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Original Article

The Employment of ATR-FTIR Spectroscopy and Chemometrics for Authentication of Bawal (*Colossoma macromopum*) Fish Oil from Palm Oil

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Abstract: Bawal fish is a product with high nutritional content, and the development of fish oil has made it easier for consumers to consume. High-quality fish oil can be a target for adulteration, such as with vegetable oils like palm oil. This study aims to develop FTIR spectroscopy for authentication of bawal fish oil (BFO) from palm oil (PO). Bawal fish oil and palm oil were prepared in binary mixtures with concentrations from 0-100%, resulting in 21 mixtures. The oil was directly measured by attenuated total reflectance (ATR) spectral measurement in the mid-infrared region (4000-600 cm^{-1}). The results were combined with linear discriminant analysis (LDA) and multivariate calibration, such as PLSR or PCR. The results showed that LDA could make clear discrimination between bawal fish oil, palm oil, and their mixtures without any misclassification observed in 4000-600 cm^{-1} region. Multivariate calibration with PLSR using the first derivative spectrum provided the best model for the relationship between actual and predicted FTIR values. At these conditions, the R2 values for the calibration and validation models obtained were 0.9966 and 0.9969, with RMSEC and RMSEP values of 0.0249 and 0.0248, respectively. Therefore, FTIR spectroscopy combined with LDA and PLSR is an effective method for authenticating bawal fish oil from palm oil.

Keywords: Bawal fish oil, palm oil, oils adulteration, chemometrics, ATR- FTIR spectroscopy

1. INTRODUCTION

Fish is one of the foods that have high nutritional content. Currently, its utilization is widely used in the form of fish oil. Fish oil has a higher price compared to fish, making it easier to be adulterated. Authentication of food supplements needs to be a significant concern due to high demand and lack of regulation [1]. Freshwater bawal fish (Colossoma macropomum) or also known as tambaqui fish is a native fish originating from the Amazon valley [2]. This fish is easily cultivated

due to its ease of breeding, fast growth, productivity, and market acceptance. The nutritional content of bawal fish is quite varied, ranging from 1-2% mineral, 15-24% protein, and 0.1-22% fat which is dominated by 60% unsaturated fatty acids with 45% monounsaturated fatty acids (MUFA) and 15% polyunsaturated fatty acids (PUFA) [3]. Currently, fish oils have emerged as promising functional foods due to its function to treat several diseases. Some analytical methods of fish oil authentication have been developed, such as chromatography method. Liquid chromatography-mass spectrometry (LCMS) and gas chromatography-mass spectrometry (GCMS) have high sensitivity and high selectivity analytical method [4,5]. Comprehensive two-dimensional tandem gas chromatography-mass spectrometry (GC x GC-MS) combined with chemometrics is able to provide a fingerprint profile of fatty acid composition and has been successfully used for the authentication of fish oil products in Brazil [1]. However, these methods require several steps of sample preparation such as the need for derivatization, skilled analysis, can damage the sample, takes longer time, and requires a lot of chemical solvents [6]. An alternative method that has been developed is infrared spectroscopy. This method is used because it has advantages such as non-destructive to the sample, fast, easy to use, simple, and does not require time-consuming sample preparation [7]. This method is also considered as a green method of analysis because it does not use hazardous solvents and there are no chemical residues or waste that can pollute the environment [8].

The application of spectroscopy in food identification is increasingly developing with the aim of controlling its quality and quantity. The spectroscopy region that is widely applied is the Mid Infrared Spectroscopy or MIRS region, which is an electromagnetic region of 4000-600 cm^{-1}. The principle of this method is the difference in the absorption characteristics produced by a material with certain chemical bonds when illuminated with a spectrum of light waves. In connecting the data set obtained from FTIR spectra with the condition of the material or sample, statistical methods, namely chemometrics, are used for authentication [9]. There have been many studies involving oil in the analysis using spectroscopy with LDA, PCR or PLS chemometrics , such as successfully identified patin fish oil from palm oil, patin fish oil from corn oil, tuna oil from pork fat, pork oil in cow's milk, tuna fish oil from palm oil and corn oil [10, 11, 12]; [6]; [13]. The application of infrared spectroscopy for the authentication of fish oil is developing and providing good results. Therefore, the aim of this study was to develop the process of authentication bawal fish oil using ATR-FTIR combined with chemometrics with linear discrimination analysis (LDA) and multivariate calibration (PLS and PCR) from palm oil.

2. MATERIALS AND METHODS

2.1. Materials

The bawal fish samples were obtained from fish farms in the Yogyakarta region, Indonesia. The researcher utilized the dry rendering method to extract the full body of bawal fish. The sample cleaning and cutting 4-5 cm are performed, followed by heating using a cabinet drying method for 24 hours at 50°C. Subsequently, the sample is subjected to pressing with a pressure of 150 kN for 5 minutes. Palm oil was used as the adulterant agent and was obtained from a supermarket in Yogyakarta, Indonesia. Palm oil was chosen because it is cheaper and more readily available in the market as food grade.

2.2. Preparation of samples

The bawal fish oil (BFO) was obtained using the dry rendering extraction method at a temperature of 50°C for 24 hours. Subsequently, a set of calibration mixtures was prepared using 21 samples. The oil of bawal fish was mixed with palm oil in binary mixtures with concentrations ranging from 0-100%. The composition of the binary mixture used can be seen in Table 1.

Table 1. The composition of Bawal Fish Oil (PFO) in biner mixtures with Palm oil

Sample	Bawal Fish Oil (BFO) (%)	Palm Oil (PO) (%)
1	100	0
2	95	5
3	90	10
4	85	15
5	80	20
6	75	25
7	70	30
8	65	35
9	60	40
10	55	45
11	50	50
12	45	55
13	40	60
14	35	65
15	30	70
16	25	75
17	20	80
18	15	85
19	10	90
20	5	95
21	0	100

2.3. ATR-FTIR Spectroscopy Measurement

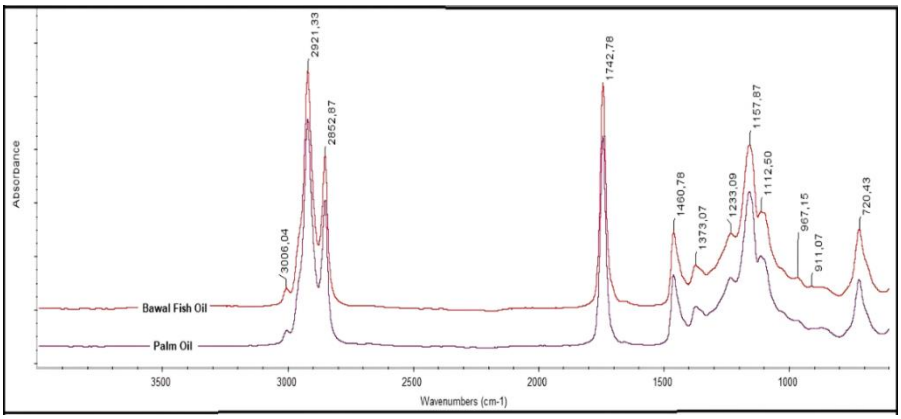
The BFO treated with bentonite as well as those mixed with Palm oil (PO) were measured using a FTIR spectrophotometer (FTIR Nicolet iS20) with a DTGS (deuterated triglycine sulfate) detector connected to the OMNIC® and Windows® software. The sample reading technique used was attenuated total reflectance (ATR). All spectra were measured in the region of 4000-600 cm-1 by placing the sample directly on the ATR crystal. The resolution used was 8 cm^{-1} with 32 scans. All spectra were recorded in absorbance mode to facilitate quantitative analysis according to the Lambert-Beer law. Each sample was read three times. Before measuring each sample, a background spectrum was recorded using an air spectrum. After measuring each sample, the ATR crystal was cleaned using acetone. The data obtained will be analyzed using the TQ-Analyst software.

2.4. Chemometrics analysis

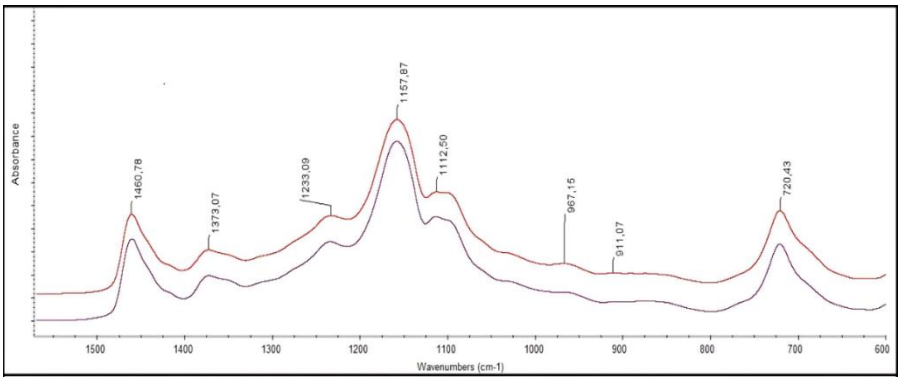
The TQ Analyst software version 9 (Thermo Fisher Scientific, Inc.) was used for chemometric analysis. Two chemometric techniques were used in this study is pattern recognition and multivariate calibration. For pattern recognition analysis, a supervised technique called LDA was used. The LDA model evaluated the discrimination between the BFO treated with bentonite and the adulterated ones according to the concentrations that were prepared using a Cooman score plot. Multivariate calibration analysis was performed using PLSR and PCR models. These models were evaluated using the root mean square error of calibration (RMSEC), root mean square of prediction (RMSEP), and coefficient of determination (R2).

3. RESULTS AND DISCUSSION

In this study, mid-infrared spectroscopy (4000-600 cm^{-1}) combined with chemometrics was used to determine the authentication of bawal fish oil (BFO) from palm oil (PO). The ATR-FTIR reading results will provide absorbance data at each wavenumber from 4000-600 cm^{-1}. The comparison spectra of BFO and PO can be seen in Figure 1 (a). It can be seen that the FTIR spectra of BFO and PO have similar spectra, but if observed in the fingerprint region (1500-600 cm^{-1}) in Figure 1 (b), there are some peaks that indicate differences in peak intensities. These differences can be used as the basis for chemometric analysis. Both spectra show typical Triglyceride (TG) spectra, as animal and vegetable oils mostly consist of TG [14].



(a)



(b)

Figure 1. ATR-FTIR spectra of Bawal fish oil and Palm Oil at (a) mid infrared region (4000-600 cm^{-1}) and finger print region (1500-600 cm^{-1})

Table 2. FTIR absorption of common bands detected in bawal fish oil

Wavenumber (cm^{-1})	Functional grup	Vibration
3006.04	Alkena	=C-H stretching
2921.33	Alkana	Methylene asymmetric C-H stretching
2852.87	Alkana	Methylene symmetric C-H stretching
1742.78	Alkana	C=O stretching
1460.78	Alkana	Methylene scissoring CH2
1373.07	Alkana	Methyl siymmetrcial C-H bending
1233.09	Alcohol and phenols	C-O stretching
1157.87	Alcohol and phenols	C-O stretching
1112.50	Esters	Aliphatic C-O stretching
967.15	Alkena	=C-H out-of-plane bending
911.07	Alkena	=C-H out-of-plane bending
720.43	Alkana	Methylene rocking

Table 2 contains information about several functional groups that can be identified. FTIR spectroscopy is a suitable method because it has fingerprinting in its analysis, so that the similarity of characteristics between samples can be distinguished in the fingerprint region, making it highly sensitive [18]. The ATR technique is used in sample reading. The principle of ATR utilizes the phenomenon of total internal reflection. The sample of oil liquid will be dropped on an ATR crystal, which is insoluble in water or organic solvents and has a high refractive index. In ATR spectroscopy technique, a crystal with excellent infrared transmitting properties and high refractive index is placed close to the sample and used as an internal reflection element (IRE) [19]. A beam of radiation that enters the crystal will undergo total internal reflection when the angle of incidence at the interface between the sample and the crystal is greater than the critical angle, where the critical angle is a function of the refractive index of both surfaces. A small portion of the wavelength penetrates outside the reflecting surface, and when a material that selectively absorbs radiation is in close contact with the reflecting surface, the beam loses energy at the wavelength absorbed by the material. The weakened radiation generated is measured and plotted as a function of wavelength by a spectrometer, and it produces spectral absorption characteristics of the sample. The ATR technique in FTIR spectroscopy allows for the measurement of samples in liquid or solid form without complex sample preparation. Therefore, this technique is often used in the analysis of oils and fats. ATR also has the advantage of minimizing interference by water or other impurities because only the surface of the sample is involved in the measurement [15]. In Figure 2, it can be seen that all 21 mixture FTIR spectra at mid infrared region (4000-600 cm^{-1}).

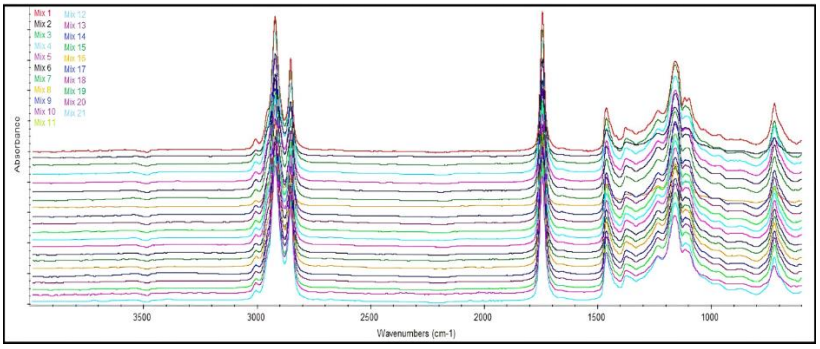


Figure 2. ATR-FTIR spectra of Bawal Fish Oil (BFO) and Palm Oil (PO) mixture at mid infrared region (4000-600 cm^{-1}) with number of scanning of 32 and at resolution of 8 cm^{-1}

The authentication analysis of fish oil using chemometrics was carried out using linear discriminant analysis (LDA) to see the classification between pure fish oil and adulterated oil. In LDA analysis, the samples were divided into three classes, bawal fish oil treated with bentonite, adulterated BFO with palm oil, and palm oil. In FTIR reading, the wavenumber was used as a variable and the absorbance data obtained from 4000-600 cm^{-1} were used as variables, which were then converted into Mahalanobis distance to classify bawal fish oil and its mixture. LDA applied to predict class membership of samples (individuals) from quantitative profiles made by several measured [20]. From Figure 3, it can be clearly seen that the classification is well-formed. This indicates that LDA successfully distinguished authentic bawal fish oil from palm oil as adulterant.

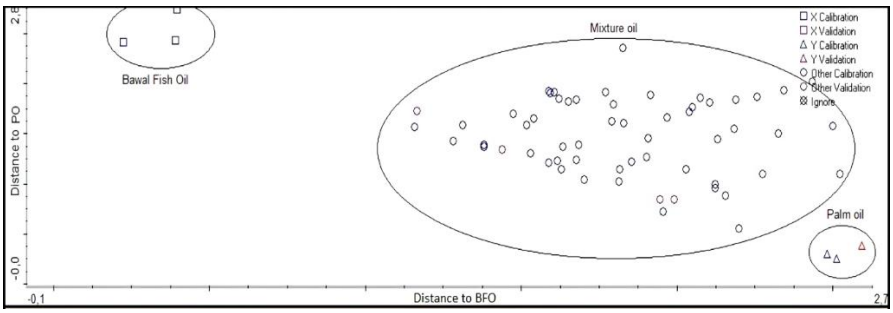


Figure 3. Linear discriminant analysis (LDA) Coomans plot for the discrimination between BFO adulterated with PO using the absorbance values of ATR-FTIR spectra at (3500-600 cm^{-1})

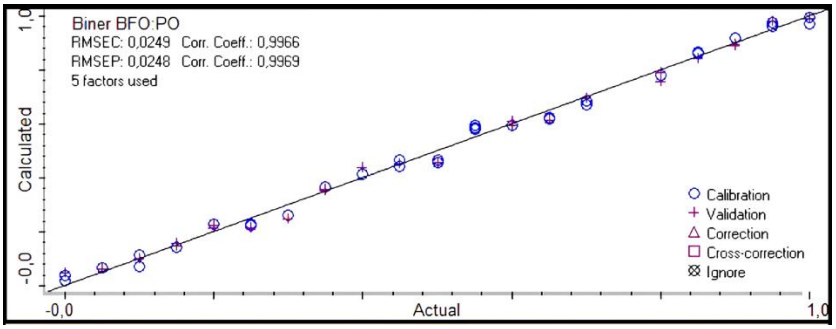
PLSR and PCR are multivariate calibration methods used for quantification of fish oil adulteration. In both methods, the concentration of the analyte (y-axis) is modeled with the principal components, which are a linear combination of absorbance values (x-axis) [21]. The combination of FTIR spectroscopy with multivariate calibration is suitable for quantitative analysis of oil samples, as it follows the principles of the Beer-Lambert law where the absorbance of specific functional groups is proportional to the analyte concentration [22]. The obtained FTIR spectra are preprocessed using spectral preprocessing, such as first and second derivative transformations. The aim of the derivative transformations is to increase the sensitivity of the analysis by eliminating interfering spectra [23]. The first derivative is used to enhance spectral resolution and simplify the baseline, while the second derivative is used to remove broad band absorption. The resulting normal and transformed spectra are then analyzed using PLSR and PCR to obtain the best prediction for PO as an adulterant agent in BFO. Statistical parameters used include the coefficient of determination (R2) to determine the

accuracy between actual and predicted values, and the root mean square of calibration (RMSEC) and root mean square error of prediction (RMSEP) to evaluate the precision of the samples (Table 3).

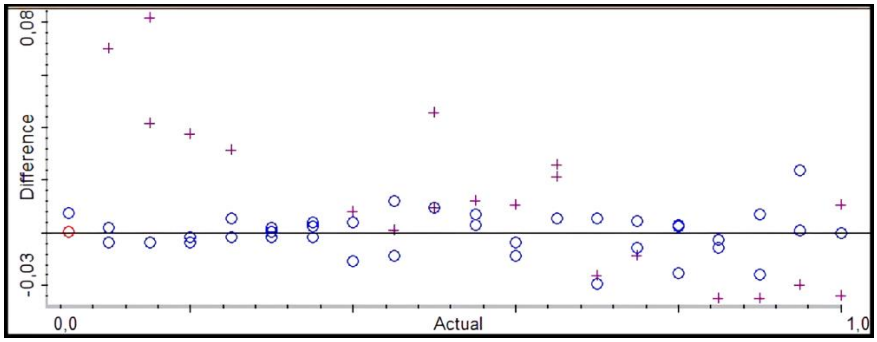
The selection of spectral was based on its ability to provide a high R2 value and low RMSEC and RMSEP values [24]. Based on the normal and derivative spectra, PLSR used the first derivative spectrum in the wavenumber range of 3500-600 cm^{-1} to provide the best model for the relationship between the actual and predicted values of BFO and PO. In the adulteration of BFO with PO, the R^2 values for the calibration and validation models were 0.9966 and 0.9969, with RMSEC and RMSEP values of 0.0249 and 0.0248. These results demonstrate that the use of FTIR combined with LDA and PLSR can be an effective method for authenticating BFO from PO as an adulterant agent. This is indicated by the accurate and precise results, as evidenced by the high R^2 value and low RMSEP and RMSEC values. Figure 4a shows the relationship between the actual and predicted values of BFO and PO, while figure 4b shows the errors that occurred during modeling. It can be seen that the errors that occurred during modeling were randomly distributed around zero difference. This indicates that there were no systematic errors and the developed model can be relied upon to predict BFO contaminated with PO.

Table 3. The wavenumber region for authentication of BFO in binary mixture with PO.

Multivariate calibration	Wavenumber (cm-1)	Spectra	Calibration		Prediction	
			RMSEC	R^2	RMSEP	R^2
PLS	**3500-600**	Normal	0.0272	0.9959	0.0300	0.9954
		1st Derivative	**0.0249**	**0.9966**	**0.0248**	**0.9969**
		2nd Derivative	0.0352	0.9933	0.0413	0.9920
	3000-600	Normal	0.0364	0.9926	0.0298	0.9960
		1st Derivative	0.0672	0.9744	0.0257	0.9970
		2nd Derivative	0.0609	0.9790	0.0361	0.9936
	1800-600	Normal	0.0732	0.9757	0.0425	0.9975
		1st Derivative	0.0678	0.9736	0.0361	0.9940
		2nd Derivative	0.0664	0.9747	0.0405	0.9922
	1500-600	Normal	0.0622	0.9846	0.0520	0.9843
		1st Derivative	0.0582	0.9806	0.0470	0.9909
		2nd Derivative	0.0541	0.9833	0.0589	0.9850
PCR	3500-600	Normal	0.0863	0.9576	0.0372	0.9934
		1st Derivative	0.0699	0.9725	0.0386	0.9930
		2nd Derivative	0.0682	0.9738	0.0476	0.9891
	3000-600	Normal	0.0543	0.9834	0.0409	0.9924
		1st Derivative	0.0681	0.9737	0.0386	0.9926
		2nd Derivative	0.0663	0.9751	0.0497	0.9889
	1800-600	Normal	0.0861	0.9571	0.0338	0.9946
		1st Derivative	0.0687	0.9729	0.0464	0.9899
		2nd Derivative	0.0628	0.9774	0.0462	0.9916
	1500-600	Normal	0.0836	0.9596	0.0664	0.9793
		1st Derivative	0.0855	0.9576	0.0382	0.9928
		2nd Derivative	0.0723	0.9700	0.0446	0.9927



(a)



(b)

Figure 4. The correlation between the actual value of PO with FTIR predicted values facilitated by partial least square calibrations 1st derivative (a) along with residual analysis (b)

FTIR-ATR is able to predict the fatty acid profile of fish oil with other components based on the vibrations of its functional groups [25]. Linear discriminant analysis (LDA) based on FTIR data is able to be the initial step in detecting potential fish oil adulteration. Once potential adulteration has been identified, the next step can be multivariate calibration in the form of PLSR or PCR to quantify it. Again, FTIR is a highly potential method to be developed in fish oil adulteration models.

