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IDENTIFICATION OF SECONDARY METABOLITES COMPOUNDS FROM NAMPU (*HOMALOMENA ROSRATA* GRIFF.)

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Abstract: Nampu (*Homalomena rosrata* Griff.) is traditionally used to increase sexual activity for men. However, the lack of scientific information on the phytochemical content of Nampu plants needs further research on the secondary metabolite compounds for isolation and identification purposes. The phytochemical screening of raw material, ethanol extract, and water fraction show positive results for flavonoid, tannin, polyphenol, quinone, monoterpenoid-sesquiterpenoid, and steroid-triterpenoid groups. The chloroform fractions were monitored by the thin-layer chromatography (TLC) method with the addition of KOH 5%. The results showed a blue spot under UV light at 365 nm after the addition of KOH 5%. The fraction was purified by a preparative thin-layer chromatography method (TLC-P), while the isolates' purity was tested by the two-dimensional TLC method and TLC with three different phases of mobile phases. The results of the analysis with the spectrophotometer UV-Visible showed a wavelength of 278.8 nm and 213.2 nm. In addition, the functional group analysis using the I.R. spectrometer showed O.H. stretch at wave numbers 3419.79 cm^{-1}, aromatic C-H at wave number 2922.16 cm^{-1}, C = O at the wave number 1672.28 cm^{-1}, strain C = C aromatic at the wave number 1585.49 cm^{-1}. Based on TLC and spectrophotometric data, the isolates were coumarin compounds.
Keywords: Nampu (*Homalomena rosrata* Griff.); isolation; thin layer chromatography; spectrophotometer UV-Visible; spectroscopy infrared; coumarin

Abstrak: Nampu (*Homalomena rosrata* Griff.) secara tradisional digunakan untuk meningkatkan aktivitas seksual pria. Namun, kurangnya informasi ilmiah tentang kandungan fitokimia tanaman Nampu, perlu penelitian lebih lanjut tentang senyawa metabolit sekunder untuk tujuan isolasi dan identifikasi. Penapisan fitokimia bahan baku, ekstrak etanol dan fraksi air menunjukkan hasil positif untuk golongan flavonoid, tanin, polifenol, kuinon, monoterpenoid-seskuiterpenoid, dan steroid-triterpenoid. Fraksi kloroform dipantau dengan metode kromatografi lapis tipis (KLT) dengan penambahan KOH 5%. Hasilnya menunjukkan bercak berwarna biru di bawah lampu U.V. 365 nm. Fraksi dimurnikan dengan metode kromatografi lapis tipis preparatif (KLT-P), sedangkan kemurnian isolat diuji dengan metode KLT dua dimensi dan KLT dengan tiga fase gerak yang berbeda. Hasil analisis dengan spektrofotometer UV-*Visible* menunjukkan panjang gelombang 278,8 nm dan 213,2 nm. Selain itu, analisis gugus fungsi menggunakan spektrometer infra merah menunjukkan adanya gugus OH, regangan pada bilangan gelombang 3419,79 cm^{-1}, C-H aromatik pada bilangan gelombang 2922,16 cm^{-1}, C=O pada bilangan gelombang 1672,28 cm^{-1}, regangan C=C

aromatik pada bilangan gelombang 1585,49 cm^{-1}. Berdasarkan data KLT dan spektrofotometri, diduga isolat merupakan golongan senyawa kumarin.
Kata kunci: Nampu (*Homalomena rosrata* Griff.); isolasi; kromatografi lapis tipis; spektrofotometer UV-Visible; spektroskopi inframerah; kumarin

