JFSP Vol.10, No.1, January-April 2024, Page: 1-11
pISSN: 2549-9068, eISSN: 2579-4558
Jurnal Farmasi Sains dan Praktis
(JFSP)
http://journal.unimma.ac.id/index.php/pharmacy

OPTIMIZATION OF DICLOFENAC POTASSIUM TRANSDERMAL PATCH FORMULA USING A COMBINATION OF POLYVYNIL PYRROLIDONE K 30, ETHYL CELLULOSE AND MENTHOL WITH SIMPLEX LATTICE DESIGN METHOD

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https://doi.org/10.31603/pharmacy.v9i3.8956

Article info:
Submitted : 06-04-2023
Revised : 25-07-2023
Accepted : 18-11-2023

Publisher:
Universitas Muhammadiyah Magelang

ABSTRACT

Diclofenac potassium is one of the NSAID drugs which can cause gastrointestinal irritation and damage to the small intestinal mucosa including erosion and ulceration. The purpose of this study was to determine the effect of the combination of PVP K 30, ethyl cellulose and menthol on organoleptic, thickness, weight uniformity, moisture and folding resistance of diclofenac potassium transdermal patch.This research is an experimental study that includes an experiment to optimize the formulation of a transdermal patch preparation with the active ingredient potassium diclofenac and a combination of PVP K 30, ethyl cellulose and menthol. The optimization method uses the simplex lattice design method. There are 13 formula designs consisting of a combination of PVP K 30, ethyl cellulose and menthol. Each formula was tested for organoleptic, thickness, weight uniformity, moisture and folding resistance. Then the optimum formula was determined and analyzed using the simplex lattice design method. The combination of PVP K 30, ethyl cellulose and menthol with a simplex lattice design has an effect on the transdermal patch of diclofenac potassium which increases the consistency of the patch surface, reduces the thickness directly proportional to the weight of the patch and increases folding resistance. The proportion of PVP K 30, ethyl cellulose and menthol that can produce the optimum formula for diclofenac potassium transdermal patches with the simplex lattice design on the critical parameters of thickness, moisture, folding resistance and penetration tests, namely PVP K 30 of 14.87%, ethyl cellulose of 10.00% and 5.13% menthol.

Keywords: Diclofenac potassium; Transdermal patch; Simplex lattice design (SLD)

1. INTRODUCTION

Diclofenac is one of the well-known Non-Steroid Anti-Inflammatory Drugs (NSAIDs) with anti-inflammatory, analgesic, and antipyretic properties, comparable to or superior to other NSAIDs. Potassium diclofenac is claimed to be soluble and absorbed faster than sodium salt and is recommended for treatments requiring a short onset of action, particularly for its analgesic properties (Barros et al., 2015). Primary dysmenorrhea and mild to moderate pain can also be treated with potassium diclofenac. Two pathophysiological pathways contribute to gastrointestinal pain caused by potassium diclofenac. In addition, current clinical research has shown that NSAIDs can harm the mucosa of the small intestine. This is because endoscopic findings indicate that NSAIDs can result in mucosal damage such as erosion and ulceration (Matsui et al., 2011).

Therefore, different administration routes have been developed to minimize the digestive system side effects associated with oral potassium diclofenac administration. The transdermal

drug administration route involves delivering the drug through the skin until it reaches the bloodstream. This route has several advantages compared to oral and injection methods (Putri, 2018). The transdermal delivery system has many advantages, including easy use, reducing the frequency of drug administration, eliminating first-pass metabolism, ensuring more uniform plasma levels, and reducing side effects such as gastric irritation and patient compliance (Puspitasari *et al*., 2016).

Purnamasari et al., 2019 have formulated and evaluated potassium diclofenac transdermal patches, and the results of the study showed that patches with PVP polymer were good both physically and homogeneously, as well as in in-vitro penetration tests. However, to determine the composition of the patch preparation that produces optimum physical characteristics, optimization is needed, including the simplex lattice design (SLD) method (Suryani et al., 2015). PVP and ethyl cellulose affect delivering the active ingredient contained in the patch preparation. The use of ethyl cellulose causes the formation of a barrier, trapping the active ingredient in the preparation, resulting in the active ingredient not easily released from its base, while PVP causes the formation of pores, so it is necessary to combine PVP and ethyl cellulose (Nurmesa et al., 2019). Maulina's research on the effect of menthol on the characteristics of sodium diclofenac gel preparations found that the addition of menthol can reduce the viscosity of the preparation.

Based on the above considerations, the researchers will formulate a transdermal patch using the solvent evaporation method which has the active ingredient of potassium diclofenac by combining PVP and ethyl cellulose as polymers and menthol as an enhancer.

2. METHODS

2.1. Material

The tools used are dropper pipettes, volume pipettes (Iwaki), glass tools (Pyrex), analytical balances (OHAUS PA323), UV-Vis's spectrophotometer (Shimadzu), pH meter (OHAUS ST3100M), Digital caliper (Mitutoyo), Magnetic stirrer (Yellow-MAG HS7), Desiccator (NORMAX), Sonicator (Elma), Software Design-Expert® version 11.0 and software SPSS Version 25.

The materials used are Diclofenac potassium (PT. Zenith Pharmaceutical), PVP K 30 (PT. Zenith Pharmaceutical), Ethylcellulose (PT. Zenith Pharmaceutical), Menthol (PT. Zenith Pharmaceutical), Dibutyl phthalate (PT. Zenith Pharmaceutical), Methylparaben (PT. Zenith Pharmaceutical), Ethanol 96% (PT. Brataco Indonesia), KH2PO4 (Merck), NaOH (Merck), Aquadest (PT. Brataco Indonesia).

2.2. Determination of Formula Using Simplex Lattice Design (SLD)

The formula of Diclofenac potassium patch was selected using SLD program to get the most optimum Diclofenac potassium patch formula containing combination of PVP K 30, ethyl cellulose and methol will be made up of 30% of the formula. The formula will be made with a weight of 5.0 grams. Response or parameters used are thickness, moisture and folding resistance.

2.3. Formulation of Film Patch Diclofenac Potassium

A matrix-type transdermal patch containing potassium diclofenac was prepared using various concentrations of PVP K 30 and ethyl cellulose polymers, as well as menthol enhancer, obtained from the SLD method. Potassium diclofenac and the polymer (PVP K 30: ethyl cellulose) were first dissolved using ethanol as a solvent in separate glass containers. Then, they were mixed together with menthol, dibutyl phthalate, and methyl paraben and homogenized using a sonicator for 20 minutes to form a thick mass. The resulting solution was poured into patch molds made of glass measuring 5 x 5 cm. The solvent was allowed to evaporate at room temperature for 24 hours (Mita et al., 2018). The variations in the concentrations of PVP K 30, ethyl cellulose, and menthol can be seen in **Table 1**.

Table 1. The Formula of Diclofenac Potassium Patch Based on SLD Method

Formula	Material (%)						
	DPO	X_1*	X_2*	X_3*	DPT**	MP**	Ethanol 95%
1	2	14	14	2	30	0.3	Add 100
2	2	10	10	10	30	0.3	Add 100
3	2	11.34	15.33	3.33	30	0.3	Add 100
4	2	10	14	6	30	0.3	Add 100
5	2	10	18	2	30	0.3	Add 100
6	2	12.66	12.67	4.67	30	0.3	Add 100
7	2	10	18	2	30	0.3	Add 100
8	2	11.34	11.33	7.33	30	0.3	Add 100
9	2	14	10	6	30	0,3	Add 100
10	2	18	10	2	30	0,3	Add 100
11	2	15.33	11.34	3.33	30	0,3	Add 100
12	2	18	10	2	30	0,3	Add 100
13	2	10	10	10	30	0,3	Add 100

DPO= Diclofenac Potassium, X_1= Polivinyl Pyrrolidon K 30, X_2= Ethyl Cellulose, X_3= Menthol, DPT= Dibuthyl Phthalat, MP= Methylparaben(*) based on result of SLD method. (**) percentage was based on dry weight of the polymer

2.4. Characteristics of Diclofenac Potassium Film Patch

2.4.1. Organoleptic Test

The characteristics of the film, including color, odor, and surface consistency, are observed (Suryani et al., 2015).

2.4.2. Thickness Test

The thickness of the film is measured using a digital caliper at three different points, and the average thickness is calculated (Suryani et al., 2015).

2.4.3. Weight Uniformity Test

The weight variation of the film in each formula is determined by weighing each patch individually, and the average weight is calculated (Suryani et al., 2015).

2.4.4. Moisture Content test

Each finished patch is weighed (initial weight) and then stored in a desiccator containing silica gel at room temperature for 24 hours. The patch is then weighed again (final weight) (Suryani et al., 2015).

$$\%\ Moisture\ Content = \frac{(initial\ weight - final\ weight)}{initial\ weight} \times 100\% \quad (1)$$

2.4.5. Folding Endurance Test

Folding endurance is the number of folds required to break the film. This test not only describes the strength of the film composed using polymers but also checks how efficient polymers and plasticizers provide flexibility. This test involves a simple phenomenon, which is repeatedly folding the film in the same place until it breaks. Thus, the number of times the film can be folded in the same place without cracking or breaking can be determined. The film is considered to meet the criteria if it can withstand folding more than 200 times (Setyawan et al., 2015).

2.5. Determination of Diclofenac Potassium Content in Film Patch

The film is cut into small pieces, weighed, and dissolved in 50 ml phosphate buffer 7.4, then sonicated for 50 minutes. The resulting solution is filtered and 1 ml of it is pipetted and diluted to 10 ml, then sonicated again for 10 minutes and filtered. To determine the drug content in each formulation, the maximum wavelength is 276 nm. The obtained absorbance is compared with the calibration curve to determine the concentration of potassium diclofenac in the patch preparation (Purnamasari et al., 2019).

2.6. Determination of The Optimum Formula

The determination of the optimum formula is done by examining the results of physical characterization tests of the patch matrix in each formula. In the physical characterization test of the patch matrix, thickness, moisture content, and folding endurance are measured. The results of each formula's response are then processed using the simplex lattice design method in Design Expert software version 11. The optimum formula is determined based on the highest desirability value obtained from the method's results.

2.7. Verification of The Optimum Formula

Verification is conducted by creating a matrix of the optimal formula predicted by the simplex lattice design in Design Expert software version 11. The production of the matrix patch is replicated three times. The observed results of the matrix are then compared with the predicted response of the optimal formula from the simplex lattice design. Verification is then carried out using the One Sample T-test in SPSS 25 software.

2.8. Data Analysis

The data for the testing of characteristics and penetration rate of potassium diclofenac transdermal patch will yield values for each response and the equation model. The simplex lattice design model will generate an optimum formula obtained after inputing the thickness, moisture, and fold resistance test values using Analysis of Variance (ANOVA) in Design Expert software version 11. The optimal formula is obtained based on the respective parameter values obtained, and contour plots are created for each parameter. The contour plots for the moisture, thickness, and folding endurance test parameters are superimposed to determine the optimal region. The testing results from the optimal formula are then compared to the predicted results obtained from Design Expert using SPSS 25 with a One Sample T-test at a confidence level of 95%.

3. RESULT AND DISCUSSION

3.1. Characteristics of Diclofenac Potassium Film Patch

The evaluation of characteristics is to determine the physical characteristics of potassium diclofenac transdermal patch, including organoleptic properties, thickness, weight uniformity, moisture content, and folding endurance. The results of the film patch characteristics can be seen in **Table 2**.

Table 2. The Result of Characteristics Diclofenac Potassium Film Patch

Formula	Thickness ± SD(mm)	Weight Uniformity ± SD(gram)	Moisture Content ± SD(%)	Folding Endurance ±SD
F1	0.71 ± 0.005	1.972 ± 0.0008	1.268 ± 0.047	226±3.559
F2	0.66 ± 0.017	1.633 ± 0.0008	1.349 ± 0.074	207±2.828
F3	0.73 ± 0.005	1.914 ± 0.0017	1.459 ± 0.064	178±4.243
F4	0.69 ± 0.009	1.785 ± 0.0022	1.544 ± 0.195	169±3.559
F5	0.75 ± 0.005	1.989 ± 0.0025	1.242 ± 0.121	158±2.828
F6	0.70 ± 0.009	1.858 ± 0.0009	1.345 ± 0.090	223±4.243
F7	0.74 ± 0.005	1.989 ± 0.0021	1.242 ± 0.081	160±3.559
F8	0.68 ± 0.009	1.764 ± 0.0005	1.379 ± 0.026	218±2.828
F9	0.68 ± 0.021	1.771 ± 0.0016	1.648 ± 0.246	226±4.243
F10	0.72 ± 0,005	1.969 ± 0.0012	1.763 ± 0.200	232±3.559
F11	0.70 ± 0.008	1.929 ± 0.0012	1.732 ± 0.109	228±4.243
F12	0.71 ± 0.005	1.971 ± 0.0021	1.778 ± 0.236	238±3.559
F13	0.66 ± 0.012	1.632 ± 0.0008	1.489 ± 0.057	218±2.828

3.1.1. Organoleptic Test

Organoleptic testing was conducted by observing the color, odor, and consistency of the surface of the potassium diclofenac film patch. Based on observations, the film patch is generally white to yellowish in color, wet, and has a distinctive menthol smell. However, there are

differences in the surface consistency of each film patch. F3, F4, F5, F7, F9, F10, F11, and F12 have non-smooth (cracked) film conditions, unlike F1, F2, F6, F8, and F13 which have smooth film conditions. This is due to the difference in polymer concentration in each formula. Formulas with non-smooth and cracked film conditions were found more in F5 and F7, which had a higher concentration of ethyl cellulose polymer. Purnamasari et al., (2019) stated in their research that a polymer combination of PVP:ethyl cellulose (1:3) produces a white, clear, and non-smooth patch. The results of the organoleptic test for diclofenac potassium film patches can be seen in the **Table 3**.

Table 3. The Result of Organoleptic Test of Diclofenac Potassium Film Patch

Formula	Color	Odor	Consistency of The Surface
F1	Off-white to pale yellow	Mentol smell	Smooth surface, not cracked and wet
F2	Off-white to pale yellow	Mentol smell	Smooth surface, not cracked and wet
F3	Off-white to pale yellow	Mentol smell	Smooth surface, slightly cracked and wet
F4	Off-white to pale yellow	Mentol smell	Smooth surface, slightly cracked and wet
F5	Off-white to pale yellow	Mentol smell	Uneven surface, cracked and wet
F6	Off-white to pale yellow	Mentol smell	Smooth surface, not cracked and wet
F7	Off-white to pale yellow	Mentol smell	Uneven surface, cracked and wet
F8	Off-white to pale yellow	Mentol smell	Smooth surface, not cracked and wet
F9	Off-white to pale yellow	Mentol smell	Smooth surface, slightly cracked and wet
F10	Off-white to pale yellow	Mentol smell	Smooth surface, slightly cracked and wet
F11	Off-white to pale yellow	Mentol smell	Smooth surface, slightly cracked and wet
F12	Off-white to pale yellow	Mentol smell	Smooth surface, slightly cracked and wet
F13	Off-white to pale yellow	Mentol smell	Smooth surface, not cracked and wet

3.1.2. Thickness Test

The results of the thickness testing were analyzed using Design Expert with simplex lattice design method. The response results can be seen in **Figure 1**.

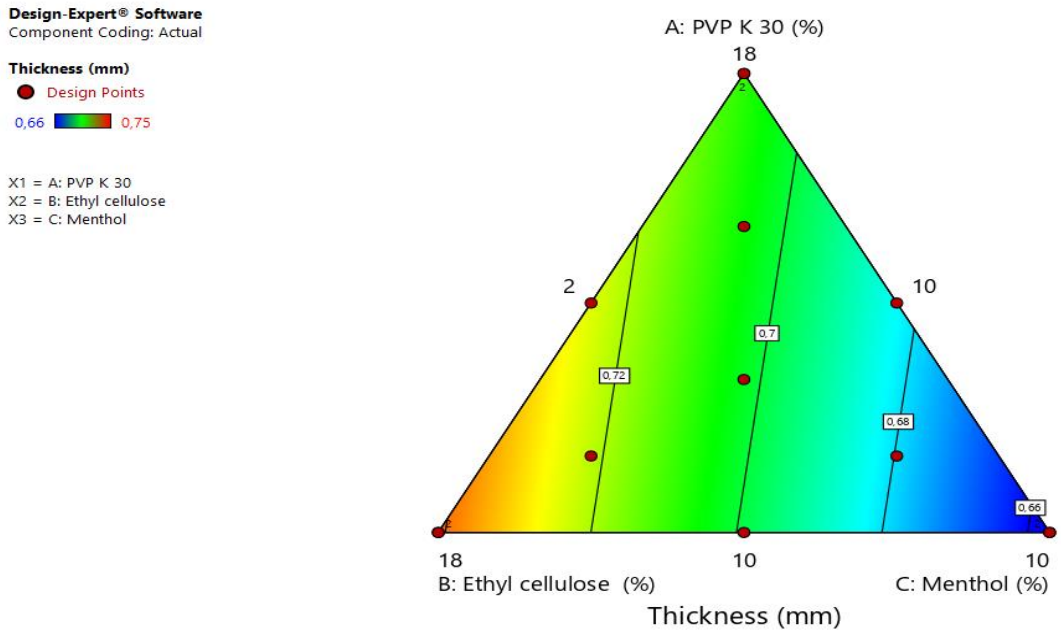

Figure 1. Contour Plot of The Thickness Response of Potassium Diclofenac Film Patch

The response of thickness based on ANOVA analysis at a 95% confidence level obtained the simplex lattice design equation for thickness as formulated in Eq (2).

$$Y = +0.7090(A) +0.7410(B) +0.6570(C) \quad (2)$$

Where: Y = Thickness; A = PVP K 30; B = Ethyl cellulose; C = Menthol

The contour plot shows the relationship between the three components, namely PVP K 30, ethyl cellulose, and menthol in the diclofenac potassium film patch towards the thickness of the film patch with the formation of color gradation, where orange represents the boundary of the highest value while dark blue represents the boundary of the lowest value. Based on the contour plot (**Figure 1**), it can be seen that ethyl cellulose affects the thickness of the film patch, as evidenced by the presence of orange color on the ethyl cellulose plot, which means that an increase in the concentration of ethyl cellulose can increase the thickness value. This can also be seen from the regression coefficient values which indicate that ethyl cellulose (+0.740) has the most significant effect in increasing the thickness of the film patch compared to PVP K 30 and menthol.

The thickness of the film patch in this test ranged from 0.66-0.76 mm (**Table 2**). The factors that influenced the difference in thickness between the formulas were the physicochemical properties of the polymer used. The test results for each formula showed that F5 and F7 had a higher average thickness compared to the other formulas. This was because F5 and F7 had the highest concentration of ethyl cellulose. According to Suryani et al., (2015), if ethyl cellulose polymer is used excessively, it will form thick and uneven fibers that can affect the thickness of the film patch. Other variables that can affect the thickness of the film patch are the size of the mold, the volume of the solution, and the total amount of solids in the solution.

3.1.3. Weight Uniformity Test

The weight uniformity test was analyzed based on the average weight of the film patch and its standard deviation. Standard deviation is a measure used to assess the variation or dispersion of a data set. According to the literature, a good standard deviation value is ≤ 0.05 (Hermanto & Nurviana, 2019). The results of the potassium diclofenac film patch weight testing can be seen in **Table 2**.

The results of each formula were considered to meet the requirements. However, the weight of each formula's film patch is different due to the different amounts of additional materials in each formula. Weight uniformity is affected by the polymer component which has a more hydrophilic property, which during the process of making the film patch and the aging process, water will easily be retained in the film patch, which will affect the resulting weight (Ermawati & Prilantari, 2019). In the potassium diclofenac patch formula, the polymer that has a hydrophilic property is PVP K 30. The weight of the film patch can also be influenced by the manufacturing method itself because it can allow the patch solution to remain partially in the container (Arifin et al., 2019). However, in this study, the highest patch weight was obtained in formulas F5 and F7, where F5 and F7 also have a higher thickness than other formulas, so it can be said that the weight of the patch formula is directly proportional to its thickness.

3.1.4. Moisture Content Test

The result of the moisture content testing was analyzed using Design Expert with a simplex lattice design method. The response can be seen in **Figure 2**. The moisture content response was analyzed using Design Expert with the simplex lattice design method. The ANOVA test results at a 95% confidence level obtained the equation for moisture content response as formulated in Eq (3).

$$Y = +1.74\ (A) +1.25\ (B) +1.45\ (C) \quad (3)$$

Where: Y = Moisture content; A = PVP K 30; B = Ethyl cellulose; C = Menthol

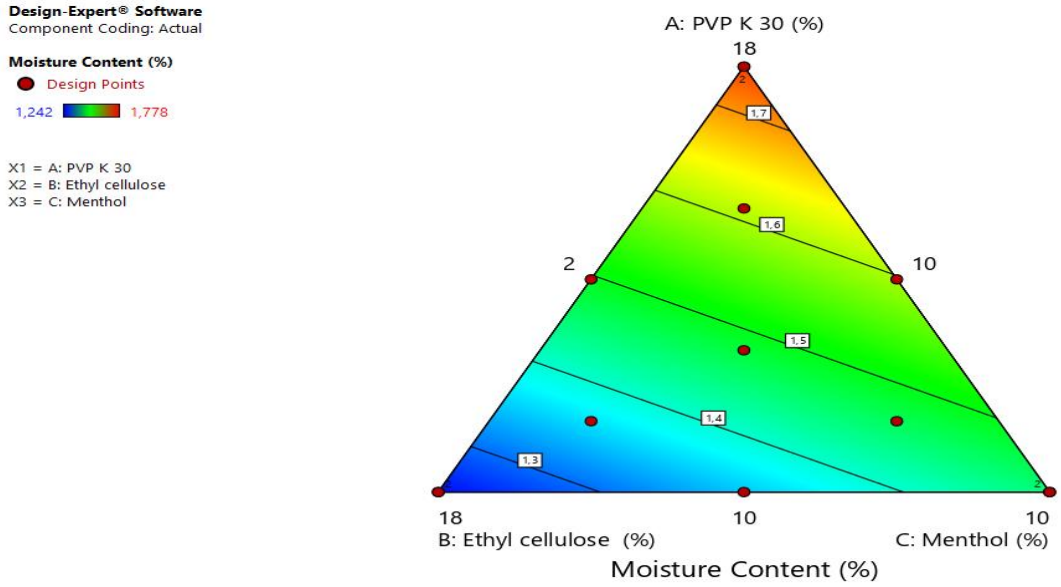

Figure 2. Contour Plot of The Moisture Content Response of Potassium Diclofenac Film Patch

The contour plot shows the relationship between the three components, namely PVP K 30, ethyl cellulose, and menthol. The color gradient is formed in shades of orange, green, and blue, where orange represents the highest value and dark blue represents the lowest value. Based on the contour plot results, it can be seen that PVP K 30 can influence the moisture content of the film patch, as indicated by the presence of orange color in the PVP K 30 plot, which means that increasing the concentration of PVP K 30 can increase the moisture content value. This is also supported by the regression coefficient values which show that PVP K 30 (+1.74) has the most significant effect on increasing the moisture content of the film patch compared to ethyl cellulose and menthol.

In general, the percentage of moisture capacity of the film will increase if the hydrophilicity of the polymer or plasticizer used also increases (Fuziyanti et al., 2022). A good film patch is said to contain little water, so the stability of the patch will be good. The required range of water content is 1-10% (Kumar et al., 2013). The results of the moisture content percentage test show that the film patch in each formula has values that meet the required range. The formula with the highest percentage of moisture content is obtained in formulas F10 and F12 because they contain a higher concentration of PVP K 30 than other formulas. This is in line with the study conducted by Fatmawaty et al., (2017), which found that the combination of PVP:EC with a higher concentration of PVP resulted in a moisture content percentage of 29%, which did not meet the required range.

The factors that can affect the humidity are the physicochemical properties of the materials used, namely the polymer and plasticizer. Dibutyl phthalate as a plasticizer is hydrophilic, similar to the polymer PVP K 30 which can increase the percentage of humidity (Fuziyanti et al., 2022). Therefore, a combination with a hydrophobic polymer is needed, otherwise there will be a high increase in humidity percentage (Puspitasari et al., 2016).

3.1.5. Folding Endurance Test

The result of the folding endurance testing was analyzed using Design Expert with simplex lattice design method. The response can be seen in **Figure 3**. The response of foldability based on ANOVA test at 95% confidence level obtained the simplex lattice design equation for foldability as formulated in Eq (4).

$$Y = +233.90(A) +157.09(B) + 213.76(C) +118.55(AB) +30.55(AC) -50.31(BC) \quad (4)$$

Where: Y = Foldability; A = PVP K 30; B = Ethyl Cellulose; C = Menthol

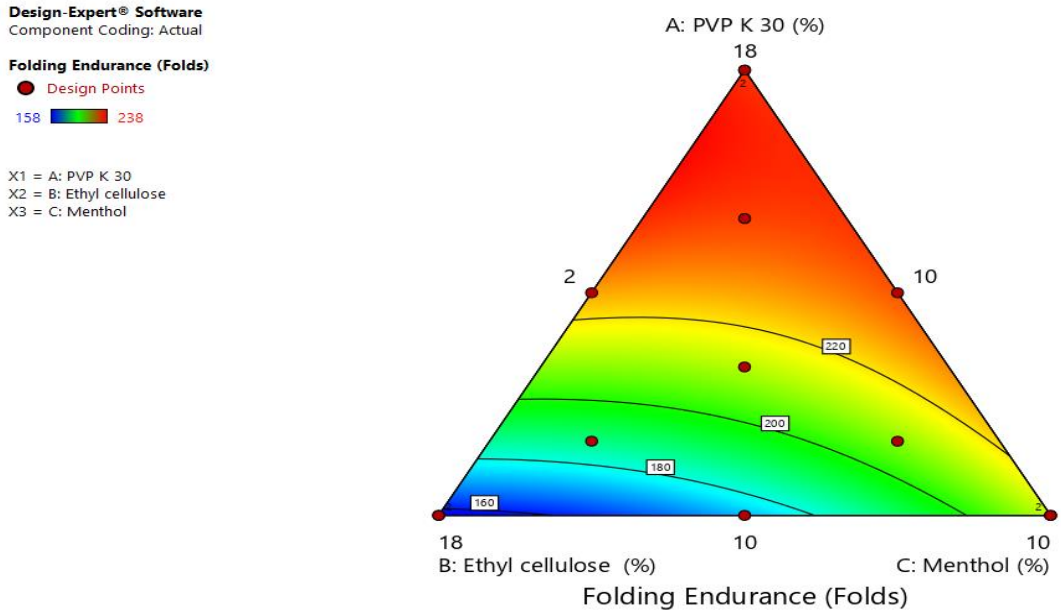

Figure 3. Contour Plot of The Fold Endurance Response of Potassium Diclofenac Film Patch

The contour plot shows the relationship between the three components, namely PVP K 30, ethyl cellulose, and menthol in the potassium diclofenac film patch against the film patch humidity, with the formation of color gradations, where the orange color is the limit of the highest value while the dark blue is the limit of the lowest value. Based on the contour plot results, it can be seen that PVP K 30 can affect the folding endurance of the film patch, as evidenced by the presence of orange color on the PVP K 30 plot, which means that an increase in PVP K 30 concentration can increase the folding endurance value. From the regression coefficient values, it is known that PVP K 30 (+233.90) has the most significant influence in increasing the film patch thickness compared to PVP K 30 and menthol. It is also known that the interactions that occur between PVP K 30: ethyl cellulose and PVP K 30: menthol provide positive values, namely (+118.55) and (+30.55), which means that these interactions can increase the folding endurance value, unlike the interaction between ethyl cellulose and menthol, which provides a negative value, namely (-50.31), which means that the interaction between the two can decrease the folding endurance value of the potassium diclofenac film patch.

The use of polymer and plasticizer combination can be seen from the good folding endurance (> 200) in F10 and F12, which are formulas that use a higher concentration of dibutyl phthalate (0.42 g) and a combination of PVP K 30 polymer compared to ethyl cellulose, while for F5 and F7, the folding endurance is < 200 times, indicating poor performance. F5 and F7 are formulas that use the same concentration of dibutyl phthalate as F10 and F12, but the polymer combination in F5 and F7 uses a higher concentration of ethyl cellulose than PVP K 30. According to the research conducted by Fatmawaty et al., (2017), the proper concentration of polymer combination has a significant effect on the folding endurance of film patches, not only plasticizers, where PVP is hydrophilic and can increase elasticity, making it less prone to breakage, and ethyl cellulose is a hydrophobic polymer that can increase the strength of the film patch, making it less prone to tearing. This indicates the importance of using PVP K 30 as a polymer and dibutyl phthalate as a plasticizer to increase the flexibility/elasticity and folding endurance of the film patch.

3.2. Determination of Diclofenac Potassium Content in Film Patch

Determination of the content (concentration) of diclofenac potassium in the film patch was carried out to measure the active ingredient in each formula. The film patch was dissolved using phosphate buffer pH 7.4 with the help of a sonicator for approximately 60 minutes, then analyzed by UV-Vis spectrophotometry at a wavelength of 276 nm. This test was carried out in triplicate

for each formula, and the average concentration and standard deviation (SD) were calculated. The results showed that the average concentration of diclofenac potassium in each formula ranged from 98.33% ± 0.036 to 101.45% ± 0.028. The results of the test for diclofenac potassium concentration in the film patch for each formula can be seen in **Table 4**.

Table 4. Diclofenac Potassium Concentration in The Film Patch

Formula	Diclofenac Potassium Concentration ± SD (%)
F1	98.56 ± 0.036
F2	99.78 ± 0.028
F3	99.18 ± 0.042
F4	101.32 ± 0.036
F5	98.79 ± 0.036
F6	99.45 ± 0.036
F7	98.86 ± 0.042
F8	99.34 ± 0.036
F9	101.45 ± 0.028
F10	99.87 ± 0.036
F11	99.65 ± 0.036
F12	98.33 ± 0.036
F13	99.67 ± 0.036

3.3. Determination of The Optimum Formula

The determination of the optimum area of potassium diclofenac patch was carried out using Design Expert 11 software in this study, which used a numerical approach to determine the optimum formula. The data input as parameters were the characteristics of the patch, including thickness, moisture content, and folding endurance. The result of the desirability contour plot obtained a value of 0.862, where the desirability value that approaches 1 indicates the more perfect the result. Desirability represents the magnitude of the value that matches the desired value (Raissi & Farsani, 2009). The achievement of the maximum value in desirability indicates that the selection of goals in the four test parameters is correct. The result of the desirability contour plot can be seen in **Figure 4**.

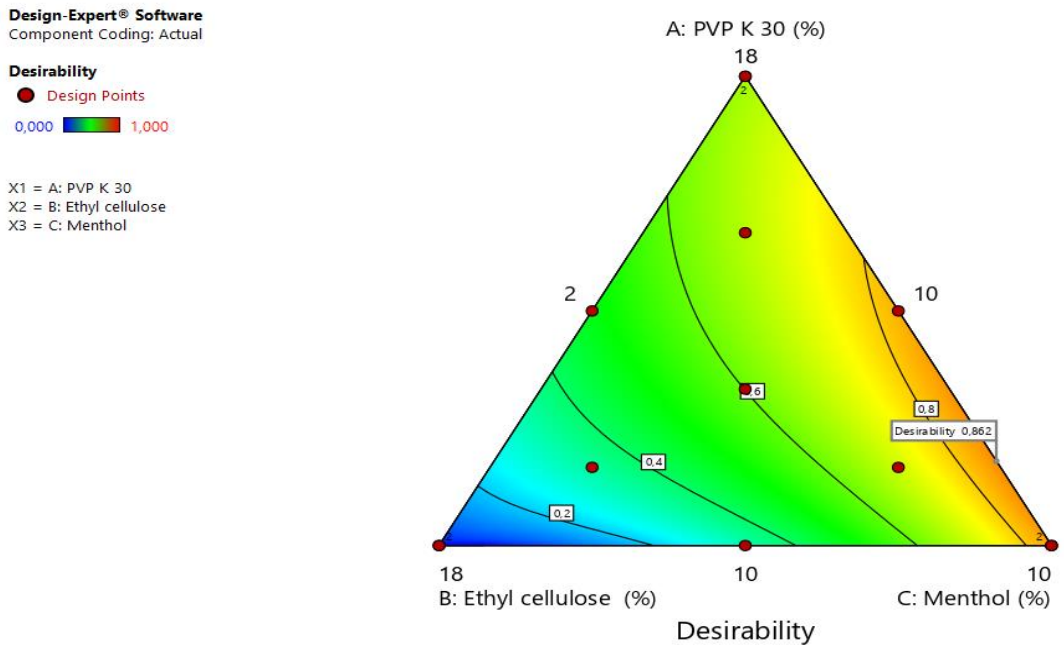

Figure 4. Contour Plot Desirability

The optimum formula for the potassium diclofenac patch components was predicted using simplex lattice design, consisting of PVP K 30 (14.87%), ethyl cellulose (10.00%), and menthol (5.13%). Three replications of the optimum formula were made and tested to obtain the thickness, moisture content, and folding endurance values of the optimum formula, which will then be verified by comparing them to the predicted values obtained from each test parameter.

3.4. Verification of The Optimum Formula

The results of the predicted values compared to the testing values of the optimum patch formula of potassium diclofenac can be seen in **Table 5**.

Table 5. Comparison of The Predicted Value with The Optimum Formula Testing Value

Parameter	Predicted Value	Test Value	*Sig.*	Explanation
Thickness (mm)	0.69	0.71	0.588	No Different
Moisture Content (%)	1.628	1.640	0.825	No Different
Folding Endurance	233	235	0.464	No Different

The comparison between the predicted values and the test values of the optimum formula was analyzed using SPSS 25 with a One Sample T-test at a 95% confidence level to determine whether there was a significant difference or not between the predicted and test values. The results of the One Sample T-test showed a sig value > 0.05 for all parameters, indicating that there was no significant difference between the predicted and test values of the optimum formula.

4. CONCLUSION

The combination of PVP K 30, ethyl cellulose, and menthol using the simplex lattice design had an effect on the transdermal patch of potassium diclofenac, where it improved the surface consistency of the patch, reduced the thickness proportionally to the weight of the patch, and increased the fold endurance. The optimum formula of the transdermal patch of potassium diclofenac was achieved by using proportions of PVP K 30 at 14.87%, ethyl cellulose at 10.00%, and menthol at 5.13% based on the critical parameters of thickness, moisture content, and folding. The data for the testing of characteristics and penetration rate of potassium diclofenac transdermal patch will yield values for each response and the equation model. endurance using the simplex lattice design.

5. ACKNOWLEDGMENT

The author would like to thank the Master Program of Pharmaceutical Science, Faculty of Pharmacy, Setia Budi University.

6. CONFLICT OF INTEREST

All authors declare no conflict of interest.