4. CONCLUSION

Combining ATR-FTIR with chemometric analysis such as LDA, PLS or PCR can classify and quantify adulteration in bawal fish oil. The LDA analysis was able to classify bawal fish oil, palm oil, and their mixtures perfectly. In addition, using multivariate calibration such as PLS or PCR can quantify the adulteration model of bawal fish oil with palm oil, which can be seen from the obtained values of RMSEC, RMSEP, and R2. Therefore, the development of ATR-FTIR spectroscopy methods is very promising for the adulteration detection of other fish oils because FTIR spectroscopy method provide a fast, non-destructive, and environmentally friendly method for the authentication of bawal fish oil and adulterants from other fish oil.

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Original Article

Formulation of Salam (*Syzygium polyanthum* (wight) walp) Leaf Ethanolic Extract Matrix Patch and Its Evaluation

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Abstract: The total flavonoid content in salam leaf has an anti-inflammatory activity at a dose of 2.1% w/w. For topical anti-inflammatory agents, matrix patches have a delivery mechanism in which drugs pass through the skin in a controlled manner for an extended period. Polymers were the patch's main component to control the drug release. Polyvinyl alcohol (PVA) and alpha-cellulose (AC) are polymers that can increase the rate of drug diffusion and form a strong film layer. Salam leaves were extracted by maceration using 96% ethanol. Matrix patches were made with a combination of PVA and AC in ratios of 1:1; 3:1; and 1:3. The patches were tested for physicochemical properties. The stability test was conducted at 4, 25, and 40 °C temperatures for 8 h, respectively. Statistical analysis of physicochemical properties test data used One-way ANOVA with a confidence level of 95%. The results showed that a high concentration of PVA significantly affected the weight and moisture content but did not affect the organoleptic thickness, folding endurance, and pH of the matrix patch. The best matrix patch of salam leaf ethanolic extract was PVA and AC of 3:1 because it met the requirements for the matrix patch, including a weight of 1.96-2.06 grams, thickness of 1.15-1.18 mm, folding endurance >300 times, humidity 25.75 – 30.17% and pH 6.3 – 7.1. The patch contains flavonoids with Rf values for extract and patch of 0.95 and 0.96, respectively. Further study, release, and in vivo anti-inflammatory tests are necessary.

Keywords: polyvinyl alcohol; alpha-cellulose; patch; physicochemical properties

1. INTRODUCTION

Salam leaves are used as food seasoning by Indonesian people [1]. Wientarsih et al. (2007) reported that salam leaves are anti-inflammatory because they contain active compounds such as tannins, flavonoids, and essential oils such as citric acid and eugenol [2]. One of the main components in salam leaves is quercetin which is included in a flavonoid group. According to The Indonesian Herbal Pharmacopoeia (2017), salam leaves contain total flavonoids of 0.40%, calculated as quercetin [3]. Quercetin in salam leaf has anti-inflammatory activity by inhibiting cyclooxygenase and lipoxygenase enzymes [4]. The anti-inflammatory mechanisms of action of salam leaf extract involved inflammatory mediators, such as interleukins (ILs), nuclear factor kappa B (NF-jB), prostaglandin E2 (PGE2), cyclooxygenase (COX) and reactive oxygen species (ROS) [32]. Research conducted by Yusuf et al. (2020) reported that the salam leaf ethanolic extract dose used for patch preparation was 2.1% [5]. The dose has an anti-hyperlipidemic activity also acts as an anti-

inflammatory. The delivery system for the active compound on the matrix patch has a delivery mechanism in which drug molecules pass through the stratum corneum in the skin in a controlled manner for an extended period [6]. This differs from topical preparations such as gels, creams, and ointments given 2-3 times a day but disappears more quickly and absorbs, so it wears off faster. Therefore, to overcome this, it is necessary to develop new formulas for other modern topical preparations, to deliver the active compound by patch delivery system. The matrix patch is one of the topical preparations with adhesive (coating adhesive), which contains a specific dose of a drug to be delivered to the action [7].

Polymers such as hydroxypropyl methylcellulose, carboxymethyl cellulose, and polyvinyl alcohol were the primary constituent components in formulating patches with a matrix system [8]. In this study, the polymers used were polyvinyl alcohol and alpha-cellulose because polyvinyl alcohol adheres well to the skin and forms a film layer that is transparent, strong, and plastic [9]. At the same time, alpha-cellulose can thicken the matrix produced and has the characteristics of being inert and suitable biocompatibility for topical preparations [10]. Previous studies on the formulation of matrix patches have been carried out using a combination of HPMC and PVP polymer ratios at a ratio of 1:1 to produce good physical characteristics [5]. A combination of PVA polymer ratios higher than ethyl cellulose at a ratio of 7:3 can provide good film characteristics and deliver the active substance through a semipermeable membrane in the skin [11]. Alpha-cellulose polymer is soluble in water but will expand if formulated in large quantities of patch preparations. Therefore, the different variations in the concentration of polymers will affect the characteristics of the patch preparation. The ratio of PVA and AC of 1:2 is the chosen formula because it meets the patch requirements, The results showed the combination of PVA and AC patch matrix of *Moringa oleifera* leaves extract had a significant effect on the physicochemical properties there are thickness, patch weight, and moisture content, but they did not affect folding endurance and pH. The percentage of total flavonoid that released from patch was 37.23% for 5 hours. The release kinetics followed the Higuchi kinetics model with a diffusion mechanism [33]. Alpha cellulose has different properties from ethyl cellulose, where AC is hydrophilic while EC is hydrophobic. Based on this, this research is expected to determine the effect of the combination of PVA polymer and alpha-cellulose on the physicochemical properties of salam leaf ethanolic extract patches and to find the combination polymer ratio of PVA and alpha-cellulose that gave the best physicochemical evaluation. Using the TLC method, the chosen formula was then used for was then used for stability test and detection of quercetin content.

2. MATERIALS AND METHODS

Materials: Salam leaves (Ngawi, East Java, Indonesia); 96% Ethanol (repackaged by PT. Bratachem); NaOH (Merck KGaA, Germany); silica gel 60 F254 (Merck KGaA, Germany); n-butanol (Merck KGaA, Germany); acetic acid (Merck KGaA, Germany); Aquadest (CV Nitra Kimia, Yogyakarta); quercetin standard (Sigma Aldrich Production, USA); polyvinyl alcohol (repackaged by PT. Bratachem, Surakarta); alpha-cellulose (Sigma Aldrich Production, USA); phenoxyethanol (repackaged by Cipta Kimia, Surakarta, batch. 028/SE/0817); 70% ethanol solvent; PEG 400 (DOW, Singapore); and propylene glycol (DOW, Singapore). Instruments: Digital balance (Precisa), water bath, stirring rod, sieve No.40, petri dish diameter 5 cm (normax), pH meter (OHAUS), caliper (TOKI), moisture analyzer (OHAUS, Japan), magnetic stirrer (IKA C-MAG HS 7), and oven (Memmert).

2.1. Sample Preparation

Determination of salam plants was conducted at the Biology Laboratory, Mathematics and Natural Science Faculty, Universitas Sebelas Maret, Surakarta, Indonesia. Salam leaves were dried in an oven at 50 °C, powdered using a blender, and then sieved using a sieve of 40 mesh sleve. Salam leaf powder was weighed at 1.0 grams and placed on a the moisture analyzer to analyze the moisture content of salam leaves powder. Salam leaf powder of 500 grams was put into a maceration jar, and then soaked with 2.5 liters of 96% ethanol solvent until the powder was completely submerged. The maceration jar was closed and protected from light for three days and stirred twice daily to homogenize the solvent. The obtained macerate was separated by filtration (filter) using filter fabric. The macerate was evaporated using a rotary evaporator at a temperature of 40 ℃ and followed by a water bath at 50 ℃ for 4 hours until a thick extract was obtained [3]. The extract obtained was weighed, and the yield, moisture content, and detection of active constituents were determined by the TLC method.

2.2. Salam Leaf Ethanolic Extract Tests

The extract yield was the percentage weight (w/w) of the division between the weight of the extract and the salam leaf powder. The yield must reach at least the requirement of the extract monograph [3]. The higher the yield value, the greater the extract that can be obtained from a salam leaves powder. *The organoleptic examination* was carried out to describe the extract's consistency, color, and odor. The purpose of this examination was for the simple initial identification of extracts. Organoleptic activity was a specific parameter of an extract. *Determining moisture content* in the extract aims to provide a minimum limit or range of the concentration of the moisture content in the extract. The higher moisture content makes contaminating the extract easier with fungus or mold. It can reduce the biological activity of the extract during storage. Generally, the required moisture content was less than 10%. Qualitative analysis of the extract was conducted to detect the flavonoid content in the extract by weighing 1.0 grams, then adding 5 drops of a NaOH solution. Identification by TLC method using standard quercetin because it includes the flavonoid group. The stationary phase was a silica gel 60 F254 plate, which was activated by heating in an oven at 105 °C for 10 minutes. The mobile phase was n-butanol: acetic acid: water (4:1:5) in a saturated chamber glass saturated. After being completely eluted, the TLC plate was evaluated for spot appearance formed by a UV lamp at wavelengths of 254 nm and 366 nm [12].

2.3. Formula of Salam Leaf Ethanolic Extract Matrix Patch

Table 1. The patch formula of the Salam leaf ethanolic extract with a variation ratio of polymer

Ingredients	Function	Grams		
		Formula 1 with PVA: Alpha-cellulose 1:1	**Formula 2 with PVA: Alpha-cellulose 3:1**	**Formula 3 with PVA: Alpha-cellulose 1:3**
Extract	Active substance	0.25	0.25	0.25
Polyvinyl alcohol	Hydrophilic polymer	0.50	0.75	0.25
Alpha-cellulose	Hydrophylic polymer	0.50	0.25	0.75
Polyethylene-glycol 400	Plasticizers	0.50	0.50	0.50
Ethanol 70%	Solvent	9.00	9.00	9.00
Propylene glycol	Penetration enhancer	0.50	0.50	0.50
Phenoxyethanol	Preservatives	0.01	0.01	0.01
Aquadest	Solvent	3.00	3.00	3.00

*each formula was replicated three times

All ingredients in Table 1 were weighted according to the matrix patch formula. Alpha-cellulose was dispersed in 70% ethanol solvent, and PVA was dissolved in water at 50 °C. The mixed

solution was stirred using a magnetic stirrer at 500 rpm. Alpha-cellulose was added to the PVA solution and stirred until homogeneous. The salam leaf ethanolic extract was dissolved in 70% ethanol solvent and then put into the mixture. The mixture was added PEG 400, propylene glycol, and phenoxyethanol were added to the mixture. The mixture was stirred until homogeneous and poured into a glass disk mold. The patch preparation was dried in an oven at 40 °C for 3 hours, and then stored at room temperature for 4 days. The patch preparation was tested on the 14th day. The data from day 0, the first week, and the second week were then processed [13].

2.4. Evaluation of physicochemical Properties of Salam Leaf Ethanolic Extract Matrix Patch

The pH test of the matrix patch was carried out by soaking the patch in 10 mL of distilled water for ± 20 minutes at room temperature. The pH meter was immersed in the patch solution. The pH meter's value was the patch's pH valueatch [13]. *The thickness test* of the patch use a caliper with an accuracy of 0.1 mm, and than the measurement results were averaged. *The weight test* of a patch was carried out using three patches that weighed respectively, and the measurement results were averaged [13]. *The folding endurance test* was carried out manually by folding the patch repeatedly on the same line until it tears. The patch meets the requirement if it can be folded up to 300 times [13]. *The moisture content test* of patch preparations was carried out using a moisture analyzer at a temperature of 105 ℃ to a constant weight. Moisture analyzers utilize infrared or halogen lamps as heat sources. The percentage of moisture content of the patch can be seen on the display monitor [13].

2.5. Stability Test

A stability test of the patch was carried out with variations in temperature at time intervals to determine the effect of temperature on the physicochemical properties of the patch [14]. The patch matrix was saved at a low temperature (4±2°C) for 8 hours and then stored at room temperature (28±2°C) for 16 hours. The test was continued in the oven at a high temperature (40±2°C) for 8 hours and then stored at room temperature (28±2°C) for 16 hours. The test counts as one cycle. This test was carried out in 6 cycles or for 12 days. Evaluation of the physicochemical stability of the patch included organoleptic, moisture content, folding endurance, and pH [13].

2.5. Data Analysis

The data was analyzed using the SPSS 21 program. One-way ANOVA test followed if the data were normally distributed to determine the presence of a significant difference between the three formulas in the physicochemical properties test. One-way ANOVA test data results with a significant difference continued with the Post-hoc test with a confidence level of 95%. The patch's physiological stability test result data were analyzed using the paired samples t-test.

3. RESULTS AND DISCUSSION

The determination result showed that the salam plant species used was *Syzygium polyanthum* (Wight) Walp. with the document number UN27.9.6.4/Lab/2021. The results of the organoleptic test of the extract showed that the salam leaf ethanolic extract produced a thick consistency, dark-brown color, and smelled salam leaf. The yield value of the extract was 7.49%; in previous research conducted by Hidayati et al. [15], the yield value of salam leaf maceration with 96% ethanol solvent was 10.21%. The yield value result of this research was lower than that of previous research. This may be because of differences in the maceration process without remaceration and soil nutrient contents that affects the content of the active compounds in salam leaves. In addition, the presence of climatic factors and rainfall can affect secondary metabolites [17]. According to the Herbal Pharmacopoeia Indonesia Edition II (2017) [3], the standard yield value of salam leaves ethanolic extract was at least 18.2%. The moisture content of salam leaf ethanolic extract

was 8.40%. This met the required moisture content of Indonesian Pharmocopeia Edition IV [16] was less than 10%.

3.1. Detection of Active Compounds in Salam Leaf Ethanolic Extract

The salam leaves extract solution was yellowish after the NaOH solution was added. This indicates that the salam leaf ethanolic extract contains flavonoids. This was due to the decomposition by bases into acetophenone molecules, which were yellow due to the cleavage of the bond to the isoprene structure [17]. The principle of UV light 254 nm detection was that the plate provided fluorescence while the sample was dark. The stain appears raised because of the interaction between UV light and the fluorescence indicator on the TLC plate. In contrast, the principle of detection by UV light 366 nm was that the stain gives fluorescence and dark-colored plates, visible stains due to the interaction between UV light and the bound chromophore group (auxochrome) on stains [18] (Figure 1).

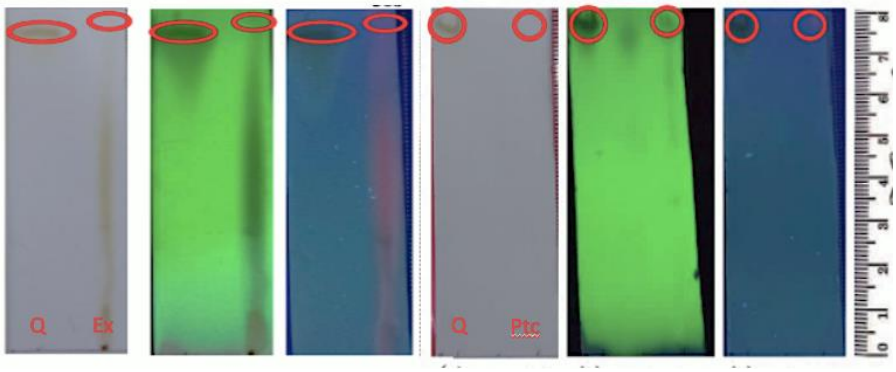


Figure 1. The Result of TLC analysis were the quercetin standard (Q), salam leaf ethanolic extract (Ex), and salam leaf ethanolic extract patch (Ptc). The stationary phase was silica gel F254, and the mobile phase was n-butanol: acetic acid: water (4:1:5). Detection of spots under the UV light at 256 and 366 nm.

The Rf values obtained from standard quercetin (Q), salam leaf ethanolic extract (Ex), and salam leaf patches were 0.95; 0.96 and 0.96, respectively. Previous research by Fitri et al. (2020) [19] obtained and the Rf value of 0.925 for the quercetin standard. The standard Rf value for quercetin was 0.80 [20]. The thin layer chromatography for phenols using a methanolic extract of Centella in the solvent system gave a retention factor (Rf) value of 0.83, similar to that of standard gallic acid [21]. Flavanoids were presence in all extracts with one spot in each (Rf 0.8 for acetone, 0.918 for methanol, 0.816 for chloroform, and 0.737 for aqueous extract) [22]. The results show that the salam leaf ethanolic extract may contain a total flavonoid compound counted as quercetin, because it was close to the Rf value of the quercetin standard.

3.2. Matrix Patch Formula of Salam Leaf Ethanolic Extract

The results of an organoleptic test on the three formulation of salam leaf ethanolic extract patches showed a dark brown appearance, smooth surface but not even, it may because the saponin compound in salam leaf that appearanced foam when it was stirred, and a salam leaves an odor. Organoleptic observations on patch preparations on day-0, days 7, and days 14 did not change color or odor during storage. These results indicate that the variation in the ratio of PVA and alpha-cellulose polymer does not affect the organoleptic properties of the three formulas, so it can be concluded that they are quite resistant to environmental changes during storage (Figure 2).

Figure 2. The patches of salam leaf ethanolic extract with variation ratios of PVA-alpha cellulose of 1:1 (Formula1); 3:1 (Formula 2); and 1:3 (Formula 3).

Table 2. The Results of physicochemical test of salam leaf ethanolic extract patches for 14 days in room temperature

Formula	Patch weight (mg)		Patch thicknes (mm)		Moisture content (%)		pH value	
	Day-0	Day-14	Day-0	Day-14	Day-0	Day-14	Day-0	Day-14
Formula 1 with PVA: Alpha-cellulose 1:1	1.66±0.10	1.52±0.10[a]	1.15±0.01	1.11±0.01 [b]	25.36±0.42	21.61±1.02	7.0±0.2	6.3±0.2
Formula 2 with PVA: Alpha-cellulose 3:1	2.06±0.03	1.96±0.05	1.18±0.01	1.15±0.02	30.17±0.68	25.75±2.16	7.1±0.2	6.4±0.1
Formula 3 with PVA: Alpha-cellulose 1:3	1.89±0.09	1.76±0.03	1.19±0.01	1.13±0.02[b]	25.71±0.63	20.86±0.24[c]	7.2±0.2	6.2±0.2

*a,b,c significantly different ($p<0.05$); mean±SE

The weight uniformity test of the salam leaf ethanolic extract patch aims to determine the uniformity of the weight of the patch to evaluate the consistency of the manufacturing process. An exemplary process of manufacture would produce uniformity of active substance content in every patch. A slim patch matrix would be preferred because it was convenient to use. The increase in the patch's weight was related to its constituents' polymer properties. Formulas with a higher PVA ratio gained weight after 14 days compared to other formulas. PVA polymer is a hygroscopic polymer. Hygroscopic properties could bind moisture during the manufacturing process and storage patch so that the weight of the patch increases [23]. In contrast, alpha-cellulose is also hydrophilic, which accelerates the diffusion process of active substances [24]. The formula with a ratio of PVA-alpha cellulose of 1: 1 and 3: 1 for 14 days of storage showed no significant difference ($p<0.05$) compared to the formula with a ratio of 1: 3. This is because 70% ethanol used as a solvent will evaporate during the drying process. Ethanol has a lower relative boiling point, so it evaporates quickly. The polymer ratio of PVA and alpha-cellulose significantly affected the increase in patch weight ($p<0.05$).

The thick patch affects the release of the active substance, which is made longer than a gel or cream [5]. The thicker patch film matrix was also less desirable because it lacked comfort when used [13]. Polymer properties influence increased patch thickness. The formula with alpha-cellulose has different properties from ethyl cellulose, where AC is hydrophilic while EC is hydrophobic. The highest PVA ratio was retained and bound in the patch preparation, thus affecting the thickness of the patch [25]. Variations in the PVA and alpha-cellulose polymer concentrations significantly affected increasing patch thickness ($p<0.05$). Due to the use of PVA polymer, this matter could improve the characteristics of elastic and strong films [9]. While the alpha-cellulose is hydrophilic, it may cause the active substance to penetrate dissolution media easily [26]. The research by Ermawati 2022 reprted that The ratio of PVA and AC of 1:2 in Moringa patch released total flavonoid from patch was 37.23% for 5 hours [33]. There was a significant difference in the formula with a ratio of

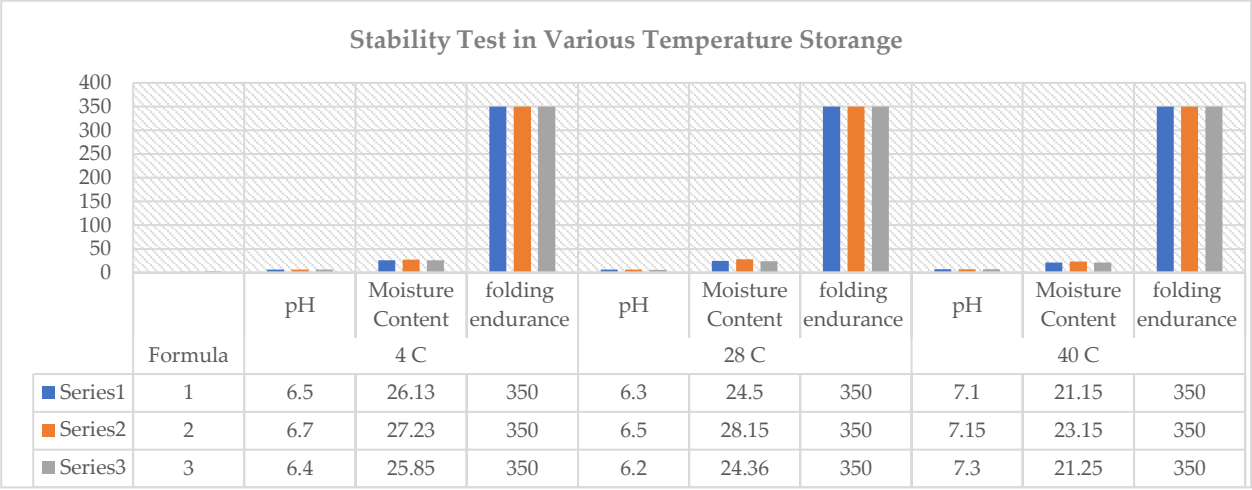
PVA and alpha-cellulose of 1: 1 and 1: 3 for 14 days of storage ($p<0.05$). This was because the PVA polymer could absorb the solvent in the patch during storage [25].