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Introduction

Homalomena rostrata Griff is a plant that grows wild on mountains, rivers, and lakeshores, or is planted as medicinal plants and ornamental, which can be widely found in Kalimantan, Sumatra and southern Thailand. *Homalomena rostrata* can grow in the lowlands, at an altitude of 3 - 90 m above sea level and in direct sunlight (Sin Yeng et al., 2011; Sin Yeng et al., 2013; Sin Yeng and Boyce, 2014; Sin Yeng and Boyce, 2020; Wong and Boyce, 2017). Traditionally, rhizomes can be used to overcome rheumatism and aches, rheumatic pain after birth, and increase sexual activity in men. The existence of these biological activities is the result of secondary metabolites activity contained in the rhizome. The compound of secondary metabolites contained in the rhizome of nampu are terpenoids, flavonoids, tannins and polyphenols (Dalimartha, 2003). Each secondary metabolite compound has its function for the plant itself and benefits as an alternative treatment, such as flavonoids. It has been reported that flavonoids contain antiallergic, antiviral, antimicrobial, can prevent carcinogenic, antioxidant, detoxification and can protect against liver damage. In addition, saponins are reported to be able to overcome hypercholesterolemia, hyperglycemia, antioxidants, anticancer, anti-inflammatory, and weight loss. Some tannins can be used as antiviral, antitumor and anti-bacterial (Olalekan and Ayodeji, 2013), and many other biological activities still need further research.

Previous reports stated that the nampu rhizome contains a class of flavonoid compounds, tannins, quinones, polyphenols, saponins, and mono-sesquiterpenes. Moreover, isolates from the n-hexane fraction are thought to be steroid compounds (Fitriani, 2015). In accordance, Brata (2015) reported that the pharmacological activity of ethanol rhizome extract affects increasing sexual activity and the number of spermatozoa in Wistar rats. Ethanol extract, n-hexane fraction, ethyl acetate fraction and water fraction had LC50 values of 97.15 µg/mL, 104.29 µg/mL, 155.47 µg/mL, and 158.01 µg/mL. Thin Layer Chromatography (TLC) results show that in ethanol extract and n-hexane fraction, there are suspects in flavonoid and steroid compounds (Sari, 2015). The aforementioned study results can provide specific and non-specific characterization data of nampu rhizome. Thus, the plant characterization of nampu

can be used as the basis for developing standardized traditional medicinal materials. It is expected that the investigation of secondary metabolites in nampu plants will provide data on where marker compounds can be used as a reference for the standardization of sample plants.

Methods

Instruments

The instruments used in this research are analytical balance (Mettler Toledo, Indonesia), microscope (Olympus, rotary evaporator (Heidolph, Germany), water bath (Jakarta, Indonesia), distillation apparatus, oven (Memmert, Germany), chromatography vessel, UV-Vis spectrophotometry (Shimadzu, Japan), Infrared spectrophotometry (Shimadzu, Japan).

Materials

The plant material used in this research was Nampu rhizome (*Homalomena rostrata* Griff.), which was obtained from South Bandung, West Java. The chemicals used included 70% ethanol (Merck), n-hexane (Merck), ethyl acetate (Merck), methanol pro analysis (Merck, Indonesia), chloroform technical grade (Merck), and silica gel 60 GF 254 (Merck).

Standardization of Raw Material

Characteristics of Raw Material

Determining the raw material characteristics was carried out using specific and nonspecific parameters. The specific parameter was the determination of the levels of water-soluble extracts and soluble concentrations of ethanol. In contrast, nonspecific parameters consisted of total ash content, water-soluble ash content, acid insoluble ash content, and water content determination done by distillation method (Anonymous, 2017).

Phytochemical Screening

The phytochemical screening phase of the nampu included examining alkaloids, flavonoids, quinones, tannins, polyphenols, saponins, steroids and triterpenes, monoterpenoids and sesquiterpenoids (Anonymous, 2017; Faramayuda et al., 2021[a]; Faramayuda et al., 2021[b]).

Extraction and Fractionation

A total of 250 g of dry material was added with 1.5 L 70% ethanol solvent and extracted using a reflux device. After being immersed in the solvent, the mixture of simplicity and solvent was refluxed for one hour, which was repeatedly done three times. Each solvent immersion was replaced with a new one.

