Analysis of Eugenol Content in Ethanolic Extract of Galangal Rhizome (*Alpinia galanga* L. Willd) Ointment Using UV-VIS Spectrophotometry Method

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Abstract: Galangal (*Alpinia galanga* L.) is a plant that commonly used by people as traditional medicines. Galangal rhizome contains various compounds such as galangin, methyl cinnamate, cineole, camphor, δ-pinene, and eugenol. Eugenol has an analgesic, antioxidant, and antibacterial activity that is usually used in topical preparations. In manufactured drugs, it is necessary to examine active compound which is one of the requirements to ensure its quality. This study aims to find out eugenol levels in the ointment of ethanolic extract from galangal rhizome. The method used to extract secondary metabolite from galangal rhizome is digestion using ethanol 70%. Eugenol separation from ointment of ethanolic extract of galangal rhizome was done by liquid-liquid extraction using chloroform as a solvent. The eugenol was analyzed using a UV-Vis Spectrophotometer because eugenol has chromophore, a benzene ring, so able to absorb ultraviolet light. Parameters of validation method used in this study are linearity, accuracy, precision, Limit of Detection (LOD) and Limit of Quantification (LOQ). The result showed that analysis method of eugenol in the ointment of ethanolic extract of galangal rhizome has good validation, with linearity, accuracy and precision occupied the requirement, LOD level is 2.388 µg/mL and LOQ level 7.235 µg/mL. Determination of eugenol level in the ointment of ethanolic extract from galangal rhizome obtained the result of 5.187 mg/gr sample.

1 INTRODUCTION

Galangal is a plant that has been used traditionally as a medicinal plant. The galangal rhizome is easily obtained and often used as food spices. Galangal rhizome contains various compounds, such as flavonoids, terpenoids, saponins, phenolic acids and essential oils (Tang *et al*., 2018). In galangal essential oils, eugenol has various activities as an analgesic, antifungal, antitermitic, antibacterial, anti-inflammatory, and antioxidant (Magalhães *et al*., 2018; Park *et al*., 2011; Xie *et al*., 2015; Zhang *et al*., 2017).

The utilization of ethanol extract of galangal rhizome as topical medicines when used directly on the skin is not optimal and is less comfortable, so it is necessary to create an ointment preparation form. This form of ointment is preferred, because it is easier to use, practical, site-specific application of drug on affected area, convenient for unconscious patients having difficulty in oral administration, chemically more stable, and avoid first-pass metabolism of drug (Shelke and Mahajan, 2015). In manufactured drugs, it is necessary to examine active compounds which is one of the requirements to ensure its quality. A good quality of drug preparation will greatly support the achievement of the expected therapeutic effect.

Eugenol has chromophore which is a group in organic compounds that is capable of absorbing ultraviolet light and visible light. The use of UV-Vis spectrophotometry for analyzing eugenol levels in drug preparation has great benefits in the industrial world. The method is simple, fast, economical, able to measure a solution with very small concentrations, and can produce results accurately. Until now, there has been no research that performed the analysis of eugenol in ointment of ethanol extract of galangal rhizome using UV-Vis spectrophotometry. Based on the reasons mentioned above, it is necessary to examine an analytical method to determine the level of eugenol in the

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ointment of ethanol extract of galangal rhizome using UV-Vis spectrophotometry method.

2 MATERIALS AND METHOD

2.1 Materials

The tools used in this study are rotary evaporator (RVO 400SD Boeco Germany), magnetic stirrer (IKA C-MAG HS 7), stirrer bar, analytical scale (KERN ALJ), UV-Vis Spectrophotometer (Thermo Scientific Genesys 10S), waterbath. Galangal rhizome obtained from Wonogiri regency, vaseline album (Bratacco), cera alba (Bratacco), stearyl alcohol (Bratacco), standard eugenol (Merck), ethanol 70%, ethanol pa (Merck), KOH (Merck), H2SO4 (Merck), n-hexane, ethyl acetate pa (Merck), chloroform pa (Merck), filter paper, and aquadest.