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JFSP Vol.10, No.1, January-April 2024, Page: 12-24
pISSN: 2549-9068, eISSN: 2579-4558
Jurnal Farmasi Sains dan Praktis
(JFSP)
http://journal.unimma.ac.id/index.php/pharmacy

IN SILICO STUDIES FOR ANTI-BREAST CANCER *Acmella Oleracea* (L.) FLOWERS

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https://doi.org/10.31603/pharmacy.v9i3.9145

Article info:
Submitted : 19-05-2023
Revised : 14-11-2023
Accepted : 24-11-2023

Publisher:
Universitas Muhammadiyah Magelang

ABSTRACT
The study of the efficacy of *Acmella oleracea* (L.) flowers on breast cancer is still in its early stages. The molecular interaction mechanisms underlying *Acmella oleracea's* anti-breast cancer activity will be elucidated using in-silico analysis. For this study, seventeen bioactive compounds were used: spilanthol, alpha- and beta-amyrin ester, stigmasterol, beta-sitosterol, alpha-1-sitosterol, 3-acetylaleuritic acid, scopoletin, vanillic acid, trans-ferulic, (72,9E)-2-oxo-undeca-7,9-dienyl 3-methylbut-2-enoate, beta-caryophyllene, beta-pinene, myrcene, caryophyllene oxide, and limone Canonical smiles were obtained from PubChem and inserted into the PASS server to determine biological activity. Several compounds were docked with protein targets, such as ESR1, MAP2K2, and PGR. We used Pyrx 0.8 software for anchoring molecular interaction and Discovery Studio software to visualize the complex binding. In terms of Antineoplastic, apoptosis agonist, caspase-3, caspase-8 stimulant, ovulation inhibitor, steroid synthesis inhibitor, and TP53 expression enhancer, all the compounds tested positive for anticancer activity. According to Swiss ADME and protox analysis, *Acmella oleracea* flowers have the potential to modulate apoptosis and cell growth. More research is required to confirm the role of *Acmella oleracea* bioactive compounds in developing target cancers. The study reveals that Acmella oleracea has numerous bioactive chemicals advantageous for cancer therapy by inducing apoptosis through interaction with ESR1, MAPK2, and PGR protein.

Keywords: *Acmella oleracea*; Anti-cancer; Breast cancer; Bioactive compounds

1. INTRODUCTION

Breast cancer is one of the most malignant cancers in women, which is increasing rapidly in about women (Johnson et al., 2018). In terms of mortality rates, cancer is only surpassed by cardiovascular disease. In this decade prevalence of breast cancer has at least 6. 6 million cases worldwide (Perdana Istyastono, 2015). Based on the newest updated data from the Global Burden of Cancer Study, in 2020, there were 354,243 new cancer cases in Indonesia, resulting in 197,894 deaths. Furthermore, Global Cancer Observatory has predicted that approximately 192,803 Indonesian women are diagnosed with breast cancer yearly. It is widely believed that the rising number of cancer patients in Indonesia is at least partially attributable to the high-dose chemotherapy and other cancer treatments that have become standard practice. Therefore, it can be helpful to create new strategies for approaching breast cancer (Vasan et al., 2019). Using bioactive compounds from natural sources in the alleys as a source of new drugs from wild plants may be the best alternative in cancer therapy. Traditional medicine has long been believed to increase the body's resistance to disease attacks (Wang et al., 2022).

Cancer of the breast typically develops when cells in the breast divide abnormally and metastasize to neighboring tissues (Perdana Istyastono, 2015). Aside from estrogen and estrogen receptors, other factors have been linked to an increased risk of developing breast cancer. This

hormone, estrogen, plays a crucial role in female reproduction. However, both menopausal hormone therapy with estrogen and high levels of endogenous estrogen are associated with an increased risk of breast cancer (Khan et al., 2022). Breast epithelial cells respond to estrogen by increasing the number of estrogen receptors, increasing the rate at which genes are transcribed. Breast cancer can develop when estrogen levels in women are artificially boosted through hyperproliferation (Khan et al., 2022).

Acmella, which belongs to the Asteraceae family, consists of 30 species and nine intraspecific taxa (Peretti et al., 2021; Sharma & Arumugam, 2021). *Acmella oleracea* is one of the most prominent members of the genus *Acmella* and has been regarded as a traditional medicine in various regions of the world, including Asia, Africa, and parts of the Americas, for generations (Sharma & Arumugam, 2021). From previous studies, every aspect of a plant, including its leaves, stems, and fruits, has been shown to alleviate various ailments and diseases. In folk medicine, inflorescences, flowers, and leaves of the *Acmella* genus are used to treat horticulture, aquaculture, insecticides, and spices in various traditional dishes (Benelli et al., 2019; Greger, 2016).

Extensive phytochemical studies on *Acmella oleracea* have been reported previously. It forms different groups of compounds. It has been found to contain many bioactive compounds important for therapeutic applications, including spilanthol, amyrin ester, stigmasterol, miriclic alcohol glycosides, sitosterol, saponins, and triterpenes. Phytochemical research has led to the discovery of alkyl amides such as 3-acetylaleuritolic acid, beta-sitosterol, scopoletin, vanillic acid, trans ferulic acid, and transisoferulic acid (Peretti et al., 2021; Sharma & Arumugam, 2021; Benelli et al., 2019; Greger, 2016; Lalthanpuii et al., 2018). The most representative compounds of this class are the alkyl amides, especially spilanthol ((2E,6Z,8E)-N-isobutyl 2,6,8-decatrienamide), also known as affinin (Sharma & Arumugam, 2021). This molecule is known for its pharmacological properties. In vitro studies have proven the bioactive compound Acmella to have anti-inflammatory and antimicrobial (Lalthanpuii et al., 2018), anesthetic (Kang et al., 2016), antipyretic, antioxidant, insecticidal, antiseptic, immune stimulation anti-obesity, and anticancer effects. Phytosterols such as -sitosterol, stigmasterol, and campesterol are well-known compounds beneficial in treating cardiovascular disease, colon cancer, and breast cancer (Suryani, 2018).

Research shows that A*cmella oleracea* is a promising source of therapeutic agents in preventing cancer growth and DNA damage (Lalthanpuii et al., 2018). The extract of *Acmella oleracea* could reduce oxidative stress and inflammatory targets related to the cancer pathway. Which then inducible nitric oxide synthase (iNOS), transcription factors of the nuclear factor-κB family (NF-κB), cyclooxygenase-2 (COX-2), and mitogen-activated protein kinase (MAPK) signaling pathways. The previous study by Lalthanpuii et al., (2018), found that the methanol extract of the plant extract is most potent on the lymphoma (Dalston'slymphoma ascites) cells with an IC50 of 147.547 µg/ml, while it does not affect lung carcinoma (V79).

The most prevalent breast cancer treatments are surgery, chemotherapy, hormone therapy, and immune therapy. However, this method has numerous drawbacks. For instance, although chemotherapy kills cancer cells rapidly, it negatively affects the body. They increase hyper-proliferation in normal cells such as hair follicles, bone marrow, and gastrointestinal tract cells. This results in typical chemotherapy side effects such as hair and skin loss or skin (Wijaya & Muchtaridi, 2017). Therefore, researchers seek more effective treatments with fewer adverse effects. In this vein, one approach is to investigate the bioactivity of natural plant compounds. This study aims to determine the in-silico relationship between 17 *Acmella oleracea* compounds and estrogen receptor alpha (ESR1), PGR, and MAP2K2.

2. METHODS

2.1. Biological Activity Analysis with PASS Server

Using the PASS server, we analyzed the biological activity of Acmella oleracea (L.) flowers. The canonical structures of the investigated bioactive compounds were retrieved from PubChem (https://pubchem.ncbi.nih.gov/) and entered into the PASS server. There will be a list of biological activities alongside the values of Pa and Pi. The Pa value in this study was set at pa > 0.5. The Pa value represents the probability of the compound's activity; the more significant the Pa value, the greater the probability of the compound's activity. Compounds with breast cancer-related activity (Pa > 0.5) were chosen for molecular Docking.

2.2. Target Protein Identification

We identified the target proteins of the 17 compounds using the Superpred (https://prediction.charite.de) and Swiss Target Prediction (http://www.targetprediction.ch) web servers. Using STRING (https://string-db.org/), we analyzed the target protein data of each compound to identify the specific breast cancer-related pathways. KEGG pathway analysis results include PD-L1 expression and the PD-1 checkpoint pathway in cancer, the estrogen signaling pathway, the p53 signaling pathway, and breast cancer. Three of the most potent target proteins were obtained: ESR1, PGR, and MAP2K2.

2.3. Modeling and Validation of the 3-dimensional Structure of the Target Protein

Then 3-dimensional structures of the three target proteins were carried out with Swiss webserver modeling (https://swissmodel.expasy.org/) by inserting the FASTA target protein from the NBCI database. The protein model was validated with a saves webserver (https://saves.mbi.ucla.edu/) based on ERRAT (Over Quality Factor and residual graph) assays and PROCEK (Ramachandran plot and residues in disallowed regions) values.

2.4. Molecular Docking

Molecular Docking was performed on 17 compounds exhibiting estrogen-related activity in breast cancer signaling pathways. Selected bio-active compounds as ligands were retrieved from PubChem and prepared to minimize the energy using open babel of PyRx software. Protein target of this study were ESR1, PGR, and MAP2K2. The proteins were pre-pared to remove water molecule and unwanted ligand using Discovery Studio 16 software. Molecular docking was done using Pyrx 0.8. Ligands docking to ESR1 was set to x= -40.5019, y = -2.5866, and z = -15.9094 with dimension (Angstrom) x = 27.9942, y = 25.9985, and z = 14.5592. Ligands docking to PGR was set to x = -1.9040, y = 15.4728, and z = -14.5936 with dimension (Angstrom) x = 24.7985, y = 15.7095, and z = 13.3525. Ligands docking to MAP2K22 protein was set to x = -10.5173, y = 3.9012, z = -0.7681 with dimension (Angstrom) x = 10.9910, y = 25.3907, and z = 14.0965. The visualization of docking results was done using Discovery Studio software.

2.5. Pharmacochemical, Physicochemical and Toxicity Analysis

Utilizing the SwissADME webserver (http://www.swissamde.ch/), the pharmaceutical and physicochemical properties of the investigated bioactive compounds were analyzed. The canonical SMILE compounds are extracted from PubChem and then added to SwissADME. While the toxicity of bioactive compounds is assessed using the Protox web server (https://tox-new.charite.de/protox II/) by inserting Canonical SMILE compounds from PubChem into Protox, with parameters such as carcinogenic, hepatotoxicity, mutagenic, cytotoxicity, and immunotoxicity.

3. RESULTS AND DISCUSSION

3.1. Apoptotic-Related Activity Screening Analyzed

Screening of the PASS online web server showed the biological activity of compounds related to antineoplastic, apoptosis agonist, caspase-3, caspase-8 stimulant, ovulation inhibitor,

steroid synthesis inhibitor, and TP53 expression enhancer (Figure. 1). Compounds with the specified activity were chosen and analyzed further. PASS analysis yielded two values of probability: Pa (to be active) and Pi (to be inactive). The greater the ratio of Pa to Pi, the greater the likelihood of a compound's biological activity. The study employed a cut-off Pa > 0.5 to obtain a probability of greater than 50 percent compound apoptosis activity. *Acmella oleracea* (L.) flowers contain 12 compounds in the cancer regulation pathway. Scopoletin displayed the most excellent TP53 expression enhancer (Pa = 0.941) compared to other compounds. Beta-Amyrin showed the highest levels of Apoptosis agonist (0.923), antineoplastic (0.916), Caspase 3 (Pa= 0.976, and caspase eight stimulant (Pa = 0.974, among others). Stigmasterol showed the highest Ovulation inhibitor activity (Pa = 0.68) and steroid synthesis inhibitor (Pa= 0.52), among others. Our current result discovered that the bioactive *Acmella oleracea* (L.) flowers might be a promising candidate for regulating cancer associated with apoptosis dan estrogen pathways based on the database server

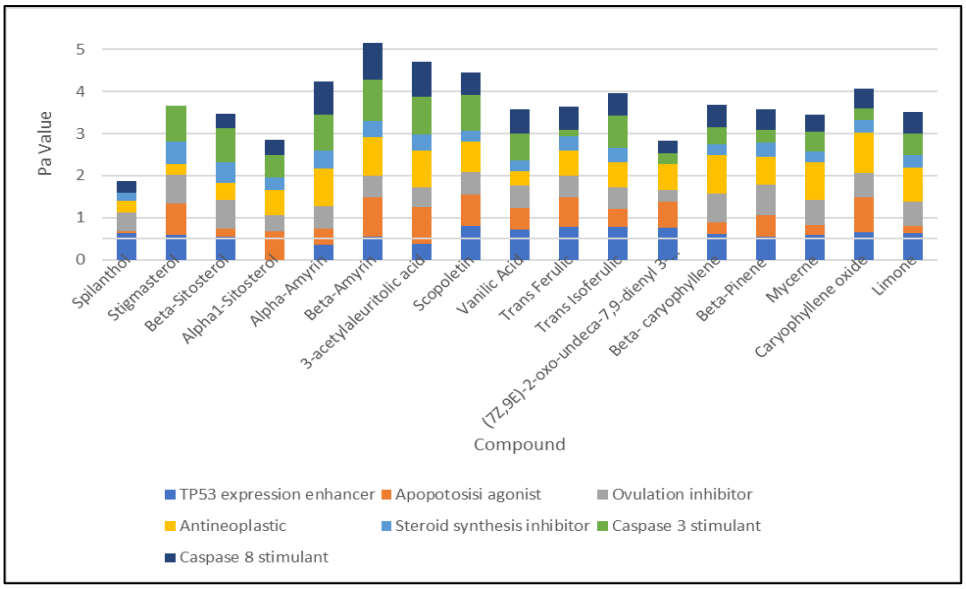

Figure 1. Apoptotic-related activity screening analyzed

3.2. Macromolecular Modelling and Validation

ESR1, MAP2K2, and PGR have been utilized as primary therapeutic targets in the treatment of Breast Cancer. The target protein receptor's 3-D structure was retrieved from the SWISS model with the highest seq-identity (Figure 2). The accuracy of the protein modeling was predicted using the Ramachandran plot. The Ramachandran plot indicates the stereochemical property of the structural structure. The PROCHECK compares the overall model geometry to the residues' geometry and calculates an expected model's stereochemical quality. As input, the PROCHECK tool requires a model protein file and generates the Ramachandran plot. The analysis of Residues in disallowed Ramachandran plots revealed minimal model protein residues. The protein model is more accurate for the fewer residues in disallowed regions. According to Table 1, the PGR protein model has a negligible number of residues (0%).

The ERRAT contains a database of highly refined protein structures and graphs the position's value. This diagram is based on the refined structure database's compilation of nonbonded interaction statistics between different atom types. The result of the ERRAT server is a graph depicting the relationship between residues and error values (Table 1). This input structure's overall quality score of 86.55% for ESR1 and 92% for MAP2K2 is good. However, the design of PGR displays excellent resolution (99%). If the input structure has the adequate resolution, its quality score should exceed 95%.

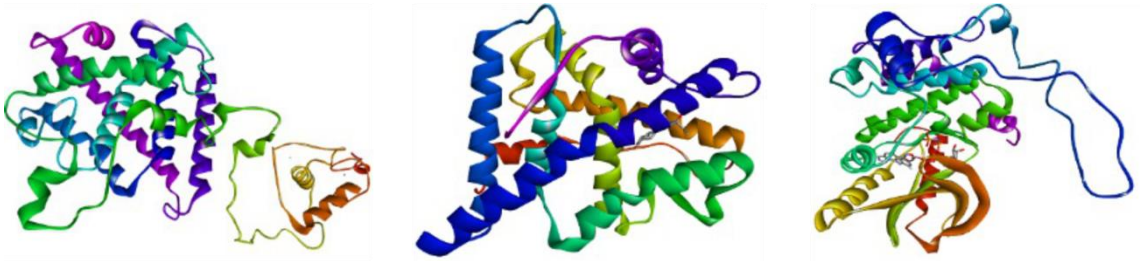

Figure 2. Macromolecular ESR, MAP2K2, and PGR

Table 1. Macromolecular Model Validation Test Results

Macromolecular	Residues in disallowed regions (PROCHECK)	Quality Factor (ERRAT)
ESR1	0.6%	86.550%
MAP2K2	1%	92%
PGR	0%	99.58%

3.3. Analysis of Molecular Docking

The best molecules for anti-breast cancer are obtained by looking at the binding energy value of docking results. The best binding energy is the most minor, meaning that the energy required to bind to the target protein is smaller so that it is more effective in binding to receptors (Islam et al., 2020). Beta-Amyrin exhibited the highest binding affinity against ESR1 (-8.5 kcal/mol) and MAP2K2 (-9.5 kcal/mol), as determined by the molecular docking analysis. In contrast, stigmasterol exhibited the highest binding affinity against PGR (-8.4 kcal/mol) (Table 2).

Table 2. The Binding Affinity of 17 Selected Compounds of Acmella oleracea.

Ligand	Binding Affinity		
	ESR1	MAP2K2	PGR
Control			
Venetoclax	**-8.9**	**-12.6**	**-9.2**
N-Alkyl amide			
Spilanthol	**-5.6**	**-5.9**	**-5.9**
Stigmasterol	**-7.3**	**-8.5**	**-8.4**
Beta-Sitosterol	**-7.0**	**-8.2**	**-7.0**
Alpha1-Sitosterol	**-7.0**	**-8.1**	**-7.6**
Alpha-Amyrin	**-8.6**	**-9.3**	**-7.0**
Beta-Amyrin	**-8.4**	**-9.5**	**-7.6**
3-acetyl aleuritic acid	**-7.7**	**-8.3**	**-7.4**
Scopoletin	**-6.5**	**-5.9**	**-6.6**
Vanillic Acid	**-5.3**	**-6.3**	**-4.7**
Trans Ferulic	**-5.1**	**-6.9**	**-5.8**
Trans Isoferulic	**-5.5**	**-8.6**	**-6.4**
Acmelonate			
(essential9E)-2-oxo-undeca-7,9-diene 3-methylbut-2-enoat)	**-4.7**	**-5.5**	**-5.0**
Triterpene			
Beta- caryophyllene	**-5.7**	**-5.8**	**-7.7**
Beta-Pinene	**-4.8**	**-6.4**	**-5.6**
Myrcene	**-4.7**	**-4.8**	**-4.7**
Caryophyllene oxide	**-5.6**	**0**	**-7.8**
Limone	**-5.7**	**-6.1**	**-5.4**

3.3.1. Molecular Docking of Bioactive Substances of Interest to the ESR1 Protein

Based on molecular docking analysis (Figure 3), the binding energy of the complex of venetoclax and ESR1 protein was -8.9 kcal/mol, involving multiple types of interaction via amino acid residues (Table 2). Seven conventional hydrogen bond interactions and seven hydrophobic ones were identified. These interactions involved the amino acid residues CYS381, PHE461,

LEU544, MET543, SER464, and MET522. These residues were then identified as the essential amino acid residues in the venetoclax-induced production of the ESR1 protein. However, the binding of stigmasterol to ESR1 protein resulted in the formation of ten hydrophobic residues, which included Leu699, PRO696, ARG766, MET692, TRP765, and HIS770. Alpha-Amyrin, Beta-Amyrin, and 3-acetyl aleuritic acid interactions with ESR1 exhibited the lowest binding affinity compared to other bioactive compounds and the closest binding energy to venetoclax, -8.6, -8.4, and -7.6 kcal/mol, respectively. The three compounds have been shown to have bioactive cancers that can play a role in regulating estrogen and breast cancer. They include TP53 expression enhancer, Apoptosis agonist, Ovulation inhibitor, Antineoplastic, Steroid synthesis inhibitor, Caspase 3 stimulant, and Caspase 8, which plays a crucial role in the final step of apoptosis and cell growth (Bellumori et al., 2022; Sharma & Arumugam, 2021). Alpha-Amyrin and ESR1 formed eight hydrophobic alkyl interactions. Beta-Amyrin, with higher binding energy, exhibited eleven hydrophobic interactions with ESR1. With the ESR1 protein, 3-acetylaleuritic acid formed one hydrogen bond and eleven hydrophobic interactions.

As a member of the nuclear receptors family, the estrogen receptor (ESR1) typically functions as a ligand-activated transcription factor. The binding of ligands induces conformational changes in receptors, leading to translocations into the nucleus and transcriptional activation of some target genes (Poirier et al., 2022). ESR1 affects the ligand-binding domains of these proteins. ESR1 is a crucial mechanism for developing endocrine resistance in breast cancer. Numerous amino acid residues, including Tyrosine, Serionin, Aspargin, Glycine, Glutamine, and Aspartic Acid, serve as activation sites for ESR ligands (Robinson et al., 2013). Through these amino acids, the catalytic activity of this protein activated mutation Estrogen. The interaction may alter the conformation of the ESR1 protein, causing it to become mature or cleaved. However, the study did not identify any residues involved in the complex interaction of investigated bioactive compounds with ESR1 protein comparable to the interaction between venetoclax and ESR1 complexes or the phytoconstituents of *Acmella oleracea* Flowers.

3.3.2. Molecular Docking of Examined Bioactive Substances with the MAP2K2 Protein

Figure. 4 depicts the molecular docking results of investigated bioactive compounds and venetoclax against the MAP2K2 protein. The interaction between venetoclax and MAP2K2 protein resulted in four hydrogen bonds (2 Conventional Hydrogen Bonds via LYS101 and SER154, Conventional Hydrogen Bond-Halogen via TYR233, 3 Carbon Hydrogen Bonds via ASP194, MET233, GLY81, and 1 Pi-Donor Hydrogen Bon via SER232). This complex formed 13 hydrophobic interactions via leucine, valine, isoleucine, alanine, methionine, and tyrosine. It was hypothesized that these amino acid residues were crucial for inhibiting MAP2K2 by venetoclax. This complex had a binding energy of -12.6 kcal/mol. An essential residue of LEU201 was involved in multiple interactions, including two hydrophobic interactions between MAP2K2 against stigmasterol, three hydrophobic interactions between MAP2K2 and Alpha-sitosterol, four hydrophobic interactions between MAP2K2 and beta-sitosterol, two hydrophobic interactions between MAP2K2 and scopoletin, and one hydrophobic interaction between MAP2K2 and vanillic acid.

A residue VAL86, as the essential residue of the venetoclax-MAP2K2 complex, is also present in the complexes of spilanthol-MAP2K2 (hydrophobic), stigmasterol-MAP2K2 (hydrophobic), Alpha and beta Amyrin-MAP2K2 (Hydrophobic), scopoletin-MAP2K2 (hydrophobic), Vanillic Acid-MAP2K2 (hydrophobic), trans iso (hydrophobic). The final essential residue, ALA99, was identified as a hydrophobic interaction in the complex of Alpha sitosterol-MAP2K2 protein, Beta sitosterol-MAP2K2 protein, scopoletin-MAP2K2 protein, and vanillic acid-MAP2K2 protein. The involvement of several key residues in the complex of bioactive compounds against MAP2K2 protein was hypothesized to inhibit MAP2K2 protein as venetoclax by interfering with its activity. The research revealed that beta-Amyrin (-9.5 kcal/mol),

Alpha Amyrin (-9.3 kcal/mol), trans isoferulic (-8.6), and stigmasterol (-8.5) have the lowest binding affinity than venetoclax (-12.6 kcal/mol) (Table 1). The interaction may alter the conformation of ESR1 so that it is either mature or cleaved by MAP2K2. Comparable to the venetoclax and MAP2K2 complexes or the compound of *Acmella oleracea* flowers. However, neither beta-Amyirin nor alpha-Amyirin contained a residue implicated in the complex interaction of investigated bioactive compounds with MAP2K2 protein.

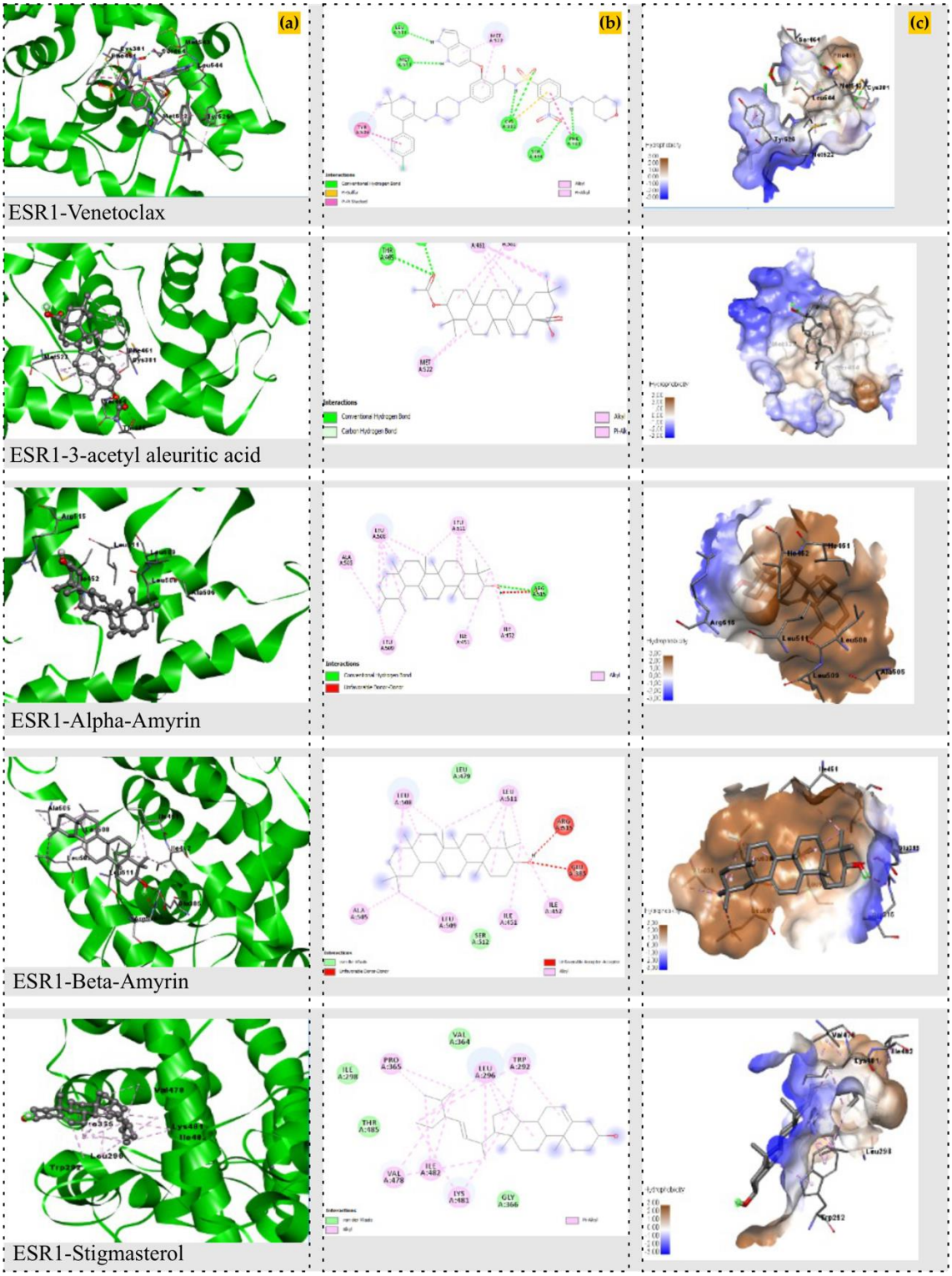

Figure 3. Bioactive compounds interaction against ESR1 protein top 5 lowest binding affinities: (a) the active site of the ligand-protein complex; (b) the complex's two-dimensional structure; (c) its hydrophobicity. The flat green ribbon represented ESR1, whereas the element ball-and-stick represented a bioactive compound

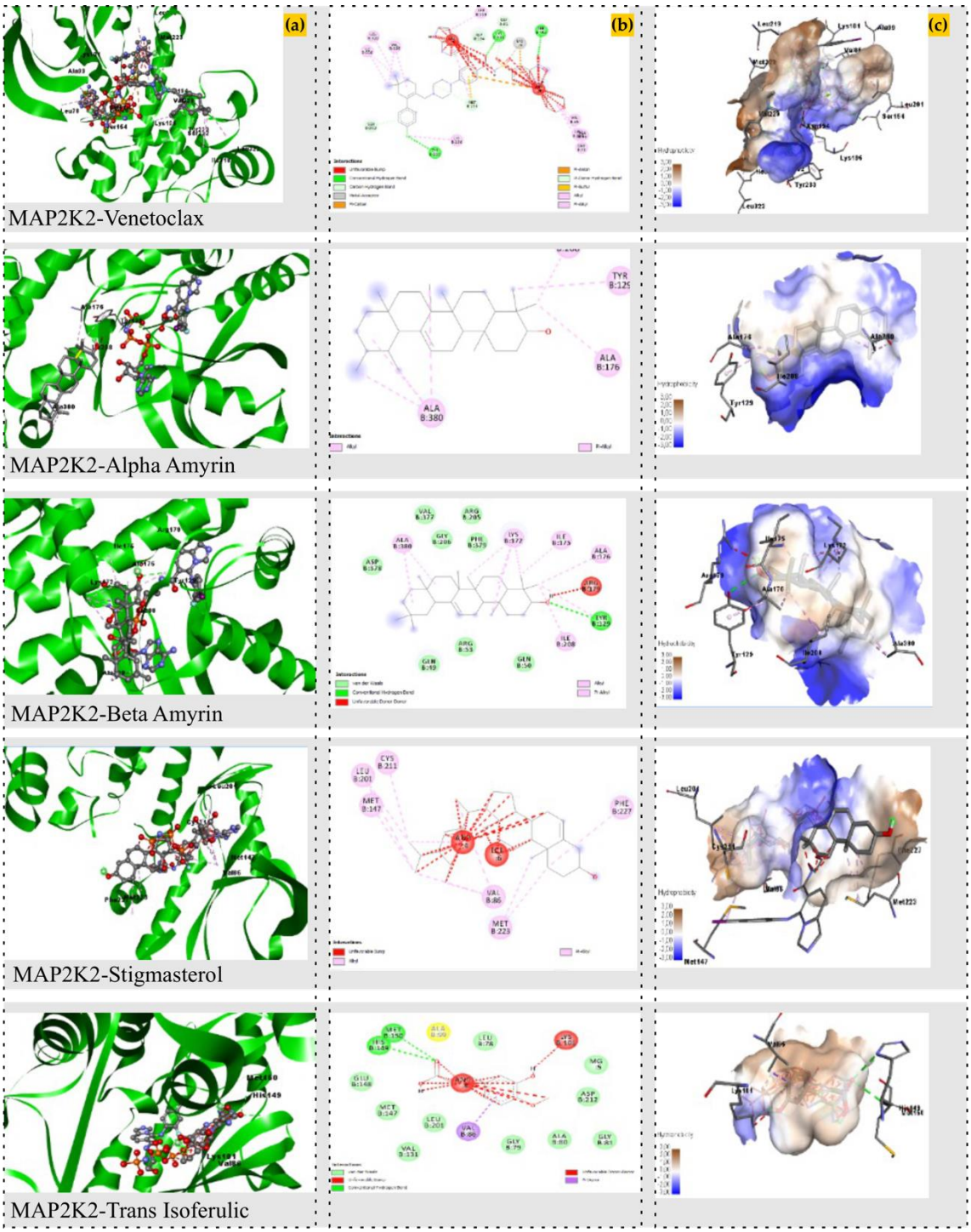

Figure 4. Bioactive compounds interaction against MAP2K2 protein top 5 lowest binding affinities: (a) the active site of the ligand-protein complex; (b) the complex's two-dimensional structure; (c) its hydrophobicity. The flat green ribbon represented MAP2K2, whereas the element ball-and-stick represented a bioactive compound.

3.3.3. Molecular Docking of Bioactive Substances of Interest to the PGR Protein

Progesterone has two types of progesterone receptors, progesterone receptor A (PRA) and progesterone receptor B (PRB). Both receptors cause the transcription of certain genes that have a specific expression of estrogen. It is suspected that PRA inhibits the effects of PRB, and the inhibition extends to the point of affecting estrogen (The impact of PRA inhibition on steroid sex is utilized to counteract the effects of endometrial proliferation by estrogen on the use of Thyroid Stimulating Hormone (TSH) (Kolatorova, et al. 2022). As a background, molecular docking study (Table 2), the binding energy of the venetoclax and PGR protein complex was -9.2 kcal/mol,

involving many types of interaction via amino acid residues (Table 4). There was one carbon-hydrogen bond interaction via SER728 and LYS731, two electrostatic (Pi-cation) interactions via ARG728 and LYS731, and seven hydrophobic interactions via SER728 and LYS731 (1 Pi-P stacked via TYR700, Alkyl via ARG724, and 7 Pi-Alkyl via ARG724, LEU727, ILE699, LYS731, and TYR700).

However, the binding affinity of complex stigmasterol-PGR to PGR protein was 84 percent lower than that of Venetoclax-PGR. Ten hydrophobic (8 alkyl and two Pi-alkyl) residues were produced, including isoleucine, proline, arginine, methionine, tryptophan, and histidine. The compound has been shown to have bioactive cancers that play a role in the regulation of estrogen and breast cancer. They include antineoplastic, apoptosis agonist, caspase-3, caspase-8 stimulant, ovulation inhibitor, steroid synthesis inhibitor, and TP53 expression enhancer, which plays a crucial role in the final step of apoptosis and cell growth (Pu et al., 2022; Siao et al., 2015). This study investigated the interaction mechanism between venetoclax and PGR protein. Four hydrogen bonds, an electrostatic interaction, and eleven hydrophobic contacts bind Venetoclax to PGR. The binding energy of residues proline, glycine, glutamate, alanine, and others were -8.8 kcal/mol. However, the distinctiveness of the three activation sites is not present in venetoclax, the control molecule. Hydrophobic interactions between the bioactive chemicals Beta-pinene and Beta-caryophyllene allowed us to locate the location (binding affinity -5.6 and -7.7). The organic component of the terpene class is found in the essential oil of numerous plants, alpha-Pinene, and beta-pinene inhibiting tumor necrosis factor (TNF)- α-induced invasiveness of MDA-MB-231 cells (De Albuquerque Barros & Henrique Morgon, 2022; Kang et al., 2016; Zang et al., 2022). Analysis revealed that α-pinene dose-dependently inhibited TNF-α-induced matrix metalloproteinase-9 gene promoter activation and mRNA expression.

Stigmasterol has the lowest binding energy (-8.4 kcal/mol), followed by caryophyllene oxide (-7.8 kcal/mol) and Alpha sitosterol and beta Amyrin (-7.6 kcal/mol). Previous research examined the impact of stigmasterol on proapoptotic signals, mitochondrial activity, formation of reactive oxygen species, and cytosolic and mitochondrial calcium levels in human ovarian cancer cells (Bae et al., 2020; Wang et al., 2022). The visualization of the interaction of molecular Docking is depicted in Figure 5.

3.4. Analysis of Pharmacochemical, Physicochemical and Toxicity

The optimal physical properties for bioactive substances to be eaten orally include molecular weight (150-500 g/mol), polarity (TPSA between 20-1302), solubility (log S not more excellent 6.0), flexibility (not greater 9.0 rotatable bonds), and lipophilicity (XLOGP3 between -0.7 and + 5.0) (Daina et al., 2017). The optimal criteria range consisted of Spilanthol, Beta-caryophyllene, Beta-Pinene, Myrcene, Caryophyllene oxide, and Limone. In contrast to Limone, which has a TPSA 20, Stigmasterol, Beta-Sitosterol, Alpha1-Sitosterol, Alpha-Amyrin, Beta-Amyrin, and 3-acetylaleuritolic acid all have >XLOGP (Table 3).

The PROTOX Toxicity test is conducted to determine the compound's toxicity. The positive test results show that the phytoconstituent is not mutagenic, so it does not cause carcinogenic for body cells. In addition, Table 4 shows that most of the bioactive compounds of *Acmella oleracea* are predicted not to cause toxicity. These parameters, including hepatotoxicity, carcinogenicity, mutagenicity, cytotoxicity, and are not extreme in Estrogen Receptor Alpha (ER), Estrogen Receptor Ligand Binding Domain (ER-LBD) effect, and become tumor phosphoprotein suppressor p53. For oral toxicity in rodents (LD50) of bioactive compounds, in silico tests and classification of compound toxicity based on the Globally Harmonized System (GHS) were carried out using the Protox online tool. By considering the prediction of toxicity effects, *Acmella oleracea* extract will be safe if it is consumed with a range of <400mg/kg body.