The process was continued with liquid-liquid extraction (fractionation) using solvents with different polarity levels, which were mixed with 100 mL of distilled water. This water extract was extracted liquid-liquid with n-hexane equally so that the n-hexane fraction and water fraction were obtained. Then the

water fraction was extracted liquid-liquid again with ethyl acetate to produce the ethyl acetate fraction. Each fraction was concentrated with a rotary evaporator at a temperature of 78 °C, speed of 80 rpm, concentration-time of 2 hours, the vacuum pressure of 20 Bar, and evaporated on a water bath at 70 °C to obtain concentrated fractions of water, n-hexane, and ethyl acetate. After that, hydrolysis of the water fraction was carried out under acidic conditions using 2N hydrochloric acid heated with the extract, dissolved in distilled water for 30 minutes, and lastly then extracted liquid-liquid using chloroform solvent.

Isolation

The chloroform fraction was dissolved and sprayed on GF254 silica gel with a size of 7x1 cm. This was developed with several single mobile phases and continued using a mobile phase mixture according to the one obtained from the process using a single mobile phase, then the mobile phase mixture was saturated in a closed vessel for 10 minutes. The plate was then inserted into the vessel to elute with the mobile phase corresponding to the upper limit specified in the silica plate. The TLC examination profile was carried out under U.V. light with 254 nm and 365 nm.

Furthermore, Thin Layer Chromatography Preparative (TLCP) plates were made with an upper borderline of 1 cm and below 1.5 cm using a plastic spoon. Before the eluting stage, the TLCP plate was activated by the oven for 15 minutes. Plates that had been given a fraction were eluted into the chamber to the upper end of the limit. After finishing the elution plate, it was left for a moment, the plate results were seen under U.V. lamp 365 nm and UV 254 nm. Areas with ribbons were marked using a plastic spoon, and the band area was scraped. Silica powder was then dissolved with a suitable solvent then filtered, and concentrated by evaporating. TLCP resulted in KLT with the appropriate Mobil Phase. The results of the plates were seen under the U.V. lamp at 254 nm and 365 nm (Faramayuda et al., 2021[b]).

Purity of Isolates Test

The chamber containing the appropriate mobile phase is saturated for 30 minutes. A plate was made with 7x7 cm plate size and with right and bottom 1 cm, left and above 0.5 cm. The plate results were seen under the 365 nm U.V. lamp. The TLC plate was reinserted into the chamber, containing the second mobile phase (the position was opposite to the first mobile phase direction). The plate results were reobserved under the 365 nm U.V. lamp to see the spot formed. The chamber containing the appropriate mobile phase was saturated for 30 minutes. In addition, the silica plate size was 7x1 cm. The isolate was sprayed on the plate and then eluted that were seen under U.V. light 254 and 365 nm. Lastly, it was sprayed with 10% H_2SO_4 patches. The process was repeated with different mobile phases.

Isolate Identification

Identification of isolate structure using UV-Visible spectrophotometer instrument was employed to determine wavelength. Isolate dissolved with methanol pro analysis. The sample was reconcentrated in the vial, and then the maximum wavelength was observed. Then, the absorbance and the resulting spectrum were recorded.

The next stage was identification with an Infrared spectrophotometer to determine the functional group started by preparing the sample. It was followed by crushing KBr, putting it into a tool and measuring it in the wavenumber. KBr was crushed then mixed with samples and measured in Infrared spectrophotometry.

Result and Discussions

Standardization of Raw Material

The total ash content obtained was 0.83% w/w, and water-soluble ash content was 3.86% w/w, which indicated the presence of physiological ash content such as alkali metals and alkaline earth in the raw materials. In comparison, acid insoluble ash content was 1.24% w/w ± 0.14, indicating that non-physiological ash such as silica was contained in the raw material. Determination of the content in water-soluble extracts and soluble levels of ethanol aimed at providing an overview of the number of compounds dissolved in water solvents and ethanol solvents. Determination of the content in water-soluble extract gave a value of 20.43 % w/w ± 0.16 and the determination of the content of soluble extracts of ethanol gave a value of 18.74 % w/w ± 0.15; which indicated that the compounds contained in raw material were more soluble in water. Determination of water content was carried out by azeotropic distillation method with toluene solvent, which had previously been saturated with water for 24 hours (Anonymous, 2017). Saturation with water is aimed at preventing water absorption by toluene when used in order to determine the actual water content in raw material. The water content of the raw material was 4% w/v, which shows that the rhizome raw material was suitable to be used in this research, as it has fulfilled the stipulated requirements that the water content in the raw material should be more than 10% w/v (see Table I).