2.2 Methods

2.2.1 Preparation of Ethanolic Extract Galangal Rhizome

The galangal rhizomes were washed, finely chopped, dried at 50ºC and then powdered. Galangal powder was extracted using 70% ethanol with 1:10 ratio. The extraction was heated in waterbath at 40-50ºC and stirred in 3000 rpm for 1 hour. The liquid extract obtained was separated, then thickened using rotary evaporator at 50ºC until viscous extract was obtained.

2.2.2 Qualitative Test for Ethanolic Extract of Galangal Rhizome

Extract and standard eugenol was spotted on TLC plate (silica gel GF254) and eluted with mobile phase (n-hexane:ethyl acetate, 4:1 v/v). Spots were observed at UV 254 nm. The separation was done and Rf value of extract was calculated and then compared to Rf value of standard eugenol.

2.2.3 Formula Ointment of Ethanolic Extract of Galangal Rhizome

Vaseline album, cera alba, stearyl alcohol were heated in waterbath until they melted. Vaseline album and cera alba were put into a warm mortar, mixed and stirred until cool. Subsequently, stearyl alcohol was added, stirred until homogeneous and formed an ointment base. Then ethanol extract of galangal rhizome was added and stirred until homogeneous. The ointment obtained was tested by organoleptic. The formula of the ointment is described in Table 1.

Table 1. Ointment formula of ethanolic extract of galangal rhizome

Ingredients	Quantity (%)
Ethanolic extract galangal rhizome	10
Vaseline album	78
Cera alba	9
Stearyl alcohol	3

2.2.4 Extraction Eugenol from Ointment

The separation process of eugenol from the ointment was carried out by liquid-liquid extraction method (LLE) using separating funnel. The sample used was the ointment of ethanolic extract of galangal rhizome. 2.5 grams of ointment was weighed and 20 mL of KOH solution 0,8 N was added to break the ointment matrix, then stirred at 2000 rpm for 30 minutes at 25ºC. The base was then separated and water-soluble phase was taken. The water phase was added with chloroform and shaken for 10 minutes to allow 2 layers to form. The layers were separated and the aqueous phase taken, added with H_2SO_4 until pH 4 then added with 10 mL of chloroform and extracted 3 times. The chloroform phase was evaporated, then the residue was dissolved in 5 mL of ethanol, filtered and inserted to the flakon.

2.2.5 Determination of Lambda Maximal of Eugenol

Stock solution was prepared by dissolving 10 mg standard eugenol in ethanol of up to 100 ml to obtain 1000 ppm of eugenol stock solution. From this stock solution, 100 µL was dissolved in 10 mL of ethanol (10 ppm), then the determination of λ max was carried out.

2.2.6 Preparation of Standard Curve of Eugenol

From eugenol stock solution, 3, 5, 15, 25, 30, 35 µg/mL were prepared and scanned in spectrophotometry UV. The corresponding absorbances were noted and then a calibration curve was plotted.

2.2.7 Validation Method

Validation of the analysis method included linearity, precision, accuracy, LOD and LOQ. Linearity was

performed by adding standard concentrations of 3, 5, 15, 25, 30, and 35 µg/mL to the samples prior to extraction process. The absorbance was measured and calibration curve was determined by linear regression equation and correlation coefficient was calculated. Precision was tested as repeatability (intraday) and intermediate precision (interday). Repeatability: analyzing the samples that have been added with the series of eugenol standard solutions 5, 15, 25, 30, 35 µg/mL, measured three times in one day. Percentage of RSD was calculated from the obtained absorbance to determine variations within a day. Intermediate precision: analyzing the samples that have been added with the series of eugenol standard solutions 5, 15, 25, 30, 35 µg/mL, measured on three different days. The percentage of RSD was calculated from the obtained absorbance to determine the variation between days. Accuracy test was performed by adding solution series of eugenol concentrations of 5, 15, 25, 30, 35 µg/mL and replicated 4 times. Accuracy was indicated as % recovery. The analysis was repeated three times. LOD and LOQ was performed by calibration curve method using linear regression line. The standard deviation was calculated using calibration curve then calculated to find the value of LOD and LOQ.