The increase in patch moisture affected the polymer properties. The high PVA polymer ratio in the formulas greatly increases the moisture value. This was due to PVA being easily soluble in water, so water would be retained in the patch for drying at the time of formulation [27]. This could retain moisture in the patch. The variation in the PVA polymer and alpha-cellulose ratio affected patch moisture ($p<0.05$). The high the concentration of PVA polymer used, the higher the value of the moisture of the patch preparation. In addition, propylene glycol in the patch could attract water to affect the skin's hydration by softening the keratin layer in the stratum corneum, thereby increasing the amount of active substance that penetrates [28]. The formula with a ratio of PVA-alpha cellulose of 1:3 for 14 days of storage had a significant difference ($p>0.05$) compared to other formulas because of the effect of the added PVA concentration, which causes fluid to be retained in the patch.

The three formulas meet the pH requirements of preparations that do not irritate the skin, namely 5-9 [29]. However, the variation in the ratio of PVA polymer and alpha-cellulose did not affect the pH of the patch ($p>0.05$). The three formulas for 14 days of storage showed no significant difference ($p<0.05$). This was because oxygen oxidized the water as a solvent due to the testing process at room temperature.

The folding endurance of the three salam leaf ethanolic extract patch formulations met the requirement because it obtained a folding endurance value of more than 300 times during storage for 14 days. The results obtained have good integrity when applied to the skin. The variation ratio of PVA and alpha-cellulose polymer in each formula does not affect patch folding endurance. However, adding PEG 400 as a plasticizer may affect the folding endurance of the patch because it could increase elasticity and reduce the risk of tearing [30].

3.3. Stability Test of Salam Leaf Ethanolic Extract Matrix Patch



	Formula	4 C			28 C			40 C		
		pH	Moisture Content	folding endurance	pH	Moisture Content	folding endurance	pH	Moisture Content	folding endurance
Series1	1	6.5	26.13	350	6.3	24.5	350	7.1	21.15	350
Series2	2	6.7	27.23	350	6.5	28.15	350	7.15	23.15	350
Series3	3	6.4	25.85	350	6.2	24.36	350	7.3	21.25	350

Figure 3. The results of the stability test of salam leaf ethanolic patches at various temperatures

Factors affecting physical stability were radiation, heat, light, and humidity [31]. Tests were carried out for each formula to determine the stability of patches ~~of~~ stored at cool (4 °C), room (28 °C), and hot temperatures (40 °C). Furthermore, the instability of a product can be observed through physical changes, including color, surface appearance, and odor by organoleptic analysis. Preparations can be

stable when they do not change during the specified storage limits. This is because the elasticity of patch storage at cool, room, and hot temperatures can be changed. The patch becomes moist and the color after being stored at cool temperatures changes to pale due to the cooling process. In contrast, the color darken when stored at room and hot temperatures. The patches stored at higher temperatures and lost water and showed a dark color at the condition of 40 ◦C. The patches stored at room temperature (28 ◦C) had no difference in color was observed. This indicated that room temperature did not cause a color change.

4. CONCLUSION

An increased ratio of PVA polymers in a combination of PVA and alpha-cellulose polymer on the physicochemical properties of the salam leaf ethanolic extract patch can increase the patch's weight, thickness, and moisture content but does not affect folding endurance and pH. The selected formula is a formula with a ratio of PVA and alpha-cellulose of 3:1 because it showed the best physicochemical properties of the patch, such as weight of 1.96 – 2.06 grams; patch thickness of 1.15-1.18 mm; patch folding endurance > 300 times; the patch moisture content of 25.75 – 30.17% and pH value of 6.3 – 7.1. The selected formula was identified using TLC analysis containing a quercetin compound with an Rf value of 0.96. Temperature variations significantly affected organoleptic (color and surface texture) and moisture content but did not affect folding endurance and pH. Further study, release and in vivo anti-inflammatory test is necessary.

Conflicts of interest: The authors declare no conflicts of interest.

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Review Article

Formaldehyde Content in Indonesian Food and the Analysis Method: A Review

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Abstract: Apart from being rich in culture, Indonesia is also rich in food culture. However, in some cases, there are illegal additions of formalin (37% formaldehyde) to food to extend shelf life. If humans consume food containing formaldehyde, it will have a harmful impact. Formaldehyde has the potential to cause cancer. In recent years, formaldehyde has still been found in several Indonesian foods. There are several analytical methods developed to detect formaldehyde levels in food. This review overview of the formaldehyde effect to human health, formaldehyde content in fish and Indonesian food, and the detection methods.

Keywords: formaldehyde; Indonesia; food; analysis

1. INTRODUCTION

Indonesia is a diverse country that has various cultures. In line with its diverse culture, food in Indonesia is also diverse. However, there are illegal acts of deliberately adding formaldehyde to food to extend its shelf life. Formaldehyde (FA) (CH_2O) is a type of colorless gas at ambient temperature. In liquid form, FA commonly known as "formalin" (37 up to 40 % w/w formaldehyde), is a low-cost chemical [1, 2]. FA is normally used in embalming to disinfect and preserve human corpses [3, 4]. In the food industry, FA is used to inhibit bacterial growth during juice production of sugar and as a bacteriostatic agent in some varieties of Italian cheese and fresh white cheese in El Salvador [1,5-6]. FA is a useful and important chemical to the global economy and is used in many industries i.e., construction (wood processing, furniture, textile, carpeting), consumable household product industries (antiseptics, medicines, cosmetics, dish-washing liquids, glues, lacquer), etc [7].

FA is a carcinogenic and hazardous substance for human health [2, 6], therefore FA is prohibited for use as food preservatives. But FA content is still found in some foods such as noodles [9], fish and seafood [10], fruits and vegetables [9, 10], fruit juice [13], mushrooms [14], and milk [15] because it has low-cost and effective as food preservatives.

The addition of formaldehyde to food poses a significant threat to Indonesia's diverse food culture in several ways:

1. Health Risks: The consumption of food contaminated with formaldehyde poses serious health risks to consumers. Given its toxic nature, prolonged exposure to formaldehyde-laced foods can lead to various health complications, undermining the well-being of the population [16-18].

2. Cultural Degradation: Indonesian cuisine is characterized by its vibrant flavors, fresh ingredients, and traditional cooking methods. The use of formaldehyde compromises the authenticity and integrity of traditional dishes, distorting their original taste and quality. This threatens to erode the cultural significance of Indonesian cuisine over time.
3. Economic Impact: Indonesia's culinary heritage is not only a source of cultural pride but also a significant driver of the economy, supporting local farmers, food producers, and businesses. The tarnished reputation resulting from the presence of formaldehyde in food products can lead to decreased consumer trust and demand, negatively impacting the livelihoods of those involved in the food industry.
4. Environmental Concerns: The illegal use of formaldehyde in food production may also have adverse environmental effects, such as contamination of water sources and soil degradation, further exacerbating ecological challenges faced by the country.

This review overview of the formaldehyde effect on human health, formaldehyde content in fish and Indonesian food, and the detection methods of formaldehyde content in Indonesian food.

2. METHODS

The articles related to the formaldehyde content in Indonesian food were identified and selected from databases. The databases used in this review were ScienceDirect, PubMed, and Google Scholar with keywords of formaldehyde+ Indonesian food. Inclusion criteria for the articles were open access and published in the last 20 years.

3. RESULTS AND DISCUSSION

3.1. Formaldehyde effect to human health

FA is highly toxic and carcinogenic [14, 15]. FA has been reported as one of the chemical mediators that caused programmed cell death (apoptosis). Since FA can generate crosslinking with proteins and DNA, this action may alter the mitochondrial membrane, open the mitochondrial transition pore, release cytochrome c into the cytosol, activate caspases and induce apoptosis [16, 17].

FA can cause respiratory symptoms, irritation of eyes, nose, and throat when inhaled by humans [23-26]. Increasing exposure can increase the risk of cancers in pharynx, nasopharynx, and brain, as well as dermatitis and allergic reactions [27-29]. Acute effects of airborne formaldehyde exposure: Odor detection, 0.05-1.0 ppm; Eye irritation, 0.01-2 ppm; Upper respiratory tract irritation (e.g., irritation of the nose or throat), 0.10-11 ppm; Lower airway irritation (e.g., cough, chest tightness, and wheezing), 5-30 ppm; Pulmonary edema, inflammation, pneumonia, 50-100 ppm; Death >100 ppm [20, 21].

In the case of food additives, the effects of CH_2O on the human body depend mainly on the volume and concentration in which the formaldehyde is present. For example, given a large quantity of CH_2O, the formic acid produced during the metabolism process can cause local corrosive action on the gastrointestinal tract; resulting in both oral and gastrointestinal mucosa. Moreover, the ingestion of 90 ml or more of 37% formaldehyde solution leads almost inevitably to death (more than 100 ppm) [20, 21].

3.2. Formaldehyde content in Indonesian food

Table 1 was shown the formaldehyde content in Indonesian food. The imported fruit (apple, pear, and grape) that was sold in Manado was analysed [9]. The research was found that the washing

fruits contain 0.060-0.136 ug/mL and unwashed fruits contained 0.080-0.195 ug/mL of formaldehyde. It was proven that imported fruit still contain formaldehyde. Noodles that were sold in Ambon contained 9.07-10.01 mg/kg of formaldehyde [7]. While in West Java, wet noodle was found containing formaldehyde in range 801-1684 mg/kg [32]. Kembung and Cucut salted fish from Bandung were found containing formaldehyde at level 0.252 and 0.482 ppm, respectively [33]. From the literature studies conducted, formalin was mostly found in wet fish samples.

Table 1. FA content in fish and Indonesian food

Place	Sample	Instrument Analysis	FA Levels	Reference
Manado	Apple, pear, and grape (Imported fruit)	UV-Vis spectrophotometry	Washed fruits: 0.060-0.136 ug/mL	[9]
Ambon	Noodle	UV-Vis spectrophotometry	9.07-10.01 mg/kg	[7]
Bandung	Kembung salted fish and Cucut salted fish	UV-Vis spectrophotometry	0.252 ppm and 0.482 ppm	[33]
Bali	Moonfish (Lampris guttatus)	UV-Vis spectrophotometry	133 mg/kg	[34]
West Java	Wet noodles	UV-Vis spectrophotometry	801-1684 mg/kg	[32]
Jakarta	Saurida tumbil fish	NMR	14 mg/kg	[35]
Hindia Ocean	Opah fish (Lampris guttatus)	UV-Vis spectrophotometry	27.82 ± 1.66 mg/kg to 133.12 ± 1.56 mg/kg	[36]
Banten	Short mackerel (Rastrelliger brachysoma	UV-Vis spectrophotometry	1.4-1.7 ppm	[37]
Jakarta	Moonfish (Lampris guttatus), hardtail scad (Megalaspis cordyla), rank goby (Saurida tumbill), and mackerel scad (Decapterus spp).	UV-Vis spectrophotometry	0 to 6.51 mg/kg	[38]
North Sumatera	Bawal, Kerapu, Kakap, Tuna, Tongkol	Formaldehyde test kit	Tongkol: 1.73 mg/L; Tuna: 1.40 mg/L; Bawal: 0.528 mg/L; Kakap: 3.42 mg/L; Kerapu: 2.47 mg/L	[39]
West Java	Rebon, Teri Medan, Teri Cue, Pakang	UV-Vis spectrophotometry	Rebon: 0.389 ppm Teri Medan: 0.156 ppm Udang Rebon: 0.389 ppm Teri Cue: 0.182 ppm	[40]
South Sulawesi	Anchovy	UV-Vis spectrophotometry	0.196-0.270 ppm	[41]
West Kalimantan	Snakehead dried salted fish	UV-Vis spectrophotometry	0.64-0.83 ppm	[42]

3.3 Analysis method to determine formaldehyde content

Some analysis method was developed to analyse formaldehyde content in fish and food. For qualitative analysis, formaldehyde can be determined using a formaldehyde test kit and TLC (thin layer chromatography) [33,34]. While quantification of formaldehyde can use UV-VIS spectrophotometry [11]; TLC-densitometry [32]; HPLC (High-performance Liquid Chromatography) [45]; NMR (nuclear magnetic resonance) [35]; and gas chromatography-mass spectrometric (GC-MS) [35]. Bianchi et al., [46] evaluated formaldehyde content in different frozen fish products using a Solid Phase Micro Extraction Gas Chromatography Ion Trap Tandem Mass Spectrometry (SPMEGC-MS) method based on fiber derivatisation with pentafluorobenzyl hydroxylamine hydrochloride. Table 1 shows the analysis of formaldehyde in Indonesian food and the analysis methods.

In the Spectrophotometric method, formaldehyde cannot be directly analysed using UV-VIS spectrophotometry because it does not have a chromophore. Formaldehyde must react with Nash reagent (acetylacetone, ammonia) to form 3,5-diacetyl-1,4-dihydrotoludine. This reaction is called the Hantzch reaction, the mechanism was shown in Figure 1[22]. This form has maximum absorption at 550 nm [9]. Another way to analyse formaldehyde can react with 4-Amino-3-hydrazino-5-mercapto-1,2,4-triazol (AHMT), pararosaniline, 3-methyl-2- benzothiazolonehydrazone (MBTH) and chromotropic acid etc., but the colorimetric methods in general are relatively slow and less sensitive [17,37].

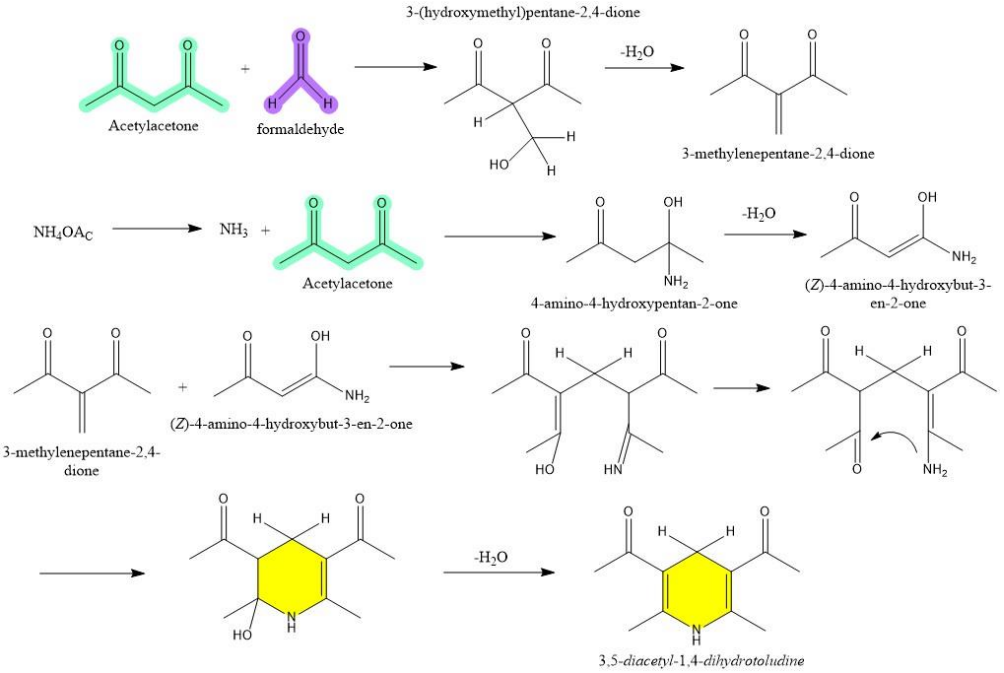

Figure 1. The mechanism in Hantzch reaction[48]

High-Performance Liquid Chromatography (HPLC) method having better selectivity, precision, and accuracy were proposed to analyse formaldehyde in squid product [49]. In the determination of formaldehyde using HPLC, the most often used derivative reagent before analysis is DNPH (2,4-dinitrophenylhydrazine), which reacted with formaldehyde to form the corresponding hydrazone [50]. The reaction between formaldehyde and DNPH was shown in Figure 2. Wang et al, [51] analyse the formaldehyde content in fruit juice samples using magnetic strong cation-exchange resin modified with DNPH. The procedures of extraction and derivatization were carried out in a single step by stirring the resins and diluted fruit juice with water. When the procedures were

completed, the resins adsorbing the HCHO–DNPH derivative were easily separated from the sample matrix by an adscititious magnet. The HCHO–DNPH derivative eluted from the resins was directly determined by high-performance liquid chromatography with UV detector at 360 nm [42].

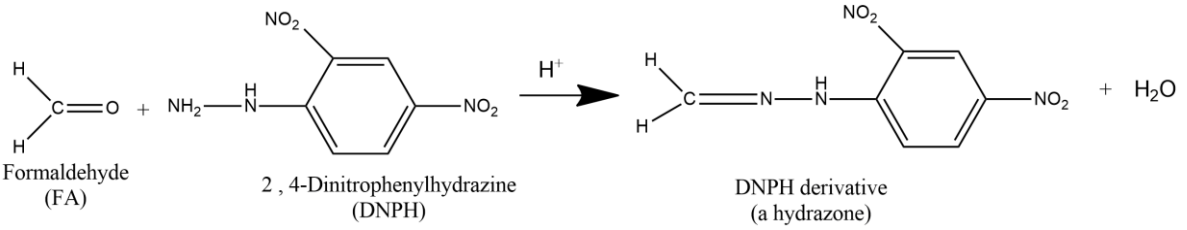

Figure 2. Reaction between formaldehyde and DNPH

3.4 Preventing the use formaldehyde in Indonesian foods

Protecting Indonesia's culinary heritage is crucial for preserving its cultural identity, promoting public health, and sustaining the country's economic development. These measures include:

a. Legislation and Enforcement: Indonesia has regulations in place that prohibit the use of formaldehyde as a food additive. The Food and Drug Supervisory Agency (BPOM) is responsible for enforcing these regulations and ensuring compliance with food safety standards. BPOM conducts inspections of food production facilities, tests food samples for chemical contaminants, and imposes penalties on violators found using formaldehyde or other harmful additives.
b. Public Awareness Campaigns: The government has also launched public awareness campaigns to educate consumers about the dangers of consuming food products contaminated with formaldehyde. These campaigns aim to raise awareness about food safety practices, encourage vigilant consumption habits, and empower consumers to report suspected cases of food adulteration to authorities.
c. Collaboration with Stakeholders: The Indonesian government collaborates with various stakeholders, including food producers, retailers, and industry associations, to promote compliance with food safety regulations and encourage responsible practices along the food supply chain. This collaboration involves providing guidance on proper food handling and storage techniques, as well as supporting initiatives to develop alternative preservative methods that are safe and effective.

Despite these regulatory efforts, challenges remain in effectively combating the illegal use of formaldehyde in food products in Indonesia. Some of these challenges include: inadequate regulatory oversight, and the presence of informal or unregistered food producers operating outside the purview of regulatory authorities. These factors can enable the continued illegal use of formaldehyde in food production despite existing regulations.

4. CONCLUSION

The illegal addition of carcinogenic formaldehyde to food is a serious concern to public health. Several analytical methods can be used to analyze the formaldehyde content in food, either for qualitative or quantitative analysis.

Foods containing formaldehyde are still found in Indonesian foods until now. So, consumers must be careful in choosing the food that they consume. Here are some recommendations and preventive actions consumers can take to avoid foods containing formaldehyde:

a. Choose Fresh Foods: Option for fresh foods over processed or packaged ones whenever possible. Fresh produce, meats, and seafood are less likely to contain added preservatives like formaldehyde.
b. Buy Organic products: Organic products are less likely to contain synthetic preservatives like formaldehyde. Look for certified organic labels when shopping for fruits, vegetables, meats, and other food items.
c. Avoid Highly Processed Foods: Processed foods, such as canned goods and ready-to-eat meals, often contain preservatives, including formaldehyde. Minimize consumption of these items.
d. Inspect Seafood: Formaldehyde is sometimes used to preserve seafood. When buying fish or shellfish, ensure they are fresh and have no off-putting odors. Avoid seafood that appears unnaturally firm or glossy.
e. Proper Storage: Properly store perishable foods in the refrigerator or freezer to prevent spoilage and the need for preservatives. Use airtight containers or wraps to maintain freshness.
f. Rinse Fruits and Vegetables: Wash fruits and vegetables thoroughly under running water to remove any surface residues, including potential traces of formaldehyde. Scrub firm produce with a brush to remove dirt and contaminants effectively.
g. Peel and Trim: Peeling and trimming fruits and vegetables can help remove surface contaminants, including any residual preservatives. However, keep in mind that some nutrients are located just under the skin, so balance this consideration.
h. Choose Trusted Suppliers: Purchase foods from reputable sources and suppliers known for their adherence to food safety regulations. Farmers' markets, local farms, and trusted grocery stores may offer fresher and less preserved options.
i. Stay Informed: Keep yourself updated on food safety guidelines and any recalls related to formaldehyde contamination. Government health agencies often provide information on food safety and recalls through websites, newsletters, and social media platforms.

By following these recommendations, consumers can reduce their exposure to formaldehyde in foods and make healthier choices for themselves and their families.

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Original Article

Study of Chemical Composition and Evaluation of Anti-Hypertensive Effect of A Fixed Oil Extracted From *Linum usitatissimum* Grains

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Abstract: Globally grown flaxseed is regarded as an oilseed with numerous medicinal and health benefits such as: lowering blood pressure, blood sugar, decreasing the risk of neurological and obesity-related illnesses. The objective of this study is to extract the flaxseed oil and to characterize it chemically and physically; by pressing the seeds into two different temperatures: 67°C and 90°Coil yields; 24% and 29% were obtained from flaxseed. The followings are the outcomes of the physico-chemical analyses: pH value: 0.476; saponification index: 168.3; KOH/g: 28.05; peroxide index: 174; density: 0.951; refractive index: 1.482 at 18.1°C and 1.485 at 18.2°C; peroxide index: 1.70 at 0.96, 1.70 mg/100; all are related to the peroxide index. In this study we tried to test the" anti-hypertension "activity of this oil by simulating a blood cycle in the laboratory which allowed us to determine the variation in blood pressure as a function of the volume of oil injected into the blood cycle from 17.4 to 14.10.