Table 3. Pharmacochemical and Physicochemical Analysis

Compound	LIPO	SIZE	POLAR	INSOLU	INSATU	FLEX	H-Bond Acceptor	H-Bond Donor
Spilanthol	3.57	221.34	29.10	-2.93	0.5	8	1	1
Stigmasterol	8.56	412.69	20.23	-7.46	0.86	5	1	1
Beta-Sitosterol	9.34	414.71	20.23	-7.9	0.93	6	1	1
Alpha1-Sitosterol	9.03	426.72	20.23	-7.84	0.87	5	1	1
Alpha-Amyrin	9.01	426.72	20.23	-8.16	0.93	0	1	1
Beta-Amyrin	9.15	426.72	20.23	-8.25	0.93	0	1	1
3-acetylaleuritolic acid	8.40	498.74	63.60	-8.03	0.88	3	4	1
Scopoletin	1.53	192.17	59.67	-2.46	0.1	1	4	1
Vanilic Acid	1.43	168.15	66.76	-2.02	0.12	2	4	2
Trans Ferulic	1.51	194.18	66.76	-2.52	0.1	3	4	2
Trans Isoferulic	1.51	194.18	66.76	-2.11	0.1	3	4	2
(7Z,9E)-2-oxo-undeca-7,9-dienyl 3-methylbut-2-enoat)	4.13	264.36	43.37	-3.42	0.5	10	3	0
Beta-caryophyllene	4.38	204.35	68.78	-3.87	0.73	0	0	0
Beta-Pinene	4.16	136.23	45.22	-3.31	0.8	0	0	0
Myrcene	4.17	136.23	48.76	-3.05	0.4	4	0	0
Caryophyllene oxide	3.56	220.35	68.27	-3.45	0.87	0	1	0
Limone	4.57	136.23	0	-3.5	0.6	1	0	0

Table 4. Analysis Toxicity

Compound	Hepato toxicity	Carcino genicity	Immuno toxicity	Muta genicity	Cyto toxicity	ER*	ER-LBD**	p53***
Spilanthol	X	X	X	X	X	X	X	X
Stigmasterol	X	X	√	X	X	X	X	X
Beta-Sitosterol	X	X	√	X	X	X	X	X
Alpha1-Sitosterol	X	X	√	X	X	X	X	X
Alpha-Amyrin	X	X	√	X	X	X	X	X
Beta-Amyrin	X	X	√	X	X	X	X	X
3-acetylaleuritolic acid	X	X	√	X	X	X	X	X
Scopoletin	X	√	√	X	X	X	X	X
Vanillic Acid	X	X	X	X	X	X	X	X
Trans Ferulic	X	X	√	X	X	X	X	X
Trans Isoferulic	X	X	√	X	X	X	X	X
(7Z,9E)-2-oxo-undeca-7,9-dienyl 3-methylbut-2-enoat)	X	X	X	X	X	X	X	X
Beta-caryophyllene	X	X	√	X	X	X	X	X
Beta-Pinene	X	X	X	X	X	X	X	X
Mycerne	X	X	X	X	X	X	X	X
Caryophyllene oxide	X	X	√	X	X	X	X	X
Limone	X	X	X	X	X	X	X	X

*ER = Estrogen Receptor Alpha; **ERLBD = Estrogen Receptor Ligand Binding Domain; ***P53 = Phosphoprotein (Tumor Suppressor)

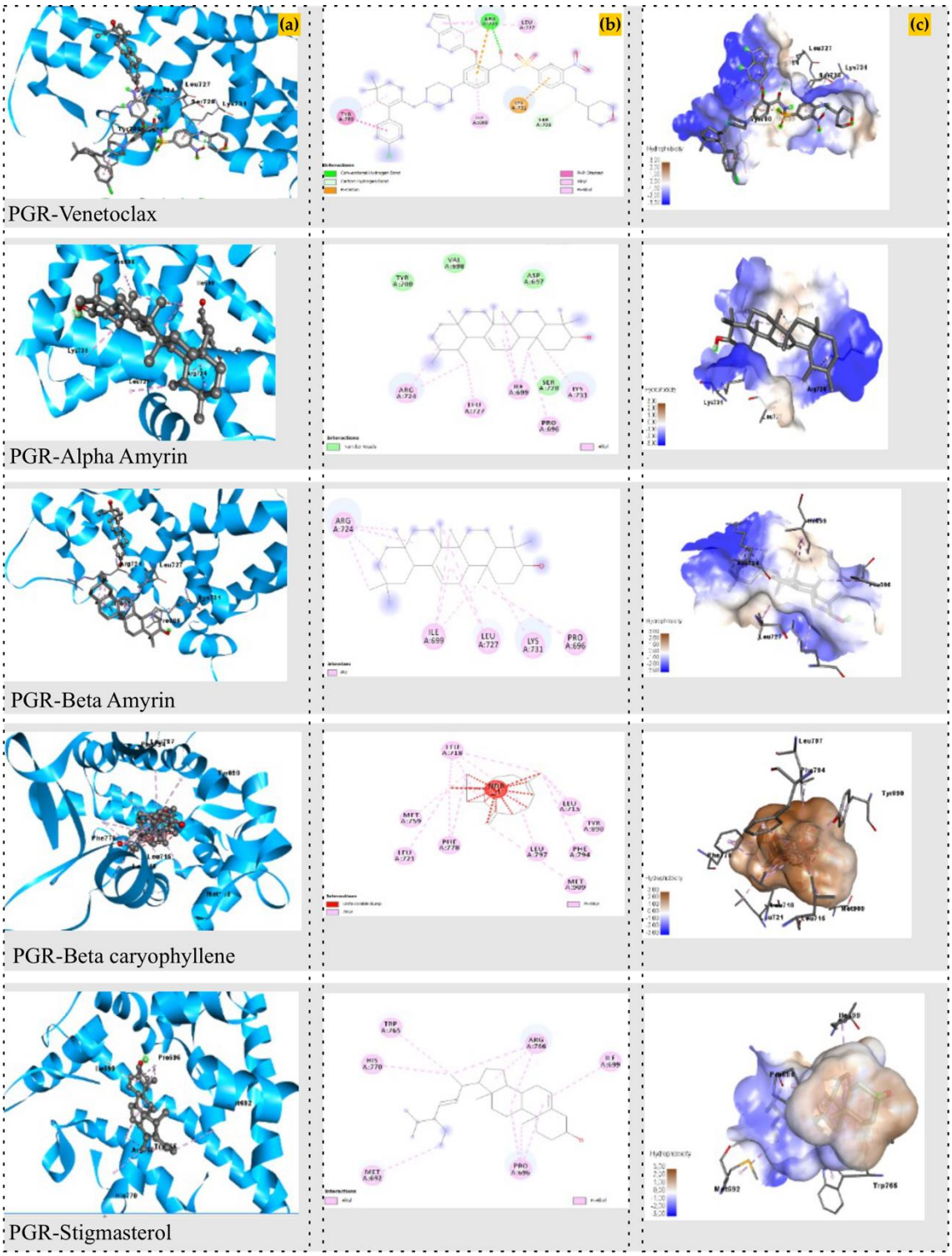

Figure 5. Top 5 lowest binding affinity interactions of the bioactive compound against PGR protein: (a) the active site of the ligand-protein complex; (b) the complex's two-dimensional structure; (c) its hydrophobicity. The flat green ribbon was PGR, whereas the ball-and-stick part was a bioactive chemical

4. CONCLUSION

In silico analysis revealed the molecular mechanisms behind *Acmella oleracea* (L.) Flowers as an anti-breast cancer agent. This study showed that bioactive chemicals derived from *Acmella oleracea* (L.) flowers may have the ability to influence apoptosis and cell proliferation. The amyrins (natural triterpene compounds), such as alpha amyrin and beta amyrin from the lignans group, were expected to be potent ESR1, MAP2K2, and PGR inhibitors. In contrast, stigmasterol, a flavonoid, was projected to be the most potent PGR inhibitor. The study reveals that *Acmella oleracea* has numerous bioactive chemicals advantageous for cancer therapy by inducing

apoptosis through interaction with ESR1, MAPK2, and PGR protein. Bioactive chemicals from *Acmella oleracea* (L.) flowers may limit cancer cell development and trigger apoptosis by interfering with antineoplastic, apoptosis agonist, caspase-3, and caspase-8 stimulant, ovulation inhibitor, steroid synthesis inhibitor, and TP53 expression enhancer. Further, to validate it, additional research is necessary.

5. ACKNOWLEDGEMENT

The author would like to thank the Department of Biology, Faculty of Mathematics and Natural Science, Universitas Negeri Malang, which has fully facilitated this study.

6. CONFLICT OF INTEREST

All authors declare no conflict of interest.

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JFSP Vol.10, No.1, January-April 2024, Page: 25-31
pISSN: 2549-9068, eISSN: 2579-4558
Jurnal Farmasi Sains dan Praktis
(JFSP)
http://journal.unimma.ac.id/index.php/pharmacy

POTENTIAL KETAPANG (*Terminalia catappa*) LEAF EXTRACT AS A DOXORUBICIN CO-CHEMOTHERAPY AGENT ON BREAST (T47D) AND CERVIX (HeLa) CANCER CELL LINES

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https://doi.org/10.31603/pharmacy.v10i1.9845

Article info:
Submitted : 08-08-2023
Revised : 24-11-2023
Accepted : 06-12-2023

Publisher:
Universitas Muhammadiyah Magelang

ABSTRACT
Doxorubicin (DOX) is chemotherapy for breast and cervical cancer with serious side effects. Ketapang (*Terminalia catappa*) is a potential plant as a co-chemotherapy agent. The purpose of this research was to examine the sensitivity of DOX as a cytotoxicity drug in combination with ethanolic extracts of ketapang leaves (EKL) against T47D and HeLa cancer cells. Cytotoxicity was determined using the MTT assay, with DOX concentration series (0.625-40 nM for T47D and 0.5-6 M for HeLa) and EKL (50-1000 µg/mL) used in combination with the study. DOX and EKL combination assays utilizing their respective IC_{50} values were performed in T47D cells and HeLa cells, and the results were used to calculate the Combination Index (CI). Furthermore, the doubling time method was used to investigate the combination of DOX and EKL proliferation inhibition on both cell lines. DOX and EKL had IC_{50} values of 158 nM and 30 µg/mL for T47D, respectively, and 3.4 M and 640 µg/mL for HeLa cell growth. While DOX and EKL have a synergistic effect on T47D cells, their combined effect on HeLa cells is cytotoxic and dose-dependent. EKL increases the inhibitory effect of DOX on the proliferation of T47D and HeLa cancer cells. In T47D cells, the combination of DOX and EKL has a higher potential for cytotoxic and antiproliferative activity than in HeLa cells.

Keywords: *Terminalia catappa*; Cytotoxicity; Doxorubicin; T47D; HeLa

1. INTRODUCTION

Breast and cervical cancers are the most common types of cancer among women worldwide. Breast cancer is the second most common type of cancer in terms of new cases and the fifth leading cause of death worldwide (Siegel et al., 2019). In comparison, cervical cancer is a kind of female reproductive system cancer that is frequently fatal (Siegel et al., 2019). Indonesia has the eighth highest cancer incidence in Southeast Asia, with breast cancer being the most prevalent in women, followed by cervical cancer.

Chemotherapy is a frequently utilized medication because it can be used to treat malignancies that have progressed to the metastatic stage by suppressing the cell cycle and apoptosis tracking processes (Hanahan, 2022). The use of doxorubicin as chemotherapy has been documented to result in cancer cell resistance and harmful effects on healthy tissues, failing cancer therapy (Braciuliene et al., 2022; Thorn et al., 2011). Resistance to chemotherapeutic treatments can develop as a result of cells developing defense mechanisms in response to the suppression of the cell regulation system during particular phases, resulting in insensitive cells (Bukowski et al.,

2020; Shah & Schwartz, 2001; Zhou et al., 2021). Apart from resistance, chemotherapeutic medicines are not selective since, in addition to cancer cells, they can damage rapidly dividing healthy cells such as hair cells, nails, skin, and blood cells, resulting in severe side effects (Altun & Sonkaya, 2018; Mayer & Burstein, 2007). One strategy for resolving these issues is to produce co-chemotherapy drugs based on natural chemotherapeutic agents, most notably chemicals derived from herbs, that may be coupled with doxorubicin chemotherapy treatments to mitigate their negative effects.

In Ketapang leaves, one of the plants may be developed as a chemopreventive agent (*Terminalia catappa*). Ketapang leaves are members of the Combretaceae family. They include important components such as phenols, flavonoids, and carotenoids that have been shown to have antibacterial, antifungal, antidiabetic, antioxidant, hepatoprotective, and anticancer properties (Morioka et al., 2005; Venkatalakshmi et al., 2016). Additionally, Ketapang extract acts as a chemopreventive agent against cancer by inhibiting colon cancer cell multiplication (Morioka et al., 2005). In vivo testing of ketapang leaf water extract (10-100 μg/mL) demonstrated a 68 percent reduction in metastasis in a mouse model of lewis lung carcinoma (LLC) via lowering TIMP-2 and PAI-1 enzyme levels (Chu et al., 2007). Ketapang leaf methanol extract is cytotoxic to EAC (*Ehrlich Ascites Carcinoma*) cells (Saroja et al., 2012). Thus, ketapang leaves are ingredients that may contain active chemicals that have the potential to be developed as anticancer agents.

However, its action in T47D breast cancer cells, HeLa cervical cancer cells, and doxorubicin has not been reported. Hence in the present study, we investigated the cytotoxic and antiproliferative effects of EKL as co-chemotherapy on T47D breast cancer and HeLa cervical cancer cell line.

2. METHODS

2.1. Material

Ketapang leaves were collected in Banyumas, Indonesia. PT. Sanbe Farma provided the doxorubicin (DOX). T47D and HeLa cancer cells were received from the Faculty of Medicine, Universitas Gadjah Mada, Laboratory of Tropical Medicine, Section of Parasitology. T47D and HeLa cells were cultured in Dulbecco's modified eagle medium (DMEM) and HeLa cells in Roswell Park Memorial Institute (RPMI) medium with 10% (v/v) Fetal Bovine Serum (FBS) and 2% (v/v) antibiotic penicillin-streptomycin. Dimethyl sulfoxide, trypsin-EDTA 0.25 percent (Gibco), MTT reagents (3- (4,5-dimethyltiazol-2-il)-2,5-diphenyltrazolium bromide), and sodium duodecyl sulfate (Merck) 10% in 0.1 N HCl are employed as cytotoxic and proliferative reagents (Merck).

2.2. Methods

2.2.1. Preparation of the Extract

To avoid destroying the chemicals contained in the leaves, 500 grams of wet ketapang leaves are dried in indirect sunlight to prevent them from turning brown. After drying, pollination of Simplicia ketapang leaves is carried out with the help of a pollinator. The maceration of ketapang leaves necessitates the use of around 70% ethanol. The filtrate and residue obtained from the maceration process are separated. A Rotary Vacuum Evaporator is then used to thicken the filtrate, which is then evaporated over a water bath at 60 degrees Celsius.

2.2.2. Cytotoxic and Proliferation Assay

The MTT (3-(4,5-dimethylthiazol-2-il)-2,5-difeniltetrazolium bromide) technique was used to culture cells, observe single and combined cytotoxic activities, and evaluate ethanolic extracts of ketapang leaves (EKL) proliferation in the presence of DOX (ISO, 2009). Cells are extracted and spread evenly throughout 96 wells at a concentration of 1 x 10^4 cells/well. By contrast, the proliferation kinetics test employs a cell density of 5 x 10^3 cells/well. For 24 hours,

cells were stimulated to adapt and adhere to wells. Following that, 100 ml of culture fluid containing only DMSO (control) or the test chemical was cultured for an additional 24 hours. The proliferation assay was conducted for 0;24;48;72 hours. Living cells react with MTT reagents to create purple formazan crystals. Following 4-6 hours, 10% SDS (Sodium Dodecyl Sulphate) is added to dissolve the formazan crystals. Cells are cultured at room temperature and sheltered from light for an overnight period. After incubation, the plate was agitated horizontally (with a shaker) for 3 minutes and then measured at λ 595 nm using an ELISA reader.

2.2.3. IC_{50} Analysis (Inhibition Concentration 50 Percent)

The absorbance data is translated to a percentage of living cells and statistically assessed using the correlation test followed by the percentage of living cells. The formula for calculating the percentage of alive cells is as follow Eq. (1) (Artanti et al., 2020).

$$\% \text{ Viability of cells} = \frac{Sample\ Absorbance - Medium\ Control\ Absorbance}{Cell\ Control\ Absorbance - Medium\ Control\ Absorbance}\ x\ 100\% \quad (1)$$

IC_{50} is determined by the logarithmic equation between the absorbance value and the extract concentration. IC_{50} is a concentration that causes the death of 50% of the cell population to be known for potential cytotoxicity. The calculation data for T47D and HeLa cells are entered into the probit program to calculate IC_{50}.

2.2.4. Combination Index (CI)

The method commonly used to evaluate drug combinations is the CI using Eq (2) (Reynolds & Maurer, 2005).

$$CI = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2} \quad (2)$$

Where:

DX, the concentration of the single component, is required to provide the same impact as the combined concentrations, D1 and D2, of the same chemical.

The concentration of the two chemicals (1 and 2) is used in conjunction with one another as a combination treatment (**Table 1**).

Table 1. Interpretation of CI value (Reynolds & Maurer, 2005)

CI Values	Interpretation
<0.1	Very strong synergism
0.1 – 0.3	Strong synergism
0.3 – 0.7	Synergism
0.7 – 0.9	Mild to moderate synergism
0.9 – 1.1	Additive
1.1 – 1.45	Mild to moderate antagonism
1.45 – 3.3	Antagonism
>3.3	Strong to very strong antagonism

2.2.5. Cell Proliferation Analysis

The data acquired following treatment were analyzed using Ms. Excel 2016 to determine the equation of the regression line between incubation time and absorbance.

3. RESULTS AND DISCUSSION

The cytotoxic assay was used to establish the IC_{50} value of the ethanolic extracts of Ketapang leaves (EKL). The IC_{50} values for T47D breast cancer cells from EKL and DOX were 30 μg/mL and 158 nM, respectively, but the IC_{50} values for HeLa cervical cancer cells from EKL

and DOX were 640 µg/mL and 3.4 M, respectively. Microscopy investigations of T47D and HeLa cells revealed the phenomena of cell death as a result of EKL therapy, as shown by changes in cell shape. Living cells appear to be linked to the plate's bottom and other cells; they are brightly colored and have elongated spherical forms. In comparison, cells that die away from the dish's bottom are dark-colored and have a more spherical shape. This occurs when cells lose cytoplasm as a result of injury to the cell membrane, rendering them incapable of transmitting light from the microscope.

The combined effect of EKL and DOX on cell viability is evaluated using the CI calculation method (**Figure 1**). The combination of EKL 3.75 µg/ml with DOX at 56.25 and 75 nM concentrations provided a cytotoxic effect by obtaining % cell viability values of 23.98% and 29% in T47D cells (**Figure 1a**). The combination of the two also showed a better reduction in cell viability in the proliferation test compared to a single treatment. Meanwhile, HeLa cells showed a high decrease in cell viability values (cell viability values <30%) at low to high EKL concentrations combined with DOX (**Figure 1b**). According to these figures, the results of a combination of EKL and DOX study on T47D demonstrated synergy, with the best CI value of 0.62 obtained at a concentration of EKL 3.75 µg/ml (1/8 IC_{50}) and DOX 56.25 nM (3/8 IC_{50}). Due to the synergy between EKL and doxorubicin, the dose of DOX required to achieve the same potential can be decreased to 3/8 IC_{50}. As a result, the dosage required is reduced, and adverse effects can be minimized. In HeLa cells, on the other hand, the CI value cannot be quantified potential synergism (**Table 2**).

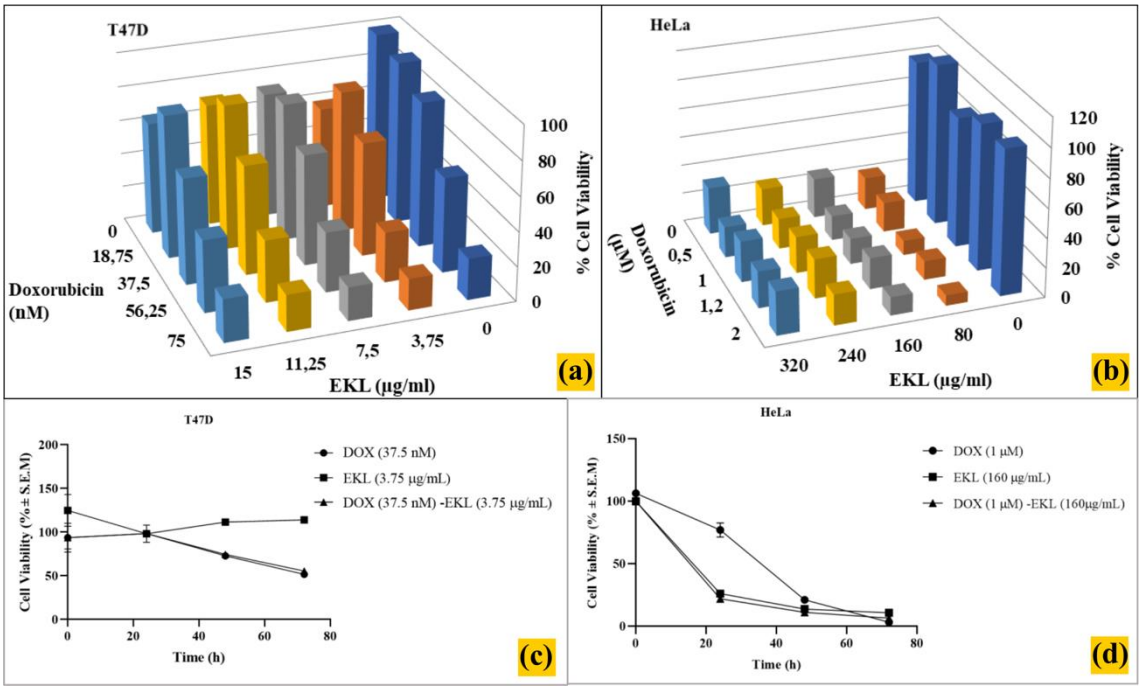

Figure 1. Graphic viability of T47D (A) and HeLa (B) cells in combination treatment of EKL and doxorubicin after incubation for 24 hours; Effects of EKL and doxorubicin treatment on T47D (C) and HeLa (D) cell proliferation

Proliferation tests can be used to measure the rate of cell growth affected by the activity of EKL and doxorubicin on the proliferation kinetics at various incubation durations. The parameter utilized in this test is the rate at which cells divide into two at the selected level. At 24 and 48 hours, a combination of EKL 3.75 µg/ml with DOX 37.5 nM inhibited cell growth more than single DOX (**Figure 1c**). However, after 72 hours, the absorbance was restored to normal levels when only DOX was used. Even when DOX is utilized as a chemotherapeutic agent, EKL's proliferation effect is more prominent, indicating that EKL has a strong proliferative effect since

it may still cause cell proliferation when combined with a cytotoxic agent. Proliferation of HeLa cells with DOX one m treatment increased from 0 to 24 hours and then reduced from 24 to 72 hours (**Figure 1d**). The combination of EKL and DOX inhibits HeLa cells more effectively than single DOX, but not considerably more effectively than single EKL, demonstrating the ability of EKL and DOX to inhibit HeLa cells.

Table 2. Combination Index Value by EKL and Dox on T47D and HeLa

T47D				
EKL (µg/mL)	**DOX (nM)**			
	18.75	**37.5**	**56.25**	**75**
3.75	1.36	0.93	0.62	0.7
7.5	1.52	1.03	0.74	0.76
11.25	nd	1.14	0.82	0.83
15	nd	1.20	0.98	0.93
HeLa				
EKL (µg/mL)	**DOX (M)**			
	0.5	**1**	**1.2**	**2**
80	1.13	nd	nd	nd
160	nd	nd	1.96	nd
240	2.86	2.07	1.43	3.24
320	3.58	1.7	2.20	1.67

*nc = no determined

The cytotoxic effect of ethanolic extracts of Ketapang leaves (EKL) alone and in combination with Doxorubicin (DOX) against T47D and HeLa cells was determined using MTT, which forms a dark blue formazan when reacting with the enzyme reductase found in living cell mitochondria (Ghasemi et al., 2021; Mahdavi et al., 2019). While MTT reagents do not affect dead cells since the mitochondria do not breathe, because the tetrazolium ring is not broken, formazan does not form, giving the color a purple hue. However, the color stays yellow (Ghasemi et al., 2021; Kusuma et al., 2010). If the purple color becomes high intense, the number of live cells increases, this absorbance data from the MTT method demonstrates the link between treatment with various dose levels and the number of live cells.

EKL cytotoxic test results on T47D cells indicate cytotoxic at low concentrations but increase cell viability at high ones. T47D cells are estrogen-producing cells that express estrogen receptors (ER) (Bouris et al., 2015; Ho et al., 2016). Because estrogenic qualities can promote cell viability, the prospect of increased viability exists due to EKL's estrogenic capabilities. Estrogenic substances have been shown to promote cell proliferation, tissue development in reproductive organs, and the transcription of specific genes (Pamplona-Silva et al., 2018). Increases cell viability as EKL levels rise, as protein accumulation for proliferation is one of the estrogenic effects generated when an estrogenic chemical is combined with estrogen receptors (Yunas et al., 2013). An additional, more focused study is required to demonstrate this. While EKL activity on HeLa cells demonstrates a lower potential effect, this is due to HeLa cells' unique properties compared to T47D cells. So, their activity on HeLa cells requires additional research.

Flavonoid and tannin molecules may have an active role in anticancer activity. Ketapang leaf extract includes tannins, and punicalagin can prevent bleomycin-induced CHO-K1 cell HGPRT cells from mutation (Chen et al., 2000). Ketapang leaves containing tannins have anticancer properties through their ability to prevent liver cancer and function as antioxidants. On EAC cells (Ehrlich Ascites Carcinoma), the flavonoid component of ketapang leaf extract has anticancer activity) (Saroja et al., 2012). Additionally, the tannin component punicalagin found in ketapang leaf extract possesses significant antioxidant activity (Sahala & Soegihardjo, 2012). The flavonoids found in ketapang are antioxidants and have been shown to suppress the proliferation of cancer cells (Lour & Meiyanto, 2007).

The IC_{50} values for EKL and DOX were utilized to adjust the cytotoxic and proliferation assays. Co-chemotherapeutic is a therapeutic method that combines EKL with a DOX chemotherapy drug. The combination of chemotherapeutic agents attempts to maximize treatment efficacy while minimizing DOX-related adverse effects. In an ideal world, the combination of drugs would have a synergistic impact on cancer cells, yet its toxicity could be tolerated in order to be clinically more effective than a single treatment. EKL has the ability to act as a co-chemotherapy agent with doxorubicin DOX in T47D cancer cells, as demonstrated by the research findings. However, this is not observed in HeLa cells, implying that additional molecular elucidation is required to develop EKL as a co-chemotherapy drug. The proliferation kinetics test provided additional proof of this combination's efficacy. According to the results obtained, cells treated with a combination of EKL and DOX appear to have the inhibitory activity of T47D and HeLa cell growth when compared to control cells, as demonstrated by the doubling time of T47D and HeLa cells treated with a combination of EKL and DOX remaining constant.

4. CONCLUSION

According to the findings of the study, a combination of Doxorubicin (DOX) and ethanolic extracts of the ketapang leaves (EKL) has more potent cytotoxic activity in T47D breast cancer cells than in HeLa cervical cancer cells, presumably due to their proliferation inhibitory activity in T47D breast cancer cells. Further investigation into the molecular mechanisms underlying these activities, on the other hand, is required.

5. CONFLICT OF INTEREST

All authors declare no conflict of interest.

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JFSP Vol.10, No.1, January-April 2024, Page: 32-41
pISSN: 2549-9068, eISSN: 2579-4558
Jurnal Farmasi Sains dan Praktis
(JFSP)
http://journal.unimma.ac.id/index.php/pharmacy

TRACING THE ANTIBACTERIAL, ANTIFUNGAL AND ANTI-BIOFILM ACTIVITIES OF ROOT EXTRACT BAJAKAH TAMPALA (SPATHOLOBUS LITTORALIS HASSK)

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https://doi.org/10.31603/pharmacy.v10i1.8804

Article info:
Submitted : 23-02-2023
Revised : 08-01-2024
Accepted : 20-01-2024

Publisher:
Universitas Muhammadiyah Magelang

ABSTRACT
Biofilm is a common causative factor for urinary tract infections due to catheter usage with a percentage of infection around 70-80%. The Bajakah tampala (Spatholobus littoralis Hassk) is one of native plants of Kalimantan which contains phenolic compounds, flavonoids and tannins which are proven to accelerate wound healing, have antibacterial activity, and have very high and strong antioxidant activity. This study aims to determine the antibacterial and anti-biofilm activity of bajakah root extract (Spatholobus littoralis Hassk) on catheter colonies of Staphylococcus aureus, Escherichia coli and Candida albicans and to determine their mechanism of action in vitro. This research is carried out with an in vitro experimental study design using a microplate reader. Extraction was carried out by maceration method using 96% ethanol solvent. The results showed that the ethanol extract of Bajakah tampala (Spatholobus littoralis Hassk) had antibacterial activity against S. aureus with a concentration of 1% w/v of 88.33% ± 0.01 and anti-biofilm activity of 82.21% ± 0.01. E. Coli bacteria had an antibacterial activity of 84.83% and an anti-biofilm activity of 80.11 at a concentration of 1% w/v. C. albicans had an antifungal activity at a concentration of 1% w/v of 82.31% ± 0.01 and anti-biofilm activity of 77.00% ± 0.01. From these results it can be concluded that the ethanol extract of Bajakah tampala (Spatholobus littoralis Hassk) has antibacterial and antifungal activities and the potential as a new anti-biofilm agent against S.aureus, E. coli and C. albicans

Keywords: Bajakah tampala; Antibiofilm; S.aureus; E.Coli; C.albicans

1. INTRODUCTION

Hospital-acquired infection seems to be difficult to avoid by health workers and patients in medical treatment. One of the nosocomial infections is in the urinary system. The cause of this infection is usually a medical equipment supporting the patient care, namely a catheter. The most common one is catheter associated urinary tract infection (CAUTI). Around 80% of urinary tract infections due to catheters are related to biofilm formation (Nurdin, E., Nurdin, G. M., & Noviyanti, 2020).

Biofilm is a causative factor for health care-related infections with a percentage of around 70-80%. The growth of biofilm on these catheters is also responsible for the death of around 7500 people per year (Nicolle, 2014). Bacteria that often cause the formation of biofilms on catheters and thus become the main cause of nosocomial infections is *Escherichia coli*. However, *Staphylococcus aureus* and *Candida albicans* also cause the formation of biofilms. *Staphylococcus aureus* is also capable of adhering to a medical device surface to form biofilms.

The prevalence of biofilm formation ranged from 65.1-69.8% and 86.7% of biofilm-producing *Staphylococcus aureus*, was multidrug resistant (Hasyrul Hamzah et al., 2023).

Even though a catheter is simple, it can provide significant benefits for patients because it is a type of modern medical device. However, long-term use of a catheter can damage the natural defenses of the urinary tract. Thus, management of patients with catheters is often complicated by infections in which biofilm formation is a major feature (Pelling et al., 2019). explains that the longer the catheter, the greater the possibility of bacteria appearing. Although not responsible for a clinical emergency, a significant increase in biofilm cells often occurs, resulting in the bacteria becoming resistant to antibacterial agents. More than 100 million urethral catheters are sold each year. In addition, according to (Darouiche, 2001), more than 30 million urinary catheters are used annually in the United States.

Antibiotic therapy in general will only kill cells that are planktonic, while the forms of bacteria that are tightly arranged in biofilms will survive. This is because antibiotics cannot penetrate the biofilm layer on the catheter (Mah & O'Toole, 2001). The use of traditional medicine in Indonesia has progressed quite rapidly because it has become an alternative treatment. (Muhlisah, 2007) states that the use of drugs from natural ingredients has a much lower level of danger and long-term risk than the use of synthetic drugs.

One of the famous native plants of Kalimantan in the last two years is Bajakah Tampala (*Spatholobus littoralis* Hassk). Bajakah Tampala contains phenolic compounds, flavonoids, and tannins (Marlina & Samad, 2013). This is reinforced by other studies which state that this plant contains phenolic compounds, flavonoids, and tannins which are proven to accelerate wound healing (Saputera & Ayuchecaria, 2018), with levels of phenolic compounds of 12.33 GAE/mg (Ayuchecaria et al., 2020), have antibacterial activity (Saputera et al., 2019) and have very high antioxidant activity (Ayuchecaria et al., 2020).

The search for anti-biofilm compounds from plants is still limited. Even though biofilm is a health problem worldwide, effective and safe antibiotics have not been found to treat it. Therefore, considering the problems above, this study aims to investigate new anti-biofilm agents from Bajakah root extract effective against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* bacteria, the main causes of biofilm.

2. METHODS

This research is carried out with an in vitro experimental study design using a microplate reader. The maceration method is used to obtain Bajakah Tampala extract (*Spatholobus littoralis* Hassk). This research was conducted from October 2021 to September 2022 at the Microbiology Laboratory of Faculty of Pharmacy at Muhammadiyah University, East Kalimantan, Faculty of Pharmacy, Universitas Muhammadiyah Kudus, Biology Laboratory of Faculty of Pharmacy and LPPT at Gadjah Mada University.

2.1. Plant Determination Results

Plant determination was carried out at the Mulawarman Herbarium, Laboratory of Ecology and Conservation of Tropical Forest Biodiversity at the Faculty of Forestry at Mulawarman University. The results showed that the plant was the Bajakah tampala with the species *Spatholobus littoralis* Hassk. from the Fabaceae family.

2.2. Sample Extraction

Extraction was carried out by maceration method using 96% ethanol solvent. The maceration method was chosen because it is the simplest method (Novriyanti et al., 2022) and 96% ethanol was chosen as a solvent because 96% ethanol is universal which can extract less polar, semi-polar to polar compounds. In addition, 96% ethanol is also able to extract flavonoids, alkaloids, saponins, antraquinones, and glycosides (Sinung et al., 2019). After the extraction

process, a viscous extract of the stem of the Bajakah Tampala (*Spatholobus littoralis* Hassk) with a weight of 49.38 g was obtained.

2.3. Secondary Metabolite Testing

2.3.1. Alkaloid Test

Extracts and fractions (2 mL) were put into test tubes. Then added with HCl 2N as much as 1 ml and then dripped with 3 drops of Dragendorff reagent, a positive test is indicated by the formation of an orange precipitate (Novriyanti et al., 2022).

2.3.2. Phenolic test

Extracts and fractions (1 mL) are put into a test tube, then 3 drops of 1% FeCl3 reagent are added, A positive test is indicated by the formation of a black color (Novriyanti et al., 2022).

2.3.3. Flavonoid Test

Extracts and fractions (2 mL). Put into a test tube, then added 0.05 mg of Mg powder and 1 mL of concentrated HCl and shaken, Positive test is indicated by the formation of red, yellow or orange color (Novriyanti et al., 2022).

2.3.4. Saponin Test

Extracts and fractions (2-3 mL) are put into a test tube, then added with 10 mL of warm water, and then shaken vigorously for 10 seconds, A positive test is indicated by the formation of a stable froth as high as 1-10 cm for 10 minutes (Novriyanti et al., 2022).

2.3.5. Steroid and Terpenoid test

Extracts and fractions (2 mL) are put into a test tube, then 1-3 drops of Lieberman Burchard reagent are added and the solution is shaken gently, A positive test for steroids is indicated by the formation of a blue or green color, while terpenoids give a brownish red color (Novriyanti et al., 2022).

2.3.6. Tannin Test

Extracts and fractions (1 ml), put into a test tube, then added 1-3 drops of 10% FeCl3 solution, Positive test is indicated by the formation of greenish-black color (Novriyanti et al., 2022)

2.3.7. Anthocyanin Test

Extracts and fractions (2 ml) were put into a test tube, then add NaOH 2N drop by drop. If the red color turns blue-green and fades slowly, it indicates the presence of anthocyanins (Trinovani et al., 2022).

2.4. Preparation of Bacterial Subcultures

Standard biofilm-forming *Staphylococcus aureus* was cultured in Brain-heart infusion broth (BHI) medium and incubated at 37ºC for 72 h. The optical densities (OD_{600}) of microbial cultures were adjusted to 0.1 (equal of the 0.5 Mc Farland standard ~1.5 x 10^8 CFU/ml), and subsequently diluted in fresh medium to OD_{600} 0.01 for each microbial species (Hasyrul Hamzah et al., 2023).

2.5. Antibacterial and Antifungal Testing

An antibacterial test was carried out using the microdilution method. The test was carried out on a microtiter plate flat-bottom polystyrene 96 wells with a series of test compound concentrations of 1%, 0.5%, 0.25%, 0.125% w/v. The control used was drug control using ciproloxacin and fluconazole. Growth control in the form of microbial suspension and solvent control was adjusted with the solvent of the test compound into each wells microplate, BHI media and bacterial suspension, and RPMI media were added for fungal suspension and then incubated at 37 °C for 24 hours for bacteria and 72 hours for fungi (Hamzah et al., 2018).

2.6. Mono-species Biofilm Test on Catheter

96% ethanol, allowed to dry, and the catheter inserted into the well. A total of 100 L of media containing bacterial suspension, normal human urine, and the test compound was added to each microtiter well plate containing the catheter, then incubated at ± 37°C for 24 hours for the middle phase. After the incubation period, the plates were washed using 150 L of sterile distilled water. The catheter was then scraped and transferred to a new plate, and 125 L of 1% crystal violet solution was added. Then the biofilm was washed with running water, and 200 L of 96% ethanol was added. The results of biofilm inhibition were read using an Optical Density (OD) 595 nm microplate reader. The test was carried out with three repetitions (Hamzah et al., 2018).

3. RESULTS AND DISCUSSION

3.1. Phytochemical Screening

As shown in **Table 1**, that the results of the qualitative phytochemical screening test showed that the ethanol extract of Bajakah Tampala (*Spatholobus littoralis* Hassk) positively contained alkaloids, flavonoids, saponins, tannins and phenols. Based on the results of the alkaloid screening, the Bajakah Tampala peel extract positively contained alkaloid compounds. According to research conducted by (Saputera & Marpaung, 2019), the factors that influence differences in compound content are differences in plant growing areas.

Table 1. Phytochemical screening results

Compound	Phytochemical Screening		
	Test Method	**Indicator**	**Results**
Alkaloids	Meyer	White precipitate	-
	Wagner	Brown precipitate	+
	Dragendroff	Orange precipitate	+
Flavonoids	NaOH	Changes color when compared to the control solution	+
	H_2SO_4 Concentrated		+
	Mg-HCL Concentrated		+
Saponins	Warm aquadest	Stable foam	+
tannins	$FeCl_3$ 1% 3-5 drops	Blackish green or blackish blue	+
Phenol	$FeCl_3$ 5% 2-5 drops	Greenish blue or green	+

Explanation: += Positive; −: Negative

3.2. Antimicrobial Test

3.2.1. Antifungals Against *C. albicans*

Antifungals are antibiotics which is able to hinder up to kills fungal growth. Antifungal has two meanings namely fungicidal and fungistatic. Fungicide is defined as a compound that can kill fungi, while fungistatic can inhibits the growth of fungi without turn it off. As seen in **Figure 1**, the antifungal test using microdilution showed that at a concentration of 1% w/v the Bajakah Tampala ethanol extract was able to provide inhibitory activity against *C. albicans* as much as 82.31% ± 0.01, while Fluconazole as a positive control provided inhibition growth of 88.10% ± 0.01. These results show that the ethanol extract of Bajakah Tampala has antifungal activity against *C. albicans*.

The ethanol extract of Bajakah Tampala has antifungal activity against *C.albicans* of 85.77% at a concentration of 1% w/v, with the control drug fluconazole providing activity of 74.20%. ± 0.01 on the fungus Candida albicans. The process of inhibition of the biosynthesis of fungal nucleic acids is caused by alkaloid compounds, which result in the growth of the fungus not developing and the fungus becoming dead and other compounds, namely flavonoids, which have pharmacological effects as antifungals (Hamzah et al., 2020). Bioactive compounds that function as antifungals are triterpenoids which are classified as terpenoids. Fungal growth will be inhibited even though it passes through the cytoplasmic membrane and the increase in fungal

spores caused by the presence of terpenoids (Mochtar, C. F., Saleh, L. O., Hamzah, H., & Ilyas, 2022).

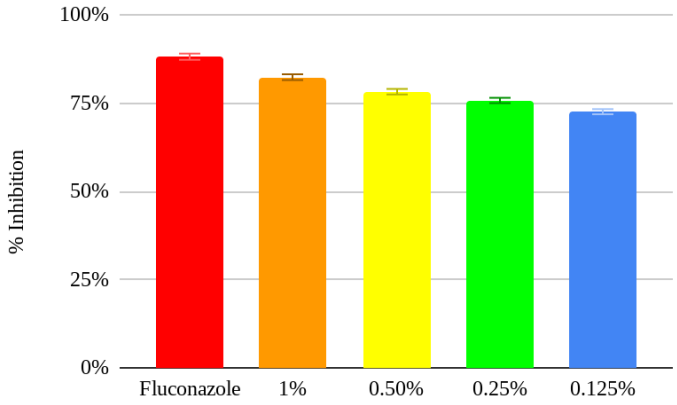

Figure 1. Antifungal activity of the ethanol extract of Bajakah Tampala

3.2.2. Antibacterial Against *S. aureus*

As seen in **Figure 2**, the results of the antibacterial test against *S. aureus* using microdilution showed that at a concentration of 1% w/v the ethanol extract of Bajakah Tampala was able to provide inhibitory activity against S. aureus of 88.33% ± 0.01, while chloramphenicol as a positive control gave growth inhibition of 89.01% ± 0.01. These results provide evidence that the ethanol extract of Bajakah Tampala has antibacterial activity against S. aureus. These results are in line with research conducted by (Mochtar, C. F., Saleh, L. O., Hamzah, H., & Ilyas, 2022). That the 1% concentration of the Bajakah Tampala ethanol extract has an antibacterial activity of S. aureus of 82.30% and (Kurniawan, 2019) reported that at a concentration of 100% it had an inhibition diameter of 12.25 ± 0.5 mm and the Bajakah Tampala Ethanol Fraction (*Spatholobus Littoralis* Hassk.) at a concentration of 80% with an inhibition diameter of 8.25 ± 0.5mm (Agustin et al., 2018).

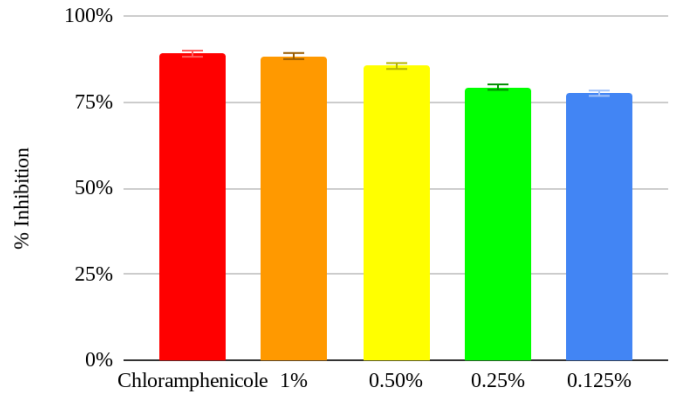

Figure 2. Antibacterial activity of the ethanol extract of Bajakah Tampala against *S. aureus*

Compounds that have antibacterial activity are alkaloids by slowing cell respiration and have a function when intercalating DNA (Hamzah et al., 2020). In addition, saponins also have antibacterial activity by interfering with the surface tension of the cell wall, so when the surface tension is disturbed, the antibacterial substance can easily enter the cell and interfere with metabolism, causing bacterial death to occur (Agustin et al., 2018).

3.2.3. Antibacterial Against *E. coli*

As seen in **Figure 3**, the ethanol extract of Bajakah Tampala (*Spatholobus littoralis* Hassk) provided an antibacterial activity of 84.83% at a concentration of 1% w/v and this activity was almost the same as the control drug chloramphenicol of 87.17%. These results indicate that the ethanol extract of Bajakah Tampala (*Spatholobus littoralis Hassk*) is able to inhibit the growth of *E. Coli* above 50%. The 50% concentration of the Bajakah Tampala ethanol extract has antibacterial activity against *E.Coli* with an average diameter of the inhibition zone of 20.32 mm (Saputera et al., 2019).

Compounds that are known to play a role in inhibiting bacterial growth include flavonoids, saponins, and tannins, where these substances function as antibacterials with different mechanisms (Febrianti et al., 2018). In tannin compounds, bacterial growth is inhibited by interfering with protein transport, inactivating cell adhesins and inactivating enzymes in bacterial cells (Agustin et al., 2018).

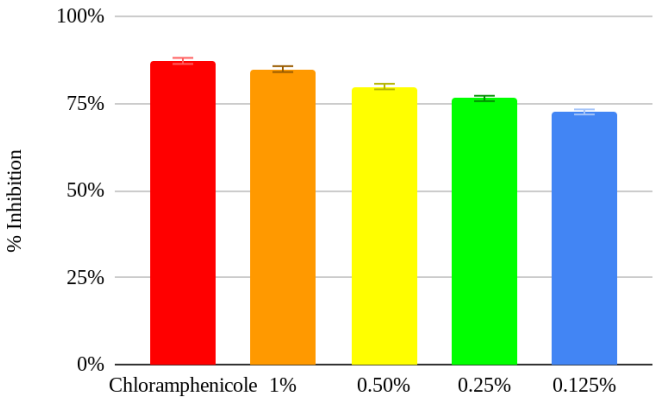

Figure 3. Antibacterial activity of the ethanol extract of Bajakah Tampala against *E. coli*

3.3. Antibiofilm Test

3.3.1. Antibiofilm Activity Against *C. albicans* (Anti-biofilm Activity of the Ethanol Extract of Bajakah Tampala in the Mid-24-hour Phase)

As seen in **Figure 4**, Bajakah tampala ethanol extract at a concentration of 1% w/v gave inhibitory activity against *C. albicans* biofilms of 77.00% ± 0.01, while Fluconazole showed inhibition of *C. albicans* biofilm formation of 80.67% ± 0.01, From the results above, it has been shown that the same extract concentration tested (1% w/v) showed weaker activity against inhibition of biofilm formation (77.00% ± 0.01) compared to planktonic (82.31 % ± 0.01).

The results showed that microbes in the form of biofilms were more difficult to inhibit than in planktonic ones. This may be because the microbes in planktonic are single cells, while the microbes in biofilms tend to live together (many colonies), attach and grow on the surface, and form multi-layered structures encased by an EPS matrix, which makes biofilms more resistant to antibiotics and antimicrobials (Hamzah, Rasdianah, et al., 2021).

3.3.2. Antibiofilm Activity Against *S. aureus* (Anti-biofilm Activity of the Ethanol Extract of Bajakah Tampala in the Mid-24-hour Phase)

In **Figure 5**, the results of the study show that the ethanol extract of the Bajakah tampala plant 1% w/v gave the highest activity of all extract concentrations as an anti-biofilm against *S. areus* in the mid phase of 80.23% ± 0.01. Meanwhile, the control drug in the form of chloramphenicol with a concentration of 1% w/v gave an activity of 82.21% ± 0.01. In this study, there was a decrease in inhibitory activity compared to inhibitory activity in planktonic. This is because biofilms produce EPS structures that function as microbial protection from drug compounds. Microbes in biofilms differ from planktonic cells in various ways of growing. One

consequence of these differences is that the microbes in biofilms have been shown to be more resistant to antibiotics and antimicrobials (Hamzah, Siregar, et al., 2021). However, the results of this study provide evidence that the ethanol extract of Bajakah Tampala has potential as a new anti-biofilm agent.

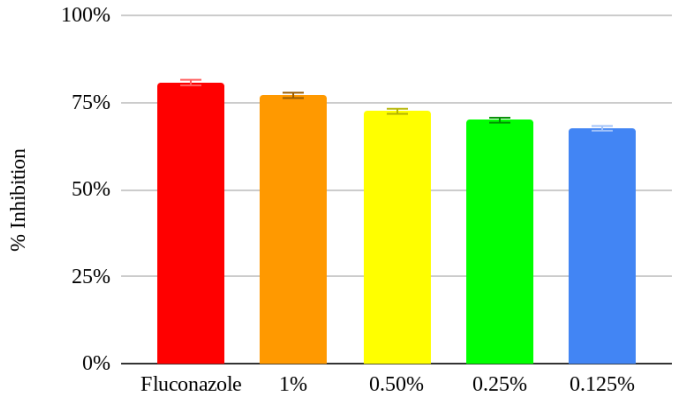

Figure 4. Middle phase anti-biofilm activity of Bajakah Tampala ethanol extract against *C.albicans* biofilm monospecies in catheters

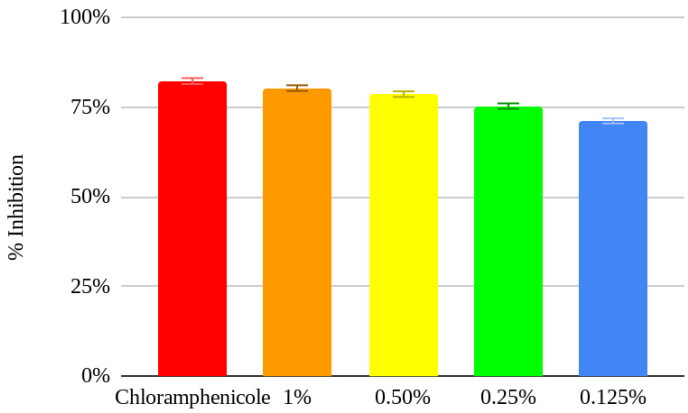

Figure 5. Middle phase anti-biofilm activity of Bajakah Tampala ethanol extract against *S. aureus* biofilm monospecies in catheters

3.3.3. Antibiofilm Activity Against E. coli (Anti-biofilm Activity of the Ethanol Extract of Bajakah Tampala in the Mid-24-hour Phase)

In **Figure 6**, it can be seen that the ethanol extract of Bajakah Tampala can inhibit the formation of E. coli biofilms in the intermediate phase from 1% to 0.125% where at 1% b/v level it provides activity of 80.11%, while the control drug chloramphenicol is 82.65%. However, there is a decrease in the inhibition of ethanol extract of bajakah tampala and chloramphenicol when biofilm formation occurs, this occurs because biofilms provide protection against E. coli from antibiotic treatment and the immune system. These bacteria can be up to 1000-fold more resistant to antibiotics than planktonic bacteria. Tolerance to antibiotics is mainly due to the following mechanisms: low antimicrobial penetration, reduced growth rate and stress response, cell survival, efflux pumps, and HGT (Ballen, Cepas., et al 2022).

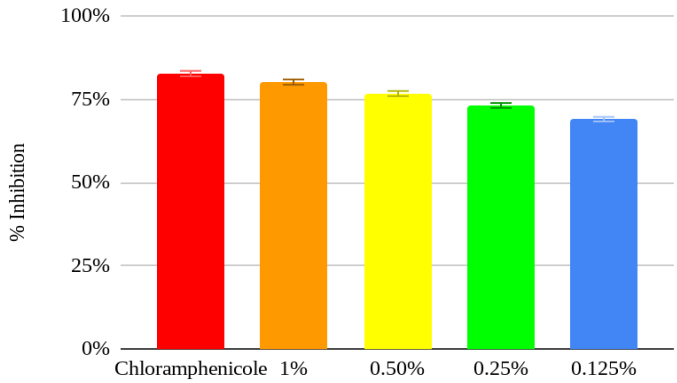

Figure 6. Mid-phase anti-biofilm activity of Bajakah Tampala ethanol extract against Middle phase anti-biofilm activity of Bajakah Tampala ethanol extract against *E. Coli* biofilm monospecies in catheters

4. CONCLUSION

From the results of the qualitative phytochemical screening, the ethanol extract of Bajakah tampala (*Spatholobus littoralis* Hassk) positively contained alkaloids, flavonoids, saponins, tannins and phenols. The ethanol extract of Bajakah tampala (*Spatholobus littoralis* Hassk) has antibacterial activity against *S. aureus* and *E. coli* bacteria. It also has antifungal activity against *C. albicans* where the higher the concentration of the extract, the higher the antibacterial and antifungal activities are produced. Microbial activity in biofilms has been shown to be more resistant to antibiotics and antimicrobials, but based on the results of the anti-biofilm activity testing, the ethanol extract of Bajakah Tampala has potential as a new anti-biofilm agent against *S. aureus, E. coli and C. albicans.*

5. ACKNOWLEDGEMENT

Thanks to the APTFMA for the grant awarded to our research team. Thank you, Chancellor of Muhammadiyah University of Kudus and Chancellor of Muhammadiyah University of East Kalimantan, for the support. Thanks to UMKU and UMKT pharmacy students who were involved in helping with this research. Thanks to the annual meeting committee for passing this research abstract, so it passed to the JFSP journal.

6. CONFLICT OF INTEREST

All authors declare no conflict of interest.

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JFSP Vol.10, No.1, January-April 2024, Page: 42-51
pISSN: 2549-9068, eISSN: 2579-4558
Jurnal Farmasi Sains dan Praktis
(JFSP)
http://journal.unimma.ac.id/index.php/pharmacy

APPLICATION OF LAKES SYSTEM IN PREPARATION OF HAIR DYES POMADE CREAM OF FREEZE-DRIED RED DRAGON (*Hylocereus polyrhizus*) FRUIT PEEL JUICE AND ACUTE DERMAL IRRITATION

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https://doi.org/10.31603/pharmacy.v10i1.8889

Article info:
Submitted : 23-03-2023
Revised : 25-12-2023
Accepted : 21-01-2024

Publisher:
Universitas Muhammadiyah Magelang

ABSTRACT
Dyes used in market products are mostly synthetic, which can irritate the skin and in the long term increase the risk of skin cancer. The peel of dragon fruit contains the red-violet pigment Betacyanin, which is potential as hair dyes but extremely sensitive to light and oxygen. The lakes system is the option to overcome the problem and increase the stability of pigments. The objective of this study was to learn about the use of the lakes system in the preparation of hair dyes pomade cream of freeze-dried red dragon fruit peel juice and its irritation aspect on skin. The lakes system was made with a 50% red dragon fruit peel juice, mixed with 5% alucol, then dried with 45% aerosil. Pomade cream was prepared by mixing oil phase and water phase at a temperature of ±60 °C with continuous mixing in 600 rpm for 10 minutes. Formula III is the bases without freeze-dried red dragon fruit peel juice. The non-lakes system of freeze-dried red dragon fruit peel juice was added to the bases for the FII, while the lakes system was added for the FI. Hair dyes pomade cream of red dragon fruit peel juice prepared in the lakes system has superior properties to the formula prepared without the lakes system. It demonstrated better homogeneity and softer texture, in addition acceptable skin pH range. The preparation do not cause skin irritation. The lakes system is suitable for the preparation of natural hair dye cream and does not irritate the skin.

Keywords: Complex; Dyes; Lakes; Pomade; Red-dragon

1. INTRODUCTION

Hair dyes preparations are cosmetics to color hair, either to restore the original hair color or to change into other colors. Hair dyeing preparation Pomade cream is a new type of temporary hair dye that can be applied directly to the hair (Auliasari et al., 2018). The dyes used in market products are mostly synthetic, such as rhodamine B, which can irritate the skin and, in the long run, increase the risk of cancer and liver damage (Syakri, 2017). Natural dyes, which are safer and have fewer side effects than synthetic dyes, may be the solution to this problem (Pardede et al., 2008). Dragon fruit peel contains pigment and can therefore be used as a natural dye. It is also commonly discarded as waste, despite accounting for nearly 30-35% of total fruit weight (Saati, 2011). Dragon fruit peel contains pigments anthocyanin and betacyanin, which can be used to create dyes (Sandy et al., 2021; Harjanti, 2016).

However, the pigment in dragon fruit peel is a type of phenolic compound that is extremely sensitive to light and oxygen. As a result, the dye's quality is deteriorating day by day (Citramukti, 2008). The lakes system can improve color pigment stability by forming a complex between reactive functional groups in pigments with hydrophobic substrates. It was created by absorbing pigments onto water-insoluble substrates like hydrated aluminum (alucol). The complex formed by the pigment and Al^{3+} from hydrated aluminum protects the pigment from light, chemicals, and heat (Cahyadi, 2006). Eventually, research will be conducted on the use of the lakes system in the preparation of hair dyes pomade cream of red dragon (*Hylocereus polyrhizus*) fruit peel juice and its irritation aspect on skin.

2. METHODS

2.1. Plant Determination

Plants are determined by matching the morphology of entire plant parts such as roots, stems, and fruits to a key of determination that refers to the literature (Backer, 1965). Plant determination was performed at the Ecology and Biosystematics Laboratory, Department of Biology, Faculty of Science and Mathematics, Diponegoro University, Semarang, Indonesia.

2.2. Freeze-Dried Red Dragon Fruit Peel Juice Preparation

Various sources cited the preparation of red dragon fruit peel juice, which is red dragon fruit peel juiced to separate the juice from the pulp. The juice was freeze-dried (Christ Alpha 1-2 LDplus), crushed, and sieved to achieve a small uniform particle size (Simanjuntak et al., 2014) (Sultan, 2017) (Husein & Lestari, 2019). The presence of secondary metabolite compounds of Alkaloids, Phenols, Flavonoids, Quinones, Saponins, and Tannins was determined using phytochemical screening on freeze-dried red dragon fruit peel juice (Kemenkes RI, 2011).

2.3. Lakes System of Freeze-Dried Red Dragon Fruit Peel Juice Preparation

The lake system is formed by pigment absorption on the hydrophobic substrate, which was created by combining 50% freeze-dried red dragon fruit peel juice with 5% alucol as the hydrophobic substrate. The mixture was then absorbed with 45% aerosil, resulting in the dried lakes. Sultan's research identified those compositions as optimal, with moisture content below 10% (Sultan, 2017).

2.4. Hair Dyes Pomade Cream of Freeze-Dried Red Dragon Fruit Peel Juice Formulation

Cream formula refers to (Husein & Lestari, 2019) with modification. Cream pomade is created by gently combining an oil phase mixture of 7.5 g of cera alba; 128.75 g Vaseline album; and 10 g of Span to an aqueous phase mixture containing 15 g of Tween 80; 75 g propylene glycol; 1.25 g methyl paraben; and aquadest at a temperature of ±60ºC. For 10 minutes, a Maspion-MT1140 mixer at 600 rpm was used to mix until a homogeneous cream base was formed. Formula III was the cream bases that did not contain freeze-dried red dragon fruit peel juice as a hair coloring agent. While in formula II (FII), an amount of 7.5 g non-lakes of freeze-dried red dragon fruit peel juice was geometrically added to the cream bases. On behalf, Formula I created by adding an amount of 7.5 g lakes system of freeze-dried red dragon fruit peel juice into the cream bases. Each formula was triple replicated and tested for its characteristics, which included:

2.4.1. Organoleptic

Conducted by visually observing the texture, color, and smell of cream preparations using the five human senses (Azkiya et al., 2017)

2.4.2. Homogeneity

The cream is shed transparently to the object-glass and its homogeneity is visually observed, with the parameters being free of coarse aggregate (Azkiya et al., 2017).

2.4.3. pH

A pH meter (HANNA HI8314-HI1612D) was employed to test the pH of 0.5 g of sample dissolved in 50 mL of aquadest. The pH meter was calibrated before measuring the sample with a standard buffer solution of pH 4, 7, and 10 (Kemenkes RI, 2020).

2.4.4. Dispersibility

A total of 0.5 g of the sample was placed on a glass surface above millimeter paper, then covered with another transparent glass for 1 minute and left. A weight of 50, 100, and 200 g was continuously added to the glass, and the diameter of the formed spread was measured (Saryanti et al., 2019).

2.4.5. Adhesion

A 0.5 g sample was placed on a glass object and then covered with another glass object for the test. For 5 minutes, a 500 g weight was placed above. A weight of 80 g is released to pull the bottom glass object, and the time it takes for the two glass objects to come off is recorded (Saryanti et al., 2019).

2.4.6. Viscosity

The viscosity of the cream was measured using a viscometer (RION VT-06 rotor no 2), which was placed in the center of the cream container. Keep an eye on the visibility needle. Once stable, the number displayed with the unit decipascal-seconds (dpas) was read (Mardikasari et al., 2020)

2.5. Hair Dyes Coloring Evaluation

Some shampoo-washed hair is dyed with a hair dye formula, then allowed to stand for 40 minutes and the color formed is observed. The coloring stability against washing and sunlight is then evaluated (Zaky et al., 2020).

2.6. Acute Dermal Irritation Test

On healthy male albino rabbits weighing 2.5-3.5 kg, a skin irritation test was performed. The experiment began with a preliminary test on one rabbit, with observations made at the third, sixty-first, and 240th minutes. If no irritation was observed, the procedure was repeated with the addition of two rabbits. Observations were administered after 24, 48, and 72 hours. The irritation score was calculated by observing the irritation parameters, which included the presence of erythema and edema (BPOM, 2020 & OECD, 2015).

3. RESULTS AND DISCUSSION

3.1. Plant Determination Result

The results of plant determination demonstrated that the plants employed were classified as Kingdom Plantae, Subkingdom Tracheobionta, Super Division Spermatophyta, Division Magnoliophyta (seed plants), Class Magnoliopsida (Dicotyledonae), Order Cactales, Family Cactaceae. Genus Hylocereus, Species *Hylocereus polyrhizus* (FAC Weber) Britton & Rose, with the local name Red Dragon Fruit.

3.2. Freeze-Dried Red Dragon Fruit Peel Juice

The freeze-dried red dragon fruit peel juice was described as red fines flakes as shown in **Figure 1**. The freeze-dried red dragon fruit peel juice yield was 2.28% of the fresh red dragon fruit peel weight. It is possible that this is due to the high-water content in the peel of fresh red dragon fruit. Red dragon fruit peel can be extracted using water or in conjunction with organic solvents such as methanol or ethanol (Sultan, 2017). The dragon fruit's red-purple hue is generated by betacyanin pigment, while the yellow tinge is caused by betaxanthin pigment. Both belong to the betalain pigment family. Several studies have shown that betacyanin pigments are more stable in aqueous solvents than in water-ethanol solvent combinations (Altamirano, 1993; Castellar et al.,

2006). This is thought to be due to the degradation of betacyanin pigments due to single or multiple decarboxylation mechanisms (Wybraniec et al., 2001).

The phytochemical screening revealed that alkaloids and flavonoids were present. It yielded the same results as phytochemical screening on super red dragon fruit peel extracted with water and dried using the freeze-drying method (Sultan, 2017). The morphology of the fruit distinguishes red dragon fruit used in this study from super red dragon fruit used in previous studies. The red dragon fruit is more oval and has dark red flesh, whereas the super red dragon fruit is round and has purple flesh (Le Bellec et al., 2006). Because betalains are a class of phenolic compounds with a hydroxyl group in their structure, which can be in the form of alkaloids or flavonoids, the results of this phytochemical screening indicate the possibility of betalain group pigments being present in the juice (Azeredo, 2009).

Figure 1. Freeze-dried red dragon fruit peel juice

3.3. Lakes System of Freeze-Dried Red Dragon Fruit Peel Juice

The lakes system has a morphology of dark red dry granules with uniform, homogeneous, and spherical particles. **Figure 2** depicts the interaction mechanism between the color pigment and the substrate at the C=O and -OH groups. Complexes between the C=O and C=O groups will form in mineral salts such as Al from alucol. A color pigment's -OH. Because the functional groups that have the potential to cause instability are used in complex formation interactions, the complex formed between the color pigment and the substrate in the lakes system can increase the color pigment's stability against light, chemistry, and heat (Kirby, 2011; Wongwad et al., 2012).

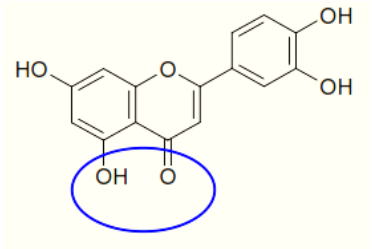

Figure 2. The position of the coordination bond between the substrate and pigment (Kirby, 2011)

3.4. Hair Dyes Pomade Cream of Freeze-Dried Red Dragon Fruit Peel Juice

The result of physical characteristics evaluation of hair dyes pomade cream of freeze-dried red dragon fruit peel juice was described below.

3.4.1. Organoleptic

Hair dyes pomade cream with red dragon fruit peel juice at FI and FII had a dark red color, was semi-solid, and smelled like dragon fruit, while FIII as bases had a milky white color. It was depicted in **Figure 3**. FII has a rougher texture than FI and FIII. It was most likely caused by the

aggregation of red dragon fruit peel juice particles that had not yet been distributed uniformly in the bases. In contrast to FII, the particles in FI which the red dragon fruit peel juice was in the form of the lakes system were coated with alucol, which makes the surface more hydrophobic and thus helps to reduce aggregation.

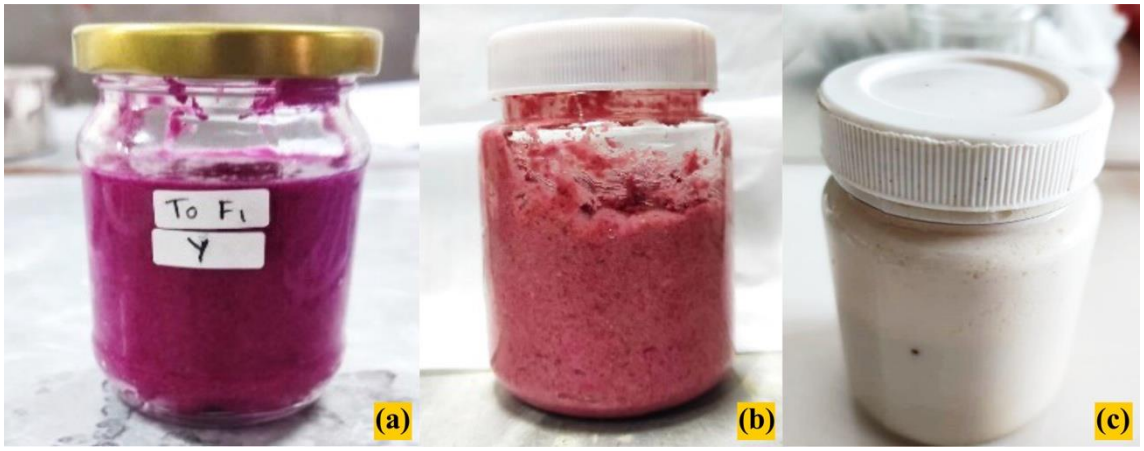

Figure 3. Pomade cream of: (a) FI (lakes of freeze-dried red dragon fruit peel juice); (b) FII (non-lakes of freeze-dried red dragon fruit peel juice) and (c) FIII (cream bases without freeze-dried red dragon fruit peel juice)

3.4.2. Homogeneity

The homogeneity of FII was lower than that of FI and FIII, most likely because the red dragon fruit peel juice could not be dispersed homogeneously in the cream bases as well as the lakes system in FI. It was related to the organoleptic results. Because it is associated with drug dose at each use, homogeneity has an impact on therapy effectiveness. If the preparation is homogeneous, the active substance content is assumed to be constant at each intake (Roosevelt et al., 2019). In the context of cosmetic uses, it influences the acceptability of the consumer.

3.4.3. pH

Figure 4 depicts the pH values for the three formulas. The ANOVA testing revealed that it was statistically different in each formula with a significance of 0.05. Due to the alkaline alucol used in the lakes system, the pH values in FI are higher than in FII and FIII, potentially increasing the pH of the product. The pH of alukol, also recognized as colloidal aluminum hydroxide, is 8-9. The pH range that the skin can tolerate is 4.5-6.5. A preparation that is too acidic and far from the pH of the skin will most likely irritate it, whereas an alkaline preparation will build the skin's scalp (Siva & Afriadi, 2019). The statistical analysis revealed that changing the lakes system affects the pH of the hair dyes pomade cream.

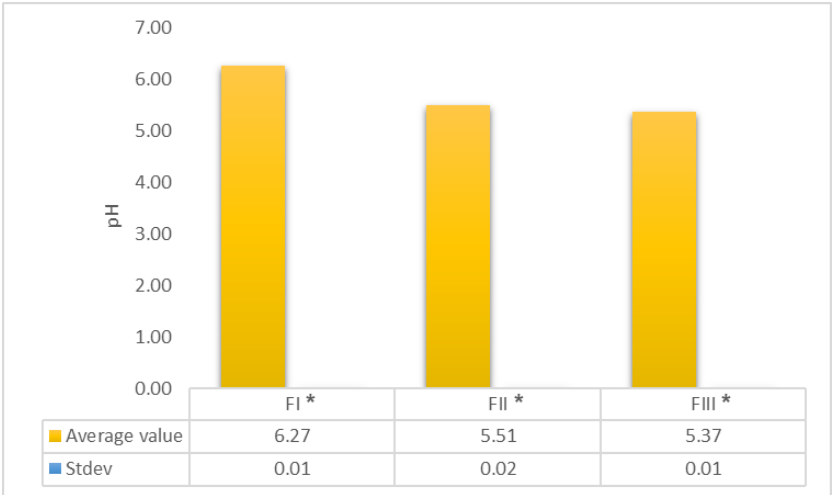

Figure 4. pH of pomade cream

3.4.4. Dispersibility

Dispersibility is inversely proportional to the value of viscosity (Zulfa & Mufrod, 2018). **Figure 5** depicts the dispersion results from the three formulas. It was discovered that the dispersion value is inversely proportional to the viscosity, with FI having the highest dispersion and FIII having the lowest. A statistical analysis revealed that each formula differed significantly from the others. It demonstrated that the lakes system was given different physical properties to the hair dyes pomade cream of red dragon fruit peel juice. According to (Rasydy et al., 2021), the 5-7 cm spread demonstrates a semi-solid consistency that is very comfortable to use. The dispersibility of the three formulas is less than 5, but they are still acceptable because the characteristics of cosmetic preparations are not strict.

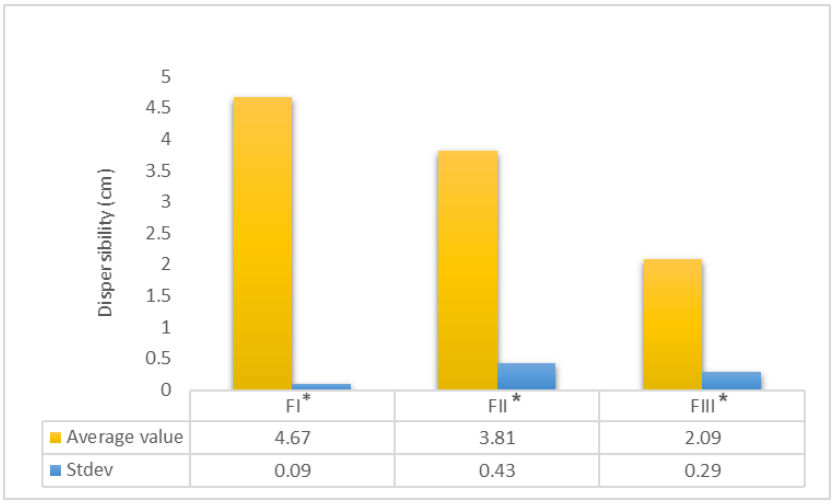

Figure 5. Dispersibility of pomade cream

3.4.5. Adhesion

The adhesion test is related to the time needed for the preparation to contact with the surface of the skin and cause physiological effects as well as related to the comfort of the preparation at this time applied (Marviani & Rochman, 2021). **Figure 6** depicts the results of the adhesion. FIII has the highest value, while FII has the lowest. It contradicts the dispersibility value, where the dispersion of FIII was lowest. It could be related to the FII homogeneity problem caused by aggregation of red dragon fruit peel juice that was not dispersed homogeneously. While in FI, the lakes system aids in particle aggregation reduction by encapsulating the red dragon fruit peel juice with alucol, which is hydrophobic in nature, causing the particle's surface to become more hydrophobic.

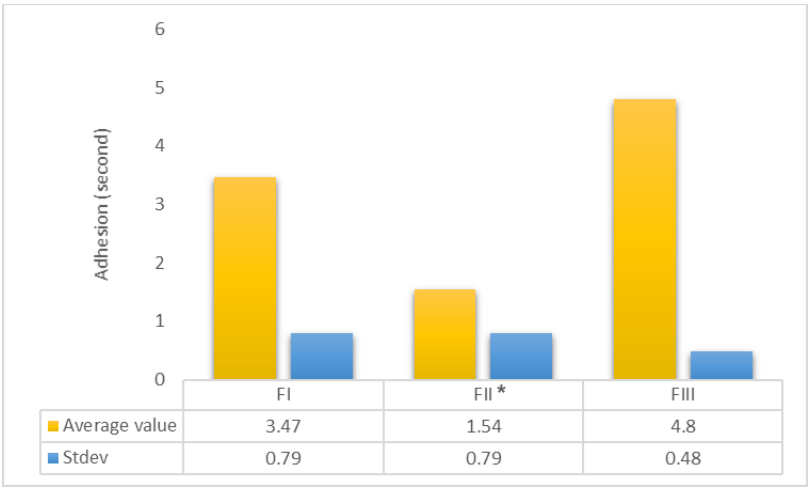

Figure 6. Adhesion of pomade cream

Statistical analysis revealed that the research treatment provided different adhesion characteristics to the hair dyes pomade cream of red dragon fruit peel juice, as demonstrated by the sig <0.05. Further statistical analysis was performed to determine whether each formula differed significantly. The results illustrated that FI (lakes system) differed significantly from FII (non-lakes system) but not from FIII (cream mass) (sig. 0.128). Furthermore, FIII demonstrated statistically significant differences to FII (sig. 0.003) but not to FI. The FII, on the other hand, demonstrated both significant differences with F1 and FII, with significant values in a row of 0.035 and 0.03.

3.4.6. Viscosity

Viscosity, spreadability, and adhesion are all characteristics of preparations that can be correlated. Viscosity is sometimes proportional to adhesion and inversely proportional to dispersion. According to the data in **Figure 7**, statistically, each formula produced a significant difference. Preparations containing the active ingredients of red dragon fruit peel juice, whether in lakes or non-lakes systems, tend to decrease viscosity, with FIII showing the highest and FI showing the lowest. It is most likely due to an increase in FI hydrophobicity caused by the impact of alucol used in the lake system, which creates hydrophobic boundaries on the surface of particles. Hydrophobicity and hydrophilicity affect viscosity as a result of dissolved gas strongly adsorbed on the surface of the particles (Sendner et al., 2009).

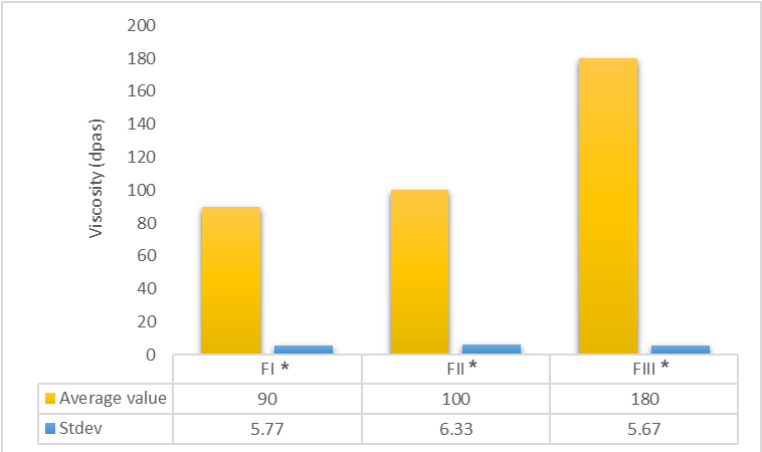

Figure 7. The viscosity of pomade cream

3.5. Hair Dyes Coloring Evaluation

Figure 8 depicts the outcome of the hair dye coloring evaluation. The figure illustrated that when exposed to direct sunlight, the color intensity of the hair dye pomade cream of red dragon fruit peel juice decreased and turned brown. Light, air, and temperature all contribute to the degradation of betaxanthin pigments (Sari, 2018). The degradation rate of betalain increased by 14.6% in air and 15.6% in light, resulting in a decrease in color intensity (Elbe et al., 1974).

Figure 8. Hair bleached condition after application of F1 (lakes), F2 (non-lakes), and F3 (only base) after a) 40 minutes observation, b) exposure of direct sunlight, and c) washing.

Furthermore, testing was also administered on washing. Water can be used to cleanly remove all three formulas. It suited the study's end goal of being a temporary hair dye. Furthermore, this can meet the needs of consumers who are always dynamic and want to change hair colors without worrying about hair damage.

3.6. Acute Dermal Irritation Test

There was no irritation on the rabbit's skin (rabbit A) based on the preliminary test results at the 3rd, 60th, and 240th minutes, as indicated by an irritation score of 0. As a result, observations were continued with two more rabbits (rabbit B and C) for 24, 48, and 72 hours. **Figure 9** depicts the results, which show that there was no irritation of the rabbit skin in the form of erythema or edema.

The consumption of temporary hair dye cream is less than 24 hours, and the testing results on the rabbit's back skin did not irritate after 24, 48, and 72 hours. Furthermore, there was no irritation or other skin damage on the 14th day of observation. The objective of this observation is to identify the possibility of corrosive and irreversible skin damage (BPOM, 2020).

Figure 9. Rabbit's back skin after the application of hair dye pomade cream preparation in: (a) rabbit A; (b) rabbit B and (c) rabbit C (K = control without treatment; NL = nonlakes system; L= lakes system; B = only base without red dragon fruit peel juice)

4. CONCLUSION

Hair dyes pomade cream in the lakes system have better characteristics than the formula prepared without the lakes system. It showed better homogeneity, softer texture, acceptable skin pH range, viscosity, dispersibility, and adhesion are consumer-acceptable than the formula in non-lakes system. The preparation can color bleached hair and can be removed when washed with water. Preparations do not irritate the skin.

5. ACKNOWLEDGMENT

The best gratitude is dedicated to the research funding assistance from the Directorate of Research and Community Service, Directorate General of Research and Development of the Ministry of Research, Technology, and Higher Education through LL DIKTI VI Central Java Region in the "Beginner Lecturer Research" scheme based on Decree Number 1867/E4/AK .04/2021 and Agreement/Contract Number 067/E4.1/AK.04.PT/2021.

6. CONFLICT OF INTEREST

All authors declare no conflict of interest.

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JFSP Vol.10, No.1, January-April 2024, Page: 52-60
pISSN: 2549-9068, eISSN: 2579-4558
Jurnal Farmasi Sains dan Praktis
(JFSP)
http://journal.unimma.ac.id/index.php/pharmacy

SUN PROTECTION FACTOR (SPF) VALUE AND PHYSICAL PROPERTIES OF PURIFIED GAMBIER GEL PREPARATION

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https://doi.org/10.31603/pharmacy.v10i1.10208

Article info:
Submitted : 10-09-2023
Revised : 30-12-2023
Accepted : 26-01-2024

Publisher:
Universitas Muhammadiyah Magelang

ABSTRACT
Gambier is an export product from Sumatera, Indonesia, that can be purified and utilized in cosmetic preparations. The content of phenolic and flavonoid compounds of purified gambier has a potential skin protection against UV rays. The chemical content of extract is closely related to the polarity of solvent, so this study aims to analyze the effect of ethanol concentration as solvent in gambier purification on phenolic content, flavonoids, and SPF value. The ethanol concentrations for this purpose were 0%, 25%, 50%, 75% and 96%. Total phenolic and flavonoid contents were analyzed by colorimetric method, whereas the SPF value of gel was measured by UV spectrophotometer. The gel preparation was evaluated for its physical properties including organoleptic test, spreadability, adhesiveness, viscosity, pH and stability. Statistical analysis performed with one-way ANOVA at 95% confidence level. As the results, ethanol concentration significantly influenced phenolic, flavonoid content and SPF values. The highest phenolic content was obtained in purification by 50% ethanol with a value of 757.2 ± 13.1 mg GAE/g, while highest flavonoid content was achieved in 96% ethanol at 5.18 ± 0.21 mg QE/g. Ethanol concentration with highest SPF value was 96% at 27.07 ± 0.33. In the gambier gel formulation with 0.2% dose had an SPF value of 6.60 ± 0.58. The gel has good homogeneity, viscosity, and pH for cosmetic preparations but poor spreadability. The stability of the gel formulation changed after accelerated stability testing for 4 weeks.

Keywords: Flavonoid; Gambier; Phenolic; Purification; SPF

1. INTRODUCTION

Indonesia is a tropical country with high-intensity UV rays which is caused high index category of ultraviolet index. UV rays has negative effects on the skin such as loss of elasticity, fine lines, wrinkles, and premature ageing (Ansary et al., 2021). To protect our skin from exposure to UV radiation, an application of sunscreen preparations is one of the effective ways. For this purpose, FDA recommended a sunscreen preparation with the SPF value of more than 15 and repeated application at least every two hours (FDA, 2022)

The active substances in sunscreen preparations are divided into two types, that are inorganic and organic compounds. Unfortunately, inorganic materials have a risk of being absorbed into certain layers of skin because they are often made in nanoparticles for aesthetic reasons (Wang & Wang, 2014). On the other hand, the active substances of organic sunscreens need to be combined to improve their protection spectrum. However, the combination of organic compounds for sunscreens is restricted by regulatory agencies due to the risk of incompatibility (Ngoc et al., 2019).

Natural compounds can be an effective alternative as sunscreen agents because they generally consist of a mixture of various compounds that can absorb UV rays. Phenolic groups and their derivatives are chemical compounds with aromatic and chromophore groups which have

potential as UV absorbers. Polyphenols are coloured compounds which can absorb UV B radiation as well as some wavelengths of UV A and UV C. Polyphenols can protect our skin from UV radiation when applied topically (Hashemi et al., 2019). Studies conducted by Stevanato et al. (2014) showed that phenolic compounds such as catechins, quercetin, rutin, kaempferol and other flavonoid compounds have absorption at UV wavelengths (290-400 nm) depending on their structure and mass.

Gambier is a popular export commodity in Sumatera region because it is widely used by other countries as an ingredient for medicine, cosmetics, food, and leather tanning (Hernani et al., 2020). Gambier is the dried extract of the leaves of *Uncaria gambir* (Hunter) *Roxb*. from *Rubiaceae* familiy (Indonesia Herbal Pharmacopoeia II, 2017). Gambier contains free phenolic compounds and flavonoids such as catechins, quercetin, kaempferol, rutin and tannin (Munggari et al., 2022). Research conducted by Winarti et al. (2022) showed that gambier has UV protection activity showed by the SPF value in lotion preparations containing its extract. The SPF produced value is proportional to the amount of gambier extract added to the lotion preparation.

The gambier produced by the traditional way has variations in catechin content ranging form 40-60% due to differences in plant sources and processing (Hernani et al., 2020). Therefore, it is necessary to purify in order to increase the chemical content and remove unnecessary substances such as cellulose. The process can be done by water as solvents or in combination with organic solvents such as ethanol (Rauf et al., 2010). Purification is a process after extraction to separate a specific or group of compounds from others substances in extract that may contain undesired activities or inert constituents (Xiao et al., 2013). The concentration of ethanol in water will affect the polarity of the solvent mixture which determines the solubility of diluted secondary metabolites of an herb. Solvent selection is one of several important aspects that need to be considered in the purification process to obtain suitable extraction conditions (Mauricino & Juliana, 2013). The effect of ethanol concentration on several responses in the gambier purification process is the focus of this study. The obtained gambier from the purification process was made into gel preparation to determine the SPF value, physical properties, and stability of the preparation.

2. METHODS

2.1. Materials

The instruments used in this research are Mettler Toledo analytical balance (Ohio, United States), BenchTop Freeze Dryer (Tokyo, Japan), quartz cuvette 1 cm, Hitachi UH5300 Spectrophotometer UV-Vis (Tokyo, Japan), and Merck pH paper (Darmstadt, German). Materials used include gambier with various forms obtained from Beringharjo Market, Yogyakarta; ethanol 96%; distilled water; methanol pro analysis; ethanol pro analysis (Smartlab); Folin-Ciocalteu reagent; sodium hydroxide, anhydrous sodium acetate (Merck), gallic acid (Woko Pure Chem), aluminum chloride hexahydrate, quercetin (Sigma-Aldrich), carbopol 940, propylene glycol, propylparaben, methylparaben, and triethanolamine.

2.2. Methods

2.2.1. Purification of Gambier

The purification of gambier involves extracting the sifted gambier powder with a mesh size of 40 by ethanol as solvents with various concentrations for 3 days at room temperature. The ratio of solvent to sample used was 1:5. Maceration was repeated once by 1:3 solvent ratio for 1 day. The filtrate was then evaporated by a water bath system and subsequently dried by freeze-drying technique. The ethanol concentrations for purification are 0%, 25%, 50%, 75%, and 96%.

2.2.2. Determination of Phenolic Content and Flavonoid Content

The determination of phenolic content in purified gambier was carried out by Folin-Ciocalteu method following the procedures outlined in the Indonesian Herbal Pharmacopoeia II

(2017), with several changes. Purified gambier was diluted with methanol to obtain a solution with a concentration of 0.1 mg/mL. A standard curve was generated by diluting gallic acid to create a series of dilution solutions at concentrations of 100, 70, 50, 30, 15, and 5 µg/mL. A volume of 1.0 mL of the sample solution was taken and mixed with 5.0 mL of 7.5% Folin-Ciocalteu reagent. The mixture was incubated for 8 minutes, added by 4.0 mL of 1% NaOH, and then reincubated for 1 hour. The sample was placed in a cuvette, and its absorbance was measured alongside a blank solution at a maximum wavelength of 730 nm. The total flavonoid content of purified gambier was determined following the procedures of the Indonesian Herbal Pharmacopoeia II (2017). A dilution series of quercetin standard was prepared by concentrations of 100, 75, 50, 25, and 5 µg/mL. Gambier samples were diluted with ethanol to get concentration of 10 mg/mL. A volume of 0.5 mL of both sample and reference solutions were taken and combined with 1.5 mL of ethanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1M sodium acetate, and 2.8 mL of distilled water. The solution was shaken and left at room temperature for 30 minutes. The absorbance of all solutions, including samples, reference standard, and blanks was measured at a maximum wavelength of 438.0 nm.

2.2.3. SPF Value Measurement of Purified Gambier

The measurement of the SPF value of purified gambier was conducted following the procedure carried out by Dutra et al., (2004) with modifications to the sample dilution. The sample was diluted by ethanol to achieve a solution concentration of 2 mg/mL. The absorbance of the solution was measured at 5 nm intervals within the wavelength range of 290 nm-320 nm by UV-Vis's spectrophotometer. Each data point was measured three times. The SPF value was calculated by the equation formulated by Mansur et al. (1986), as following Eq. (1).

$$SPF = CF \times \Sigma_{290}^{320} E(\lambda) \times I(\lambda) \times Abs(\lambda) \quad (1)$$

Where: CF: Correction Factor = 10; EE: Erythema Effect Spectrum; I: Intensity of Solar Spectrum; Abs: Absorbance of Sunscreen Formulation

The constant value of EE x I was obtained from Sayre et al. (1979), as seen in **Table 1**.

Table 1. Constants EE x I at each wavelength

Wavelength (nm)	EE x I
290	0,0150
295	0,0817
300	0,2874
305	0,3278
310	0,1864
315	0,0839
320	0,0180
Total	1,0000

2.2.4. Formulation of Purified Gambier Gel

Purified gambier with the highest SPF value was formulated into a gel preparation at a dosage of 0.2%. This powder dosage was chosen as it provides medium-level protection at a concentration of 2 mg/mL. The gel formulation follows the guidelines of Rina et al. (2019), with modifications to the type of carbopol as specified in **Table 2**.

Carbopol was dispersed in 50 mL of water and stirred homogeneously. The gel base was kept at room temperature for 1 night to fully expanded. Gambier powder was dissolved in 40 mL of water and stirred until fully dissolved. Separately, methylparaben and propylparaben were dissolved in propylene glycol and sonicated to help its solubilization. All components including the active ingredient and preservatives were added into the gel base and stirred to achieve a homogenous form. Finally, distilled water was added and stirred to reach a total amount of 100

g. Triethanolamine was added to adjust the pH value of the dosage form ranging from pH 5-6. After we got a homogeneous gel dosage form, the SPF value was measured by spectrophotometric method.

Table 2. Purified Gambier Gel Formulation

No	Gel Ingredients	Composition (w/w)
1	Carbopol 940	1.00%
2	Propylene glycol	5.00%
3	Purified gambier	0.2%
4	Methylparaben	0.18%
5	Propylparaben	0.02%
6	Triethanolamine	q.s
7	Distilled Water	Added up to 100 g

2.2.5. Assay SPF value and physical properties purified gambier gel

As SPF value measurement, approximately 1000 mg of gel was dissolved by 10 mL of ethanol p.a and assisted by sonication for 5 minutes. The resulting solution was separated from the insoluble solids by centrifugation at a speed of 8000 rpm for 5 minutes. The absorbance of the sample was measured at 5 nm intervals within the wavelength ranging from 290 nm-320 nm. Each data point was read three times (n=3), while the SPF value was calculated by the equation formulated by Mansur et al., (1986).

In this research, we also analyzed the physical properties of gel to assess the quality of the preparation. Organoleptic testing includes colour, odour, homogeneity, and texture were observed in the purified gambier gel preparation. The homogeneity was assessed by applying the gel onto two transparent glass plates to observe the presence of coarse particles or clumps within the formulation. The gel was considered homogeneous if there was no precipitate or clumps which were observed. The viscosity of the gel preparation was determined by a Brookfield Viscometer with the parameters of size 7 spindle, speed of 100 rpm, and measurement duration of 60 seconds. The spreadability of the gel was assessed by weighing 500 mg of gel onto a scaled round glass plate, then covered by second glass with a known weight and left for one minute. An additional weight of 50 grams was added to the gel every minute to be a total weight of 250 grams was reached. The spreadability of the gel was measured by calculating the average diameter of its spreading.

The adhesion test of the gel formulation was performed by weighing a 100 mg of gel onto an object glass plate and another glass plate was added on top of the gel. The glass plates holding the gel were subjected to a weight of 1 kg for 5 minutes and then placed on an adhesion testing apparatus connected to a weight of 80 grams. The time taken for the two glass plates to separate or detach since the weight was applied was recorded as an adhesion testing parameter. In the other analysis of gel, the pH of the gel preparation was measured by pH paper. Colour changes in the paper were observed to determine the pH range of the preparation based on the colour indicators specified on the packaging (Tambunan & Sulaiman, 2018).

The stability of the purified gambier gel preparation was evaluated following the accelerated testing conditions from WHO (1996) with the study duration shortened to four weeks. The gel was stored in a climatic chamber at a humidity of 75 ± 5% relative humidity (RH) and a temperature of 40 °C ± 2 °C. The evaluation of the preparation's stability included organoleptic testing, spreadability, adhesion, and pH.

2.2.6. Statistical analysis

Data were analyzed using one-way ANOVA to evaluate the influence of ethanol concentration variation on total phenolic content, total flavonoid, and SPF value. Data that did not meet the assumptions of one-way ANOVA were replaced with Kruskal-Wallis analysis. Statistical analysis was performed using SPSS® software version 25 at a confidence level of 95%.

3. RESULTS AND DISCUSSION

3.1. Chemical Content

3.1.1. The Phenolic Content

The concentration of ethanol during the gambier purification process significantly influences its phenolic content, as depicted in **Figure 1** (*sig*=0,011). Gambier contains a substantial amount of free phenolic compounds with carbon and aromatic groups that are better suited for extraction by a semi-polar solvent, such as ethanol. When the polarity of the solvent matches the nature of the compounds being extracted, more compounds will dissolve (Mauricino & Juliana, 2013). A concentration of 50% ethanol provides the highest phenolic content compared to other solvents, making it a suitable choice for gambier purification to achieve the highest total phenolic content. The findings of this study differed to Pambayun et al., (2007) because their research indicated that application of ethanol as a sole solvent resulted in a higher total phenolic content compared to an ethanol-water mixture (1:1), although the difference is not significant, as observed in this study.

3.1.2. The Flavonoid Content

The results of the analysis of total purified gambier flavonoids were shown in **Figure 1**. The total flavonoid content of gambier differs significantly due to the purification process by various ethanol concentrations (*sig*=0,000). Ethanol with high concentration produces correspondingly to high flavonoid content as well. This phenomenon occurs because gambier contains various types of flavonoid compounds, primarily catechins, as their main aglycone, which has low solubility in water (Kumar & Pandey, 2013).

3.2. SPF Value of Purified Gambier

The SPF value of purified gambier was measured to determine its protective capability against ultraviolet (UV) radiation. The purification process of gambier by various concentrations of ethanol had a significant impact on its chemical composition. Consequently, the SPF value of the purified gambier also exhibited significant differences at each ethanol concentration which was presented in **Figure 1** (*sig*=0,000). The SPF measurements were conducted on the prepared solutions by a spectrophotometer with an extract concentration of 2 mg/mL. This concentration was selected as it can provide a high SPF value without exceeding the sensitivity limits of the spectrophotometer. According to Yulianti et al. (2015), an absorbance limit of 4 is considered good for SPF measurements because absorbance values above 4 may made unstable data. Purified gambier at this concentration provided medium-level protection with an SPF value greater than 15 (European Communities, 2006). In this research, the highest SPF value was obtained by 96% ethanol in purification process. Interestingly, the purified gambier's SPF value did not correlate with its phenolic content. It was indicated that chemical compounds responsible for SPF value was not only a phenolic compound.

The purified gambier contains flavonoid aglycone compounds that are more soluble in ethanol due to their polarity nature. Flavonoid aglycone compounds are known to absorb UV radiation ranging from 275-295 nm and 300-330 nm, which is inferred as an effective UV protector. Flavonoids in the form of flavon, flavonols, and other flavonoid groups have the potential as UV radiation absorber.

3.3. The Evaluation of the Gel

3.3.1. Organoleptic Evaluation of Purified Gambier Gel

The gel had an orange colour because of the presence of phenolic compounds from purified gambier. The gel exhibited a soft texture and a slightly sticky feel due to its high viscosity. The distinct scent of the gel was a blend of the characteristic odor of carbopol and a slight gambier aroma. The addition of perfume was necessary to mask the natural scent of the gel. The gel showed good homogeneity without any visible solid particle on the two glass plates.

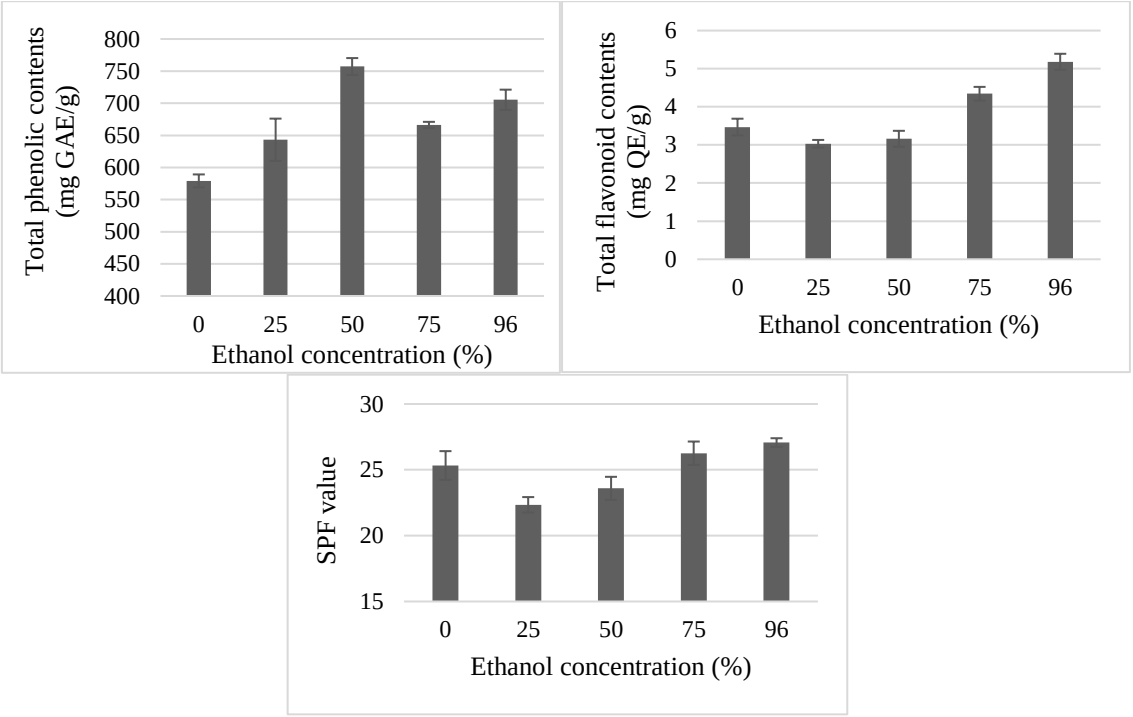

Figure 1. Effect of ethanol concentration to total phenolic contents, total flavonoid contents, and SPF value of extract

3.3.2. SPF Value of the Gel Formulation

The gel formulation containing 0.2% w/w purified gambier produced a SPF value of 6.60 ± 0.58. This result was obtained from a gel formulation with 10 times dilution, which was ready for absorbance measurement by a spectrophotometer. The SPF value of the gel was lower than the purified gambier solution, highlighting the challenges of accurately representing SPF values through spectrophotometric measurement. Many factors influence SPF measurement through spectrophotometric methods, including concentration, excipients used, formulation pH, solvent selection, interactions between components, and other factors that can influence UV absorption (Faizin, 2023).

3.3.3. Physical Characteristics of the Gel Formulation

The physical properties of the gel formulation were measured and presented in **Table 3**. The viscosity of the gel met the requirements, but its value was relatively high, resulting in a thick and sticky consistency. High viscosity impacted the spreadingability of the gel, as greater viscosity leads to increased resistance to flow. Ideally, gel formulations should possess lower viscosity to ensure easy spreading. However, higher viscosity offers the advantage of improved adhesion. Optimizing the quantities of carbopol, propylene glycol, and TEA is necessary to achieve suitable viscosity levels. The pH of the gel formulation complied with sunscreen standards and was within the range of physiological skin pH (4-6), which is minimizing the potential for skin irritation caused by the product.

Table 3. Physical Test Result of Gel

No.	Physical Parameters	Test Results	Standard value
1.	Viscosity	23.806±1137 cps	2000-50.000 cps (SNI, 1996)
2.	Spreadability	4,6±0,3 cm	5-7 cm (SNI, 1987)
3.	Adhesiveness	2,64 ± 0,38 detik	>1 s (Lieberman *et al.*, 1996)
4.	pH	5-6	4,5 – 8,0 (SNI, 1996)

3.3.4. Stability of the Gel Formulation

Organoleptic observations indicated a change of color of gel formulation from orange in the first week to be a darker red hue in the second week. This colour change is attributed to the oxidation of catechin compounds in gambier, resulting in a transition from yellow to dark brown. Other organoleptic properties such as scent, texture, and homogeneity remained unchanged. While the adhesion of the gel formulation did not exhibit significant change, there was a decreasing trend, while the spreading ability showed a slight increase. The stability changes were shown at **Figure 2**. These changes were attributed to the characteristics of carbopol viscosity, which decreases after four weeks of storage. Gel formulations can absorb moisture which is leading to decreased viscosity and increased spreading diameter. The pH of the gel formulation remained stable throughout the four-week testing period, remaining within the 5-6 pH range. Longer stability studies are necessary to determine the gel ability to maintain its physical properties and efficacy during the self-life periode.

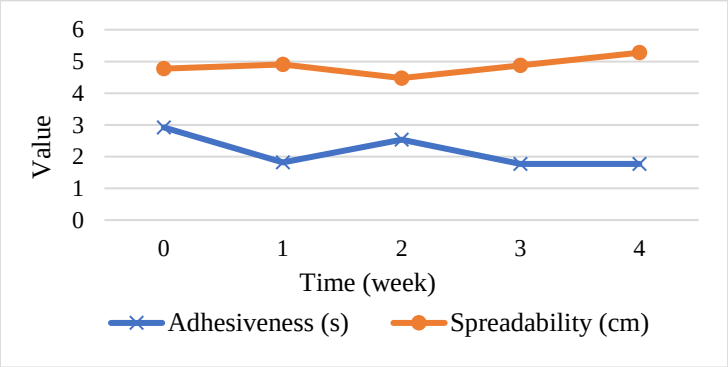

Figure 2. Stability changes of gel contains purified gambier

3.3.5. Overview Effect of Solvent Variations on Several Responses

The variation of solvent during the purification process of gambier has an impact on several responses such as phenolic content, flavonoid content, and SPF value. Additionally, the concentration of ethanol also affects the filtration process. The higher ethanol concentration makes it easier to separate solids from the filtrate because gambier contains polysaccharide compounds that can expand in water and clog the filter.

Ethanol at 75% could be a choice in the purification process of gambier because it produced a high SPF value, phenolic content, and flavonoid content, and eased in filtration. Although the highest response was obtained from 96% ethanol, it was more expensive and its response was not significantly different from 75% ethanol. However, further testing was needed for the selection of these two solvents because the industry will consider the solvent's recovery ability and the energy required for solvent removal.

4. CONCLUSION

The concentration of ethanol in the gambier purification process significantly influences the total phenolic content, total flavonoid content, and SPF value. The ethanol concentration that yields the highest total phenolic content is 50%, whereas the highest total flavonoid content and SPF value are achieved at an ethanol concentration of 96%. Ethanol 75% can be a suitable choice for gambier purification as it provides a response that is not significantly different from ethanol 96%, while also being more cost-effective. The SPF value of gel as low protection as a sunscreen. The purified gambier gel formulation meets the physical requirements for gel formulations, including organoleptic properties, viscosity, adhesion, and pH, but does not meet the spreading ability requirement. The gel formulation also experiences changes in stability, including colour, adhesion, and spreading ability.

5. ACKNOWLEDGMENT

We thank to Professor Endang and Dr. Andayana for helpful advice in this research so we could understand more in area of in-vitro test, natural product extraction and analytical method. Thank you very much also for Department of Pharmaceutical Biology and Department of Pharmaceutics which has been very helpful to give support and knowledge.

6. CONFLICT OF INTEREST

All authors declare no conflict of interest.

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JFSP Vol.10, No.1, January-April 2024, Page: 61-69
pISSN: 2549-9068, eISSN: 2579-4558
Jurnal Farmasi Sains dan Praktis
(JFSP)
http://journal.unimma.ac.id/index.php/pharmacy

EFFECTIVENESS OF MAGNESIUM CITRATE ADJUNCTIVE THERAPY ON CRAMPING PAIN INTENSITY IN NOCTURNAL LEG CRAMPS PATIENTS AT BETHESDA HOSPITAL YOGYAKARTA

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https://doi.org/10.31603/pharmacy.v10i1.9534

Article info:
Submitted : 04-07-2023
Revised : 23-11-2023
Accepted : 12-01-2024

Publisher:
Universitas Muhammadiyah Magelang

ABSTRACT

Nocturnal Leg Cramps (NLC) are involuntary lower limb contractions that are painful and occur during long rest periods. Magnesium is thought to have potential in the treatment of NLC as one of the precipitating factors of NLC is low levels of certain minerals, such as magnesium deficiency. This study aimed to assess the effectiveness of magnesium adjunctive therapy in reducing cramping pain intensity in patients with NLC. This study was a randomized clinical trial, open-label, controlled group that was followed up for 2 weeks. 30 subjects who have been diagnosed with the NLC short-form adaptation of ICSD 2005 were divided into 2 groups; (1) the intervention group who was given standard NLC therapy (calcium and gabapentin) with additional therapy of magnesium citrate 100 mg (Hi-Mg100) one tablet a day, (2) the control group who was only given standard NLC therapy. NLC cramping pain was measured using the Numeric Rating Scale (NRS) before the administration of therapy (baseline) and at week 2 after therapy. The results obtained were the addition of magnesium to standard therapy provided a significant reduction in cramping pain intensity between before and after treatment based on the Wilcoxon signed rank test (p=0.000). However, there was no statistically significant difference effect between the two therapy groups based on the Mann-Whitney test (p=0.073). In conclusion, magnesium adjunctive therapy was not significantly more effective in reducing cramping pain than standard drug therapy in patients with NLC.

Keywords: Nocturnal leg cramps; NLC; Magnesium; Cramping pain; Pain intensity

1. INTRODUCTION

Nocturnal Leg Cramps (NLC) is defined as an involuntary painful contraction of the lower limbs that occurs for several seconds to minutes during prolonged periods of rest, particularly at night (Sebo et al., 2014). NLC often occurs in the gastrocnemius muscle, although it can also occur in the smaller muscles of the lower limbs (Buttaravoli, 2022). NLC can happen at any age but is most commonly seen in adults. According to the American Academy of Family Physicians (AAFP), the prevalence of NLC in adults is around 50-60%, while in children, it is approximately 7%. The prevalence of NLC tends to increase with age (Allen & Kirby, 2012).

The etiology of NLC, considered idiopathic, has led to a lack of effective therapy for its symptoms. In NLC, one possible precipitating factor is the low levels of certain minerals, such as magnesium deficiency. A magnesium deficiency can cause increased neuromuscular transmission and excessive excitation. Therefore, magnesium supplementation is considered beneficial as a therapy for NLC, considering that one of the precipitating factors of NLC is the low intracellular magnesium absorption (Maor et al., 2017; Sebo et al., 2014). Additionally, magnesium supplementation in the context of NLC therapy aims to be a curariform agent or an inducer of

muscle relaxation at the neuromuscular junction by inhibiting acetylcholine release from motor nerve terminals. The hope is to balance inhibitory and excitatory signals transmitted to the lower motor neuron, preventing nocturnal leg cramp symptoms (Liu et al., 2021).

Previous research on the benefits of magnesium for relieving NLC pain still needs to be more extensive and conclusive. Three randomized controlled trials (RCTs) conducted by Sebo et al. showed only slight differences between the effects of magnesium and a placebo on NLC prophylaxis. This means that magnesium is less effective as a therapy for NLC (Sebo et al., 2014). However, another study by Olha Barna et al. reported that magnesium had a significant effect in reducing the intensity of NLC pain, NLC symptoms and improving the sleep quality and quality of life of NLC patients (Barna et al., 2021). Based on the above research findings, the effectiveness of magnesium for NLC remains a matter of consideration, and this study aims to test the hypothesis that magnesium adjunctive therapy is effective in reducing cramping pain intensity in patients with nocturnal leg cramps. Moreover, this study is conducted as a reference for healthcare professionals to assess the improvement in NLC pain intensity in patients who receive magnesium supplements in the future.

2. METHODS

This research utilized the Open-Label Randomized Controlled Trial method. The study used primary data taken from the stroke center of Bethesda Hospital Yogyakarta, which included the intensity of pain values in NLC patients measured using the Numeric Rating Scale (NRS). The study was conducted for 2 weeks, from February 2023 to March 2023. The sampling was done using consecutive sampling, where subjects were diagnosed with NLC based on an adapted NLC short form from ICSD 2005. The subjects were divided into two treatment groups: the intervention group, which received standard NLC therapy (calcium and gabapentin) with an additional 100 mg of magnesium citrate therapy (Hi-Mg100) once a day, and the control group, which only received standard NLC therapy. Two visits were conducted, one before the therapy (baseline) and one in the second week. The primary data results will be analyzed using the Mann-Whitney test to assess the difference in mean NRS pain values between the intervention and control groups regarding effectiveness and the Wilcoxon signed-rank test to assess significant pain improvement within each group. The secondary results will be analyzed using the Spearman rank, Chi-square, and Fischer Exact tests. This study has obtained ethical clearance from the Ethics Committee of Bethesda Hospital Yogyakarta (No. 139/KEPK-RSB/XII/22).

3. RESULTS AND DISCUSSION

3.1. Subjects Characteristic

Of the 30 subjects, 17 were assigned to the intervention group, while 13 were assigned to the control group as shown in **Table 1**. The subjects in this study were predominantly female, with a total of 22 individuals (73.3%). It possibly due to the higher risk of NLC-related varicose veins in females than in males (Bahk et al., 2012; Hallegraeff et al., 2017). Furthermore, post-menopausal women are more likely to experience metabolic disorders, leading to poorer body homeostasis (Krishnan et al., 2018; Maor et al., 2017). We found a higher proportion of older or elderly patients, with 22 subjects (73.3%) aged ≥60 years and an average age of 65.17 ± 7.53 years. NLC predominantly affects individuals over 60 years old, indicating that neurological factors cause cramps. With age, motor and medullary neurons are lost, leading to more neuromuscular incoordination in the lower limbs than the upper limbs (Bordoni et al., 2022; Rabbitt et al., 2016).

In this study, 20 subjects (66.7%) reported that the duration of NLC cramps without treatment or only with massage was ≤ 10 minutes, which is consistent with previous research (Hallegraeff et al., 2017). The comorbid history found in the subjects was predominantly

neurodegenerative (96.7%) and hypertension (86.7%). In patients with degenerative lumbar disorders, they exhibited a lack of autoregulation feedback in regulating inhibitory inputs to alpha motor neurons, leading to hyperexcitability of motor units associated with NLC (Bordoni et al., 2022; Harmsen et al., 2021). Hypertension may be related to cramps through vascular decompression mechanisms, resulting in inadequate blood flow to the lower limb muscles, especially the quadriceps femoris muscle. Insufficient oxygen supply to these muscles can lead to higher levels of muscle spasms or cramps in hypertensive patients (Breda et al., 2014; Bufford W., 2016).

Table 1. Basic characteristics of research subjects

Variable	Magnesium 100 mg + Standard Therapy (n=17)	Standard Therapy (n = 13)	Total (n=30)	p-value (Chi-Square Analysis)
Age, mean ± SD (year)	65.76 ± 7.404	64.38 ± 7.922	65.17 ± 7.53	
<60 years	4 (23.5%)	4 (30.8%)	8 (26.7%)	0.657
≥60 years	13 (76.5%)	9 (69.2%)	22 (73.3%)	
Gender				
Man	2 (11.8%)	6 (46.2%)	8 (26.7%)	0.035
Woman	15 (88.2%)	7 (53.8%)	22 (73.3%)	
Smoking History				
Do not smoke	15 (88.2%)	7 (53.8%)	22 (73.3%)	0.035
Smoke	2 (11.8%)	6 (46.2%)	8 (26.7%)	
Comorbid				
Hypertension	13 (76.5%)	13 (100%)	26 (86.7%)	0.06
Diabetes mellitus	7 (41.2%)	3 (23.1%)	10 (33.3%)	0.297
Cardiovascular Disease	6 (35.3%)	5 (38.5%)	11 (36.7%)	0.858
Neurodegenerative	17 (100%)	12 (92.3%)	29 (96.7%)	0.245
Comedy				
Antihypertensive	13 (76.5%)	13 (100%)	26 (86.7%)	0.06
Antidiabetic	7 (41.2%)	3 (23.1%)	10 (33.3%)	0.297
Antiplatelet	6 (35.3%)	5 (38.5%)	11 (36.7%)	0.858
Neuroprotectant	17 (100%)	12 (92.3%)	29 (96.7%)	0.245
Cramp Duration (without treatment)				
≤ 10 minutes	10 (58.8%)	10 (76.9%)	20 (66.7%)	0.297
> 10 minutes	7 (41.2%)	3 (23.1%)	10 (33.3%)	

The predominant comedication in this study was neuroprotectants and antihypertensive agents. Neuroprotective agents such as vitamin E and B12 complex can be used as treatments for NLC (Allen & Kirby, 2012; Brown, 2015). In cases of NLC patients with a history of hypertension, hypertension treatment is also important in addressing vascular decompression issues, and certain antihypertensive medications, such as Calcium-channel blockers, may be required (Herzberg et al., 2017; Rabbitt et al., 2016).

Based on **Table 1**, it was found that the baseline characteristics of the subjects differed significantly between the two groups only in terms of gender and smoking history using Chi-square analysis (p = 0.035). The significant results could confuse the study because the differences in subject characteristics between the two groups cause baseline differences in terms of metabolic, physical, and mental characteristics. However, it was unavoidable that the prevalence of women suffering from NLC is higher than that of men because risk factors for NLC in women were found to be less elevated than in men. One of the risk factors associated with the incidence of NLC in women is venous varicose veins. The prevalence of venous varicose veins is more often found in women than men because most jobs in women require more standing than men and women also use more high heels (Bahk et al., 2012; Hallegraeff et al., 2017). NLC was found to be slightly increased in the female population also related to the cause of NLC, one of which is metabolic disorders, where it turns out that post-menopausal women experience more metabolic disorders

so that the homeostasis system in the body is worse. This can trigger an increase in the prevalence of NLC in post-menopausal women compared to men (Krishnan et al., 2018; Maor et al., 2017). Likewise, a significant result on smoking history was found in this study due to the difference in the proportion of men and women in the two groups. In fact, the prevalence of smokers was found to be higher in men than women in Indonesia. Cigarette consumption can cause reduced blood flow to the calf muscles, making it one of the risk factors for NLC (Abate et al., 2013; Ayuningtyas et al., 2021; Fritschi et al., 2013; Maor et al., 2017).

3.2. Normality Test of Variables

Based on **Table 2**, the data distribution results show that the intensity of pain variables, including pre-intervention, post-intervention, and the difference in pain intensity between pre-and post-intervention in the two groups, are not generally distributed with a p-value < 0.05. However, the age variable follows a normal distribution with a p-value > 0.05.

3.3. Comparison of Average Pain Intensity

Based on the analysis of pain intensity data using the Wilcoxon Signed Rank Test in **Table 3**, it was found that both therapies, the additional magnesium therapy (p^b = 0.000) and the standard drug therapy (p^b = 0.001), resulted in a significant reduction in cramp pain intensity between before and after the therapy. Additionally, it was found that there was no significant difference in the value of the difference in pain intensity between the two groups (p^a = 0.073) based on the Mann-Whitney test.

Table 2. Variable normality test

	Kolmogorov-Smirnov			Shapiro-Wilk		
	Statistics	**df**	**Sig.**	**Statistics**	**df**	**Sig.**
Pre-intervention Pain Intensity	0.283	30	0.000	0.819	30	0.000
Post-intervention Pain Intensity	0.242	30	0.000	0.880	30	0.003
The difference in Pain Intensity Pre-post intervention	0.227	30	0.000	0.881	30	0.003
Age	0.111	30	0.200*	0.960	30	0.314*

Table 3. Comparison of average pain intensity

NRS Pain Level	Magnesium 100 mg + Standard Therapy (n=17)	Standard Therapy (n=13)	Total (n=30)	p^a-value
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	
Before therapy	57.06 ± 6.860	53.08 ± 6.304	55.33 ± 6.814	0.151
2 weeks after therapy	28.24 ± 11.851	30 ± 8.165	29 ± 10.289	0.481
p^b-value	0.000*	0.001*		
Pre-post therapy difference	28.82 ± 8.575	23.08 ± 8.549	26.33 ± 8.889	0.073

***p^a*: Comparison between the intervention and control groups (Mann-Whitney test)**
***p^b*: Intragroup comparison before and after intervention under the same conditions (Wilcoxon-signed rank test)**
***p-value statistically significant**

Figure 1 shows that there was a more pain intensity reduction in the magnesium supplemental therapy group when assessed subjectively (28.82 ± 8.575 vs 23.08 ± 8.549). In addition, there was also a significant difference in pain intensity between the two groups, both in pain intensity values before therapy (pa = 0.151) and 2 weeks after therapy (pa = 0.481).

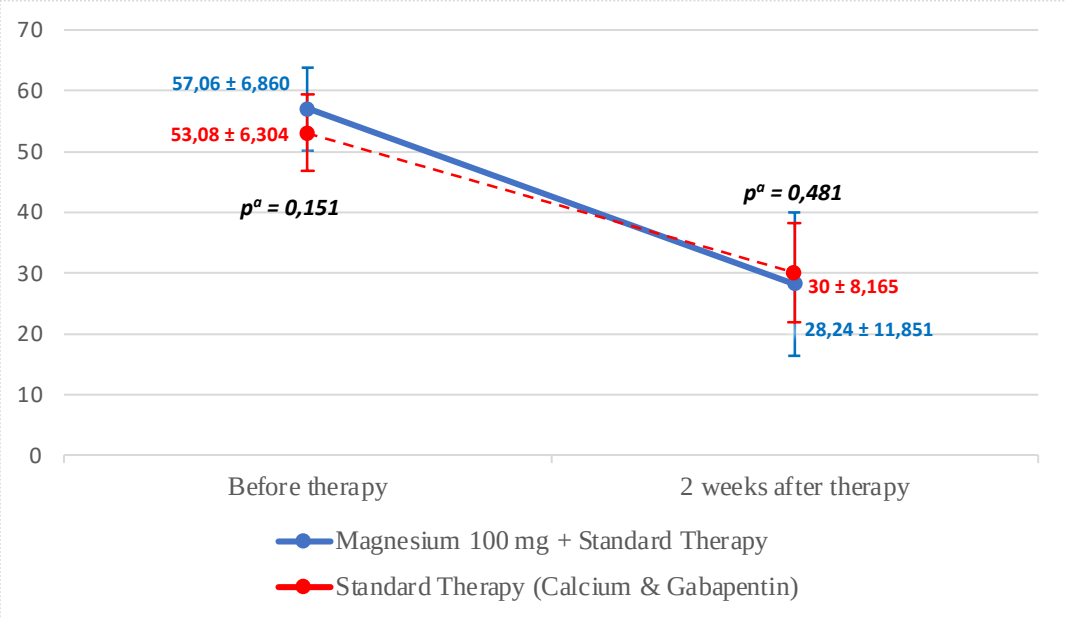

Figure 1. Graph of average NRS pain intensity

The level of improvement in subjective pain in **Table 4** was measured using the Subjective Global Assessment instrument at the end of therapy. The results showed a comparison of pain reduction: >50% reduction (5.9% vs 0%), 30-50% reduction (64.7% vs 61.5%), and a slight reduction of 10-30% (29.4% vs 38.5%). The Chi-square analysis obtained a p-value of 0.620, indicating no significant difference in the improvement of subjective pain intensity between the two groups. Based on the dose of drugs given, on average, previous studies gave higher doses of magnesium than the dose of magnesium in this study. In the previous studies, the doses of magnesium given was 226 mg and 300 mg per day (Barna et al., 2021; Supakatisant & Phupong, 2015). Meanwhile, in this study, 100 mg of magnesium was given every day. This proved that the possibility of a lack of daily doses of magnesium supplementation can affect the results of the effectiveness test. However, it should be noted that magnesium supplementation also has a maximum daily dose to avoid side effects that have been regulated in Tolerable Upper Intake Levels (UL), which for adults is a maximum of 350 mg daily outside of natural magnesium from food and beverages (Food and Drug Administration, 2016).

Table 4. Improvement of subjective pain intensity

SGA Pain Level	Magnesium 100 mg + Standard Therapy (n=17)	Standard Therapy (n=13)	Total (n=30)	P-value
	n(%)	n(%)	n(%)	
Pain is greatly reduced (>50%)	1 (5.9%)	0 (0%)	1 (3.3%)	0.620
Pain reduced (30-50%)	11 (64.7%)	8 (61.5%)	19 (63.3%)	
Slightly reduced pain (10-30%)	5 (29.4%)	5 (38.5%)	10 (33.3%)	

This study showed that the effectiveness of additional magnesium therapy in reducing or improving pain intensity is similar to standard drug therapy. These results are consistent with the study by Maor et al., (2017), where the effectiveness of magnesium supplementation on NLC severity was not significantly different from placebo, with a p-value of 0.38. This suggests that the etiology of cramps in NLC patients is not solely caused by intracellular magnesium deficiency but also by deficiencies in other mineral nutrients, such as calcium and sodium, which are involved in NLC cramp pathogenesis. Therefore, the potential of magnesium supplementation in addressing cramp intensity is likely to be low (Schwalfenberg & Genuis, 2017). One condition that leads to low intracellular magnesium levels due to magnesium depletion is pregnancy, where

the effectiveness of magnesium in reducing NLC severity is higher in this population (Sebo et al., 2014; Supakatisant & Phupong, 2015).

Additionally, the study by Garrison et al., (2011) explains that the effectiveness of magnesium itself depends on the formulation used and the characteristics of the population. This is related to magnesium's bioavailability and absorption rate, which decreases with age. In this study, an oral supplementation form with relatively low bioavailability was used, and the average age of the subjects was also geriatric (Garrison et al., 2011). In the study by Barna et al., (2021), a double-blinded RCT design was used to observe the effectiveness of monohydrate magnesium oxide on NLC episode frequency, duration, and induced pain compared to a placebo. The study was assessed during three visits: before therapy, 30 days, and 60 days after therapy. The effective results were only observed at 60 days after therapy, suggesting that the relatively short duration of treatment provided insignificant results regarding treatment effectiveness. Thus, the lack of significant results in the researcher's study could potentially be attributed to the relatively short treatment duration (< 8 weeks) (Barna et al., 2021).

In previous studies, the control group used a placebo group (Barna et al., 2021; Supakatisant & Phupong, 2015). This is different from the researcher's study, which involved an add-on approach. When using a placebo comparator, the effectiveness of test results obtained can be maximized as blinding can be performed on the intervention, and the test is single without the influence of other medications that may affect the effectiveness of test results. In the add-on group, biased effectiveness test results can occur, where standard drug therapy (gabapentin or calcium) may have a more dominant effect on pain intensity reduction (Castro, 2007; Laursen et al., 2020). Additionally, the average dose of magnesium given in previous studies was higher than the dose used in this study, which could also contribute to suboptimal test results (Barna et al., 2021; Supakatisant & Phupong, 2015).

3.4. Spearman Rank Test for Pain Intensity

Based on **Table 5**, the Spearman Rank test was conducted between age and pain intensity before therapy. The analysis revealed a p-value of 0.035 ($p < 0.05$) with a positive correlation coefficient (0.387). This result indicates a relationship between age and pain intensity before therapy, and the positive correlation suggests that as age increases, the initial pain intensity of the subjects also increases before undergoing therapy. Increased pain intensity with age is caused by increased psychological stress and a decreased pain tolerance threshold (González-Roldán et al., 2020; Yezierski, 2012).

Table 5. Spearman rank test for pain intensity

Variable		Pain Intensity NRS Score Before Therapy	Pain Intensity NRS Score After Therapy	Difference in NRS Scores Before and After Therapy
Age	Correlation coefficient	0.387	0.139	0.120
	Sig.	0.035*	0.465	0.528

In **Table 6**, Chi-square and Fischer Exact data analyses were performed to determine the relationship between gender and pain intensity. The results showed a significant association between gender and the difference in pain intensity before and after therapy, with a p-value of 0.047 ($p < 0.05$). These results show that males are more likely to experience a more considerable improvement in pain after therapy than females. Gender differences are associated with the pharmacokinetics and pharmacodynamics of consumed medications. Additionally, higher testosterone levels in males can result in a greater reduction in pain compared to females. Psychological stress is more prominent in females, and coping mechanisms also influence the level of pain reduction. Emotionally, males are expected to exhibit greater endurance and not show their pain (Khan et al., 2022; Madla et al., 2021; Templeton, 2020).

Table 6. Comparison of gender with pain intensity

Pain Intensity Before Therapy	Low (20 < x ≥ 40)	Currently (40 < x ≥ 60)	Tall (60 < x ≥ 80)	P-value
Gender				0.545
Man	0	8	0	
Woman	1	19	2	
Pain Intensity 2 Weeks Post-therapy	Very Low (≤20)	Low (20 < x ≥ 40)	Currently (40 < x ≥ 60)	*p.s*
Gender				0.369
Man	5	3	0	
Woman	8	12	2	
Difference in Pain Intensity Pre-Post Therapy	Very Low (≤20)		Low (20 < x ≥ 40)	*p.s*
Gender				0.047
Man	1		7	
Woman	12		10	

3.5. Medication Side Effects

Based on **Table 7**, it was found that there were medication side effects in 1 subject (5.9%) from the additional magnesium therapy group and 3 subjects (23.1%) from the standard drug therapy group. The reported side effects included drowsiness and mild nausea, which were considered mild and did not require hospitalization or specific treatment. The analysis found no significant difference in medication side effects between the two groups, with a p-value = 0.170.

The medication side effects in this study were mild and did not require any interventions. This demonstrates that in the management of neuropathic pain or cramp pain, gabapentin is an attractive therapy choice compared to other anticonvulsants because it causes mild and non-severe side effects (Seifollah et al., 2015). Additionally, mild side effects were found in oral magnesium therapy, with gastrointestinal symptoms such as diarrhea, nausea, vomiting, bloating, and constipation being the most commonly reported (Sebo et al., 2014).

Table 7. Comparison of the medication side effects between the 2 groups

Group	**Medication Side Effects**		**P-value**
	Yes	**No**	
Magnesium 100 mg + Standard Therapy (n=17)	1 (5.9%)	16 (94.1%)	0.170
Standard Therapy (n=13)	3 (23.1%)	10 (76.9%)	

This study has several limitations, such as the unblinding method during drug administration, which may have affected the results due to psychological factors, as patients were aware of their medications. Additionally, potential dose adjustments for magnesium were not analyzed and compared. Therefore, the optimal dose variation results could not be obtained. The duration of drug administration or intervention was also relatively short, which may explain why the significance of the results may have yet to be observed. Based on the limitations of this study, the researchers suggest that future studies use blinded methods and longer interventions with higher doses. Furthermore, it is recommended to increase the sample size to enhance the study's statistical power. Future studies are also advised not to solely focus on reducing pain scale but to consider overall aspects or total symptom score.

4. CONCLUSION

The administration of additional magnesium therapy with a supplementation dose of 100 mg daily for 2 weeks was not proven to be more effective in significantly reducing nocturnal leg cramp pain than a single standard drug therapy. Based on the results of this study, we couldn't

recommend the use of magnesium supplementation in short-term treatment yet. We might recommend the use of supplemental magnesium therapy when there are similar studies in the future with higher doses (>200 mg) and duration of treatment (>4 weeks) that prove magnesium to be effective.

5. ACKNOWLEDGEMENTS

The authors would like to thank the Research and Development (Litbang) Division, the Ethics Committee (KEPK) team, and the Department of Neurology at Bethesda Hospital Yogyakarta for their continuous support in conducting our research. We also thank the Faculty of Medicine, Duta Wacana Christian University, for providing the letter of introduction to obtain research permission at Bethesda Hospital Yogyakarta.

6. CONFLICT OF INTEREST

All authors declare no conflict of interest.

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JFSP Vol.10, No.1, January-April 2024, Page: 70-78
pISSN: 2549-9068, eISSN: 2579-4558
Jurnal Farmasi Sains dan Praktis
(JFSP)
http://journal.unimma.ac.id/index.php/pharmacy

EVALUATION OF KNOWLEDGE AND BEHAVIOR ON ANALGESIC SELF-MEDICATION IN HEALTH AND NON-HEALTH STUDENTS AT MUHAMMADIYAH UNIVERSITY, YOGYAKARTA

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https://doi.org/10.31603/pharmacy.v10i1.8828

Article info:
Submitted : 02-03-2023
Revised : 08-01-2024
Accepted : 21-01-2024

Publisher:
Universitas Muhammadiyah Magelang

ABSTRACT
Pain is the most common cause that encourages someone to do self-medication. A person's level of knowledge is known to influence a person's behavior in self-medication. The study evaluates the level of knowledge and behavior toward self-medication using analgesics among health and non-health students at the University of Muhammadiyah Yogyakarta. This research uses a non-experimental observational research design with a cross-sectional approach. The sample used was 752 health and non-health students at the University of Muhammadiyah Yogyakarta. The level of knowledge of health students is good, with a percentage of 75.3% good; 21.0% is sufficient; and 3.7% is less, while the level of knowledge of non-health students is adequate, with a percentage of 33.5% good; 50.5% is sufficient; and 16.0% is less. The behavior of health students is good, with 86.7% good, 12.5% adequate, and 0.8% less, while the behavior of non-health students is good, with 80.9% good, 17.5% enough, and 1.6% less. The difference in the level of knowledge and behavior of analgesic self-medication between health and non-health students is quite significant, with the results of the Mann-Whitney test analysis showing a value of 0.000 on the level of knowledge and 0.001 on behavior. The relationship between the level of knowledge and behavior of health students and the Spearman Rank correlation test showed a result of 0.039 with an R-value of 0.107. In contrast, non-health students showed a result of 0.027 with an R-value of 0.114, indicating a significant and weak relationship between both.

Keywords: Knowledge; Behavior; Analgesic self-medication; Health student; Non-health student

1. INTRODUCTION

Health is vital for everyone's life. One of the health supports is self-medicating behavior, or self-medication. Self-medication is the selection and use of drugs by individuals to treat self-identifiable diseases or symptoms (WHO, 2000). People usually self-medicate to treat minor complaints and illnesses, as it is a cost-effective alternative to seeking professional treatment. The results of the 2019 National Socio-Economic Survey showed that the percentage of the population who did self-medication due to health complaints was 71.46%, an increase of 0.72% from 2018. This condition indicates that self-medication behavior in Indonesia is quite large and will likely continue to increase (Badan Pusat Statistik, 2019).

Medication errors are still becoming a concern worldwide. The prevalence of medication errors is high. Medication errors can occur in implementing self-medication due to limited knowledge of drugs and their use. Improper use of drugs can result in irrational drugs, delay

seeking medical advice, and increase side effects and drug interactions (Aljadhey et al., 2013; Arundina & Widyaningrum, 2020).

Pain is one aspect that is the most common cause that drives a person to seek treatment. Pain (headaches, toothaches, aches, and menstrual pain) is the most significant percentage experienced by respondents in carrying out self-medication, as shown in Panyabunan City, which is 51.2% (Harahap et al., 2017). Among health science students, one of the most commonly used groups of drugs for self-medication is analgesic agents. “Analgesic” is a term that refers to any medication to relieve pain and is also known as a painkiller. Most nursing students self-medicate using paracetamol (57%), followed by ibuprofen (20%), diclofenac (5%), and meloxicam (2%). Use of these analgesics to treat headaches (45%), menstrual pain (23%), and fever (14%). The reason for doing self-medication is because there is little time to consult with doctors (68%), and they have received lectures about medicine. It is important to not underestimate the inappropriate use of over-the-counter painkillers given the diversity of patient knowledge and behavior that has been observed across the globe. Community pharmacists are in the best position to advise patients on self-medication or to refer them to a doctor when necessary (Faqihi & Sayed, 2021; Serge et al., 2019)

Pain is often a nuisance enough that everyone tries to treat it themselves. Islam encourages everyone to strive independently for something better for themselves. In order to handle medication independently, the behavior of drug selection, use, and storage is very influential on a person's success in self-medication. A person's behavior is strongly influenced by the knowledge he has obtained and the treatment performed by someone. The knowledge that a person has can affect the self-medication he takes. The better the knowledge and behavior of a person in self-medication will be, possible the rate of medication errors will also decrease, so further research is needed on a person's knowledge and behavior in self-medicating (Chautrakarn et al., 2021; Gupta & Chakraborty, 2022).

Students are the younger generation who have the opportunity to receive formal education in higher education. Students with essential health and non-health sciences have differences in their acceptance of knowledge, but the treatment is still independent. It depends on the individual. Health students will be public health advocates in the future who can accept public complaints and provide independent treatment solutions to someone in need, so health students will have a better level of knowledge. Ignorance of the warnings and precautions, storage requirements, recommended shelf life, and adverse responses increases the danger of side effects. Culture, ethnicity, and religion all have an impact on treatment attitudes. The roles of the family, school, medical personnel, and health authorities are of the utmost importance for adopting steps to handle this health problem more effectively in light of these findings (De Sanctis et al., 2020;Paut Kusturica et al., 2016).

The University of Muhammadiyah Yogyakarta is one of the universities located in the Special Region of Yogyakarta and consists of students majoring in health and non-health. Self-medication behavior has undoubtedly become natural for health students, but not for non-health majors. Because the level of knowledge will influence a person's decision-making, including self-medication, it is necessary to conduct research that can evaluate specific self-medication knowledge and behavior for health and non-health students regarding analgesic drugs often used in the community to deal with.

2. METHODS

2.1. Research Design

This research is non-experimental observational study with a cross-sectional approach. The data was obtained through the distribution of questionnaires, which were carried out using electronic media to facilitate their distribution. The samples used were health and non-health

students at the University of Muhammadiyah Yogyakarta, each of which amounted to 376 respondents from a total population of 17,977. Cluster random sampling determined the number of samples using the Lemeshow formula and the sampling technique in this study. The inclusion criteria was students registered as active students in the health and non-health study program at Muhammadiyah University of Yogyakarta, willing to fill out a questionnaire, and having self-medicated with analgesic drugs.The sample was then given instructions to complete the questionnaire. The data from the questionnaires were processed and edited by coding in Microsoft Excel. We analyzed the data using the Mann-Whitney test to determine the differences in the level of knowledge and behavior of health and non-health students. The researchers analyzed the data using the Spearman rank test to determine the relationship between the level of knowledge and self-medication behavior of health and non-health students. The researchers presented the test result data in tabulated form.

2.2. Ethical Approval

The study was approved by the Health Research Ethics Committee, Faculty of Medicine and Health Sciences, Universitas Muhammadiyah Yogyakarta Indonesia, No. 234/EC-KEPK FKIK UMY/VIII/2021.

3. RESULTS AND DISCUSSION

3.1. Characteristics of Respondents

The characteristics of the respondents in this study were divided into four categories, namely age, gender, faculty, and year of class. The characteristics of respondents can be seen in **Table 1**.

Table 1. Characteristics of respondents

Characteristics	Total (N = 752)	%
Age (years)		
18	111	14.8
19	132	17.8
20	155	20.6
21	210	27.9
22	98	13.0
23	38	5.1
24	8	1.1
Gender		
Man	244	32.4
Woman	508	67.6
Faculty		
Medicine and Health Sciences	376	50
Technique	56	7.4
Agriculture	32	4.3
Economics and Business	92	12.2
Social and Political Science	82	10.9
Law	35	4.7
Islam	46	6.1
Language Education	33	4.4
Force Year		
2018	380	50.5
2019	115	15.3
2020	134	17.8
2021	123	

The most significant number of respondents based on age was 21 years old (27.9%), and the gender of the majority of respondents was female, with a percentage of 67.6%. Half of the respondents are students in the Faculty of Medicine and Health Sciences, while the other 50% are

across several faculties identified as non-health. The respondents who participated the most were students in the 2018 class, with 380 respondents (50.5%). The results of this study are the same as those of Arrais et al., who found that more women self-medicate than men. This condition is because women experience more headaches, such as migraines, muscle pain, and dysmenorrhea (Arrais et al., 2016). The value of the answers determines the level of knowledge, which is divided into three categories.

3.2. Knowledge Level

The level of knowledge is said to be good if the value obtained from the respondents' answers is more than 80% of all questions; the level of knowledge is sufficient if the value obtained is 60–80%; and the level of knowledge is said to be lacking if the value is below 60% (Winarno, 2018). The results of measuring the level of knowledge can be seen in **Table 2**.

Table 2. Knowledge level

Knowledge level	Health		Non-Health	
	(n)	(%)	(n)	(%)
Not enough	14	3.7	60	16.0
Enough	79	21.0	190	50.5
Well	283	75.3	126	33.5
Total	376	100	376	100

75.3% of health student respondents belong to the category of good knowledge, 21.0% of respondents fall into the category of sufficient knowledge, and 3.7% fall into the category of less knowledge. Meanwhile, for non-health students, 33.5% of respondents belong to the excellent knowledge category, 50.5% of respondents fall into the category of sufficient knowledge, and 16.0% of respondents fall into the category of less knowledge. The table shows that, in general, health students' knowledge level is greater than that of non-health students.

In this study, the level of knowledge has several sub-variables; the first sub-variable is knowledge about drug selection according to the symptoms of the disease. 97.1% of health students correctly answered, indicating their knowledge of adjusting headache medicine to the type of headache they experience (statement number 1). As many as 84.0% of health students answered correctly, meaning they know that paracetamol can help relieve pain. Toothache (statement number 4), and as many as 58.8% of health students answered the statement correctly Statement number 3 is a negative statement, so it has an answer of "false," which means that health students know that ampicillin (an antibiotic) cannot be used to relieve headaches (statement number 3). Non-health students, totaling 90.4%, correctly answered statement number 1, indicating their knowledge of the need to adjust headache medicine according to the type of headache they experience. As many as 61.4% of non-health students answered correctly and knew that paracetamol could treat headaches. Help relieve toothaches (statement number 4). 57.7% of non-health students answered that they were unaware that ampicillin (an antibiotic) cannot be used to relieve headaches (statement number 3).

In the next sub-variable regarding knowledge about drug classes that can be used in self-medication, most health and non-health students answered the statement correctly. Statement 6 reveals that 89.1% of health students are aware that not all headache medicines require a doctor's prescription. Statement number 5 reveals that 55.9% of health students are aware that headache medicines with an over-the-counter medicine logo on their packaging can be bought without a prescription from pharmacies. Statement number 6 reveals that 67.6% of non-health students are aware that headache medicines can be purchased without a doctor's prescription, and 41.0% of them also know that these medicines have a medicine logo on their packaging. Free must not be purchased at the pharmacy (statement number 5).

The third sub-variable is knowledge about using the right medicine in self-medication; most health and non-health students answered correctly. Health students answered correctly that

headache medicine is taken according to the rules in the medicine package (statement number 8), with 96.8% of them knowing this. In contrast, statement number 2, which is a negative statement, received a "wrong" answer. However, as many as 71.3% of health students answered correctly, which means the majority of health students do not know that not all pain medications should be taken after eating (question number 2), 48.4% of health students do not know that headache medications (such as Paramex® and Saridon®) can be taken before eating (statement number 9), and in the subvariable statement number 10 is a negative statement so that it has the answer "false". The results can be seen in the graph stating that 92.8% of the majority of health students know that it is not permissible to take drugs with double doses (statement number 10). Statement number 8 reveals that 85.1% of non-health students are aware that headache medicine should be taken according to the instructions on the medicine package, while 56.4% of non-health students are unaware that not all painkillers need to be taken after eating. (Statement number 2), as many as 44.4% of non-health students also do not know that headache drugs such as Paramex and Saridon can be taken before eating (Statement number 9). In the sub-variable, number 10 is a negative statement, so it has the answer "false". The results obtained in the graph above state that as many as 77.1% of non-health students are aware of the prohibition of taking drugs in double doses (statement number 10).

Next is knowledge about drug side effects; 75% of health students know that taking Paramex® headache medicine can cause drowsiness (statement number 11), and as many as 66.0% of non-health students also know that taking Paramex® headache medicine can cause drowsiness.

Statement number 12 reveals that 91.2% of non-health students are aware that headache medicine should be stored in a place protected from direct sunlight. Statement number 7 (that headache medicine does not have to be stored in the refrigerator) is a negative statement, so it has a "wrong" answer, with a percentage of 86.7% of health students knowing. 86.7% of health students know that statement number 7, which states that headache medicine does not have to be stored in the refrigerator, is incorrect due to its negative nature.

For the sub-variable regarding knowledge about drug expiration dates, the majority of health and non-health students answered correctly on the indicator of the level of knowledge about drug expiration dates; as many as 98.1% of health students and as many as 93.6% of non-health students about that the use of pain medication. If it is expired, then the drug cannot be consumed. These results show that, in general, health students' knowledge level is greater than that of non-health students. This result is in line with research conducted by Irawati et al. regarding the level of knowledge of self-medication of analgesic drugs among students at X University who are in better health compared to non-health students (Irawati et al., 2021).

3.3. Behavior

Self-medication behavior with analgesic drugs is categorized into three categories. Behavior that is said to be good if the respondent's score from the questionnaire results ranges from 76-100% is considered sufficient if the value is 56-75% and is categorized as less if the value obtained is <55% (Nursalam, 2020). The behavior of respondents' self-medication can be seen in **Table 3**.

Table 3. Self-medication behavior

Self-medication Behavior	Health		Non-Health	
	(n)	(%)	(n)	(%)
Not enough	3	0.8	6	1.6
Enough	47	12.5	66	17.5
Well	326	86.7	304	80.9
Total	376	100	376	100

Based on **Table 3**, it is evident that out of the 376 health student respondents, three respondents (0.8%) exhibit poor behavior, 47 respondents (12.5%) exhibit good behavior, and a significant majority of 326 respondents (86.7%) exhibit moderate behavior. In this case, most health students behave well with self-medicating analgesic drugs. While the results were obtained on the behavior of non-health students, six respondents, with a percentage of 1.6% having poor behavior, 66 respondents, with a percentage of 17.5% having good behavior, and 304 non-health student respondents, with a percentage of 80.9% having a high level of good behavior, it can be concluded that the majority of non-health students also have good behavior towards self-medication of analgesic drugs.

The questionnaire divides the behavioral questions posed to health and non-health students into several sub-variables. The first sub-variable is behavior regarding drug selection according to the symptoms of the disease, statement number 1 (the selection of headache medicine according to the type of headache felt). The results obtained are that 50.0% of health students and 45.0% of non-health students always answered, as many as 28.7% of health students and 22.6% of non-health students often answered, 16.5% of health students and 25.5% of non-health students answered sometimes, and 4.8% of health students and 6.9% of students' non-health answered never. Statement number 2 (regarding taking paracetamol for headaches and toothaches) showed that only 20.0% of health students and 20.0% of non-health students answered "always," 31.4% of health students and 20.7% of non-health students answered "often," 35.1% of health students and 40.2% of non-health students answered "sometimes," and 13.6% of health students and 19.2% of non-health students answered "never."

In the second sub-variable regarding the behavior of choosing drug classes that may be used in self-medication, 37.0% of health students answered "always," 10.6% answered "sometimes," 28.2%, and never 24.2%. For non-health students, 21.8% answered "always," 14.1% answered "often," 34.8% answered "sometimes," and 29.3% answered never. Next is the behavior regarding the proper use of drugs in self-medication; as many as 73.9% of health students and 70.5% of non-health students answered "always," 17.8% of health students and 15.7% of non-health students answered "often," 6.9 % of health students and 10.1% of non-health students answered "sometimes," 1.3% of health students and 3.7% of non-health students answered: that they never read the rules for taking headache medicine on the medicine package before taking medicine. The following statement is to take medicine according to the rules of use listed on the medicine package. As many as 77.4% of health students and 77.9% of non-health students answered "always," 16.2% of health students and 13.6% of non-health students answered "often," 5.6% of health students and 7.2% of non-health students answered health students answered "sometimes", and 0.8% of health students and 1.3% of non-health students answered never. The third statement in this sub-variable is that students take more than two tablets in one drink. 95.7% of health students and 93.1% of non-health students answered "never," 3.2% of health students and 4.8% of non-health students answered "sometimes," 0.5% of health students and 0.5 % of non-health students answered often, and 0.5% of health students and 1.6% of non-health students answered always.

The following behavior is about being aware of side effects: as many as 42.0% of health students and 42.6% of non-health students answered "always," as many as 30.3% of health students and 25.8% of non-health students answered "often," as many as 24.2% of Student's Health and 28.2% of non-health students answered "sometimes," and 3.5% of health and non-health students answered that they had never read the information on drug side effects listed on the drug packaging before taking anti-pain medication.

The fifth (regarding behavior) is regarding proper drug storage; the first statement (storing medicines in the refrigerator); 80.6% of health students and 83.0% of non-health students answered "never". As many as 13.0% of health students and 9.6% of non-health students answered "sometimes," 4.5% of health students and 2.1% of non-health students answered "often"

and, 1.9% of health students and 5.3% of non-health students always kept medicines painkillers in the refrigerator. The following statement stated that as many as 85.4% of health students and 78.7% of non-health students were correct in storing medicine (headache medicine should be stored in a place that was protected from direct sunlight), 7.5% of health students and 13.6% of non-health students often store headache medicine in a place that is protected from direct sunlight, 2.4% of health students and 2.1% of non-health students sometimes store medicine in the right place, and 4.8% of health and medical students 5.6% of non-medical students never store medicine in a place that is protected from sunlight.

The last question is about the behavior of being aware of drug expiration dates. on average, respondents always check the expiration date of drugs before taking drugs (85.4% of health students and 82.2% of non-health students), 11.2% of health students and 11.7 % of non-health students check the expiration date of drugs before taking them, as many as 2.9% of health students and 4.5% of non-health students sometimes see the expiration date of drugs before taking them. As many as 0.5% of health students and 1.6% of non-medical students' health workers never check the drug's expiration date before the drug is taken. The result of this study is in line with research by Apsari et al. regarding the behavior of health and non-health student respondents who have values that are not much different, namely good behavior, because knowledge, experience, attitudes, beliefs, beliefs, values, physical condition influences a person's behavior, and a person's mental state as well as many other influences that not everyone experiences. Because many factors influence behavior, not only the level of knowledge, this causes similarities in levels of behavior (Apsari et al., 2020).

3.4. Differences in Self-Medication Knowledge and Behavior

Mann-Whitney test determines differences in knowledge between health and non-health students on analgesic self-medication. The results of the Mann-Whitney test of respondents can be seen in **Table 4**.

Table 4. Mann-whitney test results knowledge level

	Knowledge level
Asym. Sig	0.000

In **Table 4**, the test results obtained a significance value of 0.000 <0.05, the hypothesis decision is H0 is rejected, so the results obtained are differences in knowledge between health and non-health students on analgesic self-medication.

Mann-Whitney determines differences in behavior between health students and non-health students towards analgesic self-medication. In **Table 5**, the test results obtained a significance value of 0.001 < 0.05, so the hypothesis decision is H0 is rejected so that the results obtained are differences in behavior between health and non-health students towards analgesic self-medication.

Table 5. Mann-whitney test results behavior

	Behavior
Asym. Sig	0.001

The results of this study are based on Apsari et al. 's research on the knowledge and practice of self-medication carried out among Bali International University students, which stated that there were significant differences in the level of knowledge between health and non-health students. Health Students have a higher level of knowledge. The difference in knowledge is because non-health students need help understanding drug-related problems. Similar research in Saudi Arabia by Eissa also showed similar results, where pharmacy students had a better level of knowledge because they received lectures about medicine (Apsari et al., 2020; Eissa, 2013).

3.5. Relationship between Knowledge Level and Self-Medication Behavior

Spearman Rank correlation test determines the relationship between knowledge and behavior of self-medication analgesic health students. From **Table 6** it can be seen that the test results obtained the calculated r value of 0.107 and the significance value (P value) of 0.039 <0.05, it can be concluded that H0 is rejected and Ha is accepted, which means that there is a relationship between the level of knowledge and behavior of self-medication of analgesic drugs for health students.

Table 6. Results of the spearman rank correlation test for health students

r count	Sig.	Decision
0.107	0.039	Reject H_0 / Accept Ha

Spearman Rank correlation test in **Table 7** shows the calculated r-value of 0.114 with a significance value (P-value) of 0.027 <0.05; it can be concluded that H0 is rejected and Ha is accepted, which means there is also a relationship between the level of knowledge and behavior of students' self-medication of analgesic drugs.

Table 7. Results of the spearman rank correlation test for non-health students

r count	Sig.	Decision
0.114	0.027	Reject H_0 / Accept Ha

It shows that the relationship between knowledge level with behavior in self-medication drug analgesics is weak, significant, and directly proportional. The better the level of knowledge, the better the self-medication behavior of drug analgesics will be, and the better. On the contrary, if the level of knowledge is low, then the behavior of self-medication of Analgesic Drugs is lower, with a reasonably low probability for non-health students at the University of Muhammadiyah Yogyakarta. According to previous research on the relationship between the level of knowledge and behavior in students, if the level of student knowledge increases, student behavior will also increase (Perkasa et al., 2020).

4. CONCLUSION

There is a significant difference in the level of knowledge in analgesic self-medication between health and non-medical students at Muhammadiyah Yogyakarta University, with the analysis results on the Mann-Whitney test showing a sig. 0.000. There are behavioral differences in analgesic self-medication between health and non-health students at the University of Muhammadiyah Yogyakarta, which is quite significant, with the results of the analysis on the Mann-Whitney test showing a sig value. 0.001. There needs to be a stronger relationship between the level of knowledge and behavior of health and non-health students of the Muhammadiyah University of Yogyakarta.

5. ACKNOWLEDGMENT

We thank the University of Muhammadiyah Yogyakarta which has provided data facilities.

6. CONFLICT OF INTEREST

All authors declare no conflict of interest.

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JFSP Vol.10, No.1, January-April 2024, Page: 79-89
pISSN: 2549-9068, eISSN: 2579-4558
Jurnal Farmasi Sains dan Praktis
(JFSP)
http://journal.unimma.ac.id/index.php/pharmacy

MOUTHWASH FORMULATION OF ONION (ALLIUM CEPA L.) METHANOL EXTRACT FOR INHIBITING THE GROWTH OF STREPTOCCUS MUTANS BACTERIA

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https://doi.org/10.31603/pharmacy.v10i1.5492

Article info:
Submitted : 09-08-2021
Revised : 15-01-2023
Accepted : 24-01-2024

Publisher:
Universitas Muhammadiyah Magelang

ABSTRACT

Dental and oral problems experienced a significant increase from 2007 to 2018. Based on the results of basic health research, dental and oral problems increased from 23.2% to 57.6%. Dental caries is ranked sixth with a prevalence of 60% to 80%. The main cause of dental caries is Streptococcus mutans. The use of mouthwash with synthetic active ingredients can cause side effects. In addition, only a few mouthwashes were able to inhibit the growth of Streptococcus mutans bacteria. The purpose of this study was to make a mouthwash formulation from onion methanol extract and to determine its ability to inhibit the growth of Streptococcus mutans bacteria. Three mouthwash formulas were made with 10%, 20% and 30% extract concentrations respectively, then physical evaluation was carried out for 14 days on days 0, 7 and 14. Physical evaluation included stability tests (odor, taste, turbidity and precipitate), pH and diameter of inhibition. The results of the formula stability evaluation on day 14 there was a change in formulas 2 and 3 color, but not in formula 1. This was due to differences in the concentration of extracts and the sappans color stability in the formulas. The pH test results for each formula are in the range of 6.0-6.3. A good mouthwash has a pH close to neutral like the pH of the mouth, which is 6-7. The results of the diameter inhibition test ranged from 6-8 mm. A significant difference was seen between the positive controls with formulas 1 and 3, but there was no significant difference between formulas 1 and 3. The conclusion of this study was that the mouthwash of onion methanol extract had the ability to inhibit the growth of Streptococcus mutans bacteria with moderate strength and the best formula was mouthwash with 10% extract concentration.

Keywords: Dental Caries; Mouthwash; Allium cepa; Streptococcus mutans

1. INTRODUCTION

Teeth and mouth are the gateway for bacteria and germs to enter the body, but unfortunately, some people tend to overlook their dental and oral health, resulting in the disturbance of other organs' health. The results of the Basic Health Research in 2007 and 2013 showed an increase in dental and oral problems from 23.2% to 25.9% (Kementerian Kesehatan RI, 2014), even rising dramatically to 57.6% in 2018 (Kementerian Kesehatan RI, 2018). Dental caries are one of the most common dental and oral problems experienced by the community in all age groups, with a prevalence above 70% (Kusuma & Taiyeb, 2020).

Dental plaque has been proven to be the main cause of dental caries. Therefore, one way to prevent dental caries is by limiting the formation of dental plaque (Kaligis et al., 2017). Dental plaque is a thin layer consisting of various microorganisms that form on the surface of teeth shortly after they come into contact with saliva (Rezki & Pawarti, 2014). The composition of dental plaque consists of microorganisms, with more than 400 species, one of which is Streptococcus mutans, which plays a crucial role in the formation of dental plaque (Ristianti et

al., 2015). The mechanism of this bacteria in the formation of dental plaque is by fermenting sucrose into acid, causing a decrease in pH on the surface of the teeth, resulting in tooth mineralization (Anastasia et al., 2017).

Mouthwash is one of the ways that can be used to control the formation of dental plaque. However, long-term use can cause various side effects such as tooth pigmentation, changes in taste sensation, and the formation of tartar on the upper part of the gum, causing the oral cavity to turn red, mucosal damage resulting in pain, and thyroid gland function impairment (Kasuma et al., 2016). The community, in general, is also unaware that only a few brands of mouthwash are capable of inhibiting the growth of Streptococcus mutans bacteria, the cause of dental plaque, thus requiring an alternative mouthwash with antibacterial active ingredients derived from herbal ingredients (Hasanah, 2013).

One of the herbs that have antibacterial properties is shallots. Shallots are one of the main food sources containing flavonoids. The flavonoids contained in shallots are anthocyanins and quercetin. The quercetin content in shallots reaches 85-95%, while anthocyanins are around 10%, which is not dependent on the size or weight of the bulb (Rodrigues et al., 2017).

Flavonoids, as antibacterials, work by denaturing proteins and disrupting the lipid layer, resulting in damage to the cell wall (Kono et al., 2018). Flavonoids have a polar nature; therefore, the selection of solvents in extraction needs to be considered to obtain optimal results. The results of the selection of solvents in the extraction of shallots to obtain optimal total phenol content (TPC) and total flavonoid content (TFC) found that 70% methanol is the most optimal of several polar solvents used for testing (Singh et al., 2017).

The antibacterial activity of red onion methanol extract against Streptococcus mutans indicates that red onion has a strong ability to inhibit the activity of Streptococcus mutans, the cause of dental caries, both in the form of thick extract and nanoparticles (Gomaa, 2017; Shukla et al., 2013). The development of red onion into a form of medication to address the problem of dental plaque in Indonesia is still very limited. The results of literature studies on the use of red onion in Indonesia are limited to its bioactivity research (Hatijah, 2013). On the other hand, the development of red onion into a form of medication to address the problem of dental plaque is currently in the form of antibacterial dental chewing gum (Ika, 2017). Research on red onion to be developed into a mouthwash form of medication is still lacking. Therefore, it is expected that this research can produce a new product to address the problem of dental plaque from red onion extract.

2. METHODS

2.1. Materials

The equipment used in this research were glassware (Pyrex,) blender (Miyako), rotary evaporator (Biobase,) waterbath (Biobase), refrigerator (Panasonic), TLC chamber (Pyrex), petri dish (Pyrex), UV lamp, oven (Memmert), autoclave (All American), Laminar Air Flow (Biobase), incubator (Memmert), hotplate magnetic stirrer (Biobase), and a caliper.

The materials used in this research were red onion purchased from Gombong market, 70% technical grade methanol, aquadest, glycerin, propylene glycol, menthol, liquid stevia, secang wood bark, HCl, Mg powder, FeCl3, 10% NaOH, glacial acetic acid, butanol, GF254 silica TLC plate, quercetin, Mueller Hinton Agar (MHA), *Streptococcus mutans*, Whatman No. 1 paper, and 0.9% NaCl.

2.2. Procedure

2.2.1. Preparation of Simplisia

The sample used was red onion (Allium cepa L.) obtained from Gombong city. The red onion was sorted wet to remove unwanted dirt or parts, then washed with clean running water, and thinly sliced to speed up the drying process. After that, it was dried by airing it without direct

sunlight exposure until it was completely dry. The dried red onion was then sorted again to separate any remaining dirt or foreign objects.

2.2.2. Preparation of Methanol Extract of Red Onion

The method used for extracting red onion was maceration. A 50-gram sample of the dried red onion powder was added to a maceration container and 500 mL of 70% methanol solvent was added, or in a 1:10 ratio, for 3 days with occasional stirring. Next, it was filtered and the filtrate was evaporated with a rotary evaporator at 50°C, followed by using a waterbath at 60°C until a concentrated extract was obtained.

2.2.3. Qualitative Test of Flavonoid Extract

a) Tube Test

1) Alkaline Reagent Test

The extract was dissolved in aquadest and a few drops of 10% NaOH were added. The mixture turned yellow, and the yellow color faded when a dilute HCl solution was added. This indicates the presence of flavonoids in the extract (Talukdar et al., 2017).

2) Wilstater Test

4 mL of the extract solution was mixed with 1.5 mL of 50% methanol. The solution was heated and added to Mg metal. The addition of 5-6 drops of diluted HCl changed the solution to yellow, orange, or red, indicating the presence of flavonoids in the extract (Ergina et al., 2014; Jayashree et al., 2016).

b) Thin-Layer Chromatography (TLC) Test

Flavonoids in the methanol extract of red onion were detected using TLC method with quercetin as the comparator. The stationary phase used in the TLC test was GF254 silica gel, while the mobile phase used was a combination of n-butanol:acetic acid:water with a ratio of 3:1:1 (Andersen & Markham, 2006). The TLC plate was cut into a size of 4x10 cm and activated in an oven at 110°C for 30 minutes (Dewi et al., 2018). The extract and quercetin were then spotted on the plate close to each other, with a distance of 1 cm between spots. Next, it was eluted using the prepared mobile phase.

2.2.4. Formulation Design

The mouthwash formula was adapted from the formula created by Kono (Kono et al., 2018), with some modifications to the oleum menthae, calcium lactate, potassium thiocyanate, and 70% sorbitol, as shown in **Table 1**. The formula was divided into three, namely formula 1 (F1), formula 2 (FII), and formula 3 (FIII).

Table 1. The mouthwash formula

Ingredient	FI	FII	FIII
Red onion extract (Allium cepa. L) (g)	10	20	30
Propylene glycol (mL)	15	15	15
PEG-40 hydrogenated castor oil (g)	1	1	1
Menthol (g)	0.25	0.25	0.25
Benzoic acid (mg)	5	5	5
Sodium benzoate (g)	2	2	2
Flavoring	qs	qs	qs
Secang 3% (g) (Simplicia)	3	3	3
Stevia (liquid)	qs	qs	qs
Aquadest (mL)	Ad 100	Ad 100	Ad 100

Where, FI: Formula 1, FII: Formula 2, FIII: Formula 3

2.2.5. Production of Mouthwash

Secang is added to 100 mL of distilled water until the water changes color to red. This water is then used to dissolve the extract of red onion (Allium cepa. L) (water-soluble phase). Benzoic acid and menthol are dissolved with some propylene glycol (non-aqueous phase) using

a separate container and emulsified with PEG-40 hydrogenated castor oil. The remaining propylene glycol is then slowly poured and stirred until homogeneous, then slowly pour the water-soluble phase while stirring until homogeneous. Finally, add the sodium benzoate solution in water until the pH becomes 6-7 (use a pH meter to measure the pH). Aroma and Stevia are added as desired after the mouthwash is ready.

2.2.6. Physical Evaluation of Mouthwash Formulation

Formula stability includes color, odor, taste, turbidity, and sedimentation observed on days 0, 7, and 14. Observations on these days are aimed at determining the effect of time on the stability of the mouthwash formulation that has been made. The formula stability test refers to previous research conducted by Anastasia with modifications of observation time only up to 14 days (Anastasia et al., 2017).

2.2.7. Formula pH Test

The pH test uses a pH meter on days 0, 7, and 14.

2.2.8. Zone of Inhibition Test

The method used is the paper disk diffusion using Mueller Hinton Agar (MHA) media (Handayani et al., 2016). The paper disks are made using Whatmann No. 1 filter paper. Paper disks to be used for antibacterial tests are inserted into porcelain cups, each containing mouthwash formulations (FI, FII, and FIII), positive controls (total care antiseptic mouthwash), and negative controls (mouthwash formula without red onion extract). The paper disks are soaked for 30 minutes, then drained and placed into petri dishes containing MHA media that has been solidified and swabbed with the test bacteria suspension. The petri dishes are then incubated at 37°C for 24 hours, and the zone of inhibition is measured by the clear zone around the paper disk using a caliper. The zone of inhibition test is performed three times for each formula. The purpose of replication is to verify the accuracy of the obtained results.

2.2.9. Data Analysis Technique

Data analysis includes qualitative and quantitative data. Qualitative data are presented in the form of tables or graphs for easy reading and understanding. This data includes the results of stability formula tests, pH tests, and qualitative flavonoid tests (Handayani et al., 2017). Meanwhile, quantitative data will be analyzed using statistics (SPSS version 16) with the One-way ANOVA method to determine the effect of red onion extract concentration on the formula (Anastasia et al., 2017).

3. RESULTS AND DISCUSSION

3.1. The Production of Shallot Simplisia and Methanol Extract

Shallots are first prepared in the form of simplisia due to the higher content of flavonoids in shallot simplisia compared to fresh shallots (Awouafack et al., 2017). The preparation of shallot simplisia is done naturally by drying the shallots in the shade, away from direct sunlight. This is done to prevent the flavonoid content in the shallots from evaporating and becoming damaged during the drying process, which can be affected by temperature, light, and duration of the simplisia production (Aires, 2016). The obtained yield of simplisia is 16.26%.

The shallot simplisia is then blended to reduce the particle size, which will allow for more effective extraction by increasing the contact surface area between the solvent and the simplisia (Awouafack et al., 2017). The selection of solvent is also important to ensure maximal extraction of the active compounds in shallot simplisia. The solvent used in the extraction process must be chosen based on the characteristics of the active compounds.

The main active compound in shallot, quercetin, is highly soluble in polar solvents. Therefore, polar solvents are the preferred choice based on the principle of "like dissolve like" (Awouafack et al., 2017). Quercetin is also categorized as a low molecular weight polyphenol,

making methanol a more efficient solvent for the extraction process (Do et al., 2013; Fromm et al., 2012).

Another important factor to consider during the extraction process is temperature. The use of excessively high temperatures should be avoided to prevent the degradation or loss of flavonoids in the shallot extract. The recommended temperature range to maintain flavonoid content during the extraction process is 20-50°C. The use of temperatures above 70°C should be avoided as it can quickly degrade and significantly reduce the flavonoid content (Brglez Mojzer et al., 2016). This is also the basis for choosing the maceration extraction method and air-drying in the production of simplisia. The yield of the obtained extract is 60.52%. The flavonoid content of the extract was qualitatively tested.

3.2. Qualitative Flavonoid Test Results

The qualitative test of the flavonoid extract using the tube test method showed positive results, indicated by a color change in the extract sample (**Table 2**). The base reagent test on the extract also showed a positive result, as the addition of 10% NaOH to the extract resulted in a yellow color, which faded when HCl was added. The Wilstater test also showed a positive result, with a color change to orange in the sample.

Table 2. Results of flavonoid qualitative test

Test	Method	Result
Base reagent	Sample +10% NaOH changes to yellow, +HCL yellow color fades	+
Wilstater	Sample +50% methanol, heated +Mg powder+Dilute HCl changes to yellow, orange to red	+
TLC	Stationary phase Silica gel GF254, Mobile phase n-butanol: acetic acid: water (3:1:1)	+

TLC: Thin Layer Chromatography

Qualitative testing for the presence of flavonoid content in the red onion extract using TLC also showed a positive result. This is indicated by the Rf value in the extract that is parallel to the reference quercetin. Dark spots observed using a UV lamp at 254 nm, and blue fluorescence observed using a UV lamp at 365 nm also indicate the presence of flavonoid content in the extract (**Figure 1**) (Andersen & Markham, 2006).

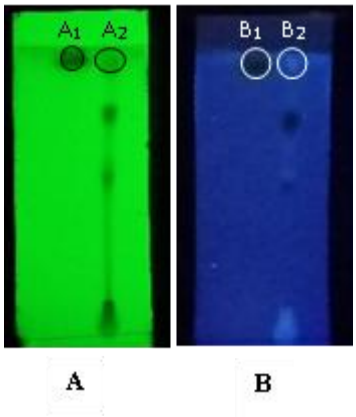

Figure 1. Results of TLC test. A. Observation at UV 254 nm B. Observation at UV 365 nm, A1 B1 Rutin Reference Kuersetin Rf 0.95, A2 B2 Red Onion Extract Rf 0.9

Red onion extract, which has been qualitatively proven to contain flavonoids, is formulated into a mouthwash preparation. Generally, the composition of mouthwash consists of ethanol and other solvents, humectants, solubilizers, flavoring agents, preservatives, and pH regulators (alkaline) (Rachma, 2010). Red onion extract functions as the main active antibacterial agent against Streptococcus mutans, which causes dental plaque. Propylene glycol, which is present at

a concentration of 15% in the formula, functions as a humectant to prolong the contact time with bacteria when used in the mouth (Anastasia et al., 2017; Rowe et al., 2009). Propylene glycol is commonly used in topical and systemic preparations and food formulations because it is generally safe to use. However, studies on the effects of routine use of propylene glycol on oral tissue and saliva properties have not yet been conducted (Radzki et al., 2022). However, a study on the use of propylene glycol in electronic cigarettes for 90 days showed no signs of toxicity in users (Phillips et al., 2017). Based on this information, the potential toxicity of using propylene glycol in mouthwash is expected to be small because mouthwash is not ingested like electronic cigarettes, which are inhaled and enter the organs in the body, such as the lungs. In addition to its role as a humectant, propylene glycol, at a concentration of 15%, also functions as a cosolvent, which is useful for dissolving phenolic compounds, and as a preservative (Anastasia et al., 2017; Sheskey et al., 2017). PEG-40 hydrogenated castor oil functions as an emulsifying agent and solubilizer, and the recommended concentration in mouthwash formula can reach 2% (Kementerian Kesehatan R.I, 2012; Rowe et al., 2009). Menthol in the formula acts as a flavoring agent to reduce the strong flavor of red onions, and the recommended concentration for mouthwash preparations is 0.1-2% (Rowe et al., 2009). Benzoic acid, together with sodium benzoate, is generally known and used as a preservative, but in this formula, both substances act as pH regulators to control the pH of the formula (Kono et al., 2018). The pH of the mouthwash solution must be controlled to match the pH of the mouth, which is 6-7. This is intended to prevent mouthwash from being corrosive to teeth due to being too acidic or interfering with the sense of taste due to being too basic (Kono et al., 2018; Mumpuni et al., 2019). Raspberry aroma helps reduce the strong aroma of red onions, secang functions as a natural red coloring agent to make it more visually appealing, and stevia is a natural sweetener that is safe to use even for people with diabetes (Raini & Isnawati, 2011; Sari & Suhartati, 2016).

3.3. Stability Test Results of the Formula

The stability test results (**Table 3**) generally indicate that all formulas remained stable in terms of odor, taste, turbidity, and sedimentation throughout the evaluation period. The only observed instability was related to color, especially for formulas 2 and 3 on day 14, while the color of formula 1 tended to remain stable during the evaluation period. Observations were conducted on days 0, 7, and 14 to assess the physical stability of the preparation. The color instability observed in secang-based formulas primarily stems from pH levels. At low pH (2-5), secang imparts a yellow color, while at pH 6-7, it exhibits a vibrant and bright red color. At alkaline pH levels, it appears purplish-red due to the presence of a compound called brazilin (Kurniati et al., 2012). The color change in secang-based formulas can be influenced by various factors, including pH, oxidizing agents, sunlight exposure, storage conditions (room temperature and cold), storage duration, and the addition of zinc metal (Kurniati et al., 2012). The stability of secang color during storage depends on the solvent used. When ethanol is used as the solvent, the color stability can last up to 9 days at room temperature, while water as the solvent provides stability for up to 8 days (Kurniati et al., 2012; Padmaningrum et al., 2012).

3.4. pH Evaluation Results

The pH evaluation results for each formula during the evaluation period also showed stability (**Table 4**). This can be observed from the pH measurements taken using a pH meter on days 0, 7, and 14, which indicated that the pH remained relatively constant for each formula, with a minimum pH of 6.0 for formulas 2 and 3, and a pH of 6.3 for formula 1. A good mouthwash generally has a pH close to neutral, similar to the pH of the mouth, which is 6-7 (Rachma, 2010). Mouthwashes with a pH $\leq$ 5.5 can cause tooth demineralization, tooth erosion, and significant enamel loss within the first few minutes of contact with the acidic mouthwash (Vivek & Shwetha, 2015). One way to maintain the pH stability of a mouthwash is by using a buffer solution (Rachma, 2010).

Table 3. Results of formula stability test

Formula	Observation	Day-		
		0	7	14
F1	Color	Light Pink	Light Pink	Light Pink
	Odor	Menthol	Menthol	Menthol
	Taste	Sweet	Sweet	Sweet
	Turbidity	Clear	Clear	Clear
	Sediment	None	None	None
F2	Color	Red	Dark Red	Dark Red
	Odor	Menthol	Menthol	Menthol
	Taste	Sweet	Sweet	Sweet
	Turbidity	Clear	Clear	Clear
	Sediment	None	None	None
F3	Color	Dark Red Purple	Dark Red Purple	Dark Red Purple
	Odor	Onion Menthol	Onion Menthol	Onion Menthol
	Taste	Sweet	Sweet	Sweet
	Turbidity	Intense Clarity	Intense Clarity	Intense Clarity
	Sediment	None	None	None

FI: Formula 1, FII: Formula 2, FIII: Formula 3

Table 4. pH evaluation results of the formula

Formula	pH on Day-		
	0	7	14
F1	6,3	6,3	6,3
F2	6,0	6,1	6,0
F3	6,0	6,0	6,0

FI: Formula 1, FII: Formula 2, FIII: Formula 3

The buffer used in this study is a combination of benzoic acid and sodium benzoate. Benzoic acid and sodium benzoate become inactive as preservatives at pH >5, and they are optimal preservatives when they are at a pH range of 2-5 for sodium benzoate and <4.5 for benzoic acid (Rowe et al., 2009). In addition to the buffer solution, the concentration of red onion extract in each formula also influences the formula's pH. This can be observed in the decreasing pH values of the formulas as the concentration of red onion extract increases. Based on the references mentioned above, it can be concluded that all formulas have good pH values, with the best pH level found in formula 1, which is 6.3. This is because formula 1 has the highest pH among the formulas.

3.5. Evaluation of Inhibition Zone Diameter Results

The evaluation of inhibition zone diameters showed that all formulas demonstrated the ability to inhibit the growth of Streptococcus mutans bacteria, with diameters ranging from 6-8 mm throughout the evaluation period. The positive control, Total Care antiseptic mouthwash, also exhibited similar results (**Table 5**). Total Care antiseptic mouthwash was chosen as the positive control due to its antibacterial properties. The assessment of inhibition zones is categorized into several groups: very strong (> 20 mm), strong (10-20 mm), moderate (5-10 mm), and weak (< 5 mm) (Kono et al., 2018). Based on these evaluation criteria, it can be concluded that both the positive control and red onion extract mouthwash have a moderate inhibitory effect.

Table 5. The results of the inhibition zone diameter test

Formula	Mean Inhibition Zone Diameter (mm) on Day-		
	0	7	14
F1	8.407	7.042	5.880
F2	7.000	6.493	6.617
F3	8.167	6.900	6.683
K+	7.200	6.533	6.500

F1 refers to Formula 1, F2 refers to Formula 2, F3 refers to Formula 3, and K+ refers to the Positive Control. The inhibition zone diameter values already include the diameter of the paper disk.

The evaluation results of the inhibitory diameter of the red onion extract mouthwash formula fell far short of expectations. Previous studies on the inhibitory effect of red onion extract on Streptococcus mutans bacteria have shown strong inhibitory activity, with inhibitory diameters ranging from 10-20 mm (Gomaa, 2017; Shukla et al., 2013). The methanol extract of red onion at a concentration of 25% exhibited an inhibitory diameter of 10.4 mm. However, in the mouthwash with a concentration of 30%, the inhibitory diameter was only 8.2 mm. The difference in inhibitory diameter results is likely due to the different test methods used. The previous studies employed the well diffusion method, while this study utilized the paper disk method. Research comparing the well diffusion and paper disk methods has shown that the well diffusion method yields better and wider inhibitory diameters compared to the paper disk method (Haryati et al., 2017; Khusuma et al., 2019; Prayoga, 2013). This is because the well diffusion method allows for a more comprehensive and homogeneous osmolar process, as each well is filled with extract according to the concentration being tested (Haryati et al., 2017).

3.6. Statistical Test Results

The inhibitory diameter data of the red onion extract mouthwash on day 0 were analyzed using SPSS 16 to determine whether there were significant differences in the concentrations of each formula compared to the positive control. The analysis used was one-way ANOVA, as there was only one variable being analyzed, which was the concentration in each formula. The one-way ANOVA test yielded a significance value of 0.001 < 0.05, indicating significant differences in inhibitory diameters among the formulas. Post hoc tests are needed because the one-way ANOVA test yielded significant results. This is done to identify which groups differ significantly. One of the post hoc tests that can be used for homogenous data variance is Tukey's test, while for non-homogeneous data, Games-Howell test can be employed. In this case, since the data variance is homogenous, Tukey's test was chosen for the post hoc analysis (**Table 6**) (Suliyono, 2010).

Table 6. Post hoc test results of One-Way ANOVA

Formula	Significance Values between Formulas (P-Value)			
	F1	**F2**	**F3**	**K+**
F1	-	0.001	0.717	0.003
F2	0.01	-	0.04	0.810
F3	0.717	0.04	-	0.011
K+	0.003	0.810	0.011	-

F1: Formula 1, F2: Formula 2, F3: Formula 3, K+: Positive Control

The results of the Tukey post hoc test revealed significant differences between the positive control and F1 as well as F3. F1 and F3 exhibited better inhibitory efficacy compared to the positive control, as indicated by the post hoc test with significance values <0.05. However, there was no significant difference between F1 and F3 (significance value >0.05), indicating that F1 and F3 have similar abilities in inhibiting the activity of Streptococcus mutans bacteria. On the other hand, there was no significant difference between F2 and the positive control, suggesting that F2 is equally effective as the positive control. However, there was a significant difference between F2 and F1 as well as F3, indicating that F1 and F3 have better inhibitory capabilities than F2. Based on this data, it can be concluded that both F1, F2, and F3 are effective in inhibiting the growth of Streptococcus mutans bacteria, with F1 and F3 having similar but superior efficacy compared to the positive control and F2.

Observations of the inhibitory zone diameter were also conducted on days 7 and 14. The results showed a decrease in the inhibitory efficacy against Streptococcus mutans bacteria, as indicated by the reduction in the diameter of the inhibitory zones. This decrease is likely due to the decrease in the quercetin content, which is the active antibacterial compound against Streptococcus mutans, in the extract caused by storage duration (Millet et al., 2012). The stability

of quercetin in red onions is influenced by temperature, pH, and storage conditions (Wang et al., 2016). Storage at a temperature of 20 °C for 28 weeks can degrade quercetin up to 100%. In this study, the extract was stored at room temperature, which potentially accelerates the degradation of quercetin due to the storage temperature exceeding 20 °C. The decrease in quercetin content will certainly affect the antibacterial activity of the red onion extract.

4. CONCLUSION

Red onion methanol extract mouthwash has the ability to inhibit the growth of Streptococcus mutans bacteria with moderate strength, and the best formula based on physical evaluation results is F1 with a 10% extract concentration. Recommendations for further researchers include conducting inhibition zone diameter tests using the well diffusion method, adding glycerin to the formula, and performing co-pigmentation when using natural dyes

5. ACKNOWLEDGMENT

Thank you to the Ministry of Research, Technology, and Higher Education (Ristekdikti) under the Student Creativity Program-Research (PKM-P) funding scheme for the year 2019, with contract number B/81/B. B3/KM.02.01/2019.

6. CONFLICT OF INTEREST

All authors declare no conflict of interest.

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JFSP Vol.10, No.1, January-April 2024, Page: 90-96
pISSN: 2549-9068, eISSN: 2579-4558
Jurnal Farmasi Sains dan Praktis
(JFSP)
http://journal.unimma.ac.id/index.php/pharmacy

AUTHENTICATION OF PATCHOULI OIL FROM VARIOUS GROWING REGIONS USING GC-MS METHOD WITH CHEMOMETRIC COMBINATION ON THE PRODUCTS IN THE MARKET

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https://doi.org/10.31603/pharmacy.v10i1.10523

Article info:
Submitted : 11-11-2023
Revised : 12-03-2024
Accepted : 14-04-2024

Publisher:
Universitas Muhammadiyah Magelang

ABSTRACT

Adulteration in patchouli oil trade is an ancient practice, where additional substances like turpentine oil are added to increase volume and odor. Therefore, this research aimed to determine the composition of patchouli oil, considering variations in growing regions and potential adulteration in the products available in the market. The analysis was carried out using the Gas Chromatography-Mass Spectrormetry (GC-MS) method with a Principal Component Analysis (PCA) chemometric combination for authentication. The sample obtained from Bantul, Kulon Progo, and Purworejo was isolated through steam and water distillation. Subsequently, it was tested in accordance with SNI standard 06-2385-2006 and analyzed using GC-MS. The results were compared to the products on the market and processed using multivariate chemometric, namely PCA with Minitab 19 software. GC-MS analysis showed the presence of various compounds contained in the oil, including alcohol, alpha-guaiene, beta-humulene, seychellene, trans-caryophyllene, neoalloocimene, and beta-patchoulene. The combination of GC-MS and PCA chemometrics was able to distinguish patchouli oil from others and the products available on the market. PCA chemometric analysis showed that the patchouli oil from various growing regions had the same chemical components as essential oil. Furthermore, PCA chemometric analysis of market products also showed similar results but varied significantly from turpentine oil. This showed that the patchouli oil product available on the market did not contain turpentine oil.

Keywords: Authentication; Essential oil; GC-MS; Patchouli oil; PCA chemometrics

1. INTRODUCTION

The market demand for patchouli oil in Indonesia is significantly increasing every year, with a stable price, contributing approximately 50% of total exports in the country. Patchouli oil is fixative (binding) in nature, presenting a good opportunity for application in several sectors due to unavailable substitutes (Hariyani et al., 2015). However, this product is still sold in its crude form (Manurung, 2003), affecting the quality, as defined by the Indonesian National Standard (SNI) 06-2385-2006.

The export of essential oil is mainly influenced by the decrease in quality due to adulteration. Moreover, adulteration is the incorporation of additional substances to increase the volume or weight, meet quality standards, and enhance the oil properties. Adulterants that are commonly used include fats, kerosene, turpentine oil, specific organic solvents, and keruing oil (Ma'mun, 2003). This makes authentication an essential process to detect and prevent exposure to adulterated products using a combination of physical and chemical methods (Rohman, 2017).

The differences in growing regions, as well as the regional variations in the source of extraction, can affect the quality and quantity of essential oil. The primary components of patchouli oil that contribute to the distinctive aroma include alcohol and norpatchoulenol. In theory, the concentration of patchouli alcohol is relatively higher (30-40%) compared to norpatchoulenol (0.3-0.4%) (Manalu et al., 2019). It is necessary to authenticate patichouli oil obtained from different growing locations because the growing location has a significant impact on the chemical content. Climate, soil, sunlight, and elevation above sea level are all factors in this (Sufriadi et al., 2021).

In previous research, gas chromatography (GC) has been applied using the principle of separating mixtures based on differences in migration speed and boiling points (or vapor pressure) of the constituent components (Gandjar & Rohman, 2007). As technology develops, this method is combined with mass spectrometry (MS) to identify compounds, and determine molecular weight, and molecular formula (David, 2005). The combination of gas chromatography and mass spectrometry (GC-MS) allows rapid and sensitive identification of essential oil components, which is commonly used in quantitative analysis to assess PA levels and other chemical components (Ramayanti et al., 2021). Chemical methods are analytical techniques used to monitor the quality of essential oil.

This research aimed to analyze the chemical component profile of patchouli oil using GC-MS combined with PCA chemometric. The analysis was carried out to group the chemical components of patchouli and turpentine oils for product authentication.

2. METHODS

2.1. Materials

The main materials used included oil obtained from patchouli plant harvested in Bantul, Kulon Progo, and Purworejo on August 29, 2021. In addition, turpentine oil was obtained from CV Multi Kimia, and patchouli oil with a brand that has received 4.9 out of 5 customer evaluations in the online market, so brand A, B, and C were chosen. Other chemicals used included anhydrous sodium sulfate (Merck) and 90% ethanol (Merck). The main instruments were refractometer (Atago Abbe) and a GC-MS (Shimadzu QP2010SE).

2.2. Patchouli Plant Preparation

Patchouli leaves and stems were collected from Bantul, Kulon Progo, and Purworejo. Subsequently, the samples collected were washed, and dried in the sun covered with a black cloth for 9 days (Ardianto & Humaida, 2020).

2.3. Water Steam Distillation Process

A total of 3 kg of patchouli plants from Bantul, Kulon Progo, and Purworejo was distilled using steam and water distillation. The oil obtained was collected and weighed, followed by drying with anhydrous sodium (Wu et al., 2019).

2.4. Oil Quality Test

The test requirements were determined based on Indonesian National Standards (SNI) (Standar Nasional Indonesia, 2006), which included:

a) Color test: observed with the eye directly.
b) The refractive index is determined using a refractometer irrigated with water, ensuring the tool reaches a stable temperature required for reading.
c) Solubility test in ethanol: 90% ethanol is dropped into a measuring cup, and the solution is shaken to obtain a clear sample at a temperature of 20 °C.

2.5. Analysis by GC-MS

Patchouli oil obtained from various growing regions, turpentine oil, and three samples of market products were placed into a 2 mL vial. This process was repeated three times, depending

on the growing regions, and placed sequentially in the sample container of the GC-MS tool. Hexane was also put into a 2 mL vial and placed in the last container. Subsequently, 0.2 µL of oil samples were injected into GC-MS, and data analysis was carried out in the form of the relative region.

2.6. Chemometric

The relative region data was analyzed by grouping patchouli oil with market products using PCA, followed by a biplot generated with MINITAB 19 software. This grouping was based on the diversity of the data, resulting in a scree, score, loading, and bi-plot (Akbar, 2020; Anggraeni et al., 2020).

3. RESULTS AND DISCUSSION

3.1. Results of Characteristics and Quality Requirements of Patchouli Oil

Dark green patchouli leaves were harvested, washed with running water, dried in the air, and distilled. The distillation process was carried out based on growing regions using water and steam methods (Tauhana, 2008). The results of patchouli oil characterization are presented in **Table 1** and **Table 2**.

Table 1. Results of steam-water distillation of patchouli oil from various growing regions

Origin of Growing Regions	Rendement (%)
Bantul	0.29
Kulon Progo	0.30
Purworejo	0.28

Table 2. Comparison of the characteristics and quality requirements of patchouli oil SNI 06-2385-2006 and research

Parameter	Indonesian National Standards (SNI 06-2385-2006)	Results		
		Bantul	Kulon Progo	Purworejo
Color	Light yellow-reddish brown	Light yellow	Light yellow	Light yellow
Refractive index	1.507 – 1.515	1.510	1.508	1.509
Solubility in ethanol	Clear or lightly opalescent solution in a volume ratio of 1:10	Clear solution 1:8	Clear solution 1 :4	Clear solution 1:7
Patchouli alcohol ($C_{15}H_{26}O$)	Min 30%	26.11 %	26.14 %	26.93 %
Alpha copaene ($C_{15}H_{24}$)	Max 0.5 %	0.09 %	0.09 %	0.07 %

In this research, the yield produced was small because patchouli oil was obtained from Javanese patchouli (Pogostemon heyneatus Benth), consisting of approximately 0.5% - 1.5% oil content (Zhao et al., 2005). This variation in yield was also attributed to differences in time and temperature in the distillation process.

The differences in the physical properties of patchouli oil obtained in this research with the quality requirements were attributed to several factors. These included climate, soil conditions, growing regions, cultivation, and harvesting processes, which resulted in decreased quality compared to standard requirements (Schaduw et al., 2012).

3.2. Profile of Patchouli Essential Oil Components Using GC-MS

Analysis of the chromatogram profile for essential oil compounds was carried out using GC-MS. The Area Under the Curve (AUC) shown in the chromatogram was directly proportional to the concentration of each component contained in the sample. The use of GC-MS allowed the determination of a potential number of essential oil components and their concentrations.

Meanwhile, the type of essential oil component is determined using Mass Spectrometry, followed by identification through spectra from NIST and WILEY.

Based on the result, out of the 25 chromatogram peaks produced by patchouli oil component profile in Bantul, 7 were selected as the main peaks. Each selected peak was estimated to contain Patchouli alcohol (26.13%), Alpha-Guaiene (14.85%), Beta-Humulene (14.41%), Seychellene (11.47%), Trans-Caryophyllene (7.02%), Neoalloocimene (5.23%), and Beta-Patchoulene (4.53%). Similarly, among the 25 chromatogram peaks produced by patchouli oil component profile in Kulon Progo, 7 were selected as the main peaks, each containing Patchouli alcohol (26.20%), Alpha-Guaiene (14.90%), Beta-Humulene (13.74%), Seychellene (11.50%), Trans-Caryophyllene (7.03%), Neoalloocimene (5.19%), and Beta-Patchoulene (4.54%). Patchouli oil component profile in Purworejo produced 25 chromatogram peaks, with 7 main peaks. Based on estimation, each main peak contained Patchouli alcohol (26.99%), Alpha-Guaiene (14.74%), Beta-Humulene (14.07%), Seychellene (11.36%), Trans-Caryophyllene (6.69%), Neoalloocimene (5.46%), and Beta-Patchoulene (4.44%).

3.3. Comparison of Essential Oil Component Profiles in Patchouli Oil, Turpentine Oil, Products A, B, and C

In this research, turpentine oil was used as a counterfeit oil, and 3 samples of essential oil products (A, B, and C) were 100% pure. The results of the component analysis of patchouli oil, turpentine oil, products A, B, and C are presented in **Table 3**.

Table 3. Results of analysis of the main components of patchouli oil, turpentine oil, products A, B, and C

Patchouli oil components	Percentage (%) of GC results						
	Bantul	Kulonprogo	Purworejo	Product A	Product B	Product C	Turpentine
Patchouli alcohol	26.11	26.14	26.93	26.53	31.30	21.53	N/A
Alpha -Guaiene.	14.74	14.85	14.87	14.63	12.86	14.73	N/A
Beta-Humulene	14.26	14.21	14.48	15.10	N/A	14.02	N/A
Seychellene	11.85	11.88	11.60	10.68	10.36	0.68	N/A
Trans-Caryophyllene	6.98	7.03	6.71	6.58	4.79	8.26	N/A
Neoalloocimene	5.35	5.3	5.34	N/A	N/A	4.83	N/A
Beta-Patchoulene	4.49	4.52	4.47	4.58	3.81	5.95	N/A
Alpha Pinene	N/A	N/A	N/A	0.97	0.067	0.06	53.59
Delta Carene	N/A	N/A	N/A	N/A	N/A	N/A	14.10
Beta Pinene	N/A	N/A	N/A	0.18	0.123	0.15	7.03
Camphene	N/A	N/A	N/A	N/A	N/A	N/A	3.01
Limonene	N/A	N/A	N/A	N/A	N/A	N/A	1.48

Note: N/A = not detected

Based on **Table 3**, the largest patchouli alcohol component was found in product B, followed by Purworejo, product A, Kulon Progo, Bantul, and product C, with proportions of 31.30%, 26.93%, 26.53, 26.14%, 26.11%, 21.53%, respectively. Alcohol is the compound that determines the smell of patchouli oil, constituting the largest component (Trifilieff, 1980). As presented in **Table 4**, the essential oil content in products A, B, and C was similar to pure patchouli oil.

3.4. Principal Component Analysis (PCA) of Patchouli Oil with Turpentine Oil and Market Products

The results of PCA analysis were evaluated using Minitab 19 software and expressed the form of principal components (PC). This showed the magnitude of variation in the initial data, where PC1 contained the largest variance. **Table 4** shows the results from the eigenanalysis score plot.

Based on **Table 4**, PC1 has an eigenvalue of 9.8822, showing 82.4% of the total original data variables, while PC 2 has 10.2% with an eigenvalue of 1.2209. The results of PCA analysis are good when a small number of main components describes a large total variation

(Purwakusumah et al., 2014). In this research, the use of two PC resulted in a cumulative value of 99.8% (Rohman, 2014).

Table 4. Eigenanalysis of the correlation matrix

Eigenvalue	9.8822	1.2209	0.5897	0.2891	0.0094	0.0035	0.0031	0.0012	0.0008	0.0001
Proportion	0.824	0.102	0.049	0.024	0.001	0.000	0.000	0.000	0.000	0.000
Cumulative	0.824	0.925	0.974	0.998	0.999	1.000	1.000	1.000	1.000	1.000
Eigenvalue	0.0000	0.0000								
Proportion	0.000	0.000								
Cumulative	1.000	1.000								

Based on **Figure 1**, the main component of turpentine oil is completely separated in a different quadrant from patchouli oil in various growing regions and market products A, B, and C. This showed that PCA chemometrics could be used to analyze or group patchouli with fakes and products available in the market.

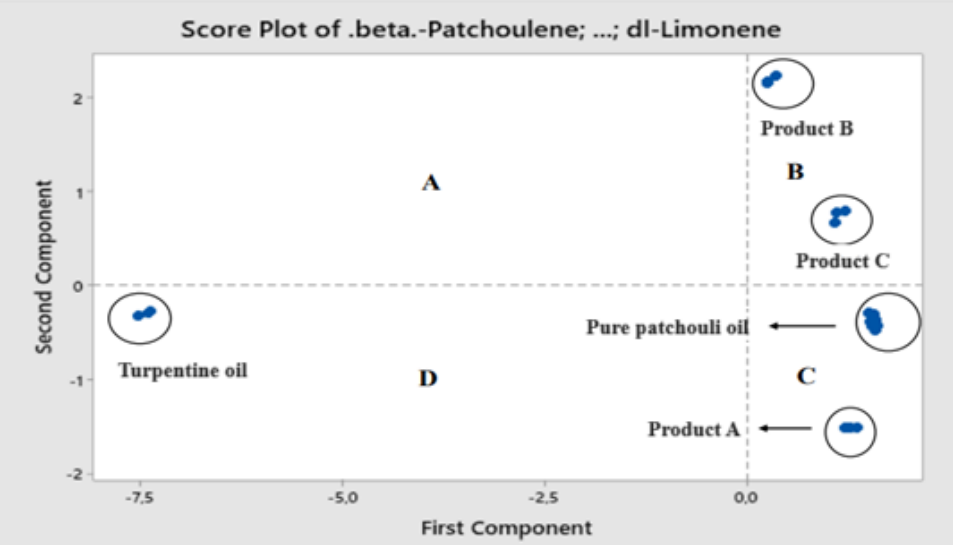

Figure 1. PCA score plot of patchouli oil, turpentine, product A, product B, and product C with type of essential oil as variable

PCA scoring between product A and others was close to pure patchouli oil but significantly varied from turpentine oil, resulting in the categorization of the score plots in different quadrants. This showed that the selected market products did not use turpentine oil as a mixture. However, products B and C are close to pure patchouli oil but are in different quadrants due to the presence of impurities (Sim et al., 2004). GC-MS can effectively analyze patchouli oil with key essential oil components, including patchouli alcohol, alpha-guaiene, beta-humulene, seychellene, trans-caryophyllene, neoalloocimene, and beta-patchoulene. When combined with PCA chemometrics, GC-MS proves useful in categorizing patchouli oil alongside other oils and various patchouli oil products available on the market. PCA chemometric analysis shows that patchouli oil from various growing regions shares common essential oil chemical components. It also confirms that patchouli oil products on the market do not contain turpentine oil.

4. CONCLUSION

GC-MS combined with PCA chemometrics successfully grouped patchouli essential oil with others and samples of products available on the market. PCA chemometric analysis showed that patchouli oil from various growing regions had the same essential oil chemical components, and market products did not contain turpentine oil.

5. ACKNOWLEDGMENT

The authors are grateful to LPPM Universitas Ahmad Dahlan for funding this research and publication.

6. CONFLICT OF INTEREST

All authors declare no conflict of interest.

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JFSP Vol.10, No.1, January-April 2024, Page: 97-106
pISSN: 2549-9068, eISSN: 2579-4558
Jurnal Farmasi Sains dan Praktis
(JFSP)
http://journal.unimma.ac.id/index.php/pharmacy

REVIEW: THE ROLE OF LIFESTYLE IN THE QUALITY OF LIFE OF TYPE 2 DIABETES MELLITUS PATIENTS

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https://doi.org/10.31603/pharmacy.v10i1.10112

Article info:
Submitted : 04-09-2023
Revised : 06-02-2024
Accepted : 28-03-2024

Publisher:
Universitas Muhammadiyah Magelang

ABSTRACT
Diabetes is a chronic disease that has increased growing from year to year in the number of sufferers from year to year. It is associated with changes in lifestyle in modern times that can affect the quality of life of the patients. The quality of life itself refers to the value assigned during a lifetime that changes with decreased functional, perceptual, and social value and can be affected by disease and treatment. The assessment of the quality of life, especially for patients with type 2 diabetes mellitus, aims to restore not only physical function in terms of mobility but also perceptions of health. This research aims to determine the role of lifestyle in improving the quality of life of type 2 diabetes mellitus patients. The non-experimental study is based on a review of various articles on the quality of life of people with type 2 diabetes mellitus published in various national journals from 2016 to 2022. The results of this study showed that exercising, consuming a balanced, nutritious diet, and monthly health check-ups greatly contribute to the improvement of the quality of life. The quality of life can also be measured using the EQ-5D-5L instrument which cover five domains mobility (the ability to move or walk), self-care, usual activities, pain or discomfort, and anxiety or depression and five domain levels. This article concludes that a good lifestyle can improve the quality of life of type 2 diabetes mellitus patients.

Keywords: Diabetes mellitus; Quality of life; Lifestyle; Type 2 diabetes mellitus

1. INTRODUCTION

Diabetes mellitus (DM) is a non-communicable disease whose development progresses slowly over a long period of time. It is characterized by increased blood sugar levels and impaired carbohydrate, lipid, and protein metabolism as a result of insulin function insufficiency (DepKes RI, 2005; Permenkes RI, 2015).The International Diabetes Federation (IDF) estimated that 463 million people aged 20–79 years in the world would suffer from DM in 2019, the equivalent of a prevalence rate of 9.3% of the total world population. Indonesia is ranked 7th out of 10 countries with the largest populations suffering from DM, with 10.7 million people living with the disease, which has contributed greatly to the prevalence of DM in Southeast Asia (Kemenkes RI, 2020).

Each individual has his/her own thoughts and tastes. The particulary in each individual's mindset influences his/her behavior in choosing something in life. Two individuals may share some similarities, but they are not the same. This leads to the diversity in the quality of life among different individuals. The World Health Organization (WHO) revealed that a person's quality of life depends on his/her perception because each individual has a different perspective on culture, goals, expectations, and living standards (WHO, 1998).

In modern times, young and old people alike increasingly pursue good quality of life, considering the rise in health problems, due to low social economy, low education, poor lifestyle, and poor food intake. One disease that is closely related to quality of life is diabetes mellitus. According to (Umam et al., 2020),one must have control over his/her quality of life. Patients of

DM constantly have needs, especially for sugar. This excessive consumption of sugar in diabetes mellitus sufferers can cause abnormal blood glucose levels (Umam et al., 2020).

Diabetes mellitus is generally defined as a disease caused by an increase in a person's consumption of sugar. It is a metabolic disorder associated with distribution (Umam et al., 2020). The increase in blood sugar levels in DM sufferers is caused by ineffective use of insulin. Of the various types of DM, type 2 DM is the most common. The American Diabetes Association (2018) reported, that type 2 DM contributed 90% of the data and has the highest relevance all types of diabetes. Research (Adikusuma et al., 2016; Teli, 2017; Putu et al., 2019; Dewi et al., 2019; Handayani et al., 2022; Nurliza et al., 2022) has demonstrated that lifestyle is important for patients. DM patients who have good quality of life will find it easier to avoid the wort risks. That being the case, this study seeks to review matters related to the quality of life of patients with type 2 diabetes mellitus using the cross-sectional method. It aims to provide readers with the knowledge of the right quality of life for people with DM.

Research reviews regarding type 2 diabetes mellitus have been carried out before, but there are differences in terms of improving the quality of life of type 2 diabetes mellitus patients. As in research conducted (Serena et al., 2023) patients who have good family support will have a comfortable feeling that can increase their motivation to obey on the management of type 2 diabetes mellitus and ultimately quality of life they increase. According to (Fitriani and Sanghati, 2021) shows that lifestyle interventions can effectively prevent the risk of developing type 2 DM in people with pre-diabetes.

2. METHODS

The present study investigated the quality of life of type 2 DM sufferers in Indonesia using a non-experimental method. Article searches in this research were achieved using Google Scholar with keywords "Diabetes mellitus; Quality of life; Lifestyle; Type 2 diabetes mellitus". It was selected based on the suitability of the title to the topic of this study. Furthermore, data were extracted from 13 articles selected and published in national journals from 2016 to 2022. Information regarding the articles' authors, titles, years of publication, objectives, methods, times of study, criteria, number of patients involved, patient characteristics, instruments used, and conclusions was retrieved. The flow of selection of articles in this study is shown in **Figure 1**.

3. RESULTS AND DISCUSSION

Age, occupation, and gender are groups that are used as measuring tools in determining the Diabetes Quality of Life Clinical Trial Questionnaire (DQLCTQ) instrument. Women are more affected by type 2 diabetes mellitus. They are 3-7 times more at risk of developing DM than men as the levels of fat in their blood is higher than that of men. The increase in sugar intolerance in the elderly makes them more susceptible to type 2 DM (Nurhaliza et al., 2022). In collecting quality of life data, physical examinations such as blood pressure checks and examinations of other anthropogenic markers useful for measuring nutritional status, and limited daily activities were performed. From psychological examinations, it was found that patients with DM tend to lack a zest for life and have no desire to live a better life. Around the age of 67, men are at higher risk of suffering from type 2 DM compared to women (Faswita, 2019). However, other research contrarily reveals that men exhibit better quality of life in terms of physical fiction, energy, mental health, and frequency of symptoms (Handayani et al., 2022). Umam et al., (2020) stated that people above 50 are at risk of developing DM because of a decreased immune state. Their leading a poor lifestyle and rarely going out for sports during their youth exacerbate their risk of developing the disease. Megawati et al., (2019) found that there is no relationship between gender and quality of life. While they face the risk of developing DM, men and women alike still have the ability to manage diabetes. Activities such as work can affect the physical state, and the consumption of high-calorie foods and a lack of exercise can cause obesity. Someone who suffers

from a disease has a fairly high emotional level compared to healthy people. This also have a role in encouraging patient's quality of life.

Adikusuma et al., (2016), discovered that patients who received monotherapy had higher quality of life than patients who received combination therapy. However, both groups of patients were in similar states of physical functioning they fell to exhaustion easily and, felt a low level of energy even though they had accepted their health condition. There was no significant difference between combination therapy and monotherapy patients on mental health. This can be attributed to the fact that all patients had accepted their health condition. They did not feel the burden of having to lead a different lifestyle. With regard to satisfaction, a significant difference was found between monotherapy and combination patients. They felt a higher degree of control over their treatment, and they wished to continue with oral antidiabetic monotherapy. However, both groups of patients had reduced quality of life due to the side effects of the drugs that they took. Meanwhile, Teli (2017) found that diabetes mellitus patients experienced a decline in physical function, mental function, pain, general health, roles, and responsibilities. They also experienced a shift in their roles. According to Nurhaliza et al., (2022), patients who received combination therapy could not fully control their lifestyle and eating patterns. In the case of housewives who dealt with various kinds of routines, physical activities could trigger stress, which ultimately affected their blood glucose levels. Moreover, the majority of the activities were carried out at home, which could lead to obesity, one of the triggering factors for type 2 diabetes mellitus.

Putu et al., (2019) in their research found that the quality of life of Prolanis (Chronic Disease Management Program) participants in psychological as well as social, environmental, and physical aspects was relatively high. Prolanis participants made regular visits to health facilities once a month. Information related to diabetes and diet management was provided to increase the participants' knowledge about diabetes and to minimize complications. In another work, (Dewi et al., (2019) used the EQ-5D-5L instrument to measure the quality of life in five domains, namely, mobility (the ability to move or walk), self-care, usual activities, pain or discomfort, and anxiety or depression. Socioeconomic and appetite changes will result in changes in eating patterns that tend to move away from the concept of nutritious food, which will have an impact on health. Smoking has been significantly linked to an increased risk of type 2 diabetes mellitus. Exercise will convert glucose into energy. It causes insulin to increase, which subsequently causes the level of sugar in the blood to decrease. Diabetics must undergo specific diet or eating arrangements, control their blood sugar, and exercise to improve their quality of life. (Hamida et al., (2019) also used the EQ-5D-5L instrument to describe health. Ratnasari et al., (2020) stated that diabetes mellitus has an impact on psychological conditions related to emotional burden, the pressure to maintain health, illness-related stress, and social relationships. To improve the quality of life, patients with diabetes mellitus should take drugs regularly and lead a healthy lifestyle. The results of the literature review of 13 articles obtained in this study can be seen in **Table 1** which contain resources, research title, location (city), total patients, length of research, variables results and conclusion, and reference.

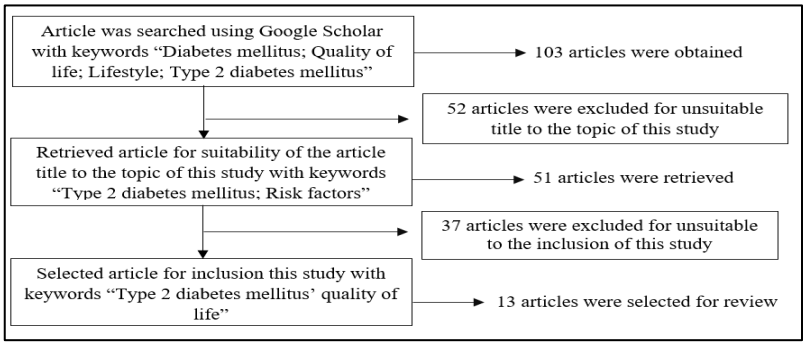

Figure 1. Scheme of the review process

Table 1. Articles review

No	Resources	Research title	Location (City)	Total patients	Length of research	Variables	Results and Conclusion	Reference
1	Jurnal Ners LENTERA	The Development of a Self-Empowerment and Quality of Life Improvement for Model for Patients with Type 2 Diabetes Mellitus	Bendo Public Health Center, Pare, Kediri, 2014	42 patients	1 month	Independent variable: Personal Factor and Self-Instructional Training Dependent variable: Self-empowerment, quality of life	1. The patient's personal factors (age, gender, education, length of suffering from DM, perceived benefits, and perceived barrier) significantly affect self-empowerment and quality of life 2. Self-empowerment has a significant influence on the quality of life of DM patients 3. Self-Instructional Training can improve self-empowerment and quality of life for type 2 DM patients	(Nuari, 2016)
2	Jurnal Ilmiah Ibnu Sina	Measurement of the Quality of Life of Type 2 Diabetes Mellitus Patients Receiving Oral Anti-Diabetic in Medication in RS PKU Muhammadiyah Bantul Yogyakarta	RS PKU Muhammadiyah Bantul Yogyakarta, 2016	56 patients	3 months	Independent variable: Patient's Characteristics (age, gender, education, job, long suffering, drug therapy) Dependent variable: Quality of life of diabetes mellitus type 2 patient	1. There were significant differences in patient quality of life (DQLCTQ) between the monotherapy and combination therapy groups in the domains of personal satisfaction and treatment satisfaction. 2. Based on the Time Trade Off (TTO) questionnaire, there was no significant difference between the monotherapy and combination therapy groups	(Adikusuma et al., 2016)
3	Jurnal Info Kesehatan	The Quality of Life of Type 2 Diabetes Mellitus Patients at the Public Health Center of Kupang City	Health centers in Kupang City, East Nusa Tenggara	65 patients	3 months	Independent variable: Long suffering from DM, gender, age, complications of DM, regularity of taking medication and regularity of checking blood sugar Dependent Variable: Quality of life of DM patient that defined as a DM patient's perspective on function, and role in life while suffering from DM	1. There is a decrease in the quality of life of type 2 DM patients in all aspects of health, including physical function, social functioning, mental health, general health, pain, role changes due to physical problems, role changes due to emotional problems with a value of < 80 2. There is no relationship between age, regularity of taking medication, regularity of checking blood sugar with quality of life of DM patients.	(Teli, 2017)

No	Resources	Research title	Location (City)	Total patients	Length of research	Variables	Results and Conclusion	Reference
							3. There is a significant relationship between sex, complications, and duration of suffering from DM with the life causality of DM type 2 patients with p-value = 0.000.	
4	Jurnal Persatuan Perawat Nasional Indonesia	The Quality of Life of Prolanis Participants with Type 2 Diabetes Mellitus in Yogyakarta	Depok Health Center, Sleman, Yogyakarta, 2019	85 patients	2 months	Independent variable: Characteristics of the patient (last education, employment status, marital status, income, length of time with diabetes, length of time following prolanis, concomitant diseases) Dependent variable: Quality of life of participants with type 2 diabetes mellitus	1. The quality of life of Prolanis participants at Puskesmas Depok Sleman Yogyakarta is good. 2. Based on demographic characteristics, good quality of life is found in the late adult age group (≥60 years), male, last junior high school education, not working, married status, earning 2 million-4 million, long time with diabetes for <5 years, following Prolanis ≥ 6 months, and not having comorbidities	(Putu et al., 2019)
5	Jurnal Oline Keperawatan Indonesia	A Description of The Quality of Life of Type 2 Diabetes Mellitus Patients At RSUD. dr. R.M. Djoelham of Binjai City In 2019	RSUD.dr.R.M. Djoelham of Binjai City, North Sumatera, 2019	24 Patients	1 month	Independent variable: Characteristics of the patient (age, gender, educational program, physical health category, physiological health category, social relationships) Dependent variable: Quality of life of people with type 2 diabetes mellitus	1. According to the characteristics of respondents, the majority are in the age group of 38-40 years as many as 8 people (33.3%), male gender as many as 13 people (54.2%), with elementary education level of as many as 9 people (37.5%) 2. The picture of the quality of life of people with type 2 Diabetes mellitus in terms of the physical health of the majority is disturbed by 54.2%, the psychological health of the majority is disturbed by 62.5%, and the majority of social relationships are disturbed by 66.6%	(Faswita, 2019)
6	Jurnal Permata Indonesia	The Quality of Life of People with Type 2	Mercusuar Pharmacy of Kaliworo,	73 patients	2 months	Independent variable: Patient characteristics such as age, sex, education,	1. Characteristics of type 2 diabetes mellitus patients aged > 45 years (80.8%), female (69.9%), married	(Dewi et al., 2019)

No	Resources	Research title	Location (City)	Total patients	Length of research	Variables	Results and Conclusion	Reference
		Diabetes Mellitus at Mercusuar Pharmacy of Kaliwiro, Wonosobo	Wonosobo, Central Java			occupation, income, marital status, family support, diet, BMI category, current blood sugar levels, exercise, insurance, medications used, type of therapy, complications, length of DM, family history, smoking history, and alcohol history, family support, and medication adherence. Dependent variable: Quality of life of type 2 DM patients	2. (83.6%), and poorly educated (75.3%). 3. The results of measuring the quality of life of type 2 diabetes mellitus patients obtained a utility value of 0.85±0.15 and a VAS (Visual Analog Scale) value of 84.38±8.163. The anxiety domain is the domain reported to have many problems in type 2 diabetes mellitus patients by 54.8%.	
7	Majalah Farmaseutik	Measurement of The Quality of Life among Prolanis in Primary Healthcare Centers using the EQ-5D-5L Instrument	Public Health Center of Pali City, Central Sulawesi and Public Health Center of Kab. West Aceh Regency, Nangroe Aceh Darussalam	200 patients	3 months	Independent variable: Factors characteristic of the patient that affect the patient's quality of life scores (gender, age, education level, employment status, income, assets owned, having other diseases, family history of illness, length of suffering, and length of control) Dependent variable: Quality of life of diabetes mellitus and hypertension patients expressed in utility value	1. The pain/discomfort domain is the domain where most reported problems occur in DM and hypertension patients. 2. There are significant differences in utility values based on age characteristics, family history of disease, length of disease, and frequency of control in hypertensive patients. 3. In DM patients only on the characteristics of having comorbidities that have significant differences in utility value	(Hamida et al., 2019)
8	Jurnal Ilmiah Medicamento	An Assessment of The Quality of Life of Type 2 Diabetes Mellitus	Ari Canti Hospital, Bali	100 patients	6 months	Independent variable: Quality of life of type 2 diabetes mellitus outpatients with quality of life	1. The average respondent feels that his quality of life is moderate and feels that his health condition is moderate.	(Megawati et al., 2019)

No	Resources	Research title	Location (City)	Total patients	Length of research	Variables	Results and Conclusion	Reference
		Inpatients in Ari Canti Hospital in 2018				questionnaire from WHOQOL BREF Dependent variable: Demographic data including name, gender, age, last education, previous occupation, marital status and length of visit	2. Judging from the dimensions of physical health, psychological dimensions, dimensions of social relationships, and environmental dimensions, the average respondent with DM who does outpatient treatment at Ari Canti General Hospital has a moderate quality of life.	
9	Jurnal Promotif Preventif	The Quality of Life of Diabetes Mellitus Patients and Its Determinants in Gorontalo District	Pulubala Public Health Center, Limboto Public Health Center, Dungaliyo Public Health Center, Tabongo Public Health Center, and Batudaa Public Health Center, Gorontalo, North Sulawesi	313 patients	2 months	Independent variable: Age, education level, employment status, economic status, and length of suffering) Dependent variable: Quality of life of people with diabetes mellitus and its determinants	1. The proportion of respondents with a high quality of life was 44.7%. 2. There is a relationship between education level, employment status, economic status, and length of suffering with the quality of life of diabetes mellitus patients 3. There is no relationship between age and quality of life of diabetes mellitus patients	(Adhayani et al., 2020)
10	Jurnal Sains Farmasi & Klinis	A Clinical Outcome Analysis Based on the Quality of Life and Direct Medical Costs of Patients With Type 2 Diabetes Mellitus	RSUD Panembahan Senopati Bantul, Yogyakarta, 2020	200 patients	1 month	Independent variable: Quality of life and direct medical costs of type 2 diabetes mellitus patients Dependent variable: Clinical outcomes of type 2 diabetes mellitus patients	1. A total of 129 out of 200 patients showed uncontrolled clinical outcomes with an average good quality of life score with direct medical costs incurred of Rp 489.005 2. There are differences in clinical outcomes based on patient quality of life in the domains of physical functioning, personal satisfaction, treatment satisfaction, frequency of disease symptoms, and based on direct medical costs	(Ratnasari et al., 2020)

No	Resources	Research title	Location (City)	Total patients	Length of research	Variables	Results and Conclusion	Reference
							3. Patients with controlled clinical outcomes show better quality of life with lower medical costs	
11	Jurnal Kesehatan Kusuma Husada	An Overview of The Quality of Life of Patients with Diabetes Mellitus at Wanaraja Public Health Center	Wanaraja Public Health Center, Garut Regency, West Java, 2020	91 patients	1 month	Independent variable: Age, gender, level of education, socioeconomic status, length of suffering, complications Dependent variable: Quality of life of patients with diabetes mellitus	1. The quality of life of diabetes mellitus patients is mostly in the moderate category. 2. Quality of life based on physical domain, psychological domain, social relationship domain, and environmental domain is in the moderate category	(Umam et al., 2020)
12	Jurnal Ilmiah Farmasi Farmasyifa	An Evaluation of The Quality of Life of Type 2 Diabetes Mellitus Patients Taking an Oral Antidiabetic at RSUD Harapan and Do'a, Bengkulu City	RSUD Harapan and Do'a Bengkulu City	98 patients	-	Independent variable: Oral antidiabetic therapy and patient characteristics (age, gender, education, occupation, long suffering from the disease) Dependent variable: Quality of Life of Type 2 Diabetes Mellitus Patients	1. There is no influence between respondents' characteristic factors on quality of life. 2. There was no difference in quality of life in patients with metformin, sulfonylureas, and acarbose therapy. 3. There is no difference in quality of life between patients with single ADO therapy and patients with combination ADO therapy.	(Handayani et al., 2022)
13	Jurnal Syifa Sciences and Clinical Research (JSSCR)	Measuring the Quality of Life of Patients with Type 2 Diabetes Mellitus Using the *Diabetes Quality of Life Clinical Trial Questionnaire* (DQLCTQ) Instrument	Siantan Tengah Public Health Center, Pontianak City, West Kalimantan	30 patients	2 months	Independent variable: Characteristics of the patient (gender, age, education, occupation, marital status) Dependent variable: Quality of Life of Type 2 Diabetes Mellitus Patients	1. The results of quality-of-life measurement in patients with type 2 diabetes tests at the Siantan Tengah Health Center were in the low category of 58.69% and the high category of 41.31% 2. The group of patients who received a single antidiabetic drug had a quality of life score above the average value	(Nurhaliza et al., 2022)

4. CONCLUSION

This article concludes that a good lifestyle can improve the quality of life of type 2 diabetes mellitus patients. Exercising, consuming a balanced, nutritious diet, and having monthly health check-ups greatly contribute to the improvement of the quality of life. By exercising, people with type 2 diabetes mellitus can convert glucose in the body into energy. The quality of life must be measured using appropriate methods. For instance, it can be measured using the EQ-5D-5L instrument. This instrument measures the quality of life in five domains mobility (the ability to move or walk), self-care, usual activities, pain or discomfort, and anxiety or depression and five domain levels.

5. CONFLICT OF INTEREST

All authors declare no conflict of interest.

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