2.2.8 Determination of Eugenol Level in Ointment of Ethanolic Extract of Galangal Rhizome

Eugenol levels were determined by liquid-liquid extraction method (LLE) using separating funnel and then measured using a UV-Vis spectrophotometer. The method used is standard additions. The absorbance of the sample without the addition of standard solution was then inserted into the calibration curve previously obtained, and the regression equation was generated.

3 RESULT AND DISCUSSION

Ethanol was chosen as a solvent because it is a universal solvent that can attract polar and non-polar compounds. Ethanol is also selected because eugenol has good solubility in alcohols (O'Neil and Budavari, 2001). Digestion method was selected because heating and stirring can reduce the viscosity, therefore increasing the dissolution of insolvent chemical content. The viscous extract obtained was thick, blackish brown, had distinctive smell and weighing 137 gr with a yield percentage of 16.407%.

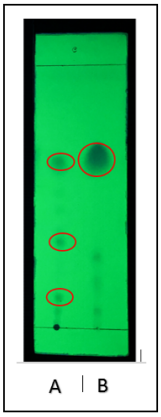

Figure 1: Qualitative test of eugenol in ethanol extract galangal rhizome (A) extract (B) eugenol standard.

The advantages of TLC were easy to use, relatively fast, and simple. The selection of mobile phase for TLC was based on the polarity of the compound. The mobile phase that was used is a mixture of some organic solvents because the elusions can be adjusted so that separation can be optimal. In this study, eugenol is a nonpolar tendency compound so that the mobile phase used was a mixture of n-hexane and ethyl acetate (4:1 v/v) (Yugatama *et al*., 2017). Based on TLC result, it can be seen that Rf value of standard eugenol was 0.662 (spot B) and Rf value of sample was 0.650 (spot A). The result of the quantification test can be seen in Figure 1.

Ointment formulation was selected because it allows longer contact with skin than other topical preparation so that the release of the active compound will be maximal. In addition, the ethanol extract of galangal rhizome can be dissolved in the suitable ointment base. The base of ointment used in this study is hydrocarbon type. The base of this ointment has properties that are difficult to be washed by water so that contact with the skin will last longer and does not allow evaporation into the air. The fat base is an occlusive cover so it can hydrate the skin (Khar *et al.*, 2013). The ointment was prepared by melting method; some of the ingredients were mixed by way of melting and cooled by constant stirring until they solidified (Allen, 2014). The obtained ointment has a rather dense, brownish shape and has distinctive odor.

Measurements of the maximum wavelength are performed because it has maximum sensitivity. The result of spectra obtained shows there are 3 peaks. The existence of several peaks is due to ethanol solvent effect with 205 nm UV cut-off 205 nm, so that the absorption peak of maximum wavelength eugenol that is at 282 nm.

The separation process of the analyte from the sample (ointment base) can be broken down by adding a chemical compound. This process is intended for analysis of the analyte so it is not disturbed by the existence of the sample matrix. In this study, we used a strong base KOH 0.8 N to break down the sample matrix, which would change the eugenol into its salt form that can dissolve in water. Eugenol has phenolic properties that are highly influential in color change because phenols are reactive to air and bases. The reaction will occur when the phenol is in contact with air, strong bases and heat, are oxidation reactions where phenol binds the oxygen causing the change of color.

The process of breaking down the sample matrix needs to be assisted by heating and stirring which aims to accelerate and maximize the process (Putri *et al*., 2014). The addition of KOH to the sample will form two parts: K-eugenolate (water layer) and other organic compounds. The KOH (strong base) will react with phenol so that it will form its salt (K-eugenolate). The soluble phenol (water layer) was added with chloroform to attract any impurities that may exist at the time of separation. It then formed two layers (water layer and chloroform layer) that was then separated and the water layer was taken. To eliminate the phenol from the salt, H_2SO_4 5 N was added to neutralize the remaining base. It was intended to regain the analytes in the whole molecule (eugenol) (Daryono, 2015; Fitri and Kawira, 2006). Then, Liquid-liquid extraction (LLE) was performed by partition using separating funnel with chloroform as non-mixing solvent. The selection of chloroform is based on the principle of '*like dissolve like*' where the nonpolar eugenol tend to be soluble in chloroform. The selection of the separation funnel method was due to the ease of separating the compound between the two non-interfering solvents. Small droplets of the solvent will create a larger surface area so that it will accelerate the equilibrium of the solute between the two solvents. The solvent obtained from the extraction (chlorophorm phase) is evaporated. The resulting eugenol was subsequently dissolved in ethanol.