Keywords: Linum usitatissimum ; linaceae ; oil ; HPLC ; Hypertension .

1. INTRODUCTION

Since antiquity medicinal plants have been the most fertile source of leads for medication development, global studies have been conducted to validate their efficacy, and some of the findings have been resulted in the manufacture of plant-based medications[1], [2]. Flaxseed is the seed from the flax plant (*Linum usitatissimum L.*), which is a member of the Linaceae family [3], [4]. The generic name "Linum" comes from Celtic word Lin means'thread' and the species name "*usitatissimum*" given by Carl Linnaeus, which means very useful [5,6]. Many Linum species are related to flaxseed but only *Linum usitatissimum L.* is grown for commercial production of oil[7]. flaxseed is among the oldest crop plants cultivated for the purpose of oil and fiber, medicines and textiles; therefore, it is been of great importance for human culture and development for more than 8,000 years[6], [8]. Flax seed lignans have shown promising results for treatment and prevention of several types of cancer[9]. The short-stemmed flaxseed bears seeds of high oil [10].The oilseed flax (linseed) (*Linum usitatissimum L.)* is predominantly the source of valuable oil, in which the most appreciated are omega-3 fatty acids where it contains cyclic hydrophobic peptides known as "cyclolinopeptides" which influence blood pressure and which are composed of eight or nine amino acid residues[11], [12]. Flaxseed is a promising candidate for improving various aspects of

health markers such as cholesterol levels, inflammation, blood pressure, cardiovascular diseases remain a leading cause of mortality worldwide, prompting researchers to investigate dietary interventions that could help reduce the risk factors associated with heart disease. The lignans found in flaxseed are known for their antioxidant properties[11], [12], which could potentially reduce inflammation and oxidative stress in the body. Overall, it is expected that regular consumption of flaxseed may have a positive impact on cardiovascular health. The objective behind this investigation was studying the chemical composition of flaxseed oil extracted by two methods (cold and hot), and comparing the quality of the oil plant based on international criteria. A preliminary in vitro study was carried out on the antihypertensive effect of flaxseed oil using an experimental device developed in our laboratory (Lab. MBSC).

2. MATERIALS AND METHODS

Flaxseed is grown in various regions around the world due to its versatility and nutritional benefits, thrives in cooler regions with temperate climates such as Canada, Russia, China, particularly the northern states, These regions provide the ideal conditions for flaxseed cultivation, including well-drained soil and moderate temperatures.

2.1. Extraction of the oil flax seed

Flaxseed oil was extracted in an amount of 2 kg using a cold or hot pressing device at a temperature of 67°C and 90 °C; 580 ml and 480 ml of oil were obtained respectively. Finally the filtration process was carried out to separate the oil from impurities.

2.2. Chemical analyses

2.2.1. Acid Index

We weigh 2 g of flaxseed oil, add 5 ml of 95% ethanol and 5 drops of 0.2% phenolphthalein (PP), then neutralize it with an ethanol solution of KOH (0.1 mol/l) until a pink color is obtained. The index acid is calculated by the following formula.

$$\mathrm{AI}(\%) = \frac{\mathrm{M(KOH)\ x\ V\ x\ C}}{m}$$

AI: Acid Index

M (KOH): Molar mass of potassium hydroxide.

V: Volume of potassium hydroxide.

C: Concentration of potassium hydroxide.

m : The mass

2.2.2. Saponification Index (SI)

The saponification index corresponds to the number of milligrams of potash necessary to saponify the fatty acids contained in a gram of fat. This involves treating the ester with sufficient concentrated and hot potash, which regenerates a following reaction. Total alcohol and potassium salt of the acid, 2g of flaxseed oil, then 25 ml of KOH added with a concentration of 0.5 mol/L, we heat it for one hour and add 0.5 ml of 0.2% phenolphthalein. Finally, the excess KOH is titrated with the hydrochloric acid (HCl) solution at 0.5 mol/l until the phenolphthalein turns colorless. The saponification index (SI) is determined as follows:

$$\mathrm{SI} = \frac{(\mathrm{V0} - \mathrm{V1})\ \mathrm{x\ C\ x\ 56.1}}{m}$$

SI : Saponification index

V0: Volume of hydrochloric acid necessary to titrate the blank.
V1: Volume of hydrochloric acid necessary to titrate the test.
C: Concentration of the standard hydrochloric acid solution.
56.1: The molecular weight of KOH
m: The mass

2.2.3. Peroxide Index (PI)

The peroxide index is the number of micrograms of active oxygen content in a large body of water and susceptible to potassium iodide. It is the first microgram of a gram or more of a millimeter of equivalent oxygen activity per kilogram, and this solution is suitable for a solution in a mixture of acetic acid (CHOOH) and chloroform, treated with one of the solutions iodure of potassium (KI). The iodine released by a solution of sodium thiosulfate ($Na_2S_2O_2$) is titrated in the presence of starch (colored indicator).

Two grams flaxseed oil was used, 10 ml of chloroform and 15 mL of acetic acid are added, then 1mL of saturated potassium iodide (KI) solution for 5 min away from light. 75 ml of distilled water and few drops of starch was added and the iode library with the solution of sodium thiosulfate $Na_2S_2O_2$ (0.002N) is agitated with the total decoloration of the solution. The index of peroxyde (IP) causes the main effect:

$$\mathrm{PI} = \frac{\mathrm{N(V1 - Vn)}}{m} \mathrm{x}\, 1000$$

PI : Peroxide Index
Vn: The indicator volume which equals 0.
V1: The volume of sodium thiosulfate necessary for the determination.
N: The exact concentration of the standard solution of sodium thiosulfate.
m: The mass

2.2.4. Iodine Index (II)

The index on the iode is the mass of the iodegrammes fixed on the doubles placed on 100 g of flesh. Addition to a solution of monochlorous iodine in a mixture of acetic acid and tetrachlorure of carbon. Titrate with the sodium thiosulfate ($Na_2S_2O_3$) solution (0.0992 N) until a yellow color is obtained. A few drops of starch and continue the titration until the blue color disappears after vigorously shaking the contents and a blank test in the same way was prepared. This device is worn as a suit:

$$\mathrm{II} = \frac{(12{,}69\mathrm{C(VI - V2)})}{M}$$

II: Iodine Index
V1: The volume of sodium thiosulfate required for the blank.
V2: The volumeof sodium thiosulfate required for the sample.
C: The normality of sodium thiosulfate solution.
M: The weight of the sample in grams

2.2.5. Indication of reaction

The index of the reaction is the rapport into the sinus of accidental angles and the reaction of the long rayon on the day, allowing the air in the air to be maintained at a constant temperature. To the

fractomètre, at 20°C for the oils. On the other hand, the shell of the drop line on the blade fractometer, the reflection index value directly.

2.3. Polarimeter and rotary power

The rotational power of a substance is measured using a polarimeter. Biot'slaw (a= $[a]_T$.l.c) makes it possible to link the specific rotating power of a molecule to the angle of deviation measured under given conditions.

2.4. UV-Vis spectroscopy analysis

The device used is callityimovation ANALYTIC JANA type with an interval ranging from 190 nm to 800 nm controlled by the software. We put a quantity of flaxseed oil with a solvent of Butanol-2 in a quartz tank with 5ml; start the final analysis in order to obtain the specific spectrogram for the sample.

2.5. IR spectroscopic analysis

The device used is Spectro of 50 DO249Tj Part N°: 56070646. Fourier transform infrared spectroscopy (FTIR) is a measurement technique for the acquisition of infrared spectra; a drop of the sample is placed on an IR-transparent material, Launches the analysis from the software and can directly obtain our infrared results

2.6. High-Performance Liquid Chromatography (HPLC)

Chromatography in liquid phase at high performance is a powerful analytical technique used to separate, identify, and quantify components in a mixture. It is a form of liquid chromatography that is widely used in various fields such as pharmaceuticals, food and beverage analysis, environmental monitoring, forensic science. The preparation of the sample for HPLC analysis is a crucial step to ensure accurate and reliable results. The HPLC system used was a SHIMADZU Scientific Instruments' system LC-20A (Shimadzu, Kyoto, Japan) with an injector of 20 µl Rheodyne 1907 sample loop, a pump LC-20A, a vacuum degasser DGU-20A5, and a Shimadzu SPD-20 with a variable wavelength ultraviolet (UV) detector. Chromatographic data were collected, stored, and analyzed using the LC Lab solution software (Shimadzu, Tokyo, Japan) [11], [12].

Chromatographic condition: We diluted a few drops of flaxseed oil in an acetonitrile solution ; then placed the sample in special HPLC tubes , The mobile phase used was acetonitrile with avolume injection of 20 µL at a flow rate of 0.5 mL/min, Chromatographic separations were conducted at ambient temperature and UV detector was set at 254 nm.

2.7. Miniature model of blood circulation system

To better discover the impact of this flaxseed oil on a possible reduction in blood pressure, we have developed a small device in the laboratory of bioactive molecules and chiral separation (LMBSC) which is used to simulate the blood circulation of an adult individual. The combination of these principles of suction and discharge results in a powerful self-priming positive displacement action. This pump is the subject of a perfect simulation of the functioning of a human heart. By activating the pump, the mixture begins to pump (150ml physiological serum + flaxseed oil) with a temperature maintained at 37°C, the mixture then passes through a pipe connected to an electronic tensiometer in order to measure the tension of the mixture in the system. Since the pump is equipped with a control that is used to adjust the flow rate, the flow rate is increased to simulate high blood pressure.

3. RESULTS AND DISCUSSION

3. 1. Flaxseed Oil Extraction

In our work we practiced the two processes at 67°C and 90°C in order to make a comparison in terms of quality of flaxseed oil and extraction yield. Flaxseed oil was extracted by the extraction method using an oil expeller with filter machine and the following results were obtained:

Table 1. Extraction yield of fixed flaxseed oils

Extraction temperature	Mass (g)	Color of HF	Yield (%)
67°C	551.58	Light yellow	24 %
90°C	456.48	Brown	29 %

From the results obtained we note that the extraction process at 90°C of flaxseed oil has a higher yield than the extraction process at 67°C (Table 1), but with different quality; initially we base ourselves on the color of the oil which changes from yellow to brown which can be explained by the oxidation of the heated oil.

3.2. Analysis characterization and chemical composition of fixed oils

3.2.1. Physico-chemical analysis of flaxseed oil

The evaluation of the physicochemical parameters of flaxseed oil according to AFNOR standards made it possible to find good results presented in Table 2.

Table 2. Physico-chemical parameters of flaxseed oil

	T=67°C	T=90°C
Acid index (mg de KOH/g oil)	0.2805	0.476
Saponification index (mg of KOH /g of oil)	168.3	28.05
Peroxide index meqdO2/kg of oil	0.96	1.70
Iodine index (g/100 g oil)	174.23	210
Refractive index	1.482(18.1°C)	1.485(18.2°C)
Density ($g.cm^{-3}$)	0.951	0.951

According to the results obtained, we notice a similarity in the density and in the refractive index since they are almost the same physical properties of this liquid, while there is an average difference in the others parameters (acid index, saponification index, peroxide index and iodine index). It can be deduced that there is a slight difference in the physicochemical properties between its two products.

a. Acid Index

Knowing the acid index of a fatty substance is a good way to determine its alteration by hydrolysis. It is a criterion for the purity of the oil flaxseed oil standards.

b. Saponification Index

The saponification index is related to the length of the fatty acids constituting the oil. Knowledge of the saponification index of a fatty substance tells us about the length of the carbon chain of the acids constituting this fatty substance. The saponification index of a fatty substance is higher when the carbon chain of the fatty acids is short [13]. The saponification index of our flaxseed oil (168.3 and 28.05) is close to the range of the codex alimentarius standard which set them between 187 and 197 [14].

c. Peroxide Index

The peroxide index is a quality criterion; it allows you to see the oxidation state of the oils and to control the first stages of oxidative alteration. The peroxide index is linked to the conservation conditions and methods of extraction. The value of the peroxide index found in this study is of the order of 0.96 and 1.7 meqO/Kg of oil.

d. Iodine Index

In the analysis of fats, it is the iodine index which represents the most useful constant because it is in relation to the values of this index that the important division of vegetable oils into drying oils, semi- drying and non-drying. Indeed, the iodine index tells us about the degree of unsaturation of the fatty acids contained in given oil. It is directly related to the degree of oxidation of oil. Thus, the more unsaturated an oil is, the higher its sodium index, and we can base ourselves on this quantity to evaluate the ease of processing given that the more unsaturations it contains, the more sensitive it will be to oxygen. The value found in this study is of the order of 174.23 and 210 (g/100g of oil). The index value iodine in our oil shows that it complies with the standard established by the Food Codex which set them between 170 and 213.

e. Refractive Index

The refractive index represents a criterion of oil purity. It depends on the chemical composition of the oils and the temperature. Generally, the index increases with unsaturation or the presence of secondary product. The refractive index value of our oil is 1.482 and 1.485.

f. Relative Density

Determining the density of oil tells us about its purity, it depends on the chemical composition of the oils and the temperature flaxseed oil has a density (0.951) almost similar to that found in marketed products (0.949), and it is between 0.925-0.955 according to the standard given by the Codex Alimentarius.

3.3. Optical Purity

Optical activity occurs in the solid state, in the liquid state or for dissolved substances. In what follows we focus on the last case. When we carry out the experiment to demonstrate the optical activity of an active compound between crossed polarizers, with non-monochromatic light, we see that it is not possible to obtain complete extinction by rotating the analyzer. This comes from the fact that the specific rotating power of the substance depends on the wavelength. In the case of our samples the polarimeter result was zero (0); so, we can say that the absorbance of polarized light for all the molecules that exist in the sample is equal on both sides and this is due to two major phenomena; the mixture is either racemic or completely achiral (absence of chiral molecules), or acids, saponosides.

3.4. Flaxseed oil analysis by UV-VIS spectroscopy

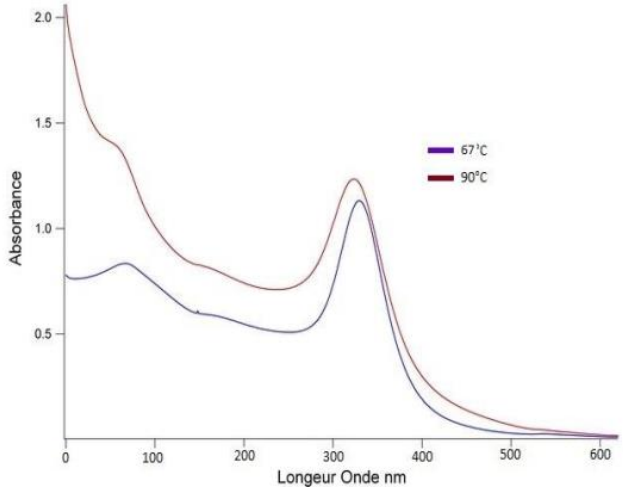

Figure 1. The UV-VIS spectrum of *linum usitatissimun* oil.

Table 3. The characteristic UV-vis bands of fixed flaxseed oil at 90°C

	Band I	**Band II**
ʎmax	190 nm	315 nm
Absorbance	1.3	1.2
ΔE (Kcal/mol)	152.06	90.84

Table 4. The characteristic UV-vis bands of fixed flaxseed oil at 67°C

	Band I	**Band II**
ʎmax	195 nm	320 nm
Absorbance	0.75	1.1
ΔE (Kcal/mol)	146.75	89.42

The UV-vis spectrum of *linum usitatissumun* oil at 90°C recorded in 2-butanol presents two characteristic absorption bands:

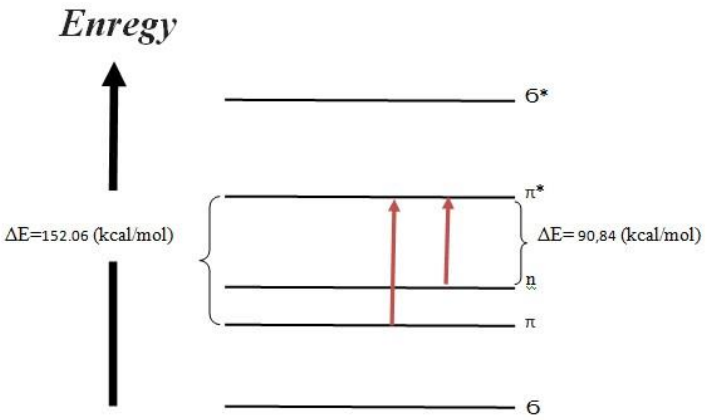

Figure 2. Energy diagram of electronic transitions (UV-Vis spectrum for flaxseed oil extracted a 90°C).

Band I: Band I is located at 190 nm with an absorbance of 1.3 and with an electronic transition energy of 152.06 kcal/mol (Table: 3), this band is attributed to the electronic transition of type π→π*, corresponds to the C=C double bond of a cetone or an aldehyde and that of carbonyl C=O.

Band II: band II is located at 315 nm with an absorbance of 1.2 and with the electronic transition energy of the order of 90.84 kcal/mol (Table 3), this band is attributed to the n→π*type electronic transition corresponds to the free C=O doublet of carboxylic acids.

The UV-vis spectrum of *linumusitatissumun* oil at 67°C recorded in 2-butanol presents two characteristic absorption bands.

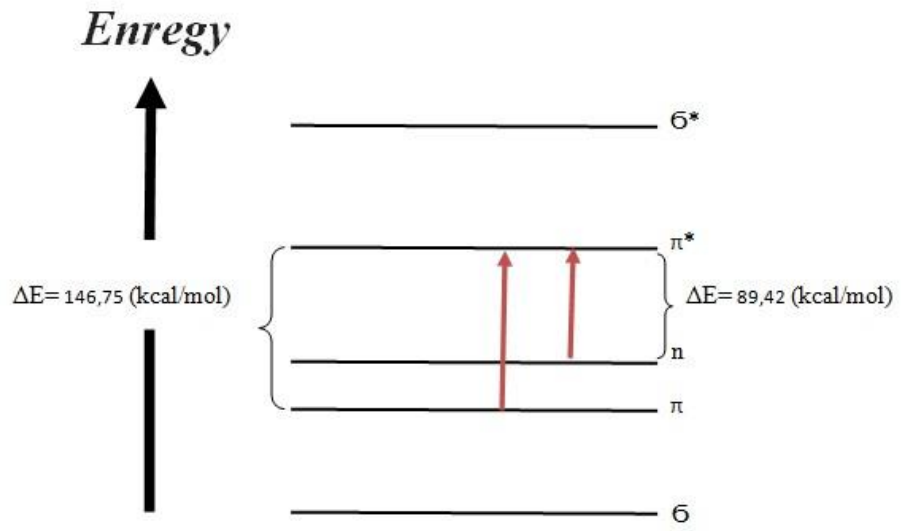

Figure 3. Energy diagram of electronic transitions. (UV-Vis spectrum for flaxseed oil extracted at 67°C.

We notice that there is a similarity in the UV-Visible spectra for the two products (T= 67°C and T= 90°C) indicating that there is almost the same chemical composition of the two products.

Band I: Band I is located at 190 nm with an absorbance of 0.75 and with an electronic transition energy of 146,75 kcal/mol (Table 4), This band is attributed to the electronic transition of type π→π*, corresponds to the C=C double bond of a cetone or an aldehyde and that of carbonyl C=O.

Band II: band II is located at 320 nm with an absorbance of 1.1 and with the electronic transition energy of the order of 89.42 kcal/mol (Table 4), this band is attributed to the n→π*type electronic transition corresponds to the free C=O doublet of carboxylic acids.

By comparison between the two UV-Vis spectra recorded from the extracted flaxseed oil (at 67°C and 90°C), we notice a bathochromic shift in the 2 max values of band II of the flaxseed oil. Extracted at 67°C compared to flaxseed oil extracted at 90°C and a hypochromic shift in the band intensity values of flaxseed oil extracted at 67°C compared to flaxseed oil extracted at 90°C.

3.5. Analysis of flaxseed oil by infrared spectroscopy

The IR spectrum recorded by Fourier transform IR-TF spectrophotometer shows nine characteristic absorption bands:

A thin, moderately intense band appears at 3009 cm^{-1} corresponding to the elongation or valence vibrations of ethylene C-H bonds which are found in the structures of unsaturated fatty

acids such as: oleic acid (C18: 1 ω9), linoleic acid (C18: 2 ω6) and a-linoleic acid (C18:3 ω3), this is confirmed by the presence of an intense and thin band around 721 cm^{-1} attributed to vibrations deformation outside the ethylenic C-H bond plane (=C-H) of these fatty acids which are part of the chemical composition of flaxseed oil (Table 5).

Table 5. The characteristic IR spectrum bands of *linumusitatissimun* oil extracted at 67°C and 90°C

No	Group	HL à 67°C	HL à 90°C
		Frequency (Cm^{-1})	Frequency (Cm^{-1})
1	δ C-H (Aromatic)	721	721
2	υ C-O (aliphatic cetone)	1160	1159
3	υ C-O(aromatic and α,β-unsaturated)	1235	1235
4	δ O-H (Alcohol, Carboxylic acid)	1377	1377
5	δ CH2	1459	1459
6	υ C=O (Carboxylic acid)	1742	1742
7	υ C=C	1620	1620
8	δ C-H (Cetone or Aldehyde)	2853, 2922	2853
9	υ -OH (Carboxylic acid)	2922	2922
10	υ C-H (Aromatic)	3008	3009

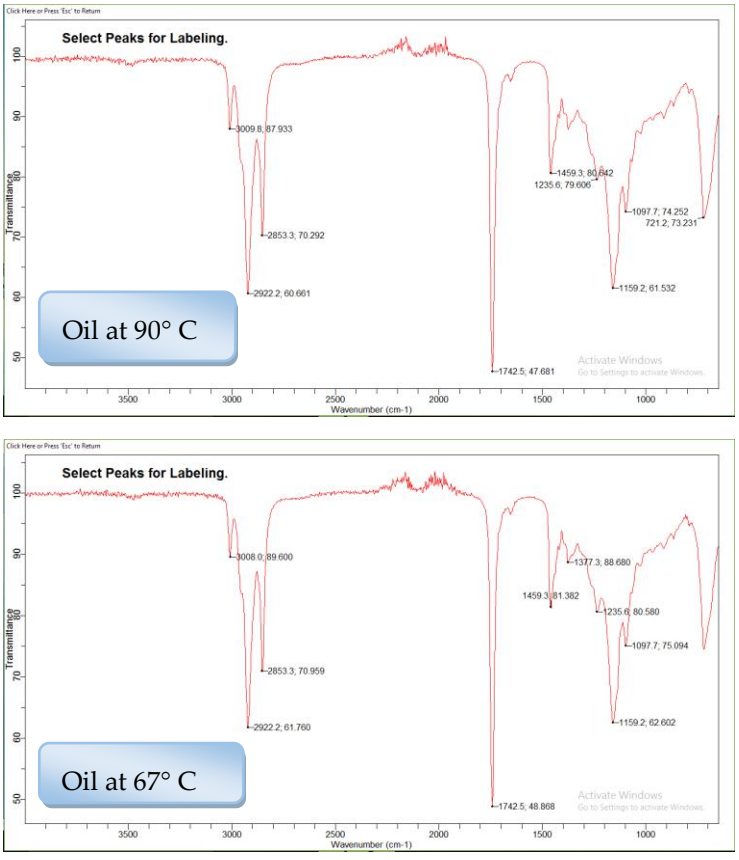

Figure 4. IR spectra of flaxseed oil used (at 67°C and 90°C)

A thin and very intense band located at 1742 cm^{-1} attributed to the valence vibrations of the double carbonyl bond (C=O) which characterize the fatty acids and aldehydes which enter into the chemical composition of flaxseed oil.