Previous studies reported total ash content, water-soluble ash content, acid-soluble ash content, Water-soluble extract content and water content were higher than the results in this research. This result was obtained due to the differences in the plant location. On contrary, the previous studies took the plant samples from Lembang, West Bandung an altitude of 1,200 m above sea level. Meanwhile, this research took the plant samples from southern Bandung at an altitude of 900 above sea level (Fitriani, 2015).

Phytochemical Screening

Phytochemical screening was intended to determine the content of compounds in the rhizome's simplicity (*Homalomena rostrata* Griff.) to make it associated with the efficacy or pharmacological activity. The groups of compounds found in the rhizome's simplicity were flavonoids, tannins, polyphenols, quinones, monoterpenoids sesquiterpenoids, steroids and triterpenoids. Phytochemical screening of ethanol extract was carried out with the groups of compounds in the rhizome's ethanol extract, which were flavonoids, tannins, polyphenols, quinones, monoterpenoids and sesquiterpenoids, steroids and triterpenoids. Phytochemical screening of the water fraction was carried out, in which the class of compounds contained in the rhizome water fraction included flavonoids, tannins, polyphenols, monoterpenoids and sesquiterpenoids, and steroids and triterpenoids. The flavonoid group test was positive with the formation of a yellow ring in the amyl alcohol layer after being reacted with 2N Mg and HCl powder (Anonymous, 2017; Faramayuda et al., 2021a; Faramayuda et al., 2021b).

In the abovementioned process, the addition of Mg powder served as a catalyst. The orange color was formed due to a reducing reaction on the carbonyl group to an alcohol group that formed a colored hydroxy compound, in which the color formed was attracted by amyl alcohol. The tannin class test was positive when precipitates were formed after the addition of a 1% gelatin reagent due to the tannin's specific nature that precipitates the protein. The polyphenol group test was positive because it formed a blackish-blue color when adding iron (III) chloride reagent. This change occurs because of the oxidation reaction of the hydroxy group in the structure of iron (III) (Effendy, 2007).

Table 1. Characteristics of Raw Material

Characteristic Type	Result
Ash content :	
a. Total ash content	6.05 % w/w ± 0.83
b. Water soluble ash content	3.86 % w/w ± 0.06
c. Acid insoluble ash content	1.24 % w/w ± 0.14
Determination of extract content:	
a. levels of water-soluble extracts	20.43 % w/w ± 0.16
b. soluble concentrations of ethanol	18.74 % w/w ± 0.15
Water content	4 % v/w ± 0

Quinone class test was positive because the yellowish-brown color was formed after the addition of potassium hydroxide. The presence of quinone compounds was indicated by their ability to form colored salts. This colored salt was formed between hydroquinone and a robust alkaline solution (NaOH or KOH). Monoterpenoid and sesquiterpenoid class tests were tested positive when

purple formed after the addition of sulfuric vanillin reagent. Steroid and triterpenoid assays tested positive because they formed green colors after the addition of the Liebermann-Burchard reagent, which would react with steroids via acetylation reaction to produce acetyl steroid complexes (see the results in Table 2).

The method used for the extraction process was a reflux method using 70% ethanol solvent. This reflux method was made because the sample used was rough-textured. The extract obtained was concentrated with a rotary evaporator to obtain a thick extract also determined the weight of 1% extract obtained results of 0.76 w/v ± 0.02. The thick extract obtained was 153.97 g, hence, the extract yield obtained was 30.79% w/w. The results of identifying secondary metabolites of the alkaloid group were not detected. These results are in line with those reported by Fitriani (2015).

Table 2. Phytochemical Screening

Class of Compound	Result		
	Raw material	Ethanol Extract	Water Fraction
Alkaloid	-	-	-
Flavonoid	