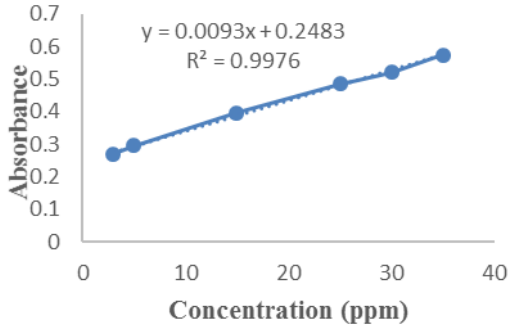

Figure 2: Calibration curve of eugenol.

Linearity test is necessary because in this area we will get the correct validation method of an analyte. The curve between absorbance and concentrations is linear because there is an increase in concentration value followed by an increase in absorbance value. At 282 nm wavelengths a linear range of concentrations of 3-35 ppm yields a linear equation y = 0.0093x + 0.2483 with R2 = 0.9976. According to ICH, the requirement of linearity is when the coefficient of determination (r^2) ≥ 0.997 (Chan *et al*., 2004). Calibration curve of eugenol can be seen in Figure 2.

Precision is a measure that indicates the suitability of individual test results as measured by the average outcome distribution if the procedure is repeatedly defined. In this study, the measurements at concentrations of 5, 15, 25, 30 and 35 ppm in each replication were then calculated to achieve the standard deviation and relative standard deviation. The variations that appear in precision results can be caused by various factors that are difficult to control such as disturbance and different conditions of each measurement. The precision result of the analytical method is described in Table 2.

In this accuracy test, standard addition method was used where a number of known standard solutions of concentration are added to the sample and then analyzed. The selection of standard solution series concentrations used in accuracy test is based on the results of calibration curve that has met the range and linearity. Given the addition of standard eugenol series to sample, at 282 nm there will be a significant increase in absorbance value. The result obtained from the calculation of % recovery is 98.195%.

Table 2: Repeatability and intermediate precision of UV-Vis spectrophotometry method.

C (µg/mL)	Repeatability (n=4)		Intermediate precision (n=4)	
	Mean Absorbance±SD	%RSD	Mean Absorbance±SD	%RSD
5	0,295±0,0019	0,639	0,297±0,0026	0,864
15	0,395±0,0026	0,646	0,399±0,0051	1,282
25	0,478±0,0054	1,130	0,481±0,0085	1,763
30	0,524±0,0024	0,466	0,525±0,0034	0,649
35	0,571±0,0033	0,586	0,573±0,0041	0,715

The method that we used in this study to determine LOD and LOQ is calculation. From the result of linear equation y = 0.0093x + 0.2483, we can calculate LOD value and LOQ value based on standard deviation and slope of a standard curve obtained. From the statistical calculation using the standard curve equation, the LOD value that was obtained was 2.388 µg/mL and LOQ value 7.235 µg/mL.

The determination of eugenol content in the sample uses standard method addition. This method is done by adding the standard eugenol to the sample. The sample absorbance of 0 ppm (without the standard addition) is plotted to the calibration curve (Fig. 3). The results obtained from the calculation of eugenol content in the ointment of ethanol extracted from galangal rhizome with accuracy 98.195% is 5.093 mg/g, so that the actual eugenol content in the ointment extract of galangal rhizome ethanol is 5.187 mg/g.

4 CONCLUSIONS

The study shows that the UV-Vis spectrophotometric method is a good method for analyzing eugenol in the ointment of ethanolic extract of galangal rhizome with validation parameters occupied the requirements. From the result, we know that the content of eugenol in ointment ethanolic extract of galangal rhizome is 5.187 mg/g.

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