral, biological, and other disorders that affect their relationship with society (Widodo *et al.*, 2022). According to the *World Health Organization* (WHO) 2016, mental disorders worldwide have become a severe problem. An estimated 264 million people are affected by depression, 45 million people are affected by bipolar disorder, 20 million by schizophrenia, and 50 million by dementia (James *et al.*, 2018). Indonesia is one of the countries with significant mental health problems. Based on the calculation of disease burden in 2017, Indonesia has experienced several types of mental disorders, including depression, anxiety, schizophrenia, bipolar disorder, behavioral disorders, autism, eating behavior disorder, intellectual disability, and *Attention Deficit Hyperactivity Disorder* (ADHD) (Tulandi, 2021).

Based on the results of the 2018 Basic Health Research (Riskesdas), there has been an increase in the prevalence of People with Mental Disorders (ODGJ) in Indonesia. The majority of households with schizophrenic mental illness, according to residents, showed that 7 $^{0}/_{00}$ in rural areas experienced more schizophrenic mental disorders than in urban areas, which is 6.4 $^{0}/_{00}$. The data show that the highest prevalence value is in the province of Bali (11.1$^{0}/_{00,}$ while in Gorontalo, the prevalence value was 6.6$^{0}/_{00}$ (Ministry of Health, 2019).

One of the most severe mental disorders is schizophrenia because the risk of death is two to three times higher; therefore, it requires relatively expensive treatment costs that trigger a high economic impact on patients, families, and health financing institutions if schizophrenic patients experience a recurrence in the hospital (Pratiwi *et al.*, 2017). A study using BPJS Kesehatan (Healthcare and Social Security Agency) sample data from 2015 to 2016 examining the financing of patients with schizophrenia in Indonesia showed that the average claim costs per patient were Rp. 427,806 for outpatients and Rp. There are 10,500,000 patients (Sangadah, 2021). Another study showed a negative difference in Rp. 136,096,659 for inpatients with schizophrenia, and 267 samples with the code F-4-10-I (Sari, 2015). A study of schizophrenia in the US from to 2005-2014 showed a significant hospitalization rate of 55.4%, with costs ranging from $10,000- $49,999 (57.1%) (Chen *et al.*, 2021). An Italian study conducted in 2009-2016, obtained the results of 15,000 social security beneficiaries showing an average annual expenditure of €160.1 million with an average cost per patient of €10,675 (Mennini *et al.*, 2021).

The high number of mental health problems in the community indicates that it is necessary to improve health services in quality medical services efficiently. Implementation of the INA-CBG programme is expected to enhance the success of health services for people who are already unhealthy (Rahem *et al.*, 2021). Increasing health financing affects health insurance costs and health quality. If the INA-CBG's claim cost is greater than the actual costs, then the hospital does not experience losses, but if the real costs are greater than the INA-CBG's rates, then the loss is shared by the hospital (Agiwahyuanto *et al.*, 2020). Several research cases, such as ischemic stroke (Muslimah *et al.*, 2017) and hypertension (Nilansari *et al.*, 2021) showed that the actual costs incurred were greater than those incurred by INA-CBG. However, studies on diabetic gangrene disease (Kinanti *et al.*, 2021) and thalassemia (Wijaya & Ariawati, 2018) have shown a positive difference between hospital real costs and INA-CBG rates. This study aimed to determine the average real costs, explore the difference between real costs and INA-CBG rates, and identify factors that affect real costs.

MATERIALS AND METHODS

Ethical considerations

The Toto Kabila Regional General Hospital issued a certificate of ethical eligibility (800/RSUD TK/1224). a/XII/2022). Permit for conducting research was issued by the Investment and Integrated One-Stop Services Agency of Bone Bolango Regency (503/DPMPTSP-BB/IPM/0338/IX/2022).

Methods

The study was conducted from October to December 2022 at Tombulilato General Hospital, using *retrospective* data from the hospital's perspective. The sampling was performed using the *total sampling* technique. The study population included 112 inpatients with schizophrenia between January 2020 and December 2021. The inclusion criteria of the sample in this study were 1) patients with a diagnosis of schizophrenia based on medical records, e-claim applications, and BPJS claims data for the January 2020-December 2021 period; 2) inpatients with schizophrenia of BPJS class 1,2,3 with the codes F-4-10-I, F-4-10-II, F-4-10-III, and 3) patients who had complete medical records. The exclusion criteria were patients who left the hospital at their request (forced return) and those who

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died. The study participants met the inclusion criteria for 112 patients.

The cost component of this study was the real costs of the hospital (laboratory costs, blood transfusion costs, nutrition installation costs, emergency department installation costs, nursing care costs, general practitioner and specialist mental health service costs, medicines and medical consumable costs, inpatient room costs, and inpatient administration) and INA-CBG rates.

This study used descriptive analysis of patient demographic data and cost components. Mann-Whitney and multiple linear regression tests were used to examine the factors affecting the hospital's real costs to see the difference between the real costs and the cost of INA-CBG inpatient schizophrenia.

RESULTS AND DISCUSSION

Patient characteristics

Between January 2020 and December 2021, 112 patients met the inclusion criteria. An overview of the characteristics of inpatients with schizophrenia is presented in Table 1.

In this study, the character data of inpatients with schizophrenia at Tombulilato Hospital showed that the number of male patients was higher than that of the 82 inpatients (73.21%). According to (Sadock *et al.*, 2014) and (Tus Ińs Ki & Lew-Starowicz, 2018), the number of patients with schizophrenia was higher in men than in women. This result is in accordance with the research conducted by (Trishna & Muhdi, 2020) that as many as 65.3% of men have schizophrenia compared to 34.7% of women. This is because sex differences in people with schizophrenia are related to reproductive hormones in men and women. Estrogen protects women against schizophrenia (Androvičová *et al.*, 2021).

Based on age, it can be seen that patients with schizophrenia experienced the highest in the early adulthood category, namely the age of 26-35 years as many as 40 patients (35.71%), which is the productive age (Badan Pusat Statistik, 2021). Early adulthood is the most vulnerable period of development to experiencing stress that impacts'individuals' well-being (Manita *et al.*, 2019).

Regarding male-dominated patients at a productive age, people with high school education appeared to have the highest education level in experiencing schizophrenia (39 patients, 34.82%) compared to other levels of education. Furthermore, 68 patients (48.21%) had a non-working status, and schizophrenic patients were influenced by intrinsic and extrinsic factors of patients, namely, people who do not work have a 6.2 times greater risk of developing schizophrenia than those who work (Zahnia & Wulan, 2016).

The prevalence of schizophrenia in this study, which is domiciled in rural areas, was 95 patients (84.82%) and 17 patients (15.18%) in urban areas. According to data from Kementrian Kesehatan RI (2019), the prevalence of schizophrenia in Indonesia based on residence is more significant in rural areas 7‰ than in urban areas 6.4‰. This contrasts with the population density theory, which states that schizophrenia is higher in areas with high population densities, such as cities. Population density is a factor that influences the occurrence of mental disorders, including psychosis (Sadock *et al.*, 2014). Based on Table 1, the highest class of treatment-experienced schizophrenia, namely class 3, with 91 patients (81.25%), showed that most patients with schizophrenia in Tombulilato Hospital came from middle to lower economic classes.

Table 1. Characteristics of Patients (n = 112)

Characteristics	Category	n =112 (%)
Gender	Male	82 (73.21)
	Female	30 (26.79)
Age (years)	12, 16	1 (0.89)
	17/25	32 (28.57)
	26-35	40 (35.71)
	36-45	26 (23.21)
	46-55	7 (6.25)
	56-55	5 (4.46)
	>65	1 (0.89)
Education	No school	1 (0.89)
	Elementary School	32 (28.57)
	Junior High School	27 (24,11)
	Senior High School	39 (34.82)
	Diploma	3 (2.68)
	Undergraduate (S1)	9 (8.04)
	Postgraduate (S2)	1 (0.89)
Occupation	Unemployed	68 (60.73)
	Students	3 (2.67)
	Working	41 (36.60)
Domicile	Village	95 (84.82)
	City	17 (15.18)
Treatment class	1	2 (1.79)
	2	19 (16.96)
	3	91 (81.25)

Compatibility of real costs and INA-CBG's rates for schizophrenic patients at Tombulilato Hospital

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Patients with schizophrenia receiving inpatient health services at the hospital were paid for using INA-CBG rates per patient per episode. Table 2 shows that for each patient treated at Tombulilato Hospital, based on all treatment classes and the severity of schizophrenia using statistical analysis of the Mann-Whitney test, the *p*-value for the overall data was 0.002 ($p<0.05$). This indicates a statistical difference between the real costs of the hospital and the INA-CBG rates. The average value of the hospital's real cost was Rp. 9,895,102 and the average INA-CBG rate was Rp. 14,820,778. A comparison between the average real costs and INA-CBG rates yielded a positive difference value. This result is different from the results of research (Sari, 2015), which shows that the cost comparison has a negative difference value of Rp.136,096,659 between INA-CBG's rates and the real costs. Also, the most influential cost component is accommodation. The difference in costs that occur in hospitals depends on the pattern of hospital rates; in Tombulilato Hospital, the hospital still refers to the 2011 regional regulations on general service levies, which are still relatively small in hospital service rates. Thus, there is still a positive difference in hospitals in the calculation of medical costs, which is achieved by directly determining the INA-CBG's rate policy in PMK 52 of 2016.

The analysis results in Table 3 show the difference between hospital real costs and INA-CBG rates, and most claims come from mild schizophrenia patients in treatment class 3 with the code INA-CBG's F-4-10-I. Treatment class 3 had the lowest premium. The treatment of mild schizophrenia (F-4-10-I) class 3 had the largest number of visits; that is, the average value of real costs was Rp. 9,854,982 and the average rate of INA-CBG was Rp. 15,722,630. Therefore, the difference in Rp was obtained. 5,867,648 with a value of $p = 0.001$ ($p<0.05$), whereas the lowest difference in schizophrenia treatment was in mild schizophrenia patients (F-4-10-I) with treatment class 1 was Rp. 288,850 ($p=1.00$). Statistically, there was no significant difference between the real costs incurred by hospitals and INA-CBG rates in mild schizophrenia patients in treatment class 1. This is because the number of patients in class 1 was only two. Overall, the difference in costs obtained based on the BPJS treatment class and severity of schizophrenia has a positive difference in value for hospitals. The results of the study indicated that the comparison of real costs and INA-CBG rates in hospitalized patients with hebephrenic schizophrenia showed positive results on the days of the acute and subchronic phases of hospitalization based on INA-CBG rates (Basirun *et al.*, 2013). Positive differences in real hospital costs with INA-CBG rates existed because the hospitals did not make cost adjustments based on standard disease management procedures with clinical pathways.

Table 2. Differences between actual costs and INA-CBG's rates of schizophrenic patients at Tombulilato General Hospital for the period of January 2020-December 2021 (n = 112)

Category	n	Average (Rp)	Total (Rp)	*P-value*
Real costs	112	9,895,102	1,108,251,500	<0.002
INA-CBG's		14,820,778	1,659,927,200	

Table 3. Compatibility of real costs with INA-CBG's rates schizophrenic patients at Tombulilato Hospital based on BPJS class and INA-CBG's code (n=112)

INA-CBG's Code		BPJS Class	n	Real Costs	INA-CBG's	Fee Difference	*P* Value
Code	Description			Average (Rp)	Average (Rp)	Average (Rp)	
F-4-10-I	Mild schizophrenia	Class 1	2	3,543,000	3,831,850	288,850	1.00
F-4-10-I	Mild schizophrenia	Class 2	19	10,780,184	12,392,531	1,612,347	0.826
F-4-10-I	Mild schizophrenia	Class 3	84	9,854,982	15,722,630	5,867,648	.001*
F-4-10-II	Moderate schizophrenia	Class 3	5	11,451,100	17,269,880	5,818,780	117
F-4-10-III	Severe schizophrenia	Class 3	2	3,998,500	4,877,550	879,050	0.439

Cost component

The real cost component of the Tombulilato Regional General Hospital includes laboratory,

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radiology, blood transfusion, medicine, medical consumables, inpatient rooms, emergency department installations, general practitioner visits, specialist mental health services, nursing care, nutrition installation, and inpatient administration costs. The currency of the cost uses the Indonesian Rupiah (IDR) for 2020-2021. Based on Table 4, medical support costs such as laboratory costs, with an average value of Rp. 98,714 and the average radiology cost was Rp. 13,571, and the average blood transfusion cost was Rp.8,178, which was the smallest cost incurred by the hospital. In general, patients undergo laboratory examinations at the beginning of hospital admission to determine the presence or absence of other accompanying diseases, whereas radiology and blood transfusions are only performed on patients who experience secondary diagnostic complaints. The most significant component of the cost of medical personnel is a general practitioner, which has an average value of Rp.1,289,732, and the average cost of specialist mental health services is Rp. 180,535. This is because the frequency of specialist visits is only once a week; therefore, the general practitioner is fully responsible for the patient. The inpatient room is the first highest-cost component, with an average value of Rp. 3,397,723, these results were influenced by the duration of the patient's length of stay. Research (Cheng *et al.*, 2022) shows that unmarried status is a risk factor for patients to stay longer. This causes an increase in severity based on the calculation of special CMG rates. Schizophrenia is a disease with a top-up system in the INA-CBG's claims. If the length of stay increases, the ADL value in the who-DAS interviews will also affect financing. The average nutritional installation cost of Rp. 2,398,741, and an average medicine cost of Rp.1,748,950. The cost of medicines for schizophrenic patients at Tombulilato Hospital is managed through a prescription system based on the use of generic drugs that adhere to the national disease and formulary guidelines. However, several psychotic medicines are branded in patients' prescriptions and manufactured to meet the needs of patients and hospital budgets. An analysis of factors that affect the real costs of the hospital in this study using multiple linear regression tests on the length of the stay (p=0.000; β=0.975) and the severity of the disease (p=0.544; β=-0.018) revealed that severity had no significant effect on the real costs, whereas the length of stay did.

Overall, this study provides an overview of the cost burden of schizophrenic patients participating in BPJS Kesehatan in Indonesia, which is expected to be a consideration in the national health insurance scheme budget and hospital financing.

Table 4. Component Cost (n = 112)

Cost Component	Average±SD (Rp)
Laboratory	98,714.29±91,319.59
Radiology	13,571.43±35,744.52
Blood Transfusion	8,178.57±80,381.21
Pharmacy	
Medicine	1,748,950.89±965552.57
Medical Material Consumables	19,107.14±33,879.85
Inpatient Room	3,397,723.21±2,093,052.84
Emergency Installation	48,785.71±15,883.26
The cost of general practitioner visit	1,289,732.14±729,323.03
The cost of Psychiatry visit	180,535.71±103,190.88
Nursing care	661,294.64±395,962.06
Nutrition Installation	2,398,741.07±1,340,712.11
Inpatient Administration	25,000.00±0.00
Average total assets	9,895,325.80±5,924,611.92

The strength of this research is that it was first performed in Tombulilato Hospital on schizophrenic patients to determine the real costs of hospitals and compared to INA-CBG rates. Research limits result from real hospital costs, affecting the number of patients due to forced reentry, including 37 patients. Therefore, hospital INA-CBG claims at actual hospital costs differ from hospital discrepancies. This study can be used to conduct a cost analysis for the treatment of patients with schizophrenia.

CONCLUSION

The real cost of the hospital was lower at Rp. 9,895,102 (p = 0.002) for INA-CBG rates of Rp. 14,820,778. The highest real cost component of the hospital was an inpatient room (34.34%), and the factor that affected the real costs was the length of stay (p = 0.000). This study shows a positive difference in real costs and INA-CBG rates in inpatients with schizophrenia in BPJS Kesehatan at Tombulilato Hospital, so it is expected to be a picture of financing schizophrenia patients through a national health insurance scheme.

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AUTHOR CONTRIBUTIONS

Conceptualization, O. M., A. R., Y. N.; Methodology, Y. N.; Software, A. R.; Validation, Y. N.; Formal Analysis, A. R.; Investigation, A. R.; Resources,

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O. M.; Data Curation, O. M.; Writing - Original Draft, Y. N.; Writing - Review & Editing, Y. N.; Visualization, A. R.; Supervision, A. R.; Project Administration A. R.; Funding Acquisition, O. M.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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In vivo Evaluation of Extracted and Fraction of *Moringa oleifera* leaves against Testosterone-Induced PCOS Model in Rattus Norvegicus

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Abstract

Background*: Polycystic ovary syndrome (PCOS) is an endocrine disorder. Parameters characteristic of the disorder include weight gain, insulin resistance and malondialdehyde (MDA). The traditional medicine used is Moringa oleifera.* ***Objectives****: The aim was to determine the effect of weight loss and blood glucose levels, MDA levels and histopathological features of the ovarian follicles in the rat model PCOS-insulin resistance induced by testosterone.* ***Methods****: Extraction process, followed by fractionation using n-hexane, ethyl acetate and water, identification of compound content using TLC, and rats were grouped into 7 groups (n=5), namely normal group, negative group, positive group, extract, fraction water, ethyl acetate fraction and n-hexane fraction by looking at the characteristic parameters and ovarian histopathology. Data analysis using ANOVA and Kruskal-wallis.* ***Results****: The yield of the extract was 30.4%, the water fraction was 85.59%, the ethyl acetate fraction was 6.64% and the n-hexane fraction was 4.05%. Positive for flavonoids, tannins, alkaloids in the ethyl acetate fraction, the water and extract fractions were positive for tannins, the n-hexane fraction was positive for steroids. The modeling sample group obtained extract body weight 195.40 g, water fraction 195.80 g, ethyl acetate fraction 194.00 g, n-hexane fraction 196.00 g, blood glucose level extract 83.00 mg/dL, water fraction 84.27 mg/dL, ethyl acetate fraction 80.00 mg/dL, n-hexane fraction 122.85 mg/dL, MDA extract content 2.704 nmol/mL, water fraction 3.547 nmol/mL, 1.685 nmol/mL, 5.308 nmol /mL and can improve ovarian histopathology.* ***Conclusion****: The most effective value is the ethyl acetate fraction because it has the highest decrease in PCOS characteristics.*

Keywords*: follicles, malondialdehyde, moringa ethyl acetate fraction, Moringa oleifera, polycystic ovary syndrome*

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INTRODUCTION

Polycystic ovary syndrome is an endocrine disorder that affects 5%-10% of women of reproductive age and is found in approximately 20%-25% in England and New Zealand (Norman *et al*., 2004). The *European Society for Human Reproduction and Embryology/American Society for Reproductive Medicine* reported a prevalence of 15% -20%. In Indonesia, the highest frequency was found in the age range of 26-30 years (45,7% (Pangastuti & Sumapradja, 2011). Prevalence estimates vary depending on the source population and the *Rotterdam Criteria 2003* diagnostic criteria. There are two of three criteria: polycystic ovaries on ultrasound examination (USG), oligoovulation/anovulation, and clinical or biochemical evidence of hyperandrogenism. Women with PCOS have disturbances in the menstrual cycle (85% -90% with oligomenorrhea and 30% -40% with amenorrhea) and at most 90% -95% infertility (Pate & Sirmans, 2014).