Two other bands matched respectively at: 1160 cm^{-1} and 1235 cm^{-1} correspond to the valence vibrations of the (C-O) α β-unsaturated bond by the C=O double bond of the function acid; this allows confirmation of the presence of an acid function.

Two other intense and fine bands appear in the area: 2800-2990 cm^{-1}, more precisely located respectively at 2853 cm^{-1} and 2922 cm^{-1}, correspond to the symmetric and asymmetric valence vibrations of C-H bonds of CH_2, and CH_3), which confirms by the significant intensity of these bands, the presence of a slow hydrocarbon chain of acids and esters which enters into the chemical composition of flaxseed oil.

The thin and intense band at 1459 cm^{-1} attributed to deformation vibrations in the same C-H bond plane of CH_2, and CH_3.

A thin and intense band appears at 1377 cm^{-1} attributed to deformation vibrations in the same plane of a hydroxy group (O-H) of the acid function.

3.6. Analyzes of linum usitatissumun oils by HPLC

From the chromatogram we can deduce that this oil contains several chemical compounds which absorb around this wavelength, in particular by way of designation; we can distinguish between 9 and 15 compounds with selectivity factors.

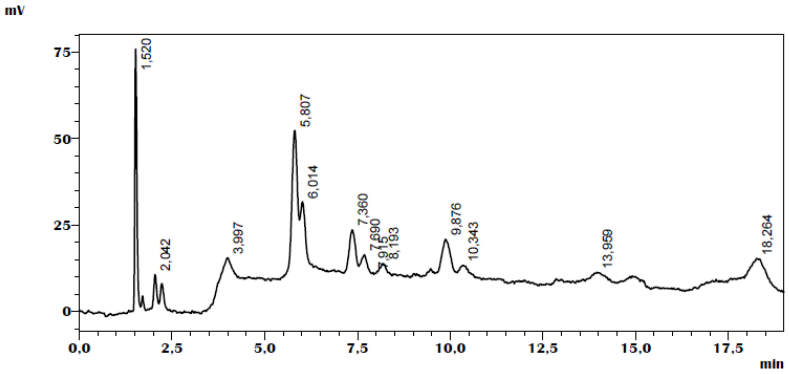

Figure 5. The HPLC chromatogram of *linum usitatissimun*oil T=90°C

Table 6. Results of analysis by HPLC of *linum usitatissimunoil* T=90°C; Vial: 1-5, 10 uL, Column: C 18, Mobile Phase: ACN, ʎ=254 nm, Flow Rate: 0.5 mL/min.

Peak	Rt (min)	A	k′	Rs	%
1	1.52	--	--	--	**15.34**
2	2.04	--	0.343	3.397	**4.17**
3	3.99	4.743	1.629	4.348	10.84
4	5.80	1.731	2.820	0.594	**21.09**
5	6.01	1.048	2.956	3.718	8.82
6	7.36	1.299	3.848	3.718	7.34
7	7.69	1.057	4.059	0.980	3.26
8	7.91	1.036	4.207	0.968	0.08
9	8.19	1.043	4.390	4.305	2.38
10	9.87	1.252	5.497	1.031	7.97
11	10.34	1.056	5.804	4.165	3.14
12	13.95	1.410	8.183	3.511	7.35
13	18.95	1.346	11.015		8.22

Rt: retention time, α: selectivity factor, K: capacity factor, Rs: resolution.

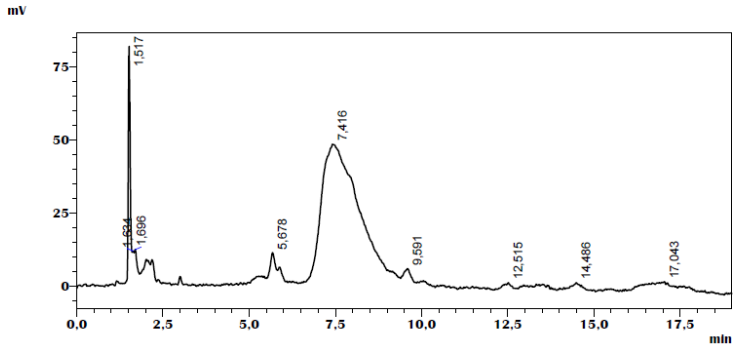

Figure 6. The HPLC chromatogram of *linum usitatissimun* oil T=67°C.

Table 7. Results of HPLC analysis of *linum usitatissimun* T=67°CVial: 1-6 In, 10 uL Column: C 18 Mobile Phase: ACN, ƛ= 254 nm, Flow Rate: 0.5 mL/min.

Peak	Rt	A	k'	Rs	%
1	1.517	--	--	--	7.754
2	1.634	--	0.077	0.141	1.082
3	1.696	1.535	0.118	0.063	1.758
4	5.678	23.195	2.742	9.934	3.371
5	7.416	1.418	3.888	1.529	83.587
6	9.591	1.369	5.322	1.943	0.986
7	12.515	1.362	7.249	7.963	0.793
8	14.486	1.179	8.549	6.095	0.240
9	17.043	1.197	10.234	9.823	0.429

Rt: retention time, α: selectivity factor, K: capacity factor, Rs: resolution.

3.7. Chemical composition of flaxseed oil

3.7.1. Fatty acids and triglycerides

The fatty acid composition is presented as it is found in the literature bringing together different origins (Europe, Canada), which has the effect of widening the ranges of values, and by comparison with the chromatographic results, we note that flaxseed oil extracted at 67°C contains 3.8% saturated fatty acids and flaxseed oil extracted at 90°C contains 7.39% saturated fatty acids. We note the absence of palmitic acid in flaxseed oil at 67°C, and the absence of Behenic acid in both flaxseed oils at 67°C and 90°C.

The results obtained show that the monounsaturated fatty acid content of the two flaxseed oils is around 21%, a content very close to international standards (between 11% and 23%). We also note that polyunsaturated fatty acids retain their presence in the two flaxseed oils according to international standards at around 67%. Given the significant proportion of alphalinolenic acid in flaxseed oil, the composition of its triglycerides shows a preponderant amount of trilinolenin (LnLnLn: 22.8% and 21.09%). In terms of the structure of triglycerides.

3.7.2. Tocopherols, sterols

Flaxseed oil contains 40 to 60 mg/100 g of tocopherols, a quantity similar to that of walnut oil, but lower than that of rapeseed and soybean oils; the gamma tocopherol form with high antioxidant protective potential is practically the only form present (96-98%); the alpha and delta-tocopherol forms are therefore present in very small quantities. The sterol contents and composition of flaxseed oil are comparable to those of most vegetable oils with beta-sitosterol in the majority.

3.8 Blood pressure and anti-hypertension activity

In this part of the study, the main objective is to evaluate the antihypertensive effects of fixed flaxseed oils (*Linum Usitatissimum*) by in vitro tests; using an experimental device that simulates the blood cycle in the human body. To achieve this objective, it was more specifically:

a. To evaluate with preliminary in vitro tests the effects of flaxseed oil (used in traditional medicine) on blood pressure.

b. Determine the dose of flaxseed oil which allows the regulation of blood circulation or the favorable dose for essential anti-hypertension activity.

3.8.1. Effect of flaxseed oil on blood pressure

The addition of flaxseed oil to the mixture is done gradually from 0.1 mL to 1mL; we generally notice that the tension measured by the tensiometer decreases with the progressive addition of the oil compared to the measurement taken by the serum alone; this means that this oil affects high blood pressure simulated by the system described previously and schematized in Figure 7.

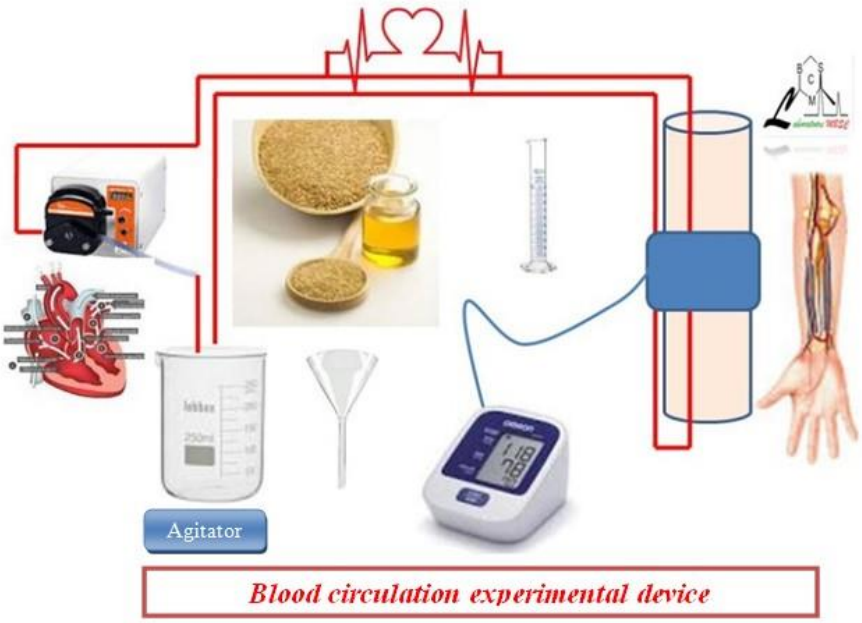

Figure 7. Presentation of the constituents of an experimental device for the in vitro evaluation of the anti-hypertension activity of flaxseed oil.

We can easily notice from the two graphs the effect of flaxseed oil on the remarkable reduction in high blood pressure; we also notice that the reduction in diastolic blood pressure is more important and more effective than systolic blood pressure. It can be interpreted by the fact that since the diastolic blood pressure indicates the residual pressure at the time of the relaxation phase of the heart, its reduction is very remarkable because the heart will be in a passive position and the arteries are not at their maximum dilation.

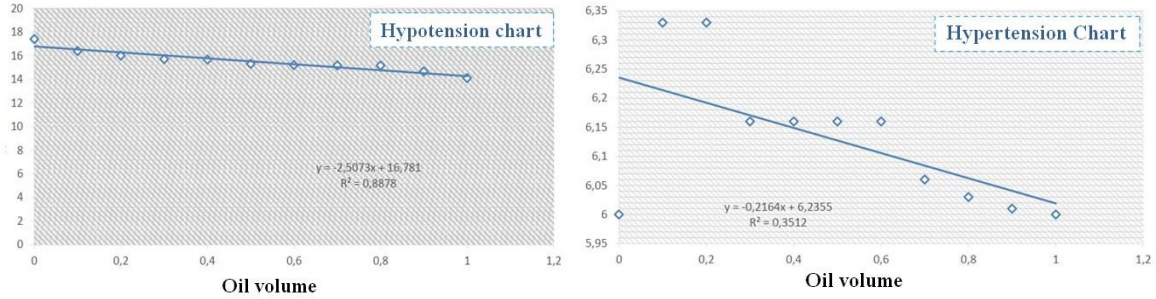

Figure 8. A curve showing the effect of oil on blood pressure

4. CONCLUSION

Flaxseed oil is considered one of the richest nutritional supplements because it contains fiber, vitamins and minerals that help strengthen the immune system. We were able to achieve a satisfactory yield of 24% to 29% in our work by applying two distinct methods of pressing at temperatures of 67°C and 90°C. The physical and chemical characteristics (density, refractive index, acid index, saponification index, iodine index, peroxide index) were determined, allowing the Food Codex to define criteria to evaluate the quality of the extracted flaxseed oil. The work's findings indicate that the two flaxseed oils' monounsaturated fatty acid content is roughly 21%, which is extremely close to the 11%–23% range that is considered worldwide. Additionally, we see that the two flaxseed oils still contain roughly 67% of the globally recognized polyunsaturated fatty acids. Furthermore, we tried to test the anti-hypertension activity of this oil by simulating a blood cycle in the laboratory which allowed us to determine the variation in blood pressure as a function of the volume of oil injected into the blood cycle from 17.4 to 14.10.

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Original Article

Enhancing Anticancer Potential: Investigating the Synergistic Impact of Doxorubicin and Curcumin on HeLa and Vero Cells in Vitro

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Abstract: Cervical cancer ranks as the fourth most prevalent cancer globally and in Asia, standing as the second most common in Indonesia. Despite its efficacy, doxorubicin chemotherapy is associated with significant side effects. To mitigate these adverse effects, a promising approach involves combining conventional drugs with curcumin. Both curcumin and doxorubicin have demonstrated cytotoxic effects against cervical cancer (HeLa). This research aims to determine the synergistic effect of the combination of doxorubicin and curcumin on HeLa and its safety in normal cells (Vero cells). This study adopted an experimental design utilizing doxorubicin and curcumin samples with HeLa and Vero cells. The investigation was initiated with cytotoxic and combination tests using the MTT method. The obtained results included IC_{50} values and combination indices, and the analysis involved a comparative examination of outcomes between HeLa and Vero cells. Cytotoxic tests revealed IC_{50} values for doxorubicin and curcumin on HeLa cells, measured at 2.17 ± 0.06 and 26.37 ± 2.00 μg/mL, and 16.57 ± 5.56 and 172.22 ± 19.93 μg/mL on Vero cells. Combination test results were represented by the combination index. The synergistic effect is observed in the combination of curcumin at a concentration of 9 μg/mL and doxorubicin at a concentration of 0.125 μg/mL, resulting in a combination index of 0.50. These findings suggest a promising avenue for enhancing the therapeutic potential of doxorubicin in cervical cancer treatment while minimizing adverse effects.

Keywords: antiproliferative, cancer, cervical, combination, doxorubicin.

1. INTRODUCTION

The incidence rate of cervical cancer in Indonesia increased slightly from 7.4 to 8.7 per 100,000 women, while the prevalence rate increased from 43.3 to 52.4 per 100.000 women from 1990 to 2017 [1]. The incidence of cervical cancer in Indonesia is estimated to reach 180.000 new cases per year [2]. Cervical cancer is the number one killer in Indonesia with an incidence rate of 100/100.000 per year [3]. Due to this, many efforts have been made to reduce the incidence of cervical cancer.

Efforts made to reduce the incidence of cervical cancer are the importannt of providing strategic and evidence-based health services to reduce the impact of cervical cancer [4]. The incidence and prevalence of cervical cancer can be reduced through education, prevention, treatment, family planning, the use of regular pap smears, and appropriate management and follow-up after cases of cancer occur [5]. Reducing cancer cases can be done with surgery, radiation, and treatment using synthetic drugs, herbs, or combinations as co-chemotherapy.

Combination of synthetic drugs and traditional medicines as co-chemotherapy for cancer treatment. The results of co-chemotherapy research provide synergistic, additive, and antagonistic information. The effects on the results of combination research are shown by the combination index. The expected combination index price is in the range that shows a synergistic effect, namely below 1. Exploration of the potential synergistic effect of chemotherapy agents with active compounds of traditional medicines to increase anti-cancer properties, reduce side effects, increase penetration of chemotherapy agents into cancer cells, and provide supportive care to improve quality of life due to chemotherapy with anticancer drugs [6].

Anticancer drugs also have toxic effects on normal cells and low bioavailability in the body. Cancer drugs that are combined to reduce the incidence of cervical cancer include cisplatin, which is a drug of choice for cervical cancer, paclitaxel, and doxorubicin. Doxorubicin has toxicity in normal cells, so research into the addition of co-chemotherapy is needed [7]. The active substances used in co-chemotherapy include flavonoids, curcuminoids, and alkaloids.

The mechanism of action of curcumin can occur both internally (in mitochondria) and externally (via receptors). Cell surface transmembrane death). The intrinsic pathway typically begins with activation of the tumor suppressor p53, a cell cycle regulator, and via members of the B cell lymphoma (Bcl-2) family [8]. Upregulation of Bcl-2-activating p53 inactivates its homolog antagonist (Bak) and Bcl-2 x-linked protein (Bax), a pro-apoptotic member of the Bcl-2 family. Bak and Bax promote apoptosis by forming pores in the mitochondrial membrane that release cytochrome c into the cytoplasm and thereby activate caspases. Nuclear factor (erythroid derivative 2) like 2 (Nrf2) is a transcription factor involved in primary defense pathways against the effects of oxidative stress [9]. The Nrf2 pathway transcription factor is a regulator of genetic variations associated with the detoxification of electrophiles and ROS as well as the repair and elimination of damaged products caused by cancer cells. Curcumin has potential as a chemopreventive and antiproliferative agent that activates the Nrf2 pathway, restores p53, and modulates inflammatory molecules [8].

Curcumin is a group of curcuminoids that are abundant in turmeric (*Curcuma longa*). The potential of curcumin as co-chemotherapy is shown in the results of research in combination with cisplatin, which showed synergistic results as co-chemotherapy [8]. However, the response to this combination provides a different effect with a small impact on restraining the growth of cancer cells, and there are still side effects [10]. A combination with other chemotherapy needs to be done, namely doxorubicin. This is supported by research showing that *C. longa* extract can reduce the side effects of doxorubicin on breast cancer in vitro in 4T1 cells, where the content of *C. longa* is curcumin [11].

Co-chemotherapy testing of curcumin and the chemotherapy agent, namely doxorubicin, on normal cells, also needs to be carried out. This is related to the toxic effect of doxorubicin on normal cells and can cause side effects such as diarrhea, nausea, vomiting, and an increased risk of infection [12]. The parameter that indicates security in normal cells is the selectivity index. The selectivity

index value is obtained by comparing the IC_{50} effect of the sample on cancer cells compared to the IC_{50} of the sample on normal cells [13].

Curcumin shows promise as a co-chemotherapy for doxorubicin in breast cancer cells, however, the potential for co-chemotherapy in other cancer cells needs to be tested. Testing doxorubicin as a co-chemotherapy in cervical cancer cells is hypothesized to provide a synergistic effect because curcumin, like in breast cancer cells, can increase bioavailability. Testing curcumin as a doxorubicin co-chemotherapy in cervical cancer cells can provide important information regarding increasing the therapeutic potential of this combination. The aim of this study was to determine the increased effect of doxorubicin with curcumin co-chemotherapy and its safety on Vero cells.

2. MATERIALS AND METHODS

The research carried out was experimental research with the active substances curcumin and doxorubicin. The synergistic effect of anticancer activity was tested with the combination of these samples: HeLa cells (cancer cervix cells) and Vero cells (normal cells). The samples used in this study were curcumin and doxorubicin. In this study, MERCK-Schuchardt brand curcumin with a purity of ≥ 90% was used. Curcumin and doxorubicin were obtained from the Phytochemical Laboratory of Ahmad Dahlan University. This research was conducted based on the ethical clearance issued by KEP UAD Number 012212203, 23 January 2023.

2.1. Cell culture and cytotoxic assay on HeLa and Vero cells

HeLa cells were grown in DMEM 1640 medium (Gibco) containing 10% volume fetal bovine serum (FBS) (Gibco) and 100 μg/ml penicillin-streptomycin (Gibco) and incubated in a CO_2 incubator (Heraeus) at 37°C with flow 5% CO_2 [14]. Cytotoxicity test with the MTT test. HeLa cells were divided into 96-well plates (Nunc), a total of 5000 cells per well, and incubated with doxorubicin and curcumin either singly or in combination using DMSO solvent for 24 hours in a CO_2 incubator (Herceus). The concentrations used for the cytotoxic test of doxorubicin in HeLa cells were 4.6; 2.3; 1.15 μM, and curcumin was 50, 25, and 12.5 μM. At the end of each HeLa cell incubation. 100 μl of MTT (Sigma) in DMEM medium (Gibco) was added to the wells. The plates were then incubated for 4 h at 37 °C until formazan crystals formed (viewed under an inverted microscope (Olympus CH-2)). After 4 hours, the MTT reaction was stopped by adding 10% SDS blocking reagent, 100 μl each, into the wells, then incubated overnight at room temperature covered with aluminum foil. The resulting violet color absorption intensity was read with an ELISA reader (Bio-Rad microplate reader reference sensor serial number 11565, Japan) at a wavelength of 595 nm to obtain the absorbance [14]. Next, the percentage of live cells was calculated, and then a linear regression of the relationship between cell concentrations and the percentage of viability cells was made. The same method is used for testing on Vero cells. The difference lies in the test concentration used. The concentrations of doxorubicin are 100, 25, and 3.125 μg/mL and the concentrations of curcumin are 50, 25, and 12.5 μg/mL.

$$\text{Percentage of viability cells} = \frac{(\text{sample absorbance} - \text{media control absorbance})}{(\text{solvent control absorbance} - \text{media control absorbance})} \text{x } 100\% \quad \ldots\ldots\ldots(1)$$

2.2. Test the selectivity

The cell selectivity test was carried out as in the cytotoxic test, with doxorubicin and curcumin samples tested on HeLa cells and Vero cells. The selectivity index is determined by comparing the IC_{50} of the test material on Vero cells to the IC_{50} of the test material on HeLa cells [15].