The pathogenesis of PCOS in women is an androgen disorder that can increase the activity of the steroidogenesis pathway in ovarian follicles, which is achieved through theca and granulosa cells. In theca cells, it increases the expression of the enzyme *17β-hydroxysteroid dehydrogenase* (17β HSD), which causes an increase in testosterone levels in granulosa cells (Magoffin, 2006). Increased adrenal activity causes insulin phosphorylation, which causes insulin resistance, affecting approximately 50% -70% of women (Pate & Sirmans, 2014). Insulin resistance increases the production of *Reactive Oxygen Species* (*ROS*) in circulating adipocytes. Increased *ROS* in adipocytes can disrupt the balance between reduction and oxidation reactions, causing *oxidative stress* which can damage cell membranes. The degree of lipid peroxidation was determined using the MDA marker parameters. The treatments available in the market for patients with PCOS include birth control pills, fertility drugs, and diabetes medication (Kashani & Akhondzadeh, 2016). The goal of treatment is to improve ovulation and reduce androgen levels. One of the selective estrogen receptor modulators used in PCOS patients is clomiphene citrate, but it is not suitable for long-term use (Dewi, 2020).

Amelia *et al*. (2017) and Wulandari *et al*. (2017) were shown to increase body weight and MDA levels of the PCOS-insulin resistance model rats induced by testosterone propionate, and moringa leaf extract was able to reduce body weight, MDA, and increase the number of follicles, related to morphometry (length, width, and weight). Ovaries) after administration of moringa leaf extract to mice at doses of 300, 400, and 500 mg/kg BW affected the morphometry of mice (Balumbi *et al*., 2021). Moringa leaves are known in Indonesia as "*the miracle tree*" because they are natural sources of nutrition and medicine, and the secondary metabolites that play an active role are flavonoids (quercetin) (Kementerian Kesehatan RI, 2017). This compound has an -OH group attached to a benzene ring, which allows it to act as an antidote to *ROS*, reduces metal ions, modulates protein phosphorylation (associated with inhibition of enzyme activity), and can inhibit lipid peroxidation, which is mostly found in flavonoid compounds such as quercetin (Halliwell & Gutteridge, 2015). The aim of this study was to evaluate the protective effects of *Moringa oleifera* leaf extract and its fractions against testosterone-induced PCOS in female *Rattus norvegicus*.

MATERIALS AND METHODS

Materials

The materials used in this study were 10% formalin and Bouin's buffer formalin solution, acetic acid (CH_3COOH) (Marck), and acetone (PT. Brataco), ammonia (NH_3) (Marck), ammonium hydroxide (NH_4OH) (Marck), and aquades (PT. Brataco), blank paper, and clomiphene citrate ($C_{26}H_{28}ClNO.C_6H_8O_7$) (PT. Kimia Farma) and chloroform ($CHCl_3$) (PT. Brataco), dragendorff reagent, ethanol (C_2H_6O) (PT. Brataco), and ethyl acetate ($C_4H_8O_2$) (PT. Brataco), ethyl acetate fraction of moringa leaf extract, ferric chloride ($FeCl_3$), formic acid (HCOOH), gallic acid (Sigma), *glucose test reagent kit*, *Hematoxylin-Eosin* (HE) staining solution, Lieberman Burchard reagent, and methanol (CH_3OH) (PT. Brataco), and moringa leaf powder were obtained from PT. Moringa Organik Indonesia (MOI), n-butanol (Marck), and n-hexane (C_6H_{14}) (PT. Brataco), n-hexane fraction of moringa leaf extract, silica gel 60 F_{254} TLC plates (Marck KGaA, Darmstadt, Germany), piperine (Sigma), quercetin (Sigma), Sodium Carboxymethyl Cellulose (Na-CMC) (Sigma-Aldrich), stigmasterol (Sigma), *TBARS assay kit*, testosterone (Wonder brand, PT. Wonderindo Phrmatama, Jakarta, Indonesia), female rats (*Rattus norvegicus*) obtained from the Center for Food and Nutrition Studies, Universitas Gadjah Mada, and water fraction of Moringa leaf extract.

Tools

The tools used in this research were an analytical balance (OHAUS PJ1003), *centrifuge* (D-37520), chamber, Eppendorf tube (OneMed), dissecting set,

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glass slides and coverslips, glass tools (Vyrex), micropipette (Dragonlab), microhematocrit (Nris-Vitrex Medical), Olympus CX23 light microscope, plain tube (OneMed), *rotary evaporator* (Laborota 4000), *Sep-Pak* C_{18} column, syringe (OneMed), and UV-Vis spectrophotometer (Shimatzu 1800).

Method

Animals

This study used 2-month-old female (the estrus cycle is observed by visual observation methods on external genitalia, such as the presence of vaginal mucus) white rats (*Rattus norvegicus*) weighing 160-200 grams, given pellets and water *ad libitum*. After acclimatization for seven days, the rats were divided into seven groups (n=5). Normal control group (CG), negative control (Na-CMC 1%) (CN), positive control (clomiphene citrate) (CP), moringa leaf extract (500 mg/kg BW) (EM), n-hexane fraction (21.04 mg/kg BW) (FN), ethyl acetate fraction (34.47 mg/kg BW) (FE), and the water fraction (444.49 mg/kg BW) (FW). Modeling uses the hormone testosterone, administered intramuscularly at a dose of 2 mg/200 g BW, volume 2 for 28 days, and giving a 14-day sample. All procedures have been described and approved by the Medical/Health Research Bioethics Commission, Faculty of Medicine, Sultan Agung Islamic University Semarang, Indonesia (350/IX/2022/Komisi Bioetik).

Extraction

Moringa oleifera leaf powder was obtained from the PT. Moringa Organik Indonesia (Jakarta, Indonesia) (SIG.LHP.II.2022.221405011). Fine powdered moringa leaves (750 g) were added to 70% ethanol at a ratio of 1:10 g/v, and then placed into the maceration vessel. Simplisia was soaked for the first 6 h while stirring occasionally and then allowed to stand for 18 h. The macerate was separated by filtration, and the remaceration process was repeated once using the type and amount of solvent, which was half the volume of solvent in the first solvent. All macerates were collected and evaporated using a rotary evaporator to obtain thick extracts. The percentage yield (w/w) was calculated. The yield must be at least as specified in each monograph (Kementerian Kesehatan RI, 2017).

Extract characterization

Organoleptic

This was done macroscopically by examining the shape, color, and smell of the Moringa leaf extract.

Determination of water content

Water content was determined using the azeotropy method (*Toluene Distillation*) with a sterling-bidwell apparatus, which was strung, and then 10 g of Moringa leaf extract was weighed and placed into a dry pumpkin. Next, 100 mL of water-saturated toluene was added to the flask, boiled over low heat so that no water drips, and the water flowed during the process. The results of the water content are recorded by looking at the volume on the tool scale, the water content is calculated in % b/v.

Determination of total ash content

Accurately weigh 2-3 grams of the test material, which has been mashed and placed in a silicate crucible that has been heated and tarred, slowly heated until the charcoal runs out, cooled, and weighed (Kementerian Kesehatan RI, 2017).

Determination of acid insoluble ash content

The ash obtained in the determination of the total ash content was boiled with 25 mL of dilute hydrochloric acid for 5 min. The parts that were not soluble in acid were collected, filtered through ash-free filter paper, washed with hot water, and heated in a crucible until the weight remained at ±600° C. The acid-insoluble ash content was calculated against the weight of the test material and expressed as % w/w (Kementerian Kesehatan RI, 2017).

Fractionation

The fraction was prepared by weighing 10 g of condensed extract and dissolved in ethanol and water at a ratio (1:9), prepared in 25 mL, which was then separated using a separating funnel with two parts of an immiscible solvent, n-hexane. The n-hexane and water parts were removed. The aqueous fraction was fractionated using an equal amount of ethyl acetate (1:1). The water portion was set aside and the ethyl acetate portion was withdrawn. Fractionate 25 mL of each solvent and repeat the process until the solution becomes clear.

Identification of the chemical content of the fraction using Thin Layer Chromatography (TLC)

Phyto components of flavonoids, tannins, alkaloids, saponins, and steroids were identified using TLC and observed at UV wavelengths of 254 and 366 nm. Flavonoid compounds were compared using a mobile phase of chloroform:acetone:formic acid (7:3:0.4), with quercetin as a comparison. The spots were then sprayed with the citroborate reagent in an oven at 100 °C for 5 min. The presence of flavonoids is indicated by green spots, and under visible light, it shows a yellow fluorescent spot. Tannin compounds were prepared using the mobile phase n-butanol:acetic acid:water (4:1:5), with gallic acid used for comparison. The spots were observed under UV light at 366 nm by spraying $FeCl_3$. The presence of tannins is indicated by the presence of brown or black spots. Alkaloid compounds

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were prepared using a mobile phase of methanol:ammonium hydroxide (100:3), with piperine as a comparison. Appearance of spots used by spraying Dragendorff reagent. The presence of alkaloids was indicated by orange-red spots. Saponin compounds were detected using the mobile phase of chloroform:methanol:water (13:7:2), and spots were detected by spraying 10% H_2SO_4. The presence of saponin compounds is indicated by purple spots. Steroid compounds using the mobile phase n-hexane:ethyl acetate (18:2), with stigmasterol as a comparison, were sprayed with Liebermann Buchard reagent. The presence of steroid compounds is indicated by blue spots.

Analysis of rat glucose levels

Measurement of fasting blood glucose levels using the GOD-PAP method, namely a blood sample taken 10 µL with 1000 µL of reagent, mixed, and incubated at 20-25℃ or 37 °C for 20 min. The intensity was measured using a spectrophotometer at a wavelength of 500 nm.

GOD-PAP $\frac{mg}{dL}$: $\frac{Sample}{Standard}$ standard × standard concentration (100 mg/dL).

Examination of *malondialdehyde* levels

Collection of Blood Specimens Weighing was done before taking Blood samples (1.5 mL) were weighed using a syringe, placed into a plain tube containing EDTA, and then centrifuged at 4000 rpm for 10 min. The liquid blood plasma separated from the solid part of the blood was transferred to an empty tube. The MDA blood test followed the method in *Indonesian food and nutrition progress, 2000 Vol. 7 no. 2*. Prepare a Standard solutions were prepared as follows:50 µL of *1,1,3,3-tetrametoxypropane* (TEP) solution, 450 µL of aquabides, 750 µL of H_3PO_4, and 250 µL of *thiobarbituric acid* (TBA). Plasma MDA levels are expressed as nmol/mL. A total of 750 µL H_3PO_4 was put into a polypropylene tube containing 250 µL TBA, 50 µL plasma was put into the tube, and 450 µL of aquabides was added. Mix in a vortex mixer for 2 min. The mixture was then placed in a water bath at 100C° for 60 min. Then, it for 1-2 hours until the temperature reached 30 °C. When the solution was cold, it proceeded to the purification stage. The method for making blanks was the same as for making samples, except that samples and standards were not used, and 50 µL of aquabides were used. MDA purification. *The Sep-Pak C18* column was prepared, rinsed with 5 mL methanol, discarded, and rinsed again with 5 mL aquabides (thrown away). The sample was then rinsed with 4 mL of aquabides (discarded), followed by elution with methanol, and the elution results were collected and placed in a cuvette. *TBARS* levels were measured using a spectrophotometer at a wavelength of 532 nm.

This study used descriptive analysis of patient demographic data and cost components. Mann-Whitney and multiple linear regression tests were used to examine the factors affecting the hospital's real costs to see the difference between the real costs and the cost of INA-CBG inpatient schizophrenia.

TBARS $\frac{nmol}{mL}$: $\frac{\Delta\, sample - a}{b}$

where Δ sample is the absorbance of the sample, a is the intercept of the regression coefficient and b is the slope of the regression constant.

Histological analysis

Ovarian specimen collection, mice were sacrificed using the cervical dislocation technique (neck dislocation), and then placed on a surgical board with pins, beginning with abdominal dissection with curved scissors. The ovarian organs were removed and separated with straight scissors, the ovarian organs were washed with distilled water until no blood was left, and then washed with 0.9% NaCl. The organs were placed on filter paper and then weighed with the organs placed in dry petri dishes, after which the weight was recorded (Amelia *et al*., 2017). For counting the number of follicles**,** tissue ovaries were fixed in 10% formalin in phosphate buffered saline (PBS), pH 7.4. Histological examination of ovaries from all groups was performed using standardized histological methods. Sections of 5 µm thickness were cut into paraffin-embedded blocks and stained with (hetoxylin and eosin HE (*Hematoxylin-Eosin*). Tissue observation was performed using an Olympus microscope to observe the real picture of the tissue and microphotography (4x magnification).

Statistical analysis

The data obtained were analyzed statistically using SPSS software version 25.0. The Shapiro-Wilk test was performed to determine whether the data were normally distributed. If the data were normally distributed, a one-way ANOVA statistical test was used. However, if there are data/groups of data that are not normally distributed or homogeneous, the non-parametric Kruskal-Wallis test will be used. This study was considered significant if the analytic variable was $p<0.05$.

RESULTS AND DISCUSSION

The calculation of the average ethanol extract yield of moringa leaves was 30.4%, which is not less than 9.2% and meets the requirements for extract production. The characterization of the extract included the following.

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Organoleptic

The extract obtained was characterized by a thick, green-brown color with a distinctive odor.

Determination of water content

The moisture content in the moringa leaf extract was 2.7%, indicating compliance with the requirements of the Indonesian Herbal Pharmacopoeia (FHI), which stipulates that it should not exceed 10.0%. High moisture content (>10.0%) can affect the stability of the extract because water is a medium for microbial growth.

Determination of acid insoluble ash content

The percentage obtained was 7.87%, indicating compliance with the FHI, which stipulated that it should not exceed 9.0%.

Determination of acid insoluble ash content

The percentage obtained was 0.98%, indicating a lower value than the total ash content because only certain minerals or metals that are insoluble in acid were present.

Fractionation

The percentage yield of each fraction of moringa leaf extract had the highest weight for the water fraction at 85.59%, followed by the ethyl acetate fraction at 6.64%, and the n-hexane fraction at 4.05%. Different compounds have different partition coefficients; therefore, if one compound is polar, its relative partition coefficient to the polar phase will be higher than that of non-polar compounds (Najib, 2018).

Identification of the chemical content of the fraction using Thin Layer Chromatography (TLC)

Identifying the secondary metabolite content using TLC showed that moringa leaves contain chemical compounds, including flavonoids, tannins, alkaloids, saponins, and steroids (Table 1). This is supported by previous research stating that moringa leaves contain a wide variety of chemical compounds with various methods of identification and analysis.

Table 1. Identifying the scondary metabolite contents of moringa leaves

Chemical Contents (Spray Agent)	Sample				Description
	Extract	n-hexane Fraction	Ethyl Acetate Fraction	Water Fraction	
Flavonoids (Citroborate)	(-)	(-)	(+)	(-)	Yellow
Tannins ($FeCl_3$)	(+)	(-)	(+)	(+)	Black
Alkaloids (dragendroff)	(-)	(-)	(+)	(-)	Reddish-orange
Saponins (H_2SO_4 10%)	(-)	(-)	(+)	(-)	Purple
Steroids (Liebermann Buchard)	(-)	(+)	(-)	(-)	Blue

Information: positive (+), negative (-)

Table 2. The levels of *malondialdehyde* in female rats with PCOS-insulin resistance model and administration of extract samples and fractionated extract samples of moringa leaves

Groups	Levels of *malondialdehyde* (nmol/mL)	
	D-28 (PCOS-Insulin Resistance)	D-42 (Sample Administration for 14 Days)
Normal Control	1.623±0.328^{bc}	1.635±1.108^{b}
Negative Control	8.214±0.173^{a}	8.277±0.178^{ac}
Positive Control	8.629±0.299^{a}	1.950±0.269^{b}
Extract	8.528±0.340^{a}	2.704±0.117^{abc}
Water Fraction	8.528±0.300^{a}	3.547±0.274^{abc}
Ethyl Acetate Fraction	8.227±0.108^{a}	1.685±0.219^{b}
n-hexane Fraction	8.390±0.134^{a}	5.308±0.259^{abc}

^{a}vs Normal Control ($p<0.05$), ^{b}vs. Negative Control ($p<0.05$), ^{c}vs Positive Control ($p<0.05$)

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Table 3. The weight of the right and left ovaries after administration of extract samples and fractionated extract samples of moringa leaves

Groups	Ovary Weight (gram)	
	Right	Left
Normal Control	0.17±0.01[b]	0.17±0.01[b]
Negative Control	0.12±0.01[ac]	0.12±0.01[ac]
Positive Control	0.17±0.01[b]	0.17±0.01[b]
Extract	0.15±0.01[abc]	0.15±0.01[abc]
Water Fraction	0.15±0.01[abc]	0.15±0.01[abc]
Ethyl Acetate Fraction	0.16±0.01[b]	0.16±0.01[b]
n-hexane Fraction	0.14±0.01[abc]	0.14±0.01[ac]

[a]vs Normal Control ($p<0.05$), [b]vs. Negative Control ($p<0.05$), [c]vs Positive Control ($p<0.05$)

Table 4. Number of histopathological ovarian follicles

Groups	Number of Follicles			
	Primary	Secondary	Tertiary	Corpus Luteum
Normal Control	3±0.8	6±3.1[b]	4±1.2[b]	15±6.0[b]
Negative Control	6±1.6	16±2.6[a]	0±0.0[ac]	1±0.8[ac]
Positive Control	3±0.8	10±1.6[b]	5±1.6[b]	15±2.1[b]
Extract	3±1.7	11±1.6[b]	2±0.5[b]	10±5.4[b]
Water Fraction	4±2.2	13±1.7[b]	2±0.8[b]	10±2.9[b]
Ethyl Acetate Fraction	3±0.8	10±3.6[b]	3±0.9[b]	13±4.6[b]
n-hexane Fraction	4±1.7	12±1.6[b]	1±0.8[ac]	8±2.4[b]

[a]vs Normal Control ($p<0.05$), [b]vs. Negative Control ($p<0.05$), [c]vs Positive Control ($p<0.05$)

The prevalence of schizophrenia in this study, which is domiciled in rural areas, was 95 patients (84.82%) and 17 patients (15.18%) in urban areas. According to data from Kementrian Kesehatan RI (2019), the prevalence of schizophrenia in Indonesia based on residence is more significant in rural areas $7^{o}/_{oo}$ than in urban areas $6.4^{o}/_{oo}$. This contrasts with the population density theory, which states that schizophrenia is higher in areas with high population densities, such as cities. Population density is a factor that influences the occurrence of mental disorders, including psychosis (Sadock *et al.*, 2014). Based on Table 1, the highest class of treatment-experienced schizophrenia, namely class 3, with 91 patients (81.25%), showed that most patients with schizophrenia in Tombulilato Hospital came from middle to lower economic classes.

Rat body weight

The average values are shown in Figure 1. of the 28-day model, based on statistical analysis, showed that the data were normally and homogeneously distributed ($p>0.05$). *Tukey's* ANOVA test showed a significant difference between the normal group and the negative and positive groups ($P <0.05$). This is because post-insulin receptors work on tyrosine phosphorylation, but what happens is serine phosphorylation occurs, which subsequently affects glucose transport to muscle or fat cells, leading to changes in body fat distribution and pushing visceral fat accumulation. This leads to insulin resistance. Supported by most cases of overweight and lean PCOS, women showing insulin resistance (Tabrizi *et al.*, 2020). Insulin resistance results in disturbances in insulin signal transduction involving two main pathways: *Phosphatidylinositol 3 Kinase* (PI3K) and *p38 Mitogen-Activated Protein Kinase* (MAPK) (Kusumastuty *et al.*, 2013). Administration of moringa leaf samples for 14 days showed statistically normal and homogenous data analysis, and *Tukey's* ANOVA test showed no significant difference for all sample groups compared to the normal group. This was due to the presence of compounds in the extract, water fraction, ethyl acetate fraction, and n-hexane fraction, in accordance with the results of compound identification. Weight change in insulin-resistant PCOS patients in this study was a contributing factor. Hong *et al.* (2018) reported that the administration of flavonoids (quercetin) prevented weight gain and caused significant weight loss in PCOS rats, whereas other compounds with positive results in compound identification include tannins, alkaloids, saponins, and steroids. Further characteristics of PCOS include blood glucose measurement on the same day as weight measurement.

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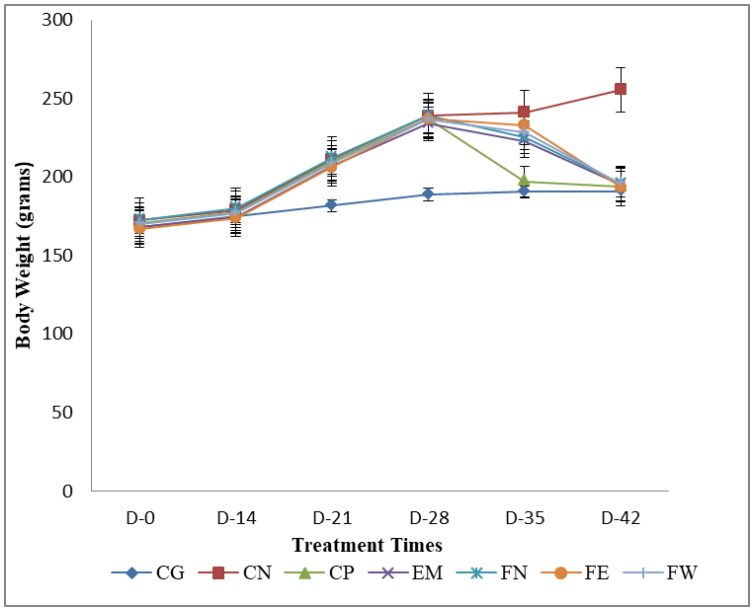

Figure 1. The results of the weight test on rats during 28 days of testosterone induction (PCOS-insulin resistance) and sample administration for 14 days (day 42). The values are represented as mean±SD

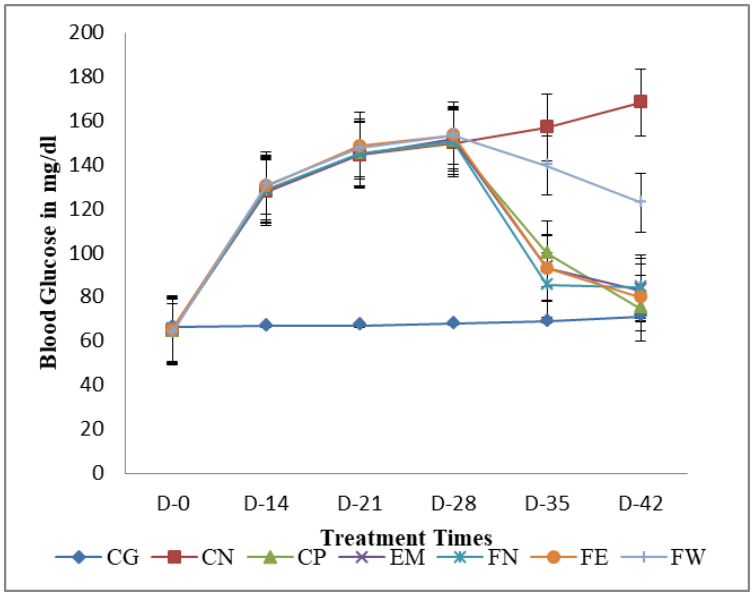

Figure 2. The results of he fasting glucose levels of rats during 28 days of testosterone induction (PCOS-insulin resistance) and sample administration for 14 days (day 42). The values are represented as mean±SD

Blood glucose measurement

The measurement of rat GDP levels after 28 days of modeling yielded GDP levels between 67.78-153.43 mg/dL. Statistical analysis showed normal and homogeneous data, followed by *Tukey*'s ANOVA test, which revealed a significant difference between the negative, positive, and normal groups ($p<0.05$). Insulin resistance in the PCOS animal model plays a major role in PCOS pathogenesis, as evidenced by the study by Wang *et al.* (2017) who showed a significant decrease in the expression of *Insulin Receptor Substrate-2* (IRS-2) protein in the ovaries of PCOS rats compared to normal control rats, which is consistent with previous research indicating that decreased expression of IRS-2 can cause insulin resistance in PCOS. IRS-2 binds to the SH2 domain of the regulatory subunit p85α of PI3K. A decrease in PI3K activity has been observed in adipose tissue, which can cause insulin resistance. P85α plays an important role and is a crucial component in insulin signaling transduction; therefore, decreased expression of p85α can cause decreased PI3K activity, which in turn inhibits most insulin metabolic functions (Figure 2).

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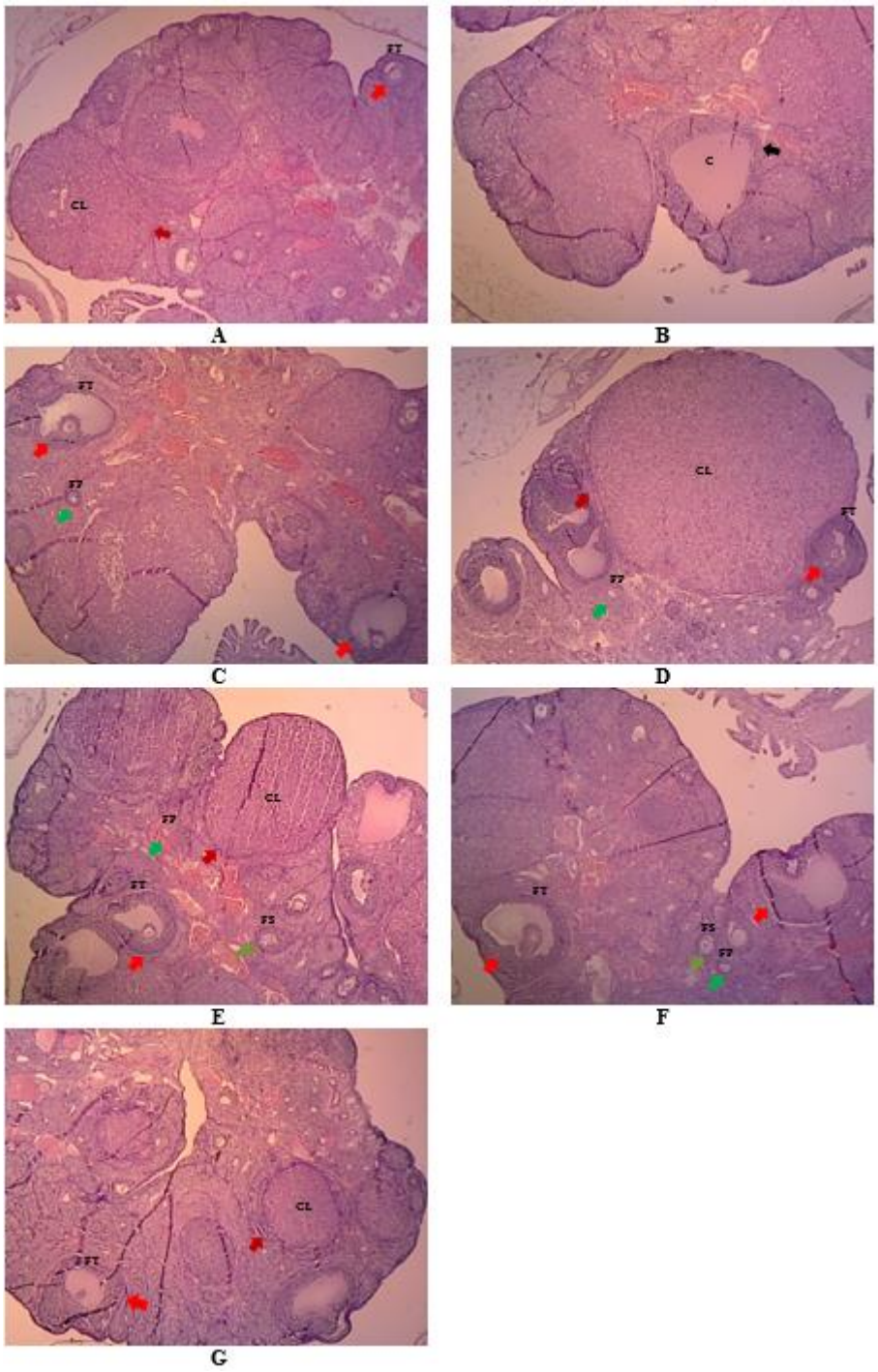

Figure 3. Ovarian histopathology of all groups. CG (A), CN (B), CP (C), EM (D), FW (E), FE (F), and FN (G). FP: Follicles Primary, FS: Follicles Secondary, FT: Follicles Tertiary, CL: Corpus Luteum

The administration of samples for 14 days showed a decrease due to the positive presence of flavonoids (quercetin) in the ethyl acetate fraction sample. In the extract and water fraction, there were positive compounds of tannins (phenolics) with antidiabetic effects similar to flavonoid compounds, namely insulin-like effects or insulin secretion stimulation, and the possibility of modulating carbohydrate metabolism enzyme function (Megawati *et al.*, 2021). One flavonoid compound with antidiabetic properties, and high glucose levels in insulin-resistant PCOS rats can maintain glucose homeostasis. In the ethyl acetate fraction, the presence of flavonoid compounds resulted in a higher percentage of decrease than in the other sample groups. Rezvan *et al.* (2017) reported that flavonoids (quercetin) stimulate glucose uptake by activating independent and AMPK-dependent pathways to increase *Glucose Transporter-4* (GLUT-4),

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subsequently decreasing enzyme regulation for gluconeogenesis and protecting pancreatic β-cell function, as well as increasing the expression of the estrogen receptor alpha (ER α) gene.

Measurement of *malondialdehyde* (MDA) level

As shown in Table 2, the negative control treatment had an average of 8.695 nmol/mL, which was the highest concentration compared with the normal control group. Based on the statistical analysis of Shapiro-Wilk normality, the treatment of samples on test animals for 28 days was normally distributed ($p>0.05$) but not homogeneous ($p<0.05$). Therefore, *Dunnett's T3* test was conducted, which showed a significant difference between the normal group and the negative, positive, extract, water, ethyl acetate, and n-hexane fractions. This indicates that the modeling of PCOS-insulin resistance was successful owing to an increase in MDA, which was used as an OS indicator. Statistical analysis was conducted 14 days after sample administration, and the data showed normality and homogeneity ($p>0.05$). The *Tukey* test showed a significant difference ($p<0.05$) in the extract sample, water fraction, n-hexane fraction, and ethyl acetate fraction groups, whereas the ethyl acetate fraction sample was not significantly different from the normal group ($p>0.05$), with an average value of 1.685 nmol/mL for the ethyl acetate fraction and 1.635 nmol/mL for the normal group. The ethyl acetate fraction showed positive identification of flavonoids, namely quercetin, which can reduce MDA levels to levels closer to normal. Flavonoids exert antioxidant effects by transferring H^+ atoms and forming *ROS*, which can be achieved by inhibiting the activity of xanthine oxidase enzymes, *Nicotinamide Adenine Dinucleotide Phosphate* (NADPH) oxidase, and chelating metals (Fe^{2+} and Cu^{2+}), thereby preventing redox reactions that can produce free radicals. This mechanism is similar to that of phenolic compounds, which also have reducing activity in the extract and water fractions. Flavonoids, such as superoxide, peroxyl radicals, and peroxynitrite, are the most effective scavengers of reactive species (Akhlaghi & Bandy, 2009). Based on the statistical analysis in Table 3, the data showed normality and homogeneity ($p>0.05$), and the ANOVA *Tukey* test showed a significant difference between the normal and negative groups ($p<0.05$), whereas the ethyl acetate fraction sample group, extract, water fraction, and n-hexane fraction showed a significant difference ($p<0.05$).

Histopathology

The number of follicles in the ovaries of rats affected the weight of the ovaries, as an increase or decrease in weight depended on the number of mature follicles (tertiary follicles), which contained a fluid-filled sac (antrum) that added weight to the ovaries. For the number of rat follicles in the PCOS insulin resistance model shown in Table 4 and Figure 3, data for primary, secondary, and corpus luteum follicles were obtained using statistical analysis, and the data were normally distributed and homogeneous ($p>0.05$). However, data for tertiary follicles were not normally distributed, so the *Kruskal-wallis* statistic was conducted, and the results showed a significant difference between the normal and negative groups ($p<0.05$). There were few tertiary follicles, as ovulation in rats occurred for only a few hours (Mardika *et al.*, 2018). The ovarian histopathology is shown in Figure 3 The diameter of the mature follicles (tertiary follicles) ranged from 0.12-0,70 mm.

The results showed that the ethyl acetate fraction group had follicle numbers that were not significantly different from the positive control group, while the n-hexane fraction group showed differences. The compound content of each fraction may significantly influence the number of follicles. As shown in Table 1, the ethyl acetate fraction contained flavonoid group compounds and the water fraction contained phenolic compounds. The activity that affects the number of follicles is influenced by compounds classified as phytoestrogens, which have two OH groups, similar to those found in estrogen hormones in the body. Flavonoids have a structure similar to 17-α estradiol, which can directly bind to estrogen receptors, thereby restoring development levels and reproductive processes, whereas steroids cannot bind directly and require synthesis (Russell *et al.*, 2000). According to FHI, the marker compound in the moringa leaf is quercetin, and the mechanism of quercetin for treating insulin-resistant PCOS can reduce testosterone levels and reverse low levels of estradiol and progesterone to levels approaching normal. Previous research has also reported that luteum causing estrus cycle recovery. After flavonoid (quercetin) administration, there was a decrease in 17β HSD steroidogenic enzyme activity, a decrease in testosterone concentration, and an increase in estradiol, which occurred because quercetin contains phenolic rings B and flavonoids have been reported to inhibit 17β HSD activity and reduce MDA activity, which acts as an anti-oxidant agent by its ability to

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inhibit xanthine oxidase through free radical reduction, antioxidant modification, and lipid peroxidation inhibition that can restore ovarian structure.

CONCLUSION

Testosterone induces changes that lead to PCOS pathogenesis. Treatment with moringa leaf extract samples and fraction extracts reduced PCOS-insulin resistance characteristics, with the highest decrease in activity observed in the ethyl acetate fraction group because it had the highest decrease in PCOS characteristics. Testosterone induction does not optimally lead to PCOS-insulin resistance pathogenesis; therefore, further research is needed on the timing and doses used in insulin-resistant PCOS rat models and determining LH, FSH, and testosterone levels to determine the levels that can cause PCOS-insulin resistance.

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AUTHOR CONTRIBUTIONS

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

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Perception of Hospital Pharmacist on Working Performance in Yogyakarta Province, Indonesia

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Abstract

***Background**: Pharmacists support the success of hospital in efforts to improve people's quality of life then the performance measurement of pharmacists is carried out**. Objectives**: This study aimed to measure pharmacist performance based on the perception of pharmacists working in hospitals. **Methods**: Perception surveys were conducted with pharmacists working in public hospitals. The assessment was performed using a closed questionnaire that was proven to be valid and reliable. This study evaluated pharmacists' perceptions of their work performance and their ability to perform their roles, duties, and functions in the hospital. A total of 192 pharmacist respondents answered 61 statements in the questionnaire that were divided into 11 dimensions as follows:1) the objectives set; 2) following the procedure; 3) initiatives; 4) performing the main task; 5) the ability to cooperate; 6) out implementing pharmaceutical standards; 7) the potential for solving problems; 8) quick response; 9) self-competence; 10) the ability to take verbal orders and writing; and 11) endurance at work. **Results**: The various answers of respondents to the questionnaire led to the conclusion that pharmacists' performance in hospitals is included in the high-performance category. **Conclusion**: This study showed that pharmacists have a high perception of their ability to work, as outlined in their assessment of their work performance in hospitals. Pharmacists' perceptions of their workplace performance in public hospitals are useful for developing pharmaceutical services. The results of this study are expected to provide a basis for improving the performance of pharmacists working in hospitals, especially hospitals in the Yogyakarta area.*

***Keywords**: performance, perception, pharmacist*

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INTRODUCTION

Pharmaceutical care is a form of service and direct responsibility of the in pharmaceutical work to improve patients' quality of life and distribution services of drugs and medical devices. To provide patient care services, pharmacists should have prior knowledge of the disease, drug therapy, non-drug therapy, laboratory and diagnostic systems, communication skills, patient monitoring skills, physical assessment skills, drug information skills, and therapeutic planning skills.

The importance of the pharmacist profession for the community is due to their ability and encyclopedic knowledge of medicine that makes them an essential part of the health care provider team. In some cases, pharmacists describe themselves as trained personnel in all matters regarding medicine. An interesting quote about the role of pharmacists has been published in Medicine Hot News: "Pharmacists are an important profession because they are trusted as health care professionals with a long history of cooperating in health care provider teams. Some patients believe that the pharmacist's ability is caused by their education, which makes them a pharmacist". This quote assumes that a pharmacist refers to the role of prescription in the collaboration context (Schindel & Given, 2013).

The hospital is one of the health facilities where pharmacists can work. Pharmacists have a considerable contribution to the hospital to support the hospital goal of providing optimal health services. The development of hospital management, both in terms of management and operational aspects, is very influenced by various demands from the environment. The hospital's success in health services provider can be measured by the hospital's performance benchmarks with all elements in it, including pharmacists who work in the hospital pharmacy installation section. This study is supported by the theory that every employee in an organization is required to make a positive contribution through good performance, considering that organizational performance depends on the performance of its employees. Also supported that the quality of service experience depends entirely upon the quality of interaction that takes place between a customer and the frontline employee. Thus, to ensure frontline employees' service performance asdesired by the customers and management is both significant and equally challenging. Considering the unprecedented growth of service sector and neck to neck competition amongst the serviceproviders, service managers have necessarily to pay greater attention to all the antecedents those improve front line employees' service performance (Mushtaq, 2015) .

Performance appraisal is a process of employee performance control that is evaluated based on certain standards carried out effectively to direct behaviour to produce high-quality services. Also, performance appraisal aims to motivate employees to carry out their duties and realize organizational goals. Performance appraisal is used to improve work performance, compensate for adjustments, and need for development, and see irregularities or errors at work (Yong *et al.*, 2020). These benefits require that performance appraisals provide an accurate and objective picture of employee work performance (Mangkunegara, 2010). In principle, a pharmacist's performance appraisal should reveal the quality of his work, the quantity of in a specific time, timeliness of work completion, the utilization of the resources, independence both individually or in teamwork, commitment to the organization, as well as the responsibility for what he has done (Flynn *et al*., 2015).

There have many types of research on the work performance of nurses (Hafizurrachman, 2011) and doctors (Hendrartini, 2011). Thus, the researchers are interested in conducting pharmacist performance assessments in hospitals based on their perception using a questionnaire instrument. Hafizurrahman carried out nurse performance measurement, Hendrartini performed physician performance, and was also carried out by several other researchers including Martin *et al.* (2020) who researched on financial performance, Chong *et al.* (2018) community pharmacist performance evaluations capture the role of modern pharmacists, mapping the competencies assessed in the Canadian community pharmacy performance evaluation template against the preparation of the General Level, Nagase reveals that a pharmacy graduate must have the ability to analyze and evaluate (Nagase, 2016).

MATERIALS AND METHODS

Study design

This study was designed using quantitative data collection methods through a questionnaire instrument filled out by pharmacists who worked in hospitals as respondents. The inclusion criteria are pharmacists who have worked in hospitals for more than two years, because it is considered two years for pharmacists to understand procedures and regulations in hospital organizations and have the ability to analyze things that are deemed to affect their performance as pharmacists and are willing to become respondents. The exclusion

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criteria were pharmacists who worked in hospitals but were not willing to be respondents or were on leave.

Research instrument

This research used a closed questionnaire as the research instrument distributed to each pharmacist to collect their responses to each statement. The statements in the questionnaire were obtained based on research about the doctors' performance (Hendrartini, 2011), research about the nurses' performance in hospitals (Hafizurrachman, 2011), and reference from performance measurement regarding Performance Aspects of Measurement. Each item of the questionnaire statement is relevant to the roles, tasks, and responsibilities of pharmacists based on Government regulations number 51 in 2009 concerning Pharmaceutical Work. Each statement in the questionnaire was given a score of 0-5. The positive statements were given a score of 5 (strongly agree), while the negative statements were given a score of 0 (strongly disagree).

There are a total of 61 statements in the questionnaire divided into 11 dimensions as follows: 1) 5 statements about the objectives set; 2) 5 statements about following the procedure; 3) 4 statements about initiatives at work; 4) 10 statements about doing the main task; 5) 5 statements about the ability to cooperate; 6) 4 statements about implementing pharmaceutical standards at the hospital, 7) 5 statements about the potential for solving problems, 8) 8 statements about the quick response, 9) 5 statements about self-competence and dynamic strengths, 10) 5 statements about the ability to take verbal orders and writing, and 11) 5 statements regarding stamina and endurance at work. The questionnaire used in this study was tested for face validity and content validity before collecting data from respondents.

The indicators that are subject to the statements in the questionnaire are categorized into 11 indicators, namely:

Setting targets

The pharmacist's perception of the set target is answered by filling in a statement that reveals indicators that they performed his work at the Pharmacy Installation in the hospital following the target, goal, instruction and priority scale of the pharmacist (Lau *et al*., 2007; Hendrartini, 2011; Hafizurrachman, 2011).

Following the procedure

Following the procedure is the second indicator in the performance appraisal. Respondents assume they have done various things related to their work according to agreed procedures. The sub-indicator statements to answer this section are: a) Every time they receive a prescription, the pharmacist filled out the prescription in an orderly and organized manner (Durfee, 2012); b) They have followed the Standard Operation Procedures prepared and made by hospitals; c) To carry out administrative tasks (reports), the pharmacist submitted the report on time; d) the pharmacists do all their work following the existing Standard Operation Procedure; e) Pharmacists require the Standard Operation Procedure in carrying out the task with the occasional permission to ignore the Standard Operation Procedure (Fernandes *et al.*, 2015).

Initiative at work

Initiative at work is the third indicator in the performance appraisal. The researcher intends to show that pharmacists have initiatives at work by giving a list of statements to respondents to do self-assessments about their ability to take initiatives in their work. The sub-indicators in the statement to answer are: a) Pharmacists have initiatives in their work; b) without being instructed, pharmacists can carry out their duties and administration properly; c) when they do not have many customers to serve or have completed administrative tasks, they should do other tasks to support the work in the hospital pharmacy; d) the pharmacists do the work according to the direction of the employer; e) they do not wait for orders from the superiors (head of the service or head of the warehouse) to carry out an emergency task (Hafizurrachman, 2011).

Doing the main tasks

Pharmacists are required to do their main duties and responsibilities at work at the hospital Pharmacy Installation. The sub-indicators in the statement to assess are: a) The pharmacists' work performance of the main tasks is good; b) Pharmacy implementation by pharmacists is good; c) the implementation of administrative tasks is good; d) they always carry out basic tasks in pharmaceutical installations and personal tasks given by superiors; e) they are loyal to their professional oath, hospital and work; f) they commit in carrying out their work; g) they comply with the hospital rules and their work; h) they become the role model for their colleagues; i) they are highly capable of carrying out the priority of pharmaceutical work; j) they completed the main tasks (Mangkunegara, 2010; Hafizurrachman, 2011).

Team working ability

Team workis an ability required by every profession, not only for pharmacists, to support the organization in achieving its target. The sub-indicators in the statement of this section are the fact that

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pharmacists do their assessment by a) being able to work together in teams, b) having no complaints about the pharmacist's inability to cooperate, c) willing to accept partners to cooperate in carrying out pharmaceutical work, d) accepting differences of opinion in carrying out cooperation; e) promoting cooperation at work (Hafizurrachman, 2011).

Performing pharmacy standards at the hospital

Employee involvement is a concept that has increasingly been prioritized in management thinking over the past decade to improve organizational performance (Harilal & Santosh, 2014). For this reason, employees and management always make work agreements to avoid confusion regarding work priorities for employees and management. Every organization has standards for each work implementation, and every worker agrees with the organization to work based on the organizational objectives. The sub-indicators of the statements used in the assessment of this section are: a) documenting the assessment/work on prescriptions based on pharmaceutical work standards regulated by the hospital; b) documenting the monitoring of drug use in patients based on pharmaceutical work standards established by the hospital; c) documenting the management of drugs and medical devices based on pharmaceutical work standards established by the hospital; d) carrying out pharmaceutical actions based on pharmaceutical work standards established by the hospital (Seto *et al*., 2015).

Potentials to solve problems

Actually, organizations must provide a conducive environment for employees to do their jobs, because a productive and conducive environment strongly supports effective learning and development in the organization so that it supports the performance of workers under the organization (Tiwari, 2014). But, every employee in doing a job must have different potential problems even though the environment has been conducive and supportive.This section requires respondents to assess their ability to solve problems, using the following sub-indicators: a) pharmacists have the potential to solve various pharmaceutical problems; b) they have the potential to solve various pharmaceutical administration problems; c) they have the potential to solve various problems that arise in the patient-hospital relationship; d) they need to be trained to solve work environment problems with their pharmaceutical techniques (Seto *et al.*, 2015 ; Rivai, 2009).

Quick response

Pharmaceutical work in a hospital is closely related to patient safety. Therefore, pharmacists need to be able to respond quickly so that patients do not have to wait for long to have drugs or medical devices to support their medication. The sub-assessment indicators in this section are: a) the level of pharmacist responsiveness is already high in doing pharmaceutical work in the workplace; b) the response of the pharmacists is good in doing their pharmaceutical work in the workplace; c) the pharmacist's accuracy is good in doing pharmaceutical work; d) Pharmacists are quick in handling cases in their workplaces; e) they have appropriate ways in handling cases in the workplace; f) they have good skills in taking administrative actions especially for patients who make up for prescriptions; g) they have skills to be placed in warehouses, in hospitals, h) they have skills to be placed in pharmaceutical services in hospitals.

Self competence and dynamic strength

The basic characteristics of competency possessed by an individual causally associated with the criteria fulfilment of a position, and dynamic strength is the ability a worker possessesto carry carrying out the work as a whole. The sub-indicators used in the assessment of this section are: a) pharmacists have competence in the field of pharmacy (in this case is having a competency certificate issued by the Indonesian Pharmacist Association); b) they have a high level of mobility in carrying out pharmaceutical work; c) they can communicate; d) their competency is in line with the task; e) they can provide a healing spirit to patients (Lau *et al*., 2007).

The ability to take oral and written commands

The Pharmacists must be able to accept orders of both oral and written because the pharmacist's work is closely related to the results of examinations and treatment decisions from the doctor as a colleague. The sub-indicators used in the assessment of this section are; a) Pharmacists can translate written orders from doctors; b) They can translate verbal orders from doctors; c) They do not make mistakes in taking verbal instructions in pharmacy; d) They do not make a mistake in taking orders as an organization; e) they have a good understanding when given oral and written commands(Hafizurrachman, 2011).

Stamina and endurance at work

The pharmacist has a heavy workload. The indicators used in the assessment of this section are: a) When the pharmacists are generally awake at night, they are always awake (not sleeping); b) they rarely take a day-off; c) they have good stamina; d) they have good endurance; e) their stamina supports their work in the

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pharmaceutical field Hafizurrachman, 2011; Fanikos *et al*., 2014).

Data collection

During data collection, pharmacists who were willing to be involved as the research respondents were explained about the purpose of this study. Next, these pharmacists were provided with an informed consent form and a questionnaire containing statements related to their perception of the performance of the pharmacists working in the hospital.

Ethical consideration

This research was approved by the Medical and Health Research Ethics Committee of the Faculty of Medicine Gadjah Mada University, Yogyakarta, Indonesia with decree number (SK) Ref: KE/FK/1383/EC/2018.

The quantitative research on the performance measurement of pharmacists working in general hospitals in the Yogyakarta Province Indonesia is presented based on the respondents' characteristics, the distribution of answers, and the work performance. The study involved 192 pharmacists working in public hospitals located in Sleman Regency, Gunung Kidul Regency, Kulon Progo Regency, Bantul Regency, Yogyakarta City, and Yogyakarta Province as the research respondents. Descriptive statistics are used to describe the pharmacists demographic data.

Characteristics of respondents

The following table explains the distribution of respondents' characteristics who participated in the study. These consist of 9 characteristics, as described in Table 1.

RESULTS AND DISCUSSION

Table 1. Characteristics of respondents

Characteristics	N (%)
Age (years)	
25-<40	176 (91.7)
40-<50	8 (4.2)
Over 50	8 (4.2)
Gender	
Male	26 (13.5)
Female	166 (86.5)
Length of Work (years)	
1-<3	106 (55.2)
3-<6	54 (28.1)
6-<9	23 (12)
15-<12	4 (2.1)
>12	5 (2.6)
Marital Status	
Married	142 (74)
Single	50 (26)
Divorce	0
The number of dependents	
No one	69(35.9)
One	29(15.1)
Two	63 (32.8)
More than two including children and wife	31 (16.1)
Participation in Training	
Participated	107 (55.7)
Never participate	83 (44.3)
Travel time	
<45 minutes	183 (95.6)
>45 minutes	9 (4.7)
Additional work sides	
Yes	168 (87.5)
No	24 (12.5)
Mode of transportation	
On foot	1 (0.5)
Motorcycle	165 (85.9)
Car	11 (5.7)
Mix	15 (7.8)

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In this study respondents who were included in the inclusion category were divided into. In this study the respondents were divided into nine characteristics including two characteristics related to congenital elements that cannot be intervened, namely age and gender; 1 characteristic concerning education, namely education/ training; and four characteristics related to socioeconomic condition, including marital status, number of dependents, length of work, and side job; and two characteristics related to transportation, namely travel time and the means of transportation used. It is conclusive that the pharmacists working in hospitals are of productive age, which is good for developing organizations, although most re females who need higher protection.

There are several factors that influence the assessment of performance, including: 1) individual factors, i.e. ability and skill (both mental and physical), background (experiences, family, etc), and demography (age, origin, etc), 2) organizational factors, i.e. resources, leadership, reward (compensation), organizational structure, and job description, 3) psychological factors, i.e. perception, attitude, personality, learning patterns, and motivation (Gibson *et al*., 2005). Several previous studies have proved that individual characteristics and organizational support have a positive relationship with employee performance, where demographic and cultural factors determine employee performance. A study conducted by (Schaftheutle *et al*., 2011), showed that the factors that affect pharmacists' performance are mental and physical factors (demographic factor related to pharmacists' health), pharmacists' demography and education, and individual characteristics, i.e. pharmacists' performance can be influenced by several factors, including individual characteristics such as age, gender, ethnicity, workplace, workplace-related factors as well as mental and physical health. This study found some evidence showing that pharmacists with certain characteristics (for example male, a part of the ethnic minority, working in a community pharmacy, participating in training abroad) are more likely to experience performance problems. Some factors related to workload and working environment are also related to performance problems, particularly concerning error. Males are more likely to have consistent professionalism than to females, who often have discipline-related problems or are given corrective action to improve performance (Cozens, 2008). Evidence from several studies showed that male pharmacists are more likely to face disciplinary processes compared to female pharmacists in terms of facing disciplinary process. In this study, the number of male pharmacists was only 3.5% (26) compared at of female pharmacists, i.e. 86.5% (166). This indicates that the pharmacists in Yogyakarta will likely to have good or improvable performance. A study found that older pharmacists are more likely to make mistakes (Szeinbach *et al*., 2007), and two studies (one was reported in two related articles) showed that pharmacists are more likely to have problems with their performance (Austin *et al*., 2004). However, a review of Australian records showed that pharmacists who face disciplinary action have a significant difference in the possibility of disciplinary action in an age group (Penm & Chaar, 2009). In this study, most pharmacists fell in the working age group, i.e. 25-<40, amounting to 176 (91.7%). Studies the risk of dispensing errors in communities, and hospitals showed that the risk of dispensing errors decreases along with increased work experiences. This is evident from the results of research showing that pharmacists who worked at hospitals in Yogyakarta had insufficient work experience since most of them, i.e. 106 (55.2%), had less than 3 years of work experience, thus leading to a higher probability of dispending error. Therefore, organizations, in this case hospitals, need to consider to improve pharmacists' performance by providing training for pharmacists who work for them. In addition, this study also showed that 83 (44.3 %) pharmacists had never received any training for the last few years.

Differences in characteristics and attitudes affect employee performance, so it is important to understand the characteristics of employees in an organization, which is useful for the decision-making process related to improving employee performance. Individual and organizational characteristics are neither different nor separable. An employee whose career plan cannot be achieved under his/her organization will leave the organisation sooner or later. This way, it is important for an organization to assist its employees in planning their career so the two parties can achieve their needs. The career path is a flexible progression an employee who follows throughout his/her employment.

In terms of study, these pharmacists have met the qualifications, but there is great potential for increasing their educational capacity by providing them with higher education, training, or coaching to broaden their insight and improve their thinking and response skills. Regarding socioeconomic, most of them have worked for more than three years, but they still need to have better work experience. The level of pharmacist

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socioeconomic status can be described through the use of transportation mode to go to the office, which shows that they are of middle-class income. Based on these nine characteristics as described above, the researcher believes that the pharmacists working in the hospital can perform better to improve the general hospital performance in the Special Region of Yogyakarta Indonesia.

Validity and reliability test of the questionnaire

The questionnaire is a list of questions distributed to respondents involved as the research sample to be directly filled by themselves. The questionnaire in this study was distributed to determine the perception and assessment of the pharmacists' performance working in the hospital. Questionnaire measurements in this study used a Likert Scale, a scale used to measure attitudes, opinions, and perceptions of a person or group of people about social phenomena (Sugiyono, 2008). In this study, respondents were asked to answer several questions in the questionnaire by choosing one of five interval scale items (Likert Scale). Each question in the questionnaire will be scored of 0 (strongly disagree) to 5 (strongly agree).

The validity test of each item of the questionnaire statement was compared with the value of r arithmetic and r table and significance <0.05 (r table = 0.138), and the results are presented in the range 0.150-0.819 (r arithmetic) and sig range 0.000-0.038. Based on that, it is clear that all items are valid statements can be used in performance measurement. The reliability test for the questionnaire instrument distributed to respondents had sig 0.969 (reliable), and based on the reliability test, it is clear that the value of Cronbach's Alpha and the value of Cronbach's Alpha Based on Standardized Items is higher than the standard 0.60, which is 0.969. Therefore it can be concluded that the construct of each statement variable is reliable.

Performance appraisal of pharmacists in hospitals

In this study performance appraisal is arranged into a questionnaire instrument containing the pharmacist's perception of the relationship between performance and the implications for a conceptual strategy of implementation as a guidance for performance appraisal using two methods: subjective method, by comparing the results of work, nature, characteristics and behaviour, and objective method by assessing the work, achievement, and data. This performance is influenced by motivational factors (achievement, work recognition, feelings of progress and development) factors and cleanliness (supervision, interpersonal relationships, working conditions, and incentives (Fuad & Ahmad, 2009).

Performance appraisal is a way to control work performance to maintain or improve work performance through evaluation based on certain standards (Frost & Adams, 2018). Pharmacist performance appraisal was done through self-assessment of pharmacists working in hospitals by filling out questionnaires. The questionnaire responded to by respondents, the pharmacists working at the hospital, was then processed and evaluated using a standard assessment by determining the categories of performance, as outlined in Table 2.

The 11 characters above are set forth in a statement filled out by the respondent and then processed quantitatively to determine the pharmacist's performance. The score of each respondent's answer is determined first. After that, the average and standard deviation are calculated using the following formula:

$$\bar{X} = \frac{\sum X}{N}$$

$$SD = \sqrt{\frac{\sum X^2}{N} + \left(\frac{x}{N}\right)^2}$$

With:

$\bar{X}$= Average score

X = answer score

N = number of respondents

SD = standard deviation

Overall SD formula:

Minimum Score : 0

Maximum Score : 5

Average : 2.5

SD : 0,833333 ((maximum value-minimum value)/6)

Performance Category:

High $X \leqslant M + SD = X \geq 3{,}333333$

Moderate $M - SD \leqslant X < M + SD = 1{,}666667 \leqslant X < 3{,}333333$

Low $X < M + SD = X < 1{,}666667$

Mean : total score/5(number of questions)

This research was conducted using a questionnaire instrument which was collected qualitatively, the data was processed quantitatively by calculating the performance level of pharmacists working in hospitals based on the answers to statements filled out by respondents. The formula is then outlined in the pharmacist's performance calculation, as presented in Table 3

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Table 2. Standard assessment by determining the categories of performance

Formula	Performance
Score < average – standard deviation	Low
Average-Standard deviation ≥ Score < Average + Standard Deviation	Medium
Score > Average + Standard Deviation	High

Tabel 3. The pharmacist's performance calculation

Variable	Total Score	Mean	Performance Category (based on formula)	Indeks (%)
Objectives set	3661	3,81	High	76,27%
Following the procedure	3551	3,70	High	73,97%
Initiatives at work	3671	4,02	High	76,47%
Doing the main task	7583	3,96	High	78,89%
The ability to cooperate	3850	4,01	High	80,2%
Implementing pharmaceutical standards at the hospital	3016	3,93	High	78,54%
The potential for solving problems	3816	3,98	High	79,5%
Quick response for taking action	6047	3,94	High	78,73%
Self-competence and dynamic strengths	3967	4,13	High	82,64%
The ability to take verbal and non-verbal orders	3641	3,74	High	75,85%
Regarding stamina and endurance at work	3791	3,96	High	79%

Based on the description in Table 3, it is apparent that performance is measured from 11 dimensions with 61 statements. It is revealed that pharmacists' performance in hospitals is in the High category. A results of this performance appraisal are based on the answers of pharmacists' respondents who self-assess their work performance. These 11 elements are quite relevant to research conducted by Bentley *et al*. (2005) and Nelson *et al.* (2020) which considers the level of ability of pharmacists to communicate, understand questions, give trust, and be able to solve problems faced by patients.

The Pharmacists' performance is closely related to the quality and success of patient treatmen (Colombo *et al*., 2017). Therefore it is very important for organizations to consider the factors that will determine the high performance of pharmacists in a health care organization, including hospitals (Chagas *et al*., 2022). Patients perceive that pharmacists should be primarily responsible for collecting medication histories (72%), identifying (96%) and solving (98%) pharmacotherapeutic problems (Fernandes *et al.*, 2020), good communication skills (Murad *et al*., 2014), and follow the procedure (Bentley *et al*., 2005)

In an organization, performance measurement has been frequently done. This is related to the need for the management of the organization itself purposely to improve the performance of the organization through the development of work performance among the employees to obtain the good and maximum output either in quality or in quantity from the organization itself following the goal of the organization. Performance can be detected through the work motivation of the workers with the quality of service, efficiency, and fairness; all of which are mediated directly by the willingness of workers to apply themselves in their task. For a pharmacist, performance refers to the work achievement for themselves as a comparison between work and work standards to be applied by the organization (Muin *et al*., 2019). A number of research on the performance measurement for the health workers in the scope of hospitals and the Centre for Public Health have been conducted as by Hafizurrahman (Hafizurrachman, 2011) measuring the performance of nurses and Hendrartini (Hendrartini, 2011) making a model of the performance of doctors based upon capitation. This was certainly done with a similar aim that is to improve the performance of health service organization.

The research on the performance measurement was conducted based upon the pharmacists perception in assessing his performance in the hospital where he works. This research used the instrument in the form of questionnaires after conducting face validity and content validity. The questionnaires used in this research were made to measure the performance based on the perception of the pharmacist (self-assessment).

The questionnaires of the performance measurement were based on the perception of the pharmacist were formed from 11 dimensions: 1) the objectives set; 2) following the procedure; 3) initiatives at work; 4) doing the main task; 5) the ability to

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cooperate; 6) implementing pharmaceutical standards at the hospital, 7) the potential for solving problems, 8) quick response, 9) self-competence and dynamic strengths, 10) the ability to take verbal orders and writing, and 11) regarding stamina and endurance at work. These were made into 61 statements using the answer from the Likert Scale of 0-5.

In the validity test obtained a result that all variables in the questionnaire statement were compared with the value of r arithmetic and r table and significance <0.05 (r table = 0.138), and the results were presented in the range of 0.150 to 0.819 (r arithmetic) and sig range 0.000-0.038. Based on this, it is clear that all items were valid statements, so that they can be used in performance measurement. The reliability test for the questionnaire instrument distributed to respondents had sig 0.969 (reliable), and based on the reliability test, it was clear that the value of Cronbach's Alpha and the value of Cronbach's Alpha Based on Standardized Items was higher than the standard 0.60, i.e. 0.969. Therefore, it can be concluded that the construct of each statement variable is reliable.

Once the test of validity and test of reliability, the questionnaires were distributed to all populations of respondents included in the inclusion scale, and their answers were processed to be the performance that could be measured based on the perception. Based on the answers of the respondents, it was found that the performance of the pharmacists working in hospitals in the Special District of Yogyakarta is found in the high category.

CONCLUSION

Based on the research, it is conclusive that pharmacists who work in hospitals in the Special Region of Yogyakarta, Indonesia, have the potential to improve their performance to support the success of hospitals in providing health services to the community. This can be seen from the distribution of pharmacists based on the following characteristics: 1) Most of them are of productive age, although most of them are female who need higher aspects of protection; 2) they have met the workforce qualifications; 3) Their average work experience is above 3 years; 4) Their level of socioeconomic status can be described through the ownership of transportation mode so that there are no obstacles to go to work. The pharmacists' performance appraisal based on their perception is in the high category, as can be seen from the average distribution of respondents' answers in the middle between 3-4 (Likert answer scale 0-5) indicating quite good performance. In addition, the average index of answers for each indicator is above 70%. Through the assessment, it can be seen that the ability of pharmacists to work together with colleagues and all employees in hospital organizations is at the highest value of 80%. The lowest value is 73.97% on the indicator of following the procedure. This study shows that pharmacists have a high perception of their ability to work, as outlined in their assessment of their work performance in hospitals. Pharmacists' perceptions about their work performance in public hospitals are helpful for developing pharmaceutical services. Improving the performance of pharmacists can be an excellent way to improve organizational performance in providing health services to the community. The results of pharmacist performance measurement based on the pharmacist's perceptions can be used as input for top management decisions in the organization in making decisions, especially regarding regulations related to improving pharmacist performance to improve hospital organizational performance.

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AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST

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Perspectives of Pharmacists, Doctors, and Nurses on Collaborative Management of Hypertension in Primary Health Centers

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Abstract

Background*: Puskesmas is a primary healthcare facility that conducts chronic disease management, such as hypertension. The role of pharmacists in team collaboration includes that of managerial and clinical pharmacies. However, doctors and nurses still need to be fully aware of the role of pharmacists, particularly in clinical pharmacies.* ***Objective****: This study aimed to determine the perspectives of pharmacists, doctors, and nurses on the collaborative management of hypertension in health centres across the Central Lombok Regency.* ***Methods****: observational qualitative method with a maximum variation sampling technique was used. Data saturation was achieved after interviewing 27 participants between April and June 2023. Participants were pharmacists, doctors, and nurses responsible for managing hypertension in the selected primary healthcare centers.* ***Results****: Five main themes were identified. The first was a perspective on pharmacists' managerial and clinical pharmacy roles. Almost all participants agreed that pharmacists played more roles in ensuring the availability of hypertension drugs than clinical pharmacies. Four themes were derived from a conceptual framework related to team readiness to collaborate: cognitive, affective/relational, behavioral, and leadership aspects. In general, doctors and nurses need to be made aware of pharmacists' role in the area of clinical pharmacy; meanwhile, pharmacists need to improve their clinical pharmacy knowledge. Meanwhile, team collaboration has not run optimally because each team member works individually rather than as a team member.* ***Conclusion****: Pharmacists need to improve their clinical pharmacy role, be more involved in team collaborations, and be more engaged in team collaborations; efforts are required to prepare for team collaboration.*

Keywords*: hypertension, perspective, pharmacist's role, team collaboration*

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INTRODUCTION

A Primary Health Center is a community-based primary health service that has existed since 1960 in Indonesia (President of the Republic of Indonesia, 1960). By prioritizing preventive and promotive aspects to improve health in an area, the vision and mission of Primary Health Centers are constantly evolving to realize optimal health status, both individually, as a family, in groups, and in society, and constantly adapt to the needs of the local community. To achieve this vision and mission, Primary Health Centers need a healthcare team consisting of health workers from various health professionals (Indonesian Ministry of Health, 2019). The healthcare team conducts various activities, including the treatment of chronic diseases. Hypertension is a chronic disease that is treated in Primary Health Centers. The treatment of patients with hypertension requires collaboration between team members, consisting of doctors, nurses, and pharmacists (Santschi *et al*., 2021). Collaboration within a team requires mutual trust, mutual knowledge, having the same goals, and working together to achieve common goals (Viani *et al*., 2021).

The involvement of pharmacists in team collaboration can improve patients' clinical, economic, and social outcomes for patients (Shrestha et al., 2022). According to various studies, the involvement of pharmacists in team collaboration can clinically reduce systolic and diastolic blood pressure (Hirsch *et al*., 2015, Smith *et al*., 2017, Margolis *et al*., 2020), improve blood pressure control (Santschi *et al*., 2021, Njonkou *et al*., 2021, Mulrooney *et al*., 2021), increase adherence to long-term therapy (Taylor *et al*., 2018) and reduce unplanned visits to primary care settings (Barreto *et al*., 2022). From an economic perspective, it has been shown to produce cost-effective care (Kulchaitanaroaj *et al*., 2017, Robins *et al*., 2013, Chung *et al*., 2020). From a social perspective, it increases access to care in rural areas through telepharmacy (Litke *et al*., 2018, Taylor *et al*., 2018) and reduces the waiting time for patients to receive hypertension treatment.

Each member of the hypertension care team has a role and activity. Based on several studies, doctors play the most role in determining patient diagnoses and establishing treatment plans (Robins *et al*., 2013, Carter *et al*., 2015, Gums *et al*., 2015, Kulchaitanaroaj *et al*., 2017). Most nurses are in charge of assessing the patient's condition by measuring blood pressure (Gums *et al*., 2015, Bui-Duy *et al*., 2018, Tetuan *et al*., 2019, Santschi *et al*., 2021, Markossian *et al*., 2021), while pharmacists are responsible for making treatment plans, designing disease-related counselling, medication, and lifestyle to patients, and providing education about medication and disease (Robins *et al*., 2013, Carter *et al*., 2015, Hirsch *et al*., 2015, Kulchaitanaroaj *et al*., 2017, Bui-Duy *et al*., 2018, Margolis *et al*., 2020). Even though the roles and activities of each member are different, interprofessional collaboration has the same goal of achieving predetermined blood pressure targets (Schrager, 2020).

According to existing literature, the common problems in interprofessional collaboration are unfavorable sentiments towards the pharmacist's role and the readiness of the team to involve pharmacists in partnership with other health workers in the team. Doctors and nurses consider other health team members less aware of the pharmacist's role, especially in carrying out clinical pharmacy activities. From their perspective, pharmacists are only responsible for drug administration. Regarding communication within the team, pharmacists were considered to have minimal communication with other health workers. Meanwhile, pharmacists consider themselves to lack clinical knowledge (Zielińska-Tomczak *et al*., 2021; Albassam *et al*., 2020, Amin and Mckeirnan, 2022; Kwak et al., 2019). Management of chronic diseases, such as hypertension, requires team collaboration involving pharmacists. This collaboration is expected to achieve the main goals in treating patients with hypertension, one of which is to control their blood pressure. However, pharmacists are not widely involved in the team because of the limited number of personnel (Rachmawati, 2021). In addition, limited time to meet and interact is a problem (Viani *et al*., 2021).

As the most common chronic disease found in Primary Health Centers across the Central Lombok Regency (Central Lombok Health Office, 2021), team-based care is recommended for hypertension management. Although each sub-district in Central Lombok Regency has two to three Primary Health Centers, and each Primary Health Center has a pharmacist, team-based care for hypertension has not been implemented. This study aimed to determine the perspectives of pharmacists, doctors, and nurses on the role and readiness of the team to involve pharmacists in managing chronic diseases, especially hypertension, in Primary Health Centers across Central Lombok Regency. Team readiness for collaboration is assessed based on cognitive, affective/relational, behavioral, and leadership aspects.

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MATERIALS AND METHODS

The research was conducted after receiving a recommendation letter from the National Unity and Politics Agency for Central Lombok Regency number 070/221/IV/R/BKBP/2023, a copy of which was sent to the Head of the Central Lombok Regency Health Office and the heads of Primary Health Centers where the research was conducted. This study also received ethical approval from the Health Research Ethics Commission, Faculty of Pharmacy, Universitas Airlangga (No. 22/L. E./2023). This study used a qualitative method with phenomenological, observational, and descriptive approaches and a purposive sampling technique. The steps of phenomenological research are finding phenomena, determining the subject under investigation and the actual context, collecting data in the field, making notes, analyzing data, and writing reports. Primary health centers were selected on a representative basis based on the year of pharmacist recruitment (2009-2015 and 2016-2018) and the number of hypertension cases handled at primary health centers (<1,000 and ≥1,000). Research locations in nine primary health centers: Mantang, Puyung, Batu Jai, Teruwai, Bonjeruk, Praya, Sengkol, Penujak, and Darek Primary Health Center. Twenty-seven participants represented each pharmacist, doctor, and nurse responsible for hypertension management activities in health centres. Before conducting the interview, we made an appointment by contacting the participants for time and place. The average interview lasted for approximately 50-65 minutes. Data saturation was obtained after interviews with 27 participants. The data collection tool used in this study was an interview guide and a recorder. The interview guide contained a list of questions that asked the participants. The questions were structured around a conceptual research framework that assessed team collaboration readiness (Shomaker et al., 2016). The questions were used to assess the perspectives of pharmacists, doctors, and nurses on the role of pharmacists and team readiness to involve pharmacists in the hypertension management team according to cognitive, affective/relational, behavioral, and leadership aspects. The recorded interviews were transcribed and coded before being analyzed and concluded. Intensive discussions between research team members were then conducted to verify the results of the data analysis and determine themes (Creswell, 2013, Zaenuddin, 2014). The interviews and data analyses were conducted from April to June 2023. The interviews were then transcribed and coded. The data were then grouped, and themes were determined. Verification of data analysis results and determination of themes jointly through intensive discussions between the research team.

RESULTS AND DISCUSSION

Characteristics of participants and research locations

Twenty-seven participants came from nine out of 28 primary health centers in the Central Lombok Regency. Each health worker (pharmacist, doctor, and nurse) in each primary health center was represented by one participant. This study conducted interviews with nine participants from each health worker (pharmacist, doctor, and nurse). The total number of participants in this study was 27 from nine primary health centers in the Central Lombok Regency. The participants' characteristics are listed in Table 1.

All pharmacists were graduates of the pharmacy education program. In addition, all the doctors were general practitioners who had graduated from medical education. Five nurses had completed their last nursing education. Three nurses had a bachelor's degree in nursing and one had a diploma III.

Results by themes

Five main themes were identified based on the interview results. The first theme was perspective on the pharmacist's role in managerial and clinical pharmacies. In contrast, the other four themes followed the conceptual framework related to team readiness to involve pharmacists in the hypertension management team in cognitive, affective/relational, behavioral, and leadership aspects (Shoemaker *et al*., 2016).

Table 1. Participants characteristics

Participants Code	Gender		Age (years)	Duration of Practise (years)
	M	F		
A	2	7	27-49	3-18
D	3	6	29-56	3-27
N	2	7	23-50	5-32

* A, pharmacists; D, doctors; N, nurses; M: Male, F: Female

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Perspectives on the pharmacist's role in managerial and clinical pharmacy aspects

All participants considered that pharmacists played a managerial role in ensuring the availability of hypertension medications at primary health centers. The availability of hypertension medication is essential, considering several programs and activities aimed at treating hypertension at primary health centers, such as the chronic disease Management Program (PROLANIS), integrated Family Service Post (POSGA), and the mobile Health Center (PUSKEL). In addition to these programs, the pharmacist's role is important in ensuring the availability of medicines in daily services at primary health centers.

""""""""""""""""""""""""We are still not involved in team-based care. It is still limited to guaranteeing the availability of the medicine"""." *""""""""""""""""""""""""Yes, (the discussion) is still about availability, availability, and availability (of medicine). We are not thinking about direct involvement in patient care yet, maybe later. Now, we are still focused on our responsibility in ensuring the availability of drugs, which is not easy either.... that (clinical pharmacy) has not yet been implemented"""."* (A4)

"There are various roles (of the pharmacists) such as ensuring the availability of hypertension medication, which is a disease that cannot be referred to a hospital according to the regulations of BPJS." (D5)

Due to the limited number of pharmacists, clinical pharmacy activities tend not to be conducted. The implemented clinical pharmacy agenda includes drug information services (PIO) and counselling. Meanwhile, home care activities do not involve pharmacists. The home care that primary health centers have routinely carried out is a home care program for treating tuberculosis (T.B.) and mental health. Home care activities for patients with hypertension were implemented at one of the primary health centers of the nine study locations.

"The clinical pharmacy that we (pharmacists) have done here (in primary health centres) is drug information service (PIO) both to health workers and the community. In addition, we have also implemented patient counselling (hypertension). Currently, we are focusing on counselling patients with diabetes mellitus and hypertension, considering that these two diseases are the most common. Therefore, we regularly chat with patients with diabetes mellitus and hypertension (for counseling" " " " " " " " " " " " " """)" (A5).

""""""""""""""""""""""""We are also involved in home visits conducted by nurses to hypertension patients whose blood pressure is unstable. We are in charge of preparing the medicines needed... We are responsible for planning the medicines that will be brought by the nurses, including those that will be brought to the mobile health center program... We are working hard given the limited funds which are more distributed to essential programs (stunting). If our planning is poor, it will be very difficult for us to guarantee the availability of the drug in question""" """ """ """ """ """"""" (D5).

Perspectives on the cognitive aspect

The cognitive aspect includes the pharmacist's ability to collaborate with the team, in terms of both clinical pharmacy knowledge and the ability to provide advice and recommendations to doctors and nurses in the team. Pharmacists assessed their ability to gather information from patients related to the consumption of antihypertensive drugs that have been consumed. Pharmacists are expected to provide advice and recommendations to doctors and nurses. In this case, the doctor delegates the nurse to prescribe the related hypertension drug substitution and the usual dosage information when the doctor is away. This suggestion is still related to the availability of antihypertensive drugs in health centres. Pharmacists are considered reliable and can communicate with doctors and nurses regarding prescription.

Clinical advice regarding drug side effects and the potential for drug-use interactions are also the responsibility of the pharmacist. However, pharmacists in the team did not carry out recommendations and suggestions for evaluating the effectiveness of therapy and assessing the achievement of therapeutic targets such as lowering and controlling blood pressure. If discussions related to this activity were carried out within the team, pharmacists would feel the need to improve their clinical pharmacy knowledge.

""""""""""""""""""""""""When patients experience problems, pharmacists ask more questions to find out if certain drugs have side effects such as captopril (dry cough). We are increasingly losing time to share knowledge of clinical pharmacy, considering that most of our time is spent doing management roles in drug warehouses"""." (A8)

Doctors and nurses are yet to realize pharmacists' potential in clinical pharmacy knowledge, such as the ability to monitor medication adherence and home care. They believed that the pharmacist's role was limited to prescribing antihypertensive drugs, the usual dosage,

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terms, and conditions for drug use, the suitability of the amount of the drug, and the substitution of drugs if the patient experienced drug side effects. Despite this, doctors and nurses consider pharmacists to possess good communication skills.

""""""""""""""""""""""""Pharmacists have excellent communication skills. They can convey drug-related information well to patients. This is not an issue, considering they are used to seeing patients daily. Conversations often revolve around drug dosages (which haven't been done), especially (assess and monitor) medication adherence. Maybe what we can improve is the patient's habit of asking for additional amounts of drugs even though the old ones are still left"""""." (N7)

Perspective on affective/relational aspects

The affective/relational aspect refers to pharmacists' participation in team discussions and joint decision-making for the care of hypertensive patients. Pharmacists have considered that collaboration has not been implemented. This is because each team member performed their role individually and did not work together. In addition, joint discussions within the team on therapeutic goals and treatment outcomes have not been conducted. Face-to-face interactions with doctors and nurses occurred, but these interactions did not address the clinical treatment of hypertensive patients but discussed the availability of hypertension drugs.

"*So far the collaboration had not been implemented. Pharmacists did not actively participate. We never hold team discussions. Doctors never asked about our role (pharmacists). At most, they only asked about the availability of drugs.*"" "(A2)

Doctors and nurses noticed problems with team members' interactions. The age difference was not a problem. However, the team's discussions focused only on surface matter and did not cover the therapeutic goal. According to doctors and nurses, this was due to the limited number of pharmacists in primary health centers and time for team discussions.

"We never made a big deal about the age difference. Not that we are seniors, and we can act however we want. It's just that, so far, we haven't touched on (discussed) more fundamental matters such as specific treatment or therapeutic goals. The obstacle to team collaboration is time and effort. We could have held a team discussion, but there were too many patients visiting, and thus the pharmacists were very busy"""""." (D2)

Perspectives on behavioral aspects

The behavioral aspect refers to everything that is needed by the team to collaborate well and achieve goals that have been set together. According to all participants, to be able to collaborate, the role of the head of the primary health center is needed to facilitate the provision of media or facilities, such as a particular room where pharmacists, doctors, and nurses discuss with each other to determine the treatment of hypertension patients. Obstacles to the lack of collaboration within the team can be caused by the limited number of pharmacists in Primary Health Centers and incomplete patient data input into the online information system owned by Primary Health Centers. Meanwhile, doctors and nurses considered that collaboration within the team could work if there were more pharmacist personnel, where one could handle drug managerial issues and the other could work on aspects of clinical pharmacy. This response is a recommendation from doctors and nurses, whose primary health centers have only one pharmacist.

""""""""""""""""""""""""We hope that there will be a team of doctors, nurses and pharmacists. As soon as the patient arrives, the doctor conducts an examination assisted by a nurse, while the pharmacist discusses which medication is right for the patient. Regarding the success of therapy, we can all know that by tracking the patient's behavior at home, to see whether the patient is taking the drug correctly or whether the diet is as recommended. We can do that through counseling"""""." (A9)

"Maybe hypertension can be a pilot case for implementing this collaboration. For this reason, the addition of pharmacist personnel is mandatory. At least two pharmacists. One pharmacist focuses on drug planning, and the other focuses on monitoring drug adherence and others related to clinical pharmacy"""""." (D5)

Perspectives on leadership aspects

The leadership aspect refers to the ability to mobilize a team to carry out its functions in the management of chronic diseases. All the participants agreed that a doctor was the right person to become a team leader. However, in the context of Primary Health Centers, doctors alone are not sufficient to encourage team collaboration. All participants agreed that the heads of Primary Health Centers should also be active in providing directions for any obstacles that exist. This can be done by the heads of Primary Health Centers by issuing a decree on the formation of a hypertension

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handling team, budget allocation for these activities, and a policy to prioritize hypertension management. Meanwhile, the policy to increase pharmacist personnel must first be discussed with the local health office.

"""""""""""""""""""""""Doctors (as team leaders) have power in many ways. Technically, it is the doctor who understands the cause of a disease. Doctors have the necessary knowledge for that. Doctors are also reliable, firm, systematic, and responsive in responding to patient needs even though they are not on duty in Primary Health Centers""""." (A3)

"The pharmacist's involvement in home care must obtain official permission from the head of Primary Health Centers. Apart from that, appreciation (funds) is also needed from Primary Health Centers so that pharmacists are more confident in communicating with the community""""." (D2)

DISCUSSION

This study provides an overview of the perspectives of pharmacists, doctors, and nurses on the role of pharmacists in the managerial and clinical pharmacy aspects of hypertension care in Primary Health Centers. The results showed that the role of pharmacists is to ensure the availability of antihypertensive drugs. If drugs are available, hypertension management programs and daily services can be run well. In terms of clinical pharmacy, due to the limited number of pharmacists, not all clinical pharmacy activities are carried out by pharmacists. The pharmacists in this study only provided drug information services (PIO) and counseling. This study also provides an overview of the perspectives of pharmacists, doctors, and nurses regarding team readiness to involve pharmacists in team care in terms of cognitive, affective/relational, behavioral, and leadership aspects. Even though pharmacists were cognitively considered capable of participating in team discussions and providing advice and recommendations regarding prescriptions, they still needed to improve their clinical pharmacy knowledge. Meanwhile, doctors and nurses are unsure about the ability of pharmacists to monitor medication adherence and home care.

From an affective/relational aspect, team members still tend to carry out their roles individually and do not work together as a team. This may be because of the pharmacist's limited time to actively participate in team discussions. From the behavioral aspect, pharmacists need instructions, media, or facilities to be able to collaborate in a team. From the leadership perspective, the involvement of the heads of Primary Health Centers is crucial to the success of the program. With their authority, obstacles can be overcome. The following is a discussion of the research results that have been obtained.

The role of pharmacists in ensuring the availability of hypertension drugs

Pharmacists play more managerial roles in drug management than in clinical pharmacies. With the existence of the regulation of the BPJS regarding 155 diseases that must be resolved in Primary Health Centers and not referred to the hospital (BPJS, 2014), the responsibility of pharmacists at Primary Health Centers to provide hypertension medication is greater. Pharmacists should be able to guarantee drug availability. In addition, for the success of hypertension management services in Primary Health Centers, pharmacists spend more time providing the needed medicines, such as in the PROLANIS program (BPJS, 2014) and PUSKEL (Indonesia Ministry of Health, 2013). For this reason, pharmacists are focused on providing antihypertensive drugs at Primary Health Centers.

The same case was found in Bandar Lampung, where the availability of antihypertensive drugs in Primary Health Centers greatly influenced treatment success of treatment (Huda *et al*., 2020). This means that hypertensive drugs must remain available to avoid faltering patient treatment. It is becoming increasingly important to note that the shortage of hypertensive drugs in Primary Health Centers often hinders the treatment process (Oktiano *et al*, 2022; Sulistiyono *et al*, 2020). Therefore, pharmacists urgently need to ensure drug availability. Previous research conducted in Madiun, East Java Province, found that pharmacists played an important role in ensuring drug availability. Drug management training, planning, and drug needs analysis can increase drug availability (Prabowo *et al.*, 2016). Based on this study, to support the guarantee of drug availability, the effort that can be made by the health office is to conduct planning and needs analysis training in drug management in Primary Health Centers. This training can be provided regularly to pharmacists in all Primary Health Centers in Central Lombok Regency, and the results of the training can be evaluated by examining the increase in drug availability before and after training. It can be used in collaboration with the health team, especially for planning drug needs in primary healthcare.

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Efforts to increase the role of pharmacists in clinical pharmacy

Unlike drug management, clinical pharmacy activities tend not to be conducted. The pharmacists performed PIO and counseling. This aligns with previous research on the role of pharmacists in hypertension care, where pharmacists are responsible for counseling, medication adjustments, and patient education at Primary Health Centers (Ayu & Syaripuddin, 2019). Conversely, from a review of several studies regarding the involvement of pharmacists in team-based care, more clinical pharmacy activities that can be carried out by pharmacists include reviewing drug dosages, potential side effects of drugs, adjusting and stopping hypertension drugs, counseling about medication and therapy, and lifestyle to patients (Kulchaitanaroaj *et al*., 2017, Taylor *et al*., 2018, Margolis *et al*., 2020, Santschi *et al*., 2021).

The pharmacist's role was to monitor patient treatment. This was also found in a study conducted by primary healthcare units in America and Canada, where pharmacists played a role in monitoring patient medication adherence (Gums *et al*., 2015, Smith *et al*., 2016, Margolis *et al*., 2020). Several attempts to improve adherence to taking medication for patients with hypertension in Primary Health Centers include home pharmacy care, medication reminder tools, and pharmacist counseling (Wibowo *et al*., 2020, Utaminingrum *et al*., 2017).

From this study, pharmacists were able to provide suggestions and recommendations, such as drug substitution, because stocks were not available and advice was given on the usual dosage for the treatment of hypertension. This finding is in line with previous research conducted in primary care facilities in America, where pharmacists were involved in preparing and providing treatment plan recommendations to doctors (Gums *et al*., 2015, Smith *et al*., 2016, Kulchaitanaroaj *et al*., 2017). The recommendations are still related to the availability of drugs in Primary Health Centers. For example, if captopril is not available, the pharmacist may suggest another drug such as amlodipine. Pharmacists were not assigned recommendations regarding the patient's clinical condition.

Clinical pharmacy recommendations that have been carried out by pharmacists pertain to the side effects of drugs experienced by patients. Pharmacists usually make drug adjustments to avoid side effects that may occur due to the combination of two types of hypertension drugs or the use of other drugs at the same time. Furthermore, recommendations from pharmacists can be used to evaluate the effectiveness of treatment and to assess the achievement of therapeutic goals. With recommendations from pharmacists, the therapeutic goals that have been determined for patients with hypertension can be achieved. According to existing research, the role of pharmacists in team-based care has been to help reduce systolic and diastolic blood pressure (Green et al., 2014, Carter *et al*., 2015, Hirsch *et al*., 2015) and control the patient's blood pressure (Ramirez et al., 2015). al., 2020, Mulrooney *et al*., 2021).

Therefore, pharmacists need to improve their clinical pharmacy knowledge. Based on the results of research in Banten province, improving clinical pharmacy knowledge can be achieved by holding clinical pharmacy training, which has been proven to increase pharmacists' knowledge and ability to provide pharmaceutical services regarding antihypertensive drugs in Primary Health Centers (Yusransyah *et al*., 2022). Efforts made by the health office include conducting routine clinical pharmacy training related to hypertension management. This training can be conducted by working together with the Indonesian Pharmacist Association (IAI) branch of the Central Lombok Regency.

Clinical pharmacy services can also be improved by adding pharmacists (Rachmawati, 2021). The lack of pharmacists is an obstacle to pharmaceutical services at Primary Health Centers. A study in Semarang, Central Java Province, recommended adding a budget to hire more pharmacists, along with clinical pharmacy education and training. Pharmacists are also added by coordinating with the health office by proposing and meeting the needs of pharmacists either through official government candidate selection (CPNS) and/or public service agencies (BLUD) (Pratiwi *et al*., 2021).

The suggestion for additional pharmacists in this study came from participants whose Primary Health Centers had only one pharmacist. If the primary health center has two pharmacists, team collaboration can work, and clear assignments can be made to divide the managerial and clinical roles of pharmacists. Thus, pharmacists will be more involved in collaboration, and programs such as PROLANIS, POSGA, PUSKEL, and Homecare will run more successfully.

Adequate provision of pharmacists in Primary Health Centers cannot easily be obtained in a short time. Efforts that can be made to overcome the shortage of pharmacists are to collaborate with community pharmacists to carry out clinical pharmacy activities

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such as home care. This finding is consistent with previous studies showing that adding community pharmacists to the care of hypertensive patients can optimize treatment to achieve blood pressure control by making treatment recommendations to doctors (Mulrooney *et al*, 2021). The same can also be applied to Primary Health Centers in the central Lombok Regency. Primary Health Centers can work with the Central Lombok IAI to help carry out home pharmacies by monitoring medication adherence. This activity can be used as a recommendation for hypertension management teams at Primary Health Centers.

Priority of hypertension treatment in primary health centers

Hypertension is not prioritized over stunting (Indonesian Ministry of Health, 2020). Therefore, Primary Health Centers prioritize funding for stunting management programs for hypertension. Data on the number of stunting cases in Central Lombok Regency in 2020 showed that 27.7% of the 73,965 toddlers in Central Lombok Regency were stunted (Informatics and Statistics Communication Department of West Nusa Tenggara, 2021). This makes the stunting management program a greater priority than hypertension management. This can be seen in the limited budget of Primary Health Centers for treating hypertension.

From the results of this study, it was found that pharmacists were not involved in home care activities for hypertension due to the minimal budget allocated. The same occurred at the Halmahera Primary Health Center, where limited funds prevented home care and PROLANIS programs from being carried out (Rosdiana *et al.*, 2017).

The treatment of hypertension in Primary Health Centers should be a priority, considering the high incidence of hypertension in Primary Health Centers across the Central Lombok Regency (Central Lombok Health Office, 2021). Adequate fund allocation is a supporting factor in creating effective and efficient performance (Sihotang, 2015). Thus, patients with hypertension will receive better services, and the need for additional pharmacist personnel will be fulfilled.

Hypertension management team leader

The team leader plays an important role as the driving force of a team. Leadership has a strong relationship with organizational commitment (Nursyamsi, 2012). Based on the results of this study, doctors are considered appropriate leaders in the technical implementation team to treat patients with hypertension. Professionalism and assertiveness are criteria for team leaders to drive their teams to achieve their goals. However, in the context of health services at Primary Health Centers, the role of the doctor as a team leader was not enough to create team collaboration, and it was considered unable to overcome various obstacles optimally. Based on the results of this study, the heads of Primary Health Centers should be able to overcome every obstacle that exists. Problems such as limited funds for hypertension treatment programs, the limited number of staff, the absence of a collaboration team, and the need for a particular room for chronic disease services at Primary Health Centers can be resolved with the heads.

Previous research has also highlighted that collaboration between hospital directors, doctors, and pharmacists increases the effectiveness of teamwork in hospitals. With the authority of a hospital director as a structural leader, a hospital director can form a clinical pharmacy team and give pharmacists authority to conduct visits, monitor drug levels, engage in team discussions, and provide recommendations on drug use. Structural leadership has a positive and significant effect on employees' organizational commitment. Organizational leaders can inspire work and determine the direction and goals of an organization (Abdulkadir, 2017). In this case, the roles of the heads of Primary Health Centers resemble those of hospital 'directors' in terms of structural authority.

CONCLUSION

Pharmacists need to improve their clinical pharmacy knowledge and be more involved in team collaborations, and efforts are needed to prepare teams to collaborate. The participants involved in this study agreed that pharmacists at primary healthcare centers across Central Lombok Regency focused on managerial aspects, such as ensuring the availability of drugs, rather than clinical pharmacy. This is because of the limited number of pharmacists available at primary healthcare centers. In addition, all participants thought that pharmacists needed to improve their knowledge of clinical pharmacy and their readiness to be more involved in team collaboration. In terms of team leadership, the heads of Primary Health Centers have the authority to overcome various obstacles that may be difficult for doctors, nurses, and pharmacists. The heads of Primary Health Centers can assist in terms of funding, adding more pharmacist personnel, forming a chronic disease management team by issuing decrees, and

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providing facilities, such as a particular room for chronic disease management.

SUGGESTIONS

Here are some suggestions from this study:

1. The heads of Primary Health Centers need to formulate policies that support and prioritize the management of hypertension at Primary Health Centers. This can be achieved by involving pharmacists in team collaboration and giving them more authority in clinical pharmacies. If this instruction is made possible through the policy of the Central Lombok Health Office, technical guidance is needed.
2. The addition of pharmacists to Primary Health Centers can be carried out in coordination with the Central Lombok Regency Health Office. If Primary Health Centers have two pharmacists, then tasks must be divided between them, where one may focus on managerial aspects or clinical pharmacies. Staff shortages can also be overcome by collaborating with the IAI to support the home-care program with additional pharmacists.
3. There is a need for capacity building related to clinical knowledge for pharmacists in Primary Health Centers. This can be achieved by conducting clinical pharmacy training programs for various chronic diseases. Training can be held by the health office in collaboration with Central Lombok IAI. Training should be performed regularly along with evaluation.

Future research can target the heads of Puskesmas as participants. This study aimed to examine the involvement of the head of the Puskesmas in collaboration with pharmacists, doctors, and nurses in managing chronic diseases at the Puskesmas.

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AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Effect of Fenofibrate as PPARα Agonist in Suppressing the Development of Oxaliplatin-Induced Peripheral Neuropathy via TRPA1 Modulation

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Abstract

Background*: CIPN (Chemotherapy-induced Peripheral Neuropathy) primarily affects the sensory system and is accompanied by pain, autonomic dysfunction, and motor impairments. Alterations of intracellular second messengers at the supraspinal level in CIPN needed to be explored more. In addition, there is a lack of evidence regarding implications for the supraspinal area through the propagation of pain via the ascending pathway.* ***Objective****: In this study, we evaluated the effect of fenofibrate as a PPARα agonist in suppressing the development of CIPN.* ***Methods****: Twenty-four mice were distributed to the normal control group, neuropathy group, and neuropathy with the treatment of fenofibrate 75 and 150 mg/kg groups, resulting in 6 animals per group. Oxaliplatin was injected on days 0, 2, 4, and 6. The hot plate test was performed before the oxaliplatin administration and then continued on the 7th, 14th, and 21st days. Thalamus tissues were collected to measure the TRPA1 mRNA expression using qPCR.* ***Results****: Fenofibrate 75 mg/kg co-treatment with oxaliplatin tended to prevent the enhancement of oxaliplatin-induced thermal hyperalgesia in hind-paw withdrawal and rubbing responses. Furthermore, fenofibrate 75 and 150 mg/kg co-treatment with oxaliplatin significantly reduced the relative TRPA1 mRNA expression but did not modulate the relative BDNF mRNA expression in the thalamus.* ***Conclusion****: PPARα agonist has a potential effect in suppressing the development of CIPN. However, given the various perspectives on the role of neurotrophins in CIPN, additional non-clinical investigations, are needed to provide more insight into other mechanisms of CIPN and the role of PPAR agonists.*

Keywords*: fenofibrate, oxaliplatin, peripheral neuropathy, PPARα agonist, TRPA1*

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INTRODUCTION

Peripheral neuropathy affects approximately 2.4 percent of the population, and the frequency increases by 8% in the elderly population (Edwards *et al*., 2022). Diabetes mellitus, nerve damage, alcohol use, genetic disorders, dietary inadequacies, and chemotherapeutic drugs are commonly identifiable causes (Castelli *et al*., 2020). The term neuropathy, commonly known as chemotherapy, is called Chemotherapy-induced Peripheral Neuropathy (CIPN). The sensory system is primarily affected by CIPN, which is accompanied by motor impairment, autonomic dysfunction, and pain. Up to 70% of patients undergoing chemotherapy develop CIPN after or during treatment completion. Additionally, 30% of these individuals experienced this problem six months after chemotherapy (Seretny *et al*., 2014). Allodynia and hyperalgesia are predominant symptoms in patients with neuropathic pain. Both affect 15–50% of individuals with neuropathic pain in different forms of peripheral neuropathy and central pain disorders. (Jensen & Finnerup, 2014).

The mechanism by which chemotherapy damages the structure of the nervous system and results in CIPN is multifactorial and involves immunological processes, neuroinflammation, microtubule disorders, oxidative stress, mitochondrial damage, changes in ion channel activity, damage to the myelin sheath, and DNA damage (Areti *et al.*, 2014). Several classes of chemotherapy drugs, such as oxaliplatin, cause damage to the sensory and motor nerves, with the most significant prevalence in the platinum group (Burgess *et al*., 2021). Oxaliplatin exposure causes glial cell activation and enhances IL-1, IL-6, and TNF-α pro-inflammatory cytokines in neuropathic pain studies (Areti *et al.*, 2018; Lee *et al*., 2022; Wang *et al*., 2017). Upregulation of these proinflammatory cytokines triggers a condition called neuroinflammation. In peripheral areas, such as the dorsal root ganglion (DRG), neuroinflammation increases the expression of markers related to nociception and inflammation, such as BDNF and TRPA1, both through regulation of transcription and translation (Kameda *et al*., 2019; Lin *et al*., 2011). Ascending pathways in the dorsal horn spinal cord project information from peripheral sensory neurons in the DRG to supraspinal regions, such as the somatosensory cortex, thalamus, anterior cingulate cortex, insular cortex, and brainstem (Baron *et al*., 2010; Kocot-Kępska *et al*., 2021). Oxaliplatin increases the phosphorylation and upregulation of PKC gamma isoforms in the thalamus and PAG (Han & Smith, 2013). Using fMRI to measure brain activity, oxaliplatin induced hyperactivity in the S2 and insula (Nagasaka et al., 2017), as well as hyperactivity in the motor cortices, cingulate, and somatosensory neurons, as measured by increased p-Erk-IR neurons (Thibault *et al*., 2012). This pathological change in the supraspinal area is associated with oxaliplatin-induced peripheral neuropathy. Therefore, it is necessary to further investigate the changes in intracellular secondary messengers at the supraspinal level in CIPN.

Pain therapy for Peroxisome Proliferator-Activated Receptors (PPARs) is being widely developed. PPARs are nuclear receptors activated by endogenous substances, such as fatty acids and their derivatives or drugs. In humans, PPARs consist of three isotypes: α, β/δ, and γ. The PPARα and PPARγ subtypes have been widely studied and are associated with neuropathic pain (Okine *et al.*, 2019). PPARα modulates the inflammatory response. Under conditions of hyperalgesia, PPARα expression in the DRG decreases, implicating the upregulation of proinflammatory cytokines (D'Agostino *et al*., 2009; Wang *et al.*, 2018). In previous studies, endogenous and synthetic PPARα agonists reduced allodynia and hyperalgesia in animal models of neuropathy (Caillaud *et al*., 2021; D'Agostino *et al*., 2009; Impellizzeri *et al*., 2016). Fenofibrate, a PPARα agonist, also reversed hyperalgesia and allodynia in mouse models (Caillaud *et al*., 2021; Oliveira *et al*., 2007)

Although exogenous agonists, such as fenofibrate, have been demonstrated to target PPAR in studies using animals for peripheral neuropathy, there is a lack of studies regarding the implications for the supraspinal area through the propagation of pain via the ascending pathway. Therefore, exploring the potential of fenofibrate as a PPARα agonist is needed to support scientific evidence regarding the role of PPARα agonists in neuropathic pain. Here, we evaluated the effect of fenofibrate in suppressing CIPN development of CIPN through a hotplate behavior test in mice. Additionally, we explored the molecular mechanisms by which fenofibrate affects TRPA1 and BDNF nociceptive biomarkers.

MATERIALS AND METHODS

Materials

Oxaliplatin (Merck, Darmstadt, Germany; CAS 61825-94-3) was solubilized in dextrose monohydrate 5% (PT. Widatra Bhakti, Pasuruan, Indonesia). Oxaliplatin was administered intraperitoneally at a dose

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of 3 mg/kg on days 0, 2, 4, and 6. Fenofibrate (pharmaceutical grade obtained from PT. Kalbe Farma Tbk., Jakarta, Indonesia) was solubilized in Tween 80 (PT. Brataco, Surabaya, Indonesia). Fenofibrate was administered at 75 and 150 mg/kg intraperitoneally for eight days since day 0. Normal saline 0,9% (PT. Widatra Bhakti) was administered subcutaneously to prevent nephrotoxicity in the platinum group (Rachman et al., 2022). Figure 1 illustrates the experimental timeline and dosing regimens.

Method

Animals

DDY mice weighing 25–30 g and aged 5-6 weeks were used in this study. Twenty-four mice were distributed to the normal control group, neuropathy group, and neuropathy with fenofibrate 75 and 150 mg/kg treatment groups, resulting in six animals per group. The animals were housed in three mice per cage, and acclimatization was performed for a week. All mice were maintained under a 12:12 h diffuse light/dark cycle at a regulated temperature (25°C ± 2°C) and humidity (60 ± 10%), with free access to food and water. All experimental protocols were approved by the ethics committee of the Faculty of Veterinary Medicine at Universitas Airlangga (ethical number 2). KEH.023.03.2023.

Hot plate test

The hot plate test was first performed one day before the induction of oxaliplatin as a baseline and then continued on days 7th, 14th, and 21st days. Before testing, the mice were habituated to the test room for 30 min. The test was carried out in a quiet room and hotplate setting at a constant temperature of 52 ± 1 ℃ to induce a response to heat. Heat inductors were applied to both plantar surfaces of the feet of the experimental mice and were placed on the metal surface of the hot plate. for 30 s as the cutoff time to avoid tissue damage (Kudla et al., 2019). The response to pain in experimental mice was based on visual observations of standing, licking, lifting the legs, jumping, and rubbing. The first latency time to a thermal stimulus was expressed as paw withdrawal latency (PWL). Three replicates of the test were performed at intervals of 15 min. (Hidayati et al., 2018).

Quantitative polymerase chain reaction (qPCR)

The animals were euthanized by decapitation 21 days after the first injection of oxaliplatin. Thalamus tissues were obtained, directly stored in liquid nitrogen, and stored at − 80 ◦C. The Total RNA Purification Kit (Jena Bioscience, Jena, Germany) was used to isolate RNA. RNA concentration was measured using a Quantus Fluorometer (Promega, Madison, WI, US). A GoScriptTM Reverse Transcriptase Kit (Promega) was used for reverse transcription to generate cDNA. Quantitative real-time PCR (qRT-PCR) was used to quantify the mRNA expression of TRPA1 (5′-GTACTTCTTGTCGTGTTTCTTGC-3′ for forward primer; 5′-ACCATCGTGTATCCAAATAGACC-3′ for reverse primer) and BDNF (5'-ATCCCATGGGTTACACGAAGGAAG-3' for forward primer; 5'-AGTAAGGGCCCGAACATACGATTG-3' for reverse primer). Using β-actin (forward primer:5'-TTCTTGGGTATGGAATCCTGT-3’; reverse primer:5’-AGCACTGTGTTGGCATAGAG-3’) as a reference gene, TRPA1 and BDNF mRNA expression levels were normalized. The GoTaq RT qPCR Master Mix Kit (Promega) was used for the qRT-PCR. Quantification of cycle threshold (Ct) values using the 2-ΔΔCt formula was used to evaluate changes in the mRNA expression of the gene of interest.

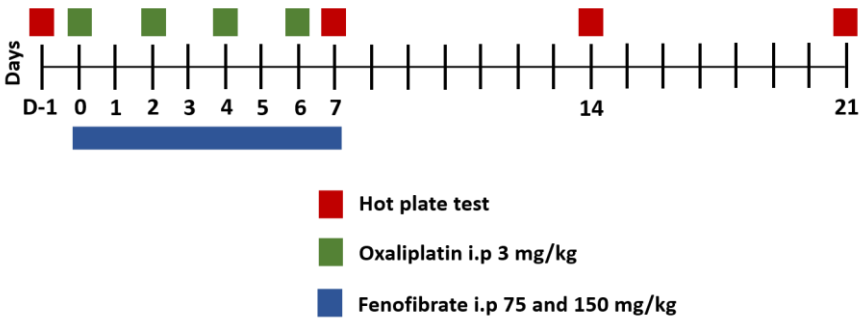

Figure 1. Timeline and dosing regiment of the experiment. Following baseline behavioral measurements on D-1, mice were given an intraperitoneal injection of oxaliplatin 3 mg/kg e on days 0, 2, 4, and 6 to induce peripheral neuropathy. On days D-1, 7, 14, and 21, behavioral assays using a hot plate test were performed. Fenofibrate of 75 and 150 mg/kg were injected for eight days since day 0 until day 7. Animals were sacrificed on day 21, and the DRGs were collected

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Statistical analysis

Data are displayed as the mean ± SEM using GraphPad Prism 9.0.2. Two-way ANOVA was used to calculate the statistics for the hot plate test, followed by Tukey's post hoc test. One-way ANOVA and Tukey post hoc tests were used to analyze the qPCR data. Differences were considered significant at p 0.05 (95% confidence).

The results of rubbing behavior showed that oxaliplatin significantly induced thermal heat hyperalgesia, as reflected by the reduced latency time on days 14 and 21, compared to the normal group (Figure 3). The neuropathy group tended to develop thermal heat hyperalgesia on days 7, 14, and 21 compared to baseline. Administration of 75 and 150 mg/kg fenofibrate reversed the reduction in rubbing response latency time induced by oxaliplatin. Meanwhile, the frequency parameter showed that exposure to oxaliplatin in the neuropathy group increased rubbing frequency on day 7 and significantly on days 14 and 21 compared to the control group. The rubbing frequency tended to increase in the neuropathy group on days 7, 14, and 21 compared to that in the neuropathy group at baseline. Meanwhile, the administration of fenofibrate at doses of 75 and 150 mg/kg tended to increase the rubbing frequency compared to the neuropathy group on days 7, 14, and 21.

The results of the jumping-off behavior test showed no significant differences in all treatment groups at each time point (Figure 4). Similar results were observed for both latency time and frequency. In this study, the administration of oxaliplatin and fenofibrate co-treatment did not affect jumping behavior when using a hot plate.

Oxaliplatin administration significantly decreased hind paw withdrawal latency time compared to that in the normal group and induced thermal heat hyperalgesia (Bhardwaj et al., 2016). Previous studies have shown that oxaliplatin reduces thermal hyperalgesia with a jumping-off response in cold hyperalgesia, but not in heat hyperalgesia (Renn *et al*., 2011). Another study showed the same result (Bouchenaki et al., 2021). Therefore, this behavioral test showed that oxaliplatin induction significantly induced thermal hyperalgesia in hind-paw withdrawal and rubbing responses, but not in jumping-off responses.

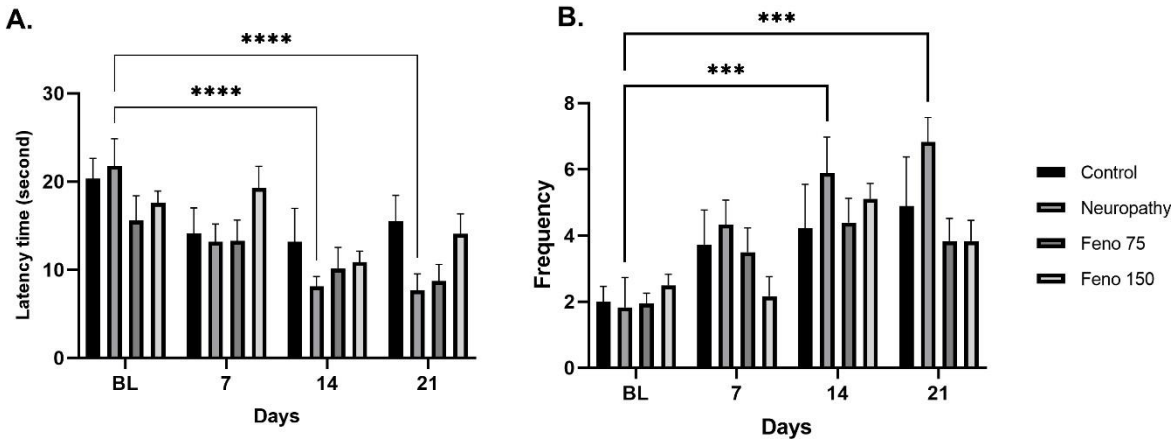

Figure 2. Fenofibrate's effect on thermal hyperalgesia caused by oxaliplatin on hind-paw withdrawal responses, with parameters for latency time (Figure 1A) and frequency (Figure 1B). Statistical significance between treatment groups is indicated by ***p<0.001 and ****p<0.0001 (two-way ANOVA followed by Tukey's post-hoc test; n = 6 mice)

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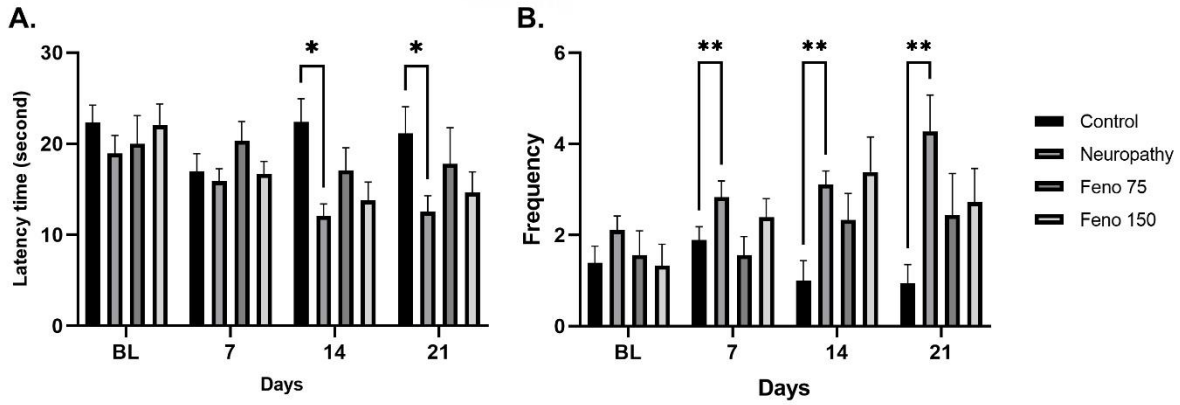

Figure 3. Fenofibrate's effect on thermal hyperalgesia caused by oxaliplatin on rubbing responses, with parameters for latency time (Figure 1A) and frequency (Figure 1B). Statistical significance between treatment groups is indicated by *p<0.05 and **p<0.01 (two-way ANOVA followed by Tukey's post-hoc test; n = 6 mice)

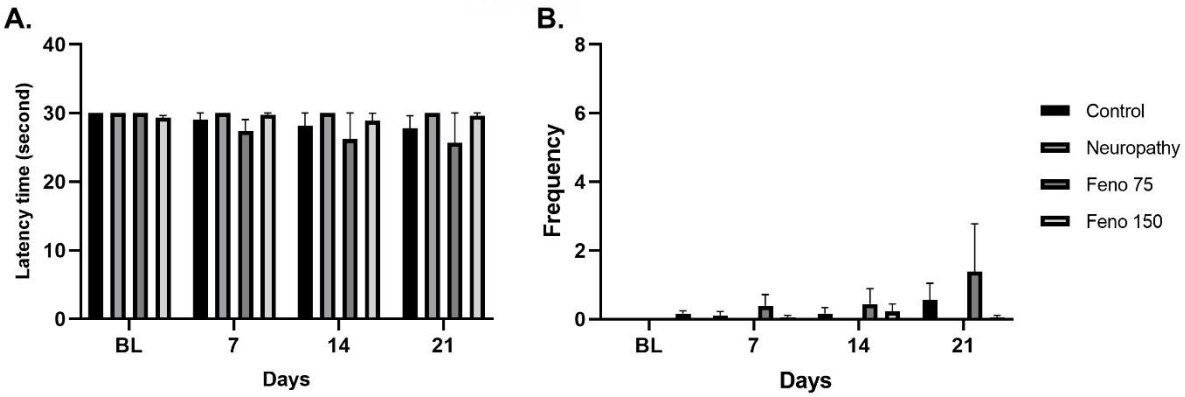

Figure 4. Fenofibrate's effect on thermal hyperalgesia caused by oxaliplatin on jumping-off responses, with parameters for latency time (Figure 1A) and frequency (Figure 1B). No significant differences in treatment groups were identified (two-way ANOVA followed by Tukey's post-hoc test; n = 6 mice)

Approximately 12 different types of behavior have been recorded in the hot-plate test, including sniffing, rearing, licking, stamping, jumping, hindleg-withdrawal, leaning posture, grooming, and freezing (Espejo & Mir, 1993). Although variances were observed depending on the type of measured activity, some of these behaviors were sensitive to particular analgesics or drugs. (Deuis et al., 2017). Forepaw withdrawal typically occurs first, and hindpaw withdrawal or licking is seen as a preferred sign of nociception. Forepaws are often biased and used in exploration because of their inconsistent contact with metal surfaces (Deuis et al., 2017; Minett *et al*., 2011). If no nocifensive behavior was observed within the 30 s cut-off time, the animal was removed from the hot plate to prevent tissue damage (Kudla et al., 2019).

Administration of 75 and 150 mg/kg fenofibrate in combination with oxaliplatin tended to prevent the enhancement of oxaliplatin-induced thermal hyperalgesia in hind-paw withdrawal and rubbing responses. This result correlates with other behavioral tests regarding the role of PPAR-alpha in neuropathy. PEA (Palmitoylethanolamide) and OEA (oleoylethanolamide) are endogenous PPARα agonists. PEA reduces mechanical and thermal hyperalgesia in mice with carrageenan-induced inflammation mice (Sasso et al., 2012). In HF-fed rats, PEA significantly decreased mechanical and thermal hypersensitivity (Wang *et al*., 2017). In a rodent model of chronic constriction injury, intraperitoneal administration of PEA for seven days reduced hyperalgesia (Bettoni et al., 2013). Moreover, mechanical and thermal thresholds in SNI mice were significantly enhanced by PEA and OEA treatments (Guida et al., 2015). The results of rubbing behavior showed that oxaliplatin significantly induced thermal heat hyperalgesia, as reflected by the reduced latency time on days 14 and 21, compared to the normal group (Figure 2). The neuropathy group tended to

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develop thermal heat hyperalgesia on days 7, 14, and 21 compared to baseline. Administration of fenofibrate (75 and 150 mg/kg) reversed the reduction in rubbing response latency time induced by oxaliplatin. Meanwhile, the frequency parameter showed that exposure to oxaliplatin in the neuropathy group increased rubbing frequency on day 7 and significantly on days 14 and 21 compared to the control group. The rubbing frequency tended to increase in the neuropathy group on days 7, 14, and 21 compared to that in the neuropathy group at baseline. Meanwhile, the administration of fenofibrate at doses of 75 and 150 mg/kg tended to increase the rubbing frequency compared to the neuropathy group on days 7, 14, and 21.

The effect of fenofibrate co-treatment against relative TRPA1 mRNA expression

To test the effects of oxaliplatin and fenofibrate co-treatment on TRPA1 mRNA expression in the thalamic tissue, we conducted a qPCR test. The results showed that oxaliplatin administration in the neuropathy group compared to the control increased TRPA1 mRNA expression in the thalamic tissue 21 days after the first oxaliplatin injection (Figure 5). This result is consistent with earlier studies that reported that oxaliplatin increased the expression of TRPA1 mRNA and protein in DRG tissue and played a crucial role in neuropathic pain in rodents (Chukyo et al., 2018; Park et al., 2015). Oxaliplatin sensitizes TRPA1 via cytosolic acidification of DRG sensory neurons (Riva et al., 2018). According to several studies, oxaliplatin increases the sensitivity of hTRPA1 by a mechanism involving the inhibiting prolyl hydroxylase and the oxidation of intracellular cysteines. (Miyake et al., 2016, 2017). In the periaqueductal region and thalamus, oxaliplatin causes mechanical allodynia by increasing the activation of PKC isoforms and causing phosphorylation of the γ/ε isoforms of PKC (Norcini et al., 2009). PKC activation increases TRPA1-mediated pain sensation (Bautista et al., 2006). These results confirm earlier research that TRPA1 expression is increased in the thalamus brain region by oxaliplatin-induced thermal hyperalgesia via a PKC-dependent mechanism, demonstrating the supraspinal role of oxaliplatin in neuropathic pain.

Administration of 75 and 150 mg/kg fenofibrate in combination with oxaliplatin significantly reduced the relative TRPA1 mRNA expression ($p<0.01$) compared to the neuropathy group. This result indicates that fenofibrate inhibited TRPA1 mRNA expression. IL-1, IL-6, and TNF were highly expressed in the DRG after treatment with oxaliplatin, and the administration of fenofibrate reduced the upregulation of these mRNAs (Caillaud *et al*., 2021; Campolo *et al*., 2021). IL-1β and TNFα induction increases TRPA1 expression in human IVD tissues (Kameda *et al*., 2019). In addition, TNFα elevated TRPA1 expression in cultured primary DRG neurons; however, at concentrations of 15 ng/ml and 50 ng/ml, it only gave less than 20% in TGNs (Meng et al., 2016). Based on the current study, the inhibition of TRPA1 mRNA expression by fenofibrate in the supraspinal area of the thalamus is possibly through the implication of the pathway in the peripheral region.

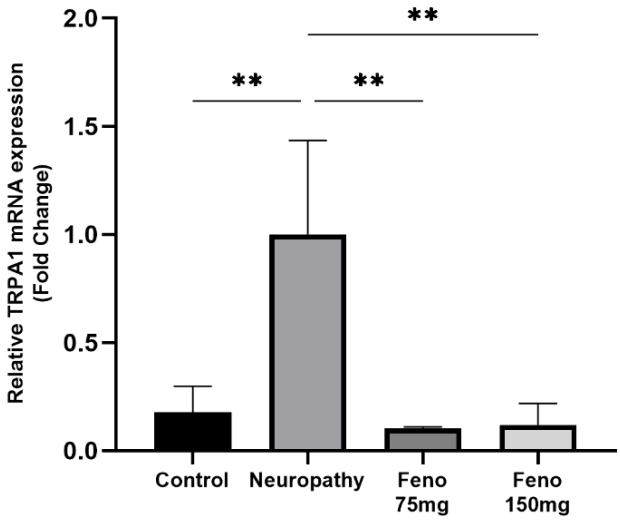

Figure 5. Quantitative RT-PCR testing of TRPA1 in the thalamus. **p < 0.01 indicates statistical significance between treatment groups (one-way ANOVA followed by Tukey's post-hoc test; n = 3 thalamus)

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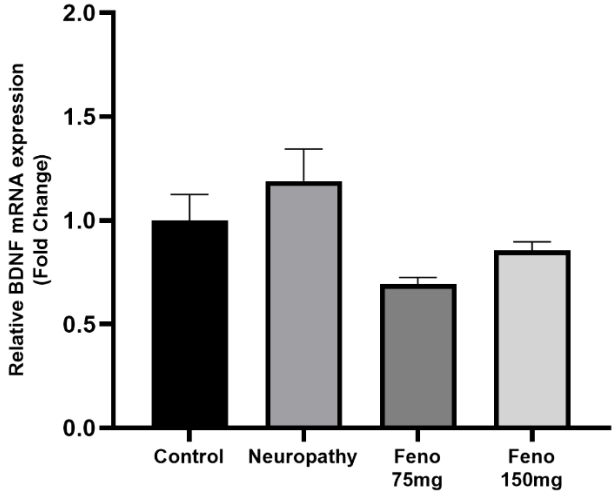

Figure 6. Quantitative RT-PCR testing of BDNF in the thalamus. No significant differences in treatment groups were identified. (one-way ANOVA; n = 3 thalamus)

The effect of fenofibrate co-treatment against relative BDNF mRNA expression

To test the effects of oxaliplatin and fenofibrate co-treatment on BDNF mRNA expression in the thalamic tissue, we conducted a qPCR test. The results demonstrated that, compared to the control group, the neuropathy group treated with oxaliplatin showed slightly enhanced BDNF mRNA expression 21 days after the initial oxaliplatin injection. (Figure 6). Administration of 75 and 150 mg/kg fenofibrate in combination with oxaliplatin decreased BDNF levels. Neurotrophic factors, such as BDNF, play a role in CIPN. However, this must be conclusively identified. Oxaliplatin significantly enhanced the expression of BDNF in the dorsal horn and DRG in previous research (Maruta et al., 2019; Ruyang et al., 2015). In a mouse model of chemotherapy-induced neuropathic pain, overexpression of BDNF in neurons contributes to the development of central sensitization (Ruyang et al., 2015). The expression of BDNF in the DRG is also increased by the elevation of pro-inflammatory cytokines under neuroinflammatory conditions (Lin *et al.*, 2011). In contrast, Campolo *et al.* (2021) found that oxaliplatin reduced BDNF and NGF in DRG samples, and treatment with PEA promoted BDNF and NGF release. These findings imply a role in facilitating the development and differentiation of new synapses and neurons. However, given the various prespectives regarding the role of neurotrophins in CIPN, additional non-clinical investigations are needed to provide more insight into other mechanisms of CIPN and the role of PPAR agonists.

TRPA1 upregulation correlates with CIPN progression. TRPA1 activation in cancer pain models increases hydrogen peroxide (H2O2) production, which maintains TRPA1 activation and sensitization; these H2O2 levels may be caused by increased NADPH oxidase and superoxide dismutase activity (Antoniazzi *et al*., 2019). Early TRPA1 inhibitor treatment prevents oxidative stress-induced CIPN, including PTX (Trevisan *et al*., 2013). As a result, the compound or drug targeting TRPA1 is promising for the prevention of CIPN. Although this research focused on the thalamus of the supraspinal area, more studies on other tissues and biomarkers provide a more integrative explanation of the CIPN mechanism.

CONCLUSION

The present findings indicate that oxaliplatin induction significantly induces thermal hyperalgesia in hind paw withdrawal and rubbing responses, but not in jumping-off responses. Administration of 75 and 150 mg/kg fenofibrate during oxaliplatin induction prevented the enhancement of oxaliplatin-induced thermal hyperalgesia. Moreover, fenofibrate significantly prevented oxaliplatin-induced upregulation of TRPA1. Therefore, PPARα agonist potential suppresses CIPN development. However, given the various prespectives regarding the role of neurotrophins in CIPN, additional non-clinical investigations need to be conducted to provide more insight into other mechanisms of CIPN and the role of PPAR agonists.

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AUTHOR CONTRIBUTIONS

Conceptualization, C. A., M. R., S.; Methodology, L. W., I. N. B. D., C. A.; Validation, A. N. A., P. A.; Formal Analysis, A. N. A., P. A., I. N. B. D.; Investigation, A. N. A., P. A., A. A. S. D. P., T. D. S., G. L. P., L. W.; Resources, C. A., M. R.; Data Curation, C. A., M. R, S.; Writing - Original Draft, A. A. S. D. P., T. D. S., G. L. P.; Writing - Review & Editing, A. N. A., P. A.; Visualization, A. N. A., P. A., A. A. S. D. P., T. D. S., G. L. P.; Supervision, C. A., M. R, S.; Project Administration, A. N. A., I. N. B. D., C. A., Funding acquisition, M. R., C. A.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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