2.3. Test the combination

The samples used were doxorubicin and curcumin, as well as doxorubicin and curcumin combination. Testing is carried out on the IC_{50} results of the samples, which are then combined at the IC_{50} concentration of the single sample. Absorbance is used to calculate living cells under the influence of a single sample, which is then included in the Combination Index calculation formula (Equation 2). Dx is the IC_{50} concentration of a single compound, and D1 and D2 are the combination concentrations that provide an effect equivalent to a single concentration [16].

$$CI = \frac{[D]1}{(Dx)1} + \frac{[D]2}{(Dx)2} \quad \text{.....(2)}$$

3. RESULTS AND DISCUSSION

The results obtained from this study were the combination index of doxorubicin and curcumin. Cytotoxic tests for single compounds are carried out first to design the combination tests to be carried out. The results of the single compound cytotoxic test are in the form of IC_{50} which is then used as the basis for the concentration used for the combination test of doxorubicin and curcumin.

3.1. Cell culture and compound cytotoxicity test on HeLa and Vero cells

HeLa and Vero cell cultures were carried out to obtain IC_{50} and see safety by comparing Vero cells and HeLa cells. Figure 1 shows a picture of HeLa cells in controls that appear to be in greater numbers compared to the effect of doxorubicin and curcumin. Figure 1 displays the findings of the log concentration vs percent viability cells relationship. This is in line with the IC_{50} results obtained on HeLa cells shown in Table 1. The IC_{50} results on Vero cells show that doxorubicin to normal cells=16.57±5.56 and curcumin=172.22±19,93, IC_{50} on HeLa cells doxorubicin=2.17±0.06 and curcumin = 26.37±2.01 µg/mL. The results of Vero and HeLa cells affected by doxorubicin and curcumin are shown in Figure 2.

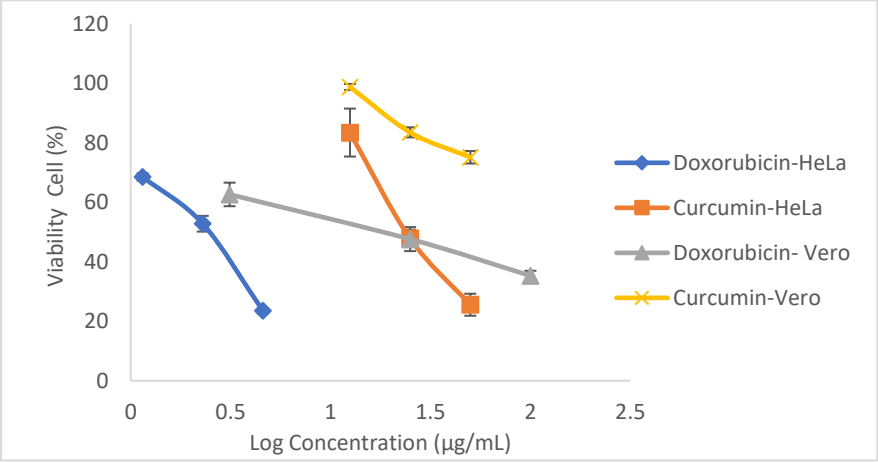

Figure 1. Correlation between doxorubicin and curcumin log concentrations vs HeLa and Vero Cell viability(%)

Doxorubicin has been shown to intercalate deoxyribonucleic acid (DNA) and bind to and subsequently inhibit DNA polymerase, both of which lead to an inhibition of DNA synthesis. It works by damaging the DNA inside the cell, which leads to cell death [17]. However, it can also cause significant side effects, such as nausea, vomiting, hair loss, an increased risk of infection, and bleeding. The optimal dose and duration of doxorubicin treatment depend on the type and stage of the cancer being treated. Based on this, this research was continued with selectivity tests on normal cells [18].

Figure 2's results demonstrate that the addition of doxorubicin does not significantly alter the image of normal cells, but it does alter the image of HeLa cells, which have fewer images. This is in line with research showing that doxorubicin has a selectivity index on cervical cells [19].

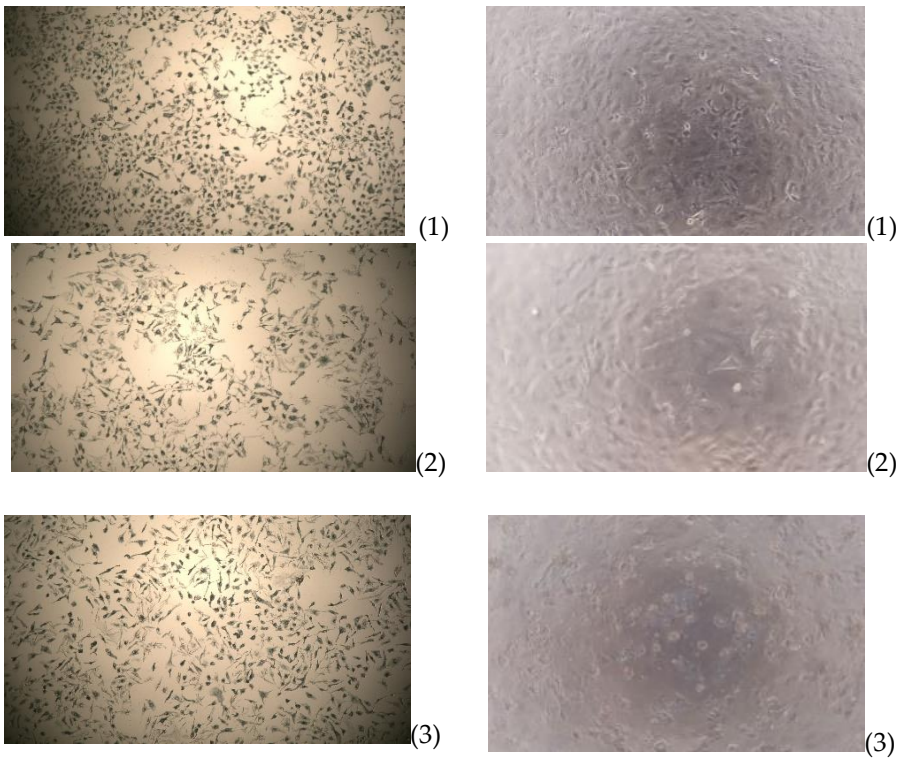

Figure 2. Image of Vero cells and HeLa cells (a) Vero cells, (b) HeLa cells, and (1) control cells; (2) doxorubicin, and (3) curcumin

3.2. Test the selectivity

The selectivity index (SI) of curcumin and doxorubicin against HeLa cells was calculated using the selectivity index parameter. The selectivity index is calculated by comparing the IC_{50} of Vero cells against HeLa cells. The selectivity index results for doxorubicin and curcumin were 7.64 and 6.53, respectively. This shows that doxorubicin and curcumin are selective in HeLa cells.

Tabel 1. IC_{50} and Selectivity Index doxorubicin and curcumin to vero and HeLa cells

Compound	IC_{50} (µg/mL)		Selectivity
	Vero	HeLa	Index
Doxorubicin	16.57±5.56	2.17±0.06	7.64
Curcumin	172.22±19.93	26.37±2.01	6.53

A high SI value (>2) of a compound gives the selective toxicity against cancer cells (SI = IC_{50} normal cell/IC_{50} cancer cell) [20]. The selectivity index for vero and HeLa cells for the compound doxorubicin was 7.64 and curcumin was 6.53. Based on these results, it is necessary to carry out tests related to the combination of the two to reduce the side effects of doxorubicin by reducing its concentration.

3.2. Test the combination

After knowing the IC_{50} of curcumin and doxorubicin against HeLa cells, a combination cytotoxic test was continued based on multiples of the IC_{50} results obtained previously. This is done to reduce the risks of using a combination of curcumin and doxorubicin. The combination series used for curcumin are 9; 4.5; 1, 2.5; and 1.25 μg/mL. Meanwhile, the concentration series used for doxorubicin are 1; 0.5; 0.25, and 0.125 μg/mL as seen in Table II.

Table 2. Combination Index of Curcumin and Doxorubicin in HeLa cells

Concentration of Doxorubicin (μg/mL)	Concentration of Curcumin (μg/mL)	Combination Index
1	9	2.20
0.5	9	1.27
0.25	9	0.93
0.125	9	**0.50**
1	4.5	2.49
0.5	4.5	3.32
0.25	4.5	3.39
0.125	4.5	6.31
1	2.5	2.04
0.5	2.5	3.41
0.25	2.5	3.10
0.125	2.5	1.44
1	1.25	2.02
0.5	1.25	3.60
0.25	1.25	1.96
0.125	1.25	3.17

The results of calculating the combination index values obtained from 16 combinations of curcumin and doxorubicin against HeLa cells at a concentration of 9 μg/mL curcumin and 0.25 μg/ml doxorubicin are 0.93 and a concentration of curcumin of 9 μg/mL and doxorubicin of 0.125 μg/ml with a combination index value of 0.50. The interpretation of the combination index value is that curcumin and doxorubicin have a mild-moderate synergistic effect and have a synergistic effect on HeLa cells on the combination curcumin of 9 μg/mL and doxorubicin of 0.125 μg/mL.

The synergistic effect of the combination of curcumin of 9 μg/mL and doxorubicin of 0.125 μg/mL is shown by the combination index based on previous research, which states that when the combination index is <1, then the combination is in the synergistic effect range [21]. This is in line with research that states that the combination of curcumin and doxorubicin showed a synergistic

effect in enhancing the anticancer activity in gastric adenocarcinoma cells [22]. Curcumin is used as a co-chemotherapy against doxorubicin because curcumin can increase the anticancer effect of doxorubicin, possibly by increasing the absorption of doxorubicin. Apart from that, curcumin also protects normal cells that are damaged by doxorubicin through the mechanism of reducing oxidative stress, which can damage DNA. Thus, curcumin has the potential to act as a co-chemotherapy for doxorubicin in reducing the toxic effects [22]. Curcumin has been known to reduce the adverse effects of DOX on normal cells and tissues by reducing inflammation, oxidative stress, and apoptosis. The current development related to the development of curcumin as a co-chemotherapy is the development of nanoparticles [23]. The potential of curcumin as co-chemotherapy needs to be developed, considering that chemotherapy has toxicity to normal cells.

4. CONCLUSION

The combination of doxorubicin at a concentration of 0.125 μg/mL and curcumin at a concentration of 9 μg/mL produces a synergistic effect with a combination index of 0.50.

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Conflicts of interest: The authors declare no conflict of interest.

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Original Article

Formulation and Determination of Tannin and Flavonoid Levels of Anti-Acne Gel Formulation from Red Betel Leaf Extract (*Piper crocatum*)

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Abstract: Acne is a common skin condition that often occurs among teenagers and adults aged 18-23 years. Acne can be triggered by several factors, genetic, psychological, weather, stress, cosmetics, and bacterial infections. Red betel leaf contains phytochemical compounds, namely tannins and flavonoids, which function as antibacterial agents. The formulation of anti-acne gel is designed by varying the concentration extract, F0 (0%), F1 (10%), F2 (15%), and F3 (20%), to determine the best anti-acne gel formulation. Red betel leaf extract (RBLE) is tested for tannin and flavonoid levels using the UV-Vis Spectrophotometer test method. The gel preparation is examined, including organoleptic testing, pH testing, homogeneity testing, and spreading testing. The research results show that RBLE has a tannin content of 4.7056% and a flavonoid content of 0.0934%. The results of the anti-acne gel preparation study meet the requirements for organoleptic properties, pH, homogeneity, and spreading. Based on the results of various extract concentrations, satisfactory results are obtained, with formulations F2 and F3 meeting the requirements.

Keywords: *Acne; Gel; Ethanol Extract; Red Betel Leaf Extract*

1. INTRODUCTION

The healthy condition of the skin significantly contributes to one's self-confidence; when the skin is unhealthy, it can affect self-perception and become a health issue that needs attention [1].One part of the skin that needs attention to cleanliness and health maintenance is the facial skin. Maintenance of hygiene behaviour, especially on facial skin, is described through the awareness and initiative taken by individuals to maintain cleanliness and health and prevent disease [2]. One problem that arises when facial skin hygiene is not maintained is the occurrence of acne. Acne is a common skin disorder found among both teenagers and adults, with an average prevalence in the age range of 18-23 years [3]. Acne is an accumulation of overly active sebaceous gland on the skin that are clogged with dirt and become infected due to the presence of bacteria such as *Staphylococcus aureus* and *Propionibacterium acnes* [4], [5]. Acne can be triggered by several factors including genetic, psychological, weather, stress, diet, cosmetics, and bacterial infections.

In addressing acne problems, the most preferred dosage form by consumers is in the form of a gel preparation [6]. The advantage of gel preparations is that they are non-sticky and also evaporate quickly, facilitating the efficient delivery of medication to the skin, allowing acne to dry quickly [7].

The solubility of the extract in the gel formulation might affect skin penetration since it influences the rate of active component absorption by the skin. The better the extract dissolves in the gel formulation, the more the formulation can enter the skin layers.

Red betel leaf (*Piper crocatum*) is one of the potential plants empirically known to have efficacy in curing various diseases [8]. Red betel leaves have also been used in cosmetics including hand antiseptic gels, peel-off masks, clay masks, sheet masks, lip balms, and loose powders. Red betel leaf is a plant belonging to the *Piperaceae* family [9]. Red betel leaves (*Piper crocatum*) contain phytochemical compounds such as essential oils, alkaloids, saponins, tannins, and flavonoids with antioxidant and antibacterial activities [10]. Flavonoids can form complex compounds with extracellular proteins that have bacterial cell membrane integrity [11]. The mechanism of action of flavonoids as antimicrobials can be divided into 3 categories: inhibiting nucleic acid synthesis, inhibiting cell membrane function, and inhibiting energy metabolism [12].

2. MATERIALS AND METHODS

2.1. Material

The material utilized in this study is red betel leaves obtained from Lubuk Minturun, Padang, Indonesia. Carbopol (Lubrizol, USA), TEA, Methyl Paraben, Propilenglikol, Aquadest. This research was conducted in the Chemistry Laboratory of the Faculty of Mathematics and Natural Sciences, Universitas Negeri Padang.

2.2. Extraction Method

This extraction method refers to the study conducted by Rosari et al., 2021. Fine powder of red betel leaves weighing 200 grams was extracted using 70% ethanol at a ratio of 200 grams to 2 liters (1:100). The maceration process was carried out in glass jars for 3x24 hours. All the macerates were collected and concentrated using a rotary evaporator to obtain the extract, which was then placed in a water bath to thicken the extract. The yield of RBLE obtained from 2 liters was 48.7704 grams. Based on the research findings [13], the anti-acne gel formula with a concentration of 15% black betel leaf extract resulted in the best anti-acne gel preparation. All anti-acne gel formulations did not cause irritation, indicating that the anti-acne gel is safe for use. Therefore, this study will utilize an innovative approach by using different concentrations of RBLE compared to the aforementioned study, namely the use of RBLE concentrations of F1 (10%), F2 (15%), and F3 (20%). The innovation in extract concentration aims to determine whether reducing or increasing the concentration of extract in the previously studied anti-acne gel formula will yield better results, or if the 15% extract concentration remains the most effective.

2.3. Determination of Tannin And Flavonoid

The test for tannin and flavonoid content utilizes the UV-Vis spectrophotometry technique. The qualitative testing of flavonoids and tannins is conducted using the tube method. The presence of flavonoid compounds is indicated by a yellow color change after the addition of Mg powder and concentrated HCl. The presence of tannins is indicated by a color change in the extract from green to dark green after the addition of FeCl3 [14].

2.4. Formulation of Anti-Acne Gel with RBLE

Table 1. Formulation of Anti-Acne Gel with RBLE

Material	Formulation (%)			
	F1	F2	F3	Control
RBLE	10	15	20	-
Carbopol	2	2	2	2
Triethanolamine (TEA)	2.5	2.5	2.5	2.5
Methyl Paraben	0.2	0.2	0.2	0.2
Propilenglikol	15	15	15	15
Aquadest ad	100	100	100	100

The process of making the anti-acne gel involves several stages. The first stage is to prepare the equipment and materials, where the required materials are weighed accordingly. Next, Carbopol is dispersed in distilled water at a temperature of 70℃, then 2.5% TEA is added, followed by dissolving 0.2% methylparaben in 15% propylene glycol. The dissolved methylparaben is then added to the gel base and stirred until homogeneous. Subsequently, RBLE is added according to the predetermined formulations F1 (10%), F2 (15%), and F3 (20%). The final stage involves testing the gel preparation, including pH testing, organoleptic testing, homogeneity testing, and spreading power testing.

2.5. pH Testing

The determination of the formulation's pH is conducted using a universal pH indicator that is dipped into the diluted gel sample. After being completely immersed, the universal pH indicator paper is observed for color changes and matched with the universal pH standard. The pH of the gel formulation should be in accordance with the pH of the skin, which is 4.5 – 6.5 [15].

2.6. Organoleptic Testing

Organoleptic assessment of the anti-acne gel involves the direct observation of its form, color, and odor. The gel typically appears clear with a semi-solid or thick consistency [16].

2.7. Homogeneity Testing

Homogeneity testing is conducted by evenly and thinly applying 0.1 g of the gel formulation onto a glass slide. Another glass slide is then placed on top and pressed together. The test formulation should exhibit a homogeneous structure with no coarse particles present [15].

2.8. Spreadability Testing

The spreadability test is performed by placing 1 gram of gel in the center of a petri dish, then applying a 150-gram weight and waiting for 1 minute. The spread of the gel is measured by the diameter of the gel spread from two sides. Good spreadability of the formulation typically falls between 5-7 cm [13]

3. RESULTS AND DISCUSSION

3.1. Determination of Tannin and Flavonoid Levels

Table 2. Analysis Results of Tannin and Flavonoid Levels in RBLE

Analyte of Interest	Result (100 gr sample)	Test Method
Tanin	4.7056%	UV-Vis Spectrophotometry
Flavonoid	0.0934%	

The identification results of tannins and flavonoids in this study showed positive results containing tannins and flavonoids. The determination of tannin and flavonoid levels in RBLE was conducted using the UV-Vis spectrophotometer test method. The levels of tannins and flavonoids in RBLE were found to be 4.7056% for tannins and 0.0934% for flavonoids. This research was conducted to determine the best formula for anti-acne gel formulations of RBLE through 4 formulations. The difference between these four formulations lies in the concentration of RBLE used (Table 1). In formulation F0, no RBLE was used, in formulation F1, red betel leaf extract was used at a concentration of 10%, in formulation F2, RBLE was used at a concentration of 15%, and in formulation F3, RBLE was used at a concentration of 20%.

3.2. pH Test

Table 3. Results of pH Test of RBLE Anti-Acne Gel

Formulation	Range pH	pH
F0	4.5-6.5	6
F1		5
F2		5.5
F3		6

The anti-acne gel formulation containing RBLE was tested for pH using universal pH paper with the aim of determining the pH level of the anti-acne gel. The required pH range for skin is 4.5-6.5. Formulation F0 (base without the addition of red betel leaf extract) and F3 had the highest pH at 6 but still fell within the standard pH range. The pH value for formulations F1 and F2 of the anti-acne gel was 5.5. Therefore, it can be concluded that the anti-acne gel containing RBLE has a pH value within the standard range for skin pH.

3.3. Organoleptic Test

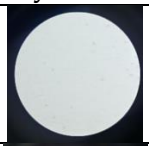
Table 4. Results of Organoleptic Test

Formulation	Organoleptik		
	Consistency	Color	Aroma
F0	Thick	Transparent	Characteristic of Base
F1	Thick	Light Brown	Characteristic of Red Betel Leaf
F2	Thick	Brown	Characteristic of Red Betel Leaf
F3	Thick	Dark Brown	Characteristic of Red Betel Leaf

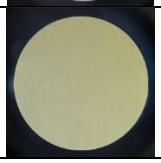
The results of the organoleptic test for the consistency of formulations F0, F1, F2, and F3 showed a thick consistency. The best color among the formulations was observed in formulation F3 (dark brown). Additionally, the most distinctive aroma was found in formulation F3 (characteristic scent of the extract).

3.4. Homogeneity Test

Table 5. Results of Homogeneity Test

Formulation	Analysis Results
F0	
F1	

continued Table 5…

F2	
F3	

The anti-acne gel formulation with RBLE was tested for homogeneity using a microscope to determine the level of homogeneity. The test preparation shows a homogeneous arrangement with no coarse particles present in the preparation. Formulations F0 (base without the addition of RBLE), F1, F2, and F3 showed homogeneity.

3.5. Spreadability Test

Table 6. Results of Spreadability Test of RBLE Anti-Acne Gel

No	Formulation	Spreadability
1	F0	6.1 cm
2	F1	6.7 cm
3	F2	6.3 cm
4	F3	6.8 cm

The anti-acne gel formulation with red betel leaf extract was tested for its spreadability by placing 1 gram of gel in the center of a petri dish, then applying a 150-gram weight and waiting for 1 minute. The spread of the gel was measured by the diameter of the gel spreading from two sides. The ideal spreadability for the formulation is between 5-7 cm. The spreadability of the anti-acne gel in formulation F0 was 6.1 cm, formulation F1 was 6.7 cm, formulation F2 was 6.3 cm, and formulation F3 was 6.8 cm. It can be concluded that the least spreadability was found in formulation F0, while the widest spreadability was found in formulation F3.

4. CONCLUSION

The extract of red betel leaves (Piper crocatum) can be formulated as an anti-acne gel that meets the requirements for anti-acne gel formulations with formulations F1 10%, F2 15%, and F3 20%. Based on the results of the research, discussion, and conclusions outlined above, the formulation with the highest evaluation and in accordance with the standard for anti-acne gel is formulations F2 and F3. Based on the above research results, the researcher suggests for further research to conduct stability testing of the anti-acne gel formulation of RBLE and to add other suitable ingredients for acne facial skin care.

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Review Article

Kaempferia galanga (L.): An Updated Overview of In Vitro and In Vivo Antioxidant Properties

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Abstract: *Kaempferia galanga* L. (*K. galanga*) locally called aromatic ginger or "*kencur*" in Bahasa is traditionally used for the treatment of various diseases related to antioxidants such as anti-inflammatory, anti-cancer, and immunomodulator. This study aimed to provide a critical review of the current antioxidant activity of *K. galanga* using *in vitro* and *in vivo* assays. The information and data on *K. galanga* were collected from various sources such as Scopus, PubMed, Science Direct, and Google Scholar. This review is reported according to PRISMA, 21 articles were included in this review. The results showed that *K. galanga* plays a role in the defense systems against oxidative stress. Several *in vitro* assays have been used to measure the antioxidant activity of *K. galanga*, namely, 1-diphenyl-2-picrylhydrazyl (DPPH), ferric reduction of antioxidant power (FRAP), and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS). The *in vivo* antioxidant activity of *K galanga* included lifespan, survival life, intracellular reactive oxygen species (ROS) levels, malondialdehyde (MDA) levels, and antioxidant enzyme activities, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px). Based on these findings, it can be concluded that *K. galanga* has powerful antioxidant activities with IC_{50} of 7.93 μg/mL-3.09 ± 0.34 mg/mL, and therefore, could have potential as a natural antioxidant.

Keywords: antioxidant, *Kaempferia galanga* (L.), *in vitro*, *in vivo*, oxidative stress

1. INTRODUCTION

A Global Burden of Disease study, in Indonesia showed a decrease in the prevalence of infectious diseases between 1990 and 2019, despite communicable diseases such as lower respiratory infections, diarrheal illnesses, and tuberculosis. Otherwise, non-communicable diseases, especially stroke, ischaemic heart disease, and diabetes become prioritized by policymakers [1]. Numerous neurological conditions (such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis, memory loss, and depression), pulmonary conditions (such as asthma and chronic obstructive pulmonary disease), rheumatoid arthritis, nephropathy, ocular diseases, cancer, diabetes, ageing, infection, inflammation, and the foetus are all linked to oxidative stress [2–4]. In the human body, oxidative stress is caused by an imbalance between reactive oxygen species (ROS) and antioxidants [5].

Reactive oxygen species (ROS) are produced during biological processes in living organisms [6]. ROS plays a role in the immune system against pathogens such as fungi and bacteria [7]. However, the overproduction of ROS leads to biomolecular damage, including DNA, lipids, and proteins [8]. In addition, ROS induces the breakdown of the peptide chains, cross-linking proteins,

oxidizing amino acids, and lipid peroxidation, thus causing cell death [9]. Free radicals, commonly known as ROS, contain one or more unpaired electrons. Non-free radicals, such as hydrogen peroxide and singlet oxygen, can be reactive free radicals [10]. The odd number of electrons in free radicals causes instability and is a highly reactive molecule. Radical ions are notated with the • symbol [11]. ROS include hydroxyl (HO•), superoxide ($O_2•^-$), nitrogen dioxide ($NO_2•$), and nitric oxide (NO•). The ROS and non-free radical species are summarized in Table 1 [10].

Table 1. The reactive oxygen species (ROS) and non-free radical species

Non-free radical species		Reactive oxygen species	
Hydrogen peroxide	H_2O_2	Hydroxyl radical	HO•
Singlet oxygen	$^1O_2$	Superoxide radical	$O_2•^-$
Nitrous acid	HNO_2	Nitrogen dioxide radical	$NO_2•$
Nitryl chloride	NO_2Cl	Nitric oxide radical	NO•
Nitroxyl anion	NO^-	Nitrosyl cation	NO^+
Ozone	O_3	Hydroperoxyl radical	HOO•
Lipid hydroperoxide	LOOH	Lipid radical	L•
Peroxynitrite	$ONOO^-$	Peroxyl radical	ROO•
Hypochlorite	HOCl	Lipid peroxyl radical	LOO•
Dinitrogen trioxide	H_2O_3	Lipid alkoxyl radical	LO•
Nitrous oxide	N_2O	Protein radical	P•
Peroxynitrous acid	ONOOH	Thiyl radical	RS•

Antioxidants are compounds that directly or indirectly inhibit oxidation and reduce the overexpress of free radicals [12]. Normally, the human body produces antioxidants to protect against the harmful effects of ROS, which are called endogenous antioxidants. Endogenous antioxidants include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px). SOD can be converted the superoxide anion to H_2O_2, CAT metabolizes H_2O_2 to water and oxygen, and GSH-Px reduces H_2O_2. Otherwise, exogenous antioxidants are produced from diet or food supplements as non-enzymatic antioxidants, such as vitamin C, vitamin E, carotenoids, and flavonoids. Vitamin C is a polar antioxidant and vitamin E is a non-polar antioxidant that protects against lipid peroxidation. In addition, the popular synthetic antioxidants include butylated hydroxyanisol (BHA), butylated hydroxytoluene (BHT), and tert-butylhydroquinone (TBHQ) [13].

The use of herbal plants with high antioxidant activity has been explored to prevent disease severity. *K. galanga* is one of the traditional medicines used for the treatment of various diseases, including anti-inflammatory [14], anti-cancer [15], antimicrobial [16], anti-sedative [17], and immunomodulator [18]. Several diseases are associated with oxidative stress and antioxidant activity. Measurement of antioxidant activity is important in studying the efficiency of antioxidants in preventing and treating diseases related to oxidative stress. Therefore, studies on natural antioxidants and food supplements that act against oxidative stress will exist in the future. This review highlights the antioxidant activities of *K. galanga in vitro* and *in vivo* using several assays. In addition, it can provide strong evidence that antioxidants can contribute to the improvement of some infectious conditions and chronic degenerative diseases to improve quality of life.

2. MATERIALS AND METHODS

The literature search was performed using the plant name using search engines including Scopus, PubMed, ScienceDirect, and Google Scholar. The following keywords were used "*Kaempferia*

galanga" AND "antioxidant or oxidative stress". The inclusion criteria in this review were only *in vitro* and *in vivo* published data from 2018-2023 and references without English-language titles were excluded. The databases were identified, analyzed, and chosen based on their relevance to the topic. The name of the plant was cross-checked with http://www.worldfloraonline.org website on August 3, 2023.

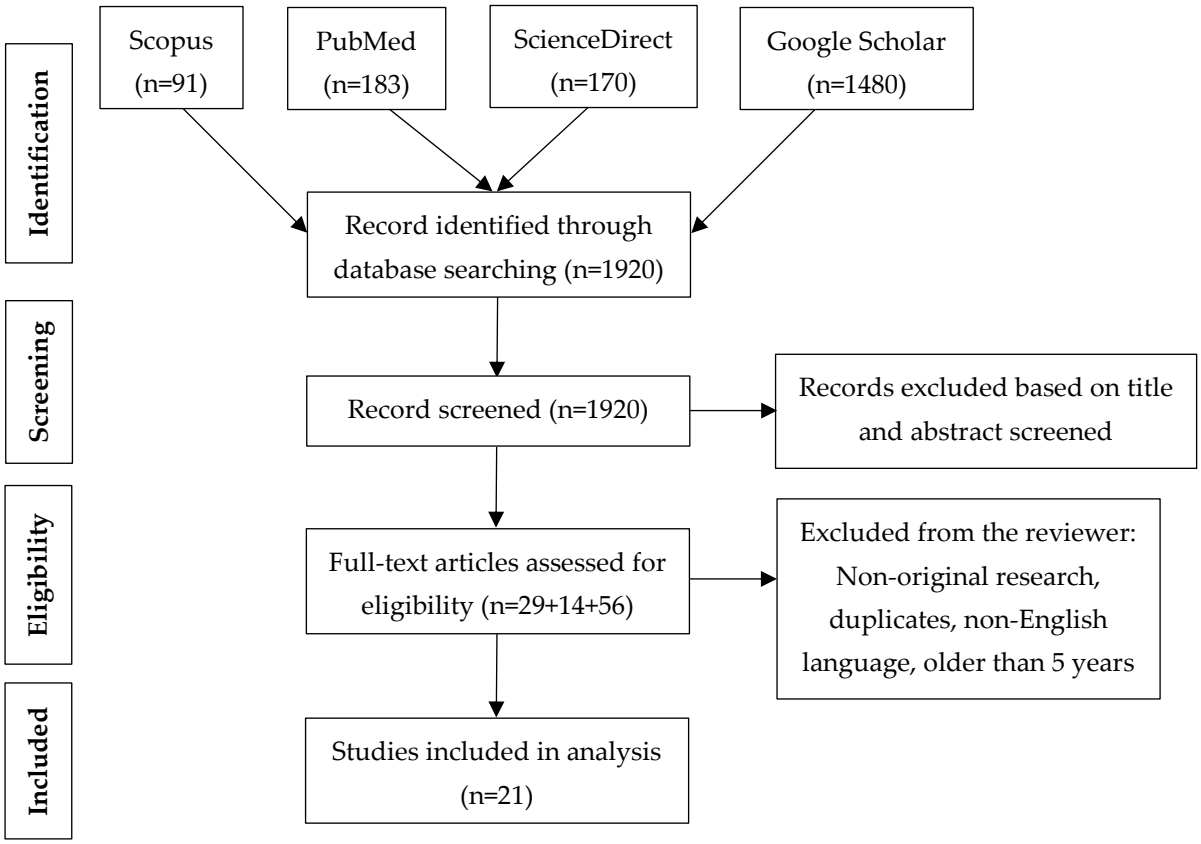

Figure 1. Flowchart of literature review

3. RESULTS AND DISCUSSION

Kaempferia galanga L. (*K. galanga*) is commonly called aromatic ginger, grouped under the Zingiberaceae family. *K. galanga* is probably a native plant from Asian countries including Indonesia, China, Malaysia, Thailand, and Nigeria [19]. Indonesian local people call as "*kencur*" in Bahasa and mix it with white rice as a beverage or "*Jamu Beras Kencur*"[20]. In recent decades, studies have reported that *K. galanga* extracts exhibit various pharmacological effects. The classification of *K. galanga* is explained as follows [21]:

Kingdom : Plantae
Division : Magnoliophyta
Class : Liliopsida
Family : Zingiberaceae
Order : Zingiberales
Genus : Kaempferia
Species : *Kaempferia galanga* L.

According to a previous study, the methanol extract of *K. galanga* increased total antioxidant and ferrous reducing capacities in a concentration-dependent manner. The methanol extract of *K. galanga* reported an IC_{50} value of 16.58, 8.24, and 38.16 μg/mL using 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and nitric oxide radical scavenging assays, respectively. In addition, the total phenolic and flavonoid content of *K. galanga* was 15.40 ± 0.35 mg/g of gallic acid equivalent and 37.72 ± 0.50 mg/g of catechin equivalent, respectively [22]. The DPPH radical scavenging activity of *K. galanga* crude extracts showed IC_{50} values of 831.82, 492.75, and 424.44 μg/mL in hexane, ethyl acetate, and methanol, respectively. Here, the study concluded that the methanol crude extracts had higher antioxidant activity [23]. The methanol and acetone extracts of *K. galanga* leaves showed IC_{50} values of 611.82 and 702.79 μg/mL, whereas the total phenolic contents were 1.012 and -0.066 mg of gallic acid equivalents/gm [24]. *K. galanga* showed antioxidant activity as determined by various assays of 53.39 ± 0.54, 26.16 ± 2.81, and 42.30 ± 2.96 μM Trolox equivalent/g DW in ABTS, DPPH, and ferric reduction of antioxidant power (FRAP) assays, respectively [25].

Flavonoid-rich *K. galanga* ethanolic extract using ultrasound-assisted extraction shows a 50% scavenging concentration of 0.1084 and 0.1273 mg/mL in DPPH and ABTS assays, respectively [26]. In addition, the antioxidant capacities of *K. galanga* essential oils with an IC_{50} of 7.93 μg/mL for both the DPPH assay and the Trolox C assay [27]. *K. galanga* extraction in 80% methanol solvent exhibited 1.10 ± 0.015 mg AAE/g, 22.15 ± 0.83 %, 1.53 ± 0.02 μg GAE/mg extract, and 0.82 ± 0.01 mg/QE/g of dried sample in ferric reducing antioxidant power activity, free radical scavenging activity, total phenolic, and total flavonoid content, respectively [18].

Kiptiyah *et al*., 2021 [28], evaluated that the ultrasonic extraction method with blanching treatment showed a higher effect on the total phenolic, total flavonoid, and radical scavenging activity of *K. galanga* (67.32 ± 3.05 mg GAE/L, 452.76 ± 9.68 mg EK/L, 56.20 ± 5.04%, respectively). Based on a correlation study, the total phenolic and flavonoid content shows a significant correlation with antioxidant activity. Jamil and Hasyim, 2022 [29], reported the comparison of the rhizome and leaves of *the K. galanga* extraction method. The IC_{50} values of 128.91 and 175.64 μg/mL in *K. galanga* rhizomes with ultrasonic-assisted extraction and hydrodistillation methods, respectively. Whereas, the IC_{50} values of 209.12 and 239.41 μg/mL in *K. galanga* leaves ultrasonic-assisted extraction and hydrodistillation methods, respectively. Muderawan *et* al., 2022 [30], evaluated some varieties of *K. galanga* from Bali, Indonesia. The first one is bigger rhizomes with dark brown epidermis and smaller rhizomes with light brown epidermis, which are thus varieties extracted using steam distillation and maceration methods. The DPPH antioxidant activity found IC_{50} values of 86.10 ± 1.51, 85.24 ± 1.48, 89.19 ± 1.72, and 86.49 ± 2.03 μg/mL in bigger rhizomes using steam distillation, bigger rhizomes using maceration, smaller rhizomes using steam distillation, and smaller rhizomes using maceration, respectively.

Recently, the level of total phenolic content using the Folin-Ciocalteu method and the total flavonoid total of *K. galanga* ethanol extract was 50.35 ± 0.05 mg GAE/100g, and 20.98 ± 0.09 mg QE/100 g, respectively. *K. galanga* ethanol extracts showed IC_{50} values of 626.308 ± 5.06 μg/mL and 139.92 ± 0.51 μg/mL using DPPH antioxidant and xanthine oxidase inhibitory activity, respectively [31]. Nonglang *et* al., 2022 [32], reported that freeze-dried ethanolic extract of *K. galanga* showed IC_{50} values of 1.824 mg/mL and 0.307 mg/mL for the DPPH and the ABTS assays, respectively. Whereas, the

results obtained showed a total polyphenol content of 23.55 ± 0.5 mg GAE/g dry weight of the extract and a flavonoid content of 100 ± 1.414 mg rutin equivalent/g dry weight of the extract.

The current findings indicate that the antioxidant activity of *K. galanga* at 100-500 μg/mL inhibits radical scavenging in the DPPH assay [33]. The total amount of phenolics and flavonoids in the ethanolic extract of *K. galanga* was 55.58 ± 0.54 mg GAE/g extract and 56.16 ± 3.15 mg quercetin equivalent/g, respectively. *In vitro* antioxidant effects of *K. galanga* ethanolic extract with IC_{50} values of 240.80 ± 5.50, 151.64 ± 2.51, 116.49 ± 4.76, and 214.80 ± 6.42 μg/mL for DPPH, ABTS, reducing power, and total antioxidant capacity assays, respectively [34].

Begum *et al*., 2023 [35] showed that the antioxidant activity of *K. galanga* with an IC_{50} value of 15.64 ± 0.263 μg/mL and 21.24 ± 0.413 μg/mL for the DPPH assay, 16.93 ± 0.228 μg/mL and 21.156 ± 0.345 μg/mL for the ABTS assay of ethyl *p*-methoxycinnamate rich *K. galanga* and ascorbic acid, respectively. The reducing power assay showed that ethyl *p*-methoxycinnamate-rich *K. galanga* was superior to ascorbic acid. In addition, the IC_{50} of the metal chelating assay was 19.29 ± 0.805 μg/mL for samples and 30.72 ± 0.834 μg/mL for EDTA. *In vitro*, the antioxidant activity of *K. galanga* extracts showed strong free radical scavenging capacity in DPPH, ABTS, and hydroxyl radical scavenging assays (IC_{50} values of 19.77 ± 1.28, 1.41 ± 0.01, and 3.09 ± 0.34 mg/mL, respectively) and weak reducing capacity in the reducing power assay with an EC_{50} value of 389.38 ± 4.07 mg/mL. In addition, *K. galanga* essential oil at 10 mg/mL showed DPPH radical scavenging activity of 52.70% [36]. Therefore, *K. galanga* has the potential to donate electrons to reactive free radicals.

Srivastava *et al*., 2019 [15], evaluated the ethyl *p*-methoxycinnamate of *K. galanga* inhibited ROS production in H_2O_2-stimulated J774.1 macrophage cells in a dose-dependent manner using an oxidation-sensitive dye (DCFH-DA) assay. In addition, the result of this study showed that the ethyl *p*-methoxycinnamate at 10 μg/mL decreased by more than 50% of the ROS level. Thus, ethyl *p*-methoxycinnamate of *K. galanga* potentially protects the cells from oxidative cellular damage. *K. galanga* rhizome oil and microemulsion formulation indicated moderate UV protective activity and reduced nitric oxide production in lipopolysaccharides (LPS)-induced RAW 264.7 macrophage cells [37]. Moreover, *Kaempferia galanga* L. rhizomes gel formulation has been examined for its wound healing activity by increasing cell viability, cell migration, collagen content, and decreasing nitric oxide production in H_2O_2-induced human dermal fibroblasts [38].

Oxidative stress leads to lipid peroxidation, which produces malondialdehyde (MDA) [39]. Dwita *et al*., 2021 [40], examined the ethanol extract, water fraction, ethyl acetate fraction, n-hexane fraction, and ethyl *p*-methoxycinnamate isolate of *K. galanga* decreases MDA concentration in serum, exudate, and tissue in carrageenan-induced granuloma air pouch inflammation rats.

Based on Men et al., 2022 [34], an *in vivo* antioxidant study showed *K. galanga* ethanolic extract in both 20 mM paraquat and H_2O_2 assays using the *Drosophila melanogaster* model. Under 20 mM paraquat, *K. galanga* increased the longevity of fruit flies through life expectancy (at 18.4 ± 0.69 h), maximum lifespan (at 29.0 ± 1.0 h), and 50% survival (at 15.67 ± 0.58 h). Meanwhile, under the 10% H_2O_2 condition, *K. galanga* shows 50% survival at 30.0 ± 2.0 h, mean lifespan at 36.87 ± 1.35 h, and maximum lifespan at 59.67 ± 0.58 h, as well as under paraquat conditions.

Recently, *K. galanga* essential oil extracts were shown to play a role in antioxidant activity by attenuating ROS generation, cell death, lipid peroxidation, and malondialdehyde [36]. According to Munda et al. (2018), the main essential oils of K. galanga are ethyl p-methoxycinnamate, ethyl cinnamate, 1,8-cineole, borneol, camphene, linoleoyl, methyl-cinnamate, and pentadecane [41].

Treatment of the zebrafish model with H_2O_2-induced oxidative stress reduced intracellular ROS generation, cell death, and lipid peroxidation by 103.31%, 105.04%, and 108.00%, respectively. In addition, the extracts increased superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) levels, as well as the survival rate and heart rate in H_2O_2-treated zebrafish embryos. In contrast, the MDA level decreased by 63.00% compared with that in the H_2O_2-treated group [36]. Shao *et al*., 2023 [42] demonstrated that kaempferide in *K. galanga* inhibited oxidative stress in renal tubule cells to protect against cisplatin-induced acute kidney injury by regulating intracellular ROS, MDA, and SOD.

Here, we summarize the popular design used to evaluate the antioxidant activity of *Kaempferia galanga*. Among *in vitro* antioxidant assays, radical scavenging methods are the most popular. This review found that DPPH, ABTS, FRAP, ROS, SOD, GSH, MDA, and CAT are commonly used for the antioxidant assays of *K. galanga*. Table 2. Commonly, the pharmacology effect of *K. galanga* is on the rhizome. However, this review found that leaves could be used for antioxidant activity.

Table 2. *In vitro*, the antioxidant activity of *K. galanga* using a chemical reaction

No.	Assays	Extracts/Fractions/Compounds	Results		Ref.
			[IC_{50}]	**[EC_{50}]**	
1.	DPPH	Methanol extract	16.58 μg/mL		[22]
		Methanol extract	424.44 μg/mL		[23]
		Hexane extract	831.82 μg/mL		[23]
		Ethyl acetate extract	492.75 μg/mL		[23]
		Methanol extract	611.82 μg/mL		[24]
		Acetone extract	702.79 μg/mL		[24]
		Ethanolic extract	26.16 ± 2.81 μM Trolox equivalent/g		[25]
		Flavonoid-rich ethanolic extract	0.1084 mg/mL		[26]
		Essential oils	7.93 μg/mL		[27]
		80% methanol extract	22.15 ± 0.83 %		[18]
		Water extract (ultrasonic extraction method with blanching treatment)	56.20 ± 5.04 %		[28]
		Essential oil rhizome extract (ultrasonic-assisted extraction pretreatment)	128.91 μg/mL		[29]
		Essential oil rhizome extract (hydrodistillation method)	175.64 μg/mL		[29]
		Essential oil leaves extract (ultrasonic-assisted extraction pretreatment)	209.12 μg/mL		[29]
		Essential oil leaves extract (hydrodistillation method)	239.41 μg/mL		[29]
		Rhizome oil (bigger rhizomes using steam distillation method)	86.10 ± 1.51 μg/mL		[30]
		Rhizome oil (bigger rhizomes using maceration method)	85.24 ± 1.48 μg/mL		[30]
		Rhizome oil (smaller using steam distillation method)	89.19 ± 1.72 μg/mL		[30]
		Rhizome oil (smaller rhizomes using the maceration method)	86.49 ± 2.03 μg/mL		[30]
		Ethanolic extract	626.308 ± 5.06 μg/mL		[31]
		Freeze-dried ethanolic extract	1.824 mg/mL		[32]
		Ethanolic extract	100-500 μg/mL		[33]
		Ethanolic extract	240.80 ± 5.50 μg/mL		[34]

…continued Table 2

		Ethyl *p*-methoxycinnamate-rich essential oil	15.64 ± 0.263 µg/mL		[35]
		Essential oil		19.77 ± 1.28 mg/mL	[36]
2.	FRAP	Ethanolic extract	42.30 ± 2.96 µM Trolox equivalent/g		[25]
		80% methanol extract	1.10 ± 0.015 mg AAE/g		[18]
		Ethanolic extract	116.49 ± 4.76 µg/mL		[34]
		Essential oil		389.38 ± 4.07 mg/mL	[36]
3.	ABTS	Methanol extract	8.24 µg/mL		[22]
		Ethanolic extract	53.39 ± 0.54 µM Trolox equivalent/g		[25]
		Flavonoid-rich ethanolic extract	0.1273 mg/mL		[26]
		Freeze-dried ethanolic extract	0.307 mg/mL		[32]
		Ethanolic extract	151.64 ± 2.51 µg/mL		[34]
		Ethyl *p*-methoxycinnamate-rich essential oil	16.93 ± 0.228 µg/mL		[35]
		Essential oil	1.41 ± 0.01 mg/mL		[36]
4.	Hydroxyl radical scavenging assays	Essential oil	3.09 ± 0.34 mg/mL		[36]
5.	Metal chelating assay	Ethyl *p*-methoxycinnamate-rich essential oil	19.29 ± 0.805 µg/mL		[35]
6.	Phosphomolybdate	Ethanolic extract	214.80 ± 6.42 µg/mL		[34]
7.	Xanthine oxidase assay	Ethanolic extract	139.92 ± 0.51 µg/mL		[31]
8.	NO radical scavenging assays	Methanol extract	38.16 µg/mL		[22]

Note: DPPH (1,1-diphenyl-2-picrylhydrazyl); FRAP (ferric reduction of antioxidant power), ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)); NO (nitric oxide); IC_{50} (inhibition concentration 50%); EC_{50} (effective concentration at which the absorbance was 0.5).

Table 3. Antioxidant activity of *K. galanga* in cell lines and animal models

No.	Compounds	Models	Induction	Results	Ref.
1.	Ethyl *p*-methoxycinnamate	J774.1 macrophage cells	H_2O_2	↓ROS production	[15]
2.	Rhizome oil and microemulsion formulation	RAW 264.7 macrophage cells	LPS	↓NO production	[37]
3.	Rhizomes gel formulation	Human dermal fibroblast	H_2O_2	↑cell viability, cell migration, collagen content	[38]
4.	Ethanol extract, water fraction, ethyl acetate fraction, n-hexane fraction, ethyl *p*-methoxycinnamate isolated	*Wistar* rats	Carrageenan	↓NO production ↓MDA	[40]

…continued Table 2

5.	Ethanolic extract	*Drosophila melanogaster*	20 mM paraquat, 10% H_2O_2	↑life expectancy, lifespan, survival	[34]
6.	Essential oil	Zebrafish	H_2O_2	↓MDA, ROS generation, cell death, lipid peroxidation	[36]
7.	Kaempferide	C57BL/6 mice, HK-2 cells	Cisplatin	↑SOD, CAT, GSH ↓ROS, MDA ↑SOD	[42]

Note: ↑(increase); ↓(decrease); LPS (lipopolysaccharides); ROS (reactive oxygen species); SOD (superoxide dismutase), CAT (catalase); GSH (glutathione peroxidase); MDA (malondialdehyde); NO (nitric oxide); H_2O_2 (hydrogen peroxide)

Generally, the main mechanism of antioxidants is to prevent or detect chain oxidative propagation, stabilize the radicals, and thus reduce oxidative damage in the body [43]. Several assays have been used to evaluate antioxidant activities. Mainly, based on chemical reactions, the mechanisms of antioxidant assay are hydrogen atom transfer (HAT) and single electron transfer (SET). HAT assesses the ability of antioxidants to transfer hydrogen atoms, including the total peroxyl radical trapping antioxidant parameter (TRAP), oxygen radical absorption capacity (ORAC), and total oxy-radical scavenging capacity (TOSC) assays. On the other hand, SET measures the ability of antioxidants to transfer an electron in a pH-dependent manner. SET assays include ferric reduction of antioxidant power (FRAP), cupric ion-reducing antioxidant capacity (CUPRAC), and the Folin-Ciocalteu test (FC). Mixed assays (HAT/SE) were used to eliminate stable chromophores, including 1,1-diphenyl-2-picrylhydrazyl (DPPH) free-radical scavenging activity, Trolox equivalent antioxidant capacity (TEAC)/ABTS radical cation decolorization, and *N*, *N*-dimethyl-p-phenylenediamine dihydrochloride (DMPD) [9, 39].

DPPH is a stable, long-lived radical that reacts with other radicals or reducing agents, leading to a decrease in absorption at 515 nm. Unfortunately, contrary to peroxyl radicals, antioxidants may react slowly or inertly with DPPH. *K. galanga* may inhibit the color development of the ABTS radical. The formation of ABTS cation radicals from 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) intensively decreases absorption at 734 nm. However, neither DPPH nor ABTS radicals are exact models of radical reactions in biological systems; instead, they are stable radicals. Fe(TPTZ)2]Cl3 (TPTZ ligand 2,4,6-tripyridyl-triazine) as an oxidizing agent is reduced by a transfer electron from *K. galanga* to produce [Fe(TPTZ)2]Cl2 that absorbs at a wavelength of 593 nm. In addition, the mechanism of the hydroxyl radical scavenging assays of *K. galanga* is reduced to the transfer of a hydrogen atom from *K. galanga* to the peroxyl radicals (ROO•) [45].

K. galanga plays a crucial role in the protection of cells from oxidative damage by inhibiting lipid oxidation. MDA is an indicator of lipid peroxidation caused by oxidative stress, particularly ROS. Hydroxyl radicals and peroxynitrite cause cell membrane and lipoprotein damage via lipid peroxidation. SOD, CAT, and GSH-Px are enzymes that play a role in defense against oxidative stress. SOD, as the first line of defense against free radicals, transforms the dismutation of $O_2^{\bullet -}$ into H_2O_2 and is then converted into H_2O and O_2 by CAT or GSH-Px [46-47].

As mentioned previously, *K. galanga* can stimulate several antioxidant enzymes, such as SOD, CAT, and GSH [36, 40]. On the one hand, *K. galanga* is shown to reduce MDA and NO production

levels [37, 40]. In addition, the parameter of intracellular antioxidant enzymes plays a key role in homeostasis and the mechanism of ROS signaling pathways [48]. Antioxidant enzymes counterbalance the effects of reactive oxygen species (ROS) and reactive nitrogen species (RNS). In between, radical nitric oxide is produced by nitric oxide synthases, which react with $O_2^{\bullet -}$ to result in peroxynitrite formation. The peroxynitrite then reacts with CO_2 to lead to nitrosoperoxycarboxylate, which then breaks down into carbonates and $NO_2\bullet$ radicals [49]. Briefly, *K. galanga* inhibits oxidative stress through the suppression of the NF-κB signaling pathway [50]. NF-κB plays a role in cell death, ROS, RNS, and immune responses. Therefore, the regulation of NF-κB as a target may be proposed for protection from ROS. *K. galanga* acts as an antioxidant by reducing ROS production, hence decreasing the activation of IκBα Kinase β (IKK β), as a regulator of the transcription factor NF-κB [51].

Interestingly, a review found that the zebrafish is the modern *in vivo* model that is used to evaluate the bioactivity of compounds because of its easy handling, short generation time, and transparent embryos [52]. In a previous study, an H_2O_2-induced zebrafish model was used to evaluate the *in vivo* antioxidant effect of *K. galanga* [36]. *Drosophila melanogaster* (*D. melanogaster*) fruit flies were used to evaluate the antioxidant activity of herbal medicine. A previous study reported that approximately 75% of *Drosophila* genes are homologous to human disease genes. *Drosophila* has been successfully applied as an *in vivo* model to assess antioxidant studies [53, 54].

The high antioxidant activity of *K. galanga* may be attributed to its high *ethyl p-methoxycinnamate* concentration [35]. This review is an effort to recapitulate recent and well-established research on effective *K. galanga* antioxidants, which would be helpful for the modern prevention of acute and chronic disease advancement in this field.

4. CONCLUSION

K. galanga contained phenolic and flavonoid compounds that exhibited antioxidant activities. The antioxidant activity of *K. galanga* occurs through several mechanisms, such as hydrogen atom transfer and single electron transfer, with the radical scavenging mechanism being the most popular. In addition, *K. galanga* exhibited antioxidant effects by regulating ROS, MDA, SOD, CAT, and GSH-Px levels. However, the feasibility of the antioxidant activity of *K. galanga* is based on the combination of various mechanisms. In the future, *K. galanga* could be used as a preventive agent against oxidative stress-associated diseases.

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Original Article

Formulation and Evaluation of Ketoconazole Nano-suspension

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Abstract : The use of antifungals that are widely used by the public is ketoconazole. Ketoconazole is included in the Biopharmaceutical Classification System (BCS) II category, where the drug is poorly soluble in water but has high permeability. In this study, ketoconazole was prepared in a nanosuspension formulation increase solubility. The method for preparing 2% ketoconazole nanosuspension used a sonicator bath with variations of tween 80 (F1 8%, F2 10%, F3 12%) and transcutol as surfactant and co-surfactant. The results showed that the particle size of the ketoconazole nanosuspension F1 was 102 ± 4.3 nm, F2 was 105 ± 3.2 nm, and F3 was 90 ± 2.4 nm on day 0. Testing on day 28 showed an increase in particle size in F1 115 ± 3.1 nm, F2 129±4.5nm, F3 97±2.4nm ($p>0.05$). In testing the morphology of the nanosuspension using Scanning Electron Microscopy (SEM), The results showed that the irregular spheres were scattered. Organoleptic testing showed clear and homogeneous preparations. pH testing for 28 days showed a pH range of 5.6-6 which is included in the physiological skin pH range ($p>0.50$). Nanosuspension preparations can be said to meet the physical evaluation requirements.

Keywords: BCS II, Ketoconazole, Nanosuspension, SEM, Permeability

1. INTRODUCTION

The fungal disease of the skin is a disease that is still commonly found in Indonesia [3]. Fungus treatment that is often used by the community is the azole group, one of the drugs is ketoconazole 2%. Azole antifungals have a broad spectrum that can be well tolerated by the body. Ketoconazole can inhibit the growth or death of fungal cells by causing irregularities in the fungal cytoplasmic membrane [6]. Ketoconazole is included in the Biopharmaceutical Classification System (BCS) class II, which is a class of drugs that are poorly soluble in water, have high permeability [2]. Ketoconazole preparations on the market are in the form of creams and tablets. Absorption of ketoconazole orally is not maximized due to low solubility and side effects. Drug ingredients that are poorly soluble in water show imperfect absorption [11].

Nanotechnology is a form of new technique in the world of health that can optimize a drug delivery system [7]. One of the nanotechnology that has been developed for drug delivery is nanosuspension [13]. Nanosuspension can help increase the solubility and bioavailability of class II BCS drugs. Nanosuspension is a colloidal dispersion system that contains drugs with a particle size of 10-1000 nm dispersed with surfactants or polymeric materials as stabilizers [12]. In a previous [14] study regarding the manufacture of Paracetamol suistained release nanosuspension preparations, the particle size distribution of formulation was obtained in narrow ranges from 145nm to 86 nm and the average particle size was found to be 95±8 nm. At 15 minutes,

54.081% of drug was released from the pure drug in water, 29.050% of drug released from NS1, 27.250 % drug released from NS2 and 14.725% of drug released from NS1.

In this study, ketoconazole nanosuspension was prepared using a sonicator bath to reduce particle size. The preparation of this preparation requires consideration in the selection of stabilizers in the form of surfactants, polymers, or a combination of both to increase solubility by reducing the surface tension of a drug [9]. This study used a non-ionic surfactant, namely tween 80 which is non-toxic and non-irritant, and co-surfactant in the form of transcutol. This study aims to evaluate the variation of tween 80 in ketoconazole nanosuspension preparations related to particle size, polydispersity index, organoleptic, SEM, pH, and room temperature stability.

2. MATERIALS AND METHODS

2.1. Materials

The tools used in this study include magnetic stirrer (SH-2), sonicator bath (WL-963), Particle Size Analyzer (PSA) (LS 13-320), pH meter (Atc), SEM (FEI-S50), analytical balance (M5-M214A), and other glassware. The materials used in this study included Ketoconazole (Dexa Medica), tween 80 (MagLab), Transcutol (AvenaLab), 96% ethanol (Onemed), and distilled water (MagLab).

2.2. The Nanosuspension Preparation

In the process of making nanosuspension preparations, a modified procedure was carried out from research conducted by Dzakwan [4]. The design of the nanosuspension spray preparation formula:

Table 1. Ketoconazole Nanosuspension Formula Design

Ingredients	Formula 1	Formula II	Formula III
Ketoconazole	2%	2%	2%
Tween 80	8%	10%	12%
Transcutol	2%	2%	2%
Aquadest	ad 100%	ad 100%	ad 100%

The process of making 30 ml of nanosuspension spray preparations, the ingredients are weighed according to the concentration of each ingredient. The ketoconazole and tween 80 materials were put into a beaker and mixed using a magnetic stirrer for 15 minutes. Then the transcutol was put into the mixture in the beaker glass, then stirred again with a magnetic stirrer for 15 minutes until all the ingredients were mixed homogeneously. Furthermore, the mixture was reduced in particle size using a sonicator bath frequency 50 Hz with temperature 25-30℃ for 60 minutes. Then, aAfter the nanosuspension is formed, visually the preparation is transparent, up to 30 ml of aquadest is added to the nanosuspension preparation and stirred using a magnetic stirrer for approximately 15 minutes until it is homogeneous.

2.3. The Evaluation of Ketokonazole Nanosuspension :

2.3.1 The Organoleptic Test

The organoleptic test of the nanosuspension was carried out by visually observing the shape, aroma, and color of the nanosuspension preparation [16].

2.3.2 The Particle Size Test and Polydispersion Index

Particle size testing was carried out with a PSA tool by means of 1 ml of nanosuspension preparation added with 10 aquadest then put into the cuvette up to the mark, then read with the PSA tool. The results obtained are in the form of particle size values and polydispersity index graphs [6].

2.3.3 The Particle Morphology Test

The morphology of the nanosuspension particles was measured using an SEM tool by preparing a platinum plate and placing the sample on its surface. Then the plate is inserted into the SEM tool for approximately 15 minutes. SEM tools that are connected to a computer and observed at a voltage of 7 kV at a magnification of 40,000 times [6].

2.3.4 The pH test

The pH test was carried out using a pH meter beginning with calibrating the electrode with a standard pH buffer of 4.7 and 9. After the pH display was appropriate and stable, the electrode was dipped into the preparation. The pH value will appear on the screen when the number is stable. The pH value required for topical preparations on the skin is 4.5-6.5 [15].

2.3.5 The Stability Test

Testing the stability of the ketoconazole nanosuspension preparations was carried out by placing the preparations at room temperature conditions and evaluating organoleptic, particle size, and pH on days 0, 14, and 28 [14]. In the stability test, the nanosuspension preparations were tested for organoleptic, particle size, polydisspecificity index, and pH stored at room temperature.

2.3.6 Data analysis

The research data were analyzed descriptively and statistically. Statistical testing using the one-way ANOVA method. The value of $p > 0.05$ indicates that there is no difference in the effect of the variables between the formulas. While the value of $p < 0.05$ indicates that there is a difference due to the influence of variables in the study, namely variations in the concentration of tween 80 surfactant.

3. RESULTS AND DISCUSSION

In this study, ketoconazole was obtained from PT. Dexa Medica Palembang as a test sample. Evaluation of the preparations carried out included organoleptic tests, particle size and PI tests, pH tests, particle morphology tests, and stability tests. Optimization of the preparation of ketoconazole nanosuspension preparations with varying concentrations of tween 80. Tween 80 is a non-ionic surfactant, non-toxic, non-irritating to the skin, and safe for topical application. The process of reducing the size of ketoconazole particles using a sonicator bath. The sonicator device utilizes sonic vibrations (sound) to break the particles down to nano size. Ultrasonic waves when in a liquid medium will cause acoustic cavitation which causes the particles to disintegrate due to sound [17]. The particles in the nanosuspension have a large enough surface area so that the surface free energy is also high. This causes the particles in the nanosuspension to tend to interact with each other so that agglomeration or aggregation can occur [1]. In this study, the stabilization of nanosuspensions used tween 80 with a mechanism of steric hindrance, in which the particles in the nanosuspension preparations would be covered by a stabilizer so that interactions between particles could be minimized [20]. The organoleptic test results obtained are as follows.

Table 2. Organoleptic Test Results

No	Formula	Observation		
		Form	**Colour**	**Homogenity**
1	F1	dispersed	slightly turbid	homogeneous
2	F2	dispersed	slightly turbid	homogeneous
3	F3	dispersed	transparent	homogeneous

This organoleptic test was carried out on day 0. The size of the nanosuspension particles can be seen visually, showing relatively clear and transparent dispersion results [12]. Optimization of nanosuspension preparations was carried out by comparing nanosuspension preparations F1, F2, and F3, where in the three formulas there were differences in Tween 80 concentration. From the test results, it was found that the F3 formula produced a clear solution because it was influenced by the high concentration of tween 80, which was 12%. Whereas for F1 and F2 the results obtained were slightly turbid preparations, and when left to stand for 1 week experienced fine particle settling which could still be dispersed when shaken. This could be due to the fact that the concentration of tween 80 to stabilize the particles is still not optimal [6].

Figure 1. Optimization Results of Making Ketoconazaol NanosuspensionF1(left), F2(middle), F3 (right**)**

Tests for particle size and distribution were carried out to see whether the nanosuspension preparations entered the range of less than 1000 nm [12]. Following are the results of testing the particle size and polydispersity index. The polydispersity index value of below 0.5 indicating that nanosuspensions of ketokonazole have the homogeneous dispersion system, so it could have a better stability.

Table 3. Particle Size Test Results and Polydispersity Index (n-3)

Formula	**Particle Size**		**Polydispersity Index**	
	0 Day	**28th day**	**0 day**	**28th day**
1	106±2.11	115±1.11	0.3±0.04	0.3±0.04
2	102±1.24	129±3.44	0.3±0.05	0.3±0.05
3	95±2.67	97±2.14	0.1±0.045	0.1±0.04

The measurement results showed changes in particle size from day 0 and day 28. Factors causing changes in particle size could be because the particles are unstable during storage and experience particle deposition resulting in agglomeration [4]. The changes in particle size up to day 28 were also visible as there was a change in color which became visually slightly cloudy. The polydispersity index value obtained for each formula, on the 0th and 28th day tests obtained a value less than 0.5 which indicates a uniform distribution of particles [8]. Statistical tests also showed no significant difference in the results of the particle size and polydidercity index tests on the 0th and 28th days for each sample ($p>0.05$).

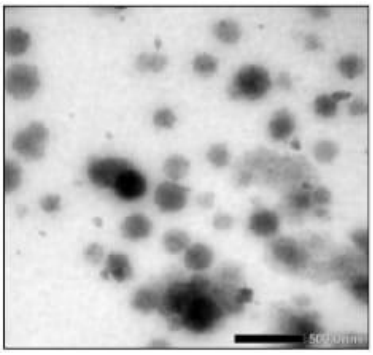

Figure 2. The Results of Particle Morphology Test of Ketoconazole Nanosuspension F3 (magnification 10000x)

In testing the morphology of the nanosuspension particles using the SEM tool, shown in the Figure 2, it can be seen that the particles are almost spherical in shape which are irregular. The formula used in this test is formula 3 because the particle size is the smallest among the other formulas. SEM testing of this nanosuspension wanted to see the shape resulting from the nano manufacturing process. Based on the results obtained, some particles tend to combine which may result in agglomeration or settling of large particles for a certain period of time [18]. This is possible visually it may not be visible because the color is clear, but microscopically it becomes visible. Thus, these events can be considered in determining the concentration of surfactants and co-surfactants used [9].

The results of the nanosuspension pH test showed a pH value in the range of 5.6-6. The pH value shows that all the formulas made meet the requirements for the pH range criteria that are tolerated by the skin, namely 4.5-6.5 [1]. The increase in pH in each formula can be caused by an increase in tween concentration, where the pH of tween is 5.5-7.2. The degree of acidity or pH is a value that is used to see the level of acidity or alkalinity of a substance, solution, or object.

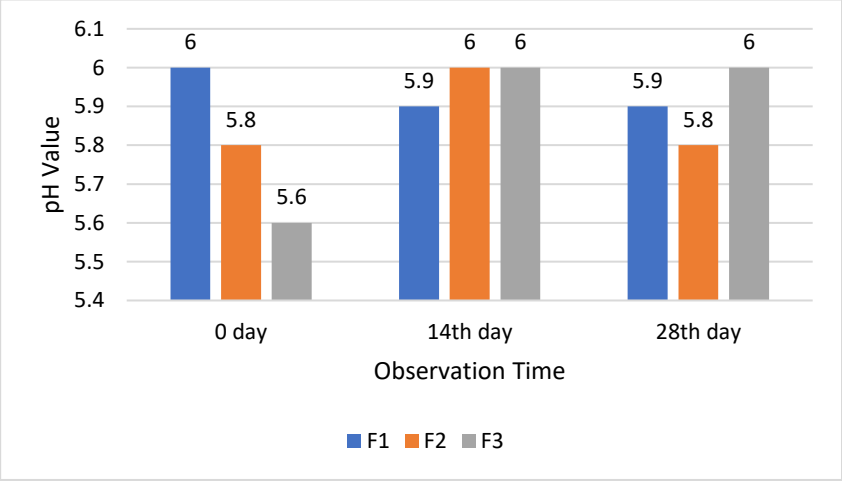

Figure 3. The Results of pH testing of ketoconazole nanosuspension preparations

The pH value of the nanosuspension preparation tends to be acidic because of the acidic nature of kecoconazole [10]. The ketoconazole nanosuspension was tested for 28 days to see if there was a change in pH during storage at room temperature. The results of the statistical tests showed that there was no significant difference from the results of the tests on days 0, 14 and 28 on the pH value of each preparation ($p>0.05$).

4. CONCLUSIONS

Based on the results of the research that has been done, it was found that of the 3 formulas for the ketoconazole nanosuspension, the particle size entered the nano range, namely 95-129 nm, the pH entered the range, namely 5.6-6, the particle morphology test showed irregular spherical particle shapes, and visually the appearance of the nanosuspension is clear and there is no precipitate. The test was carried out for 28 days and each preparation was tested. There is no significant difference from any results evaluated based on statistical tests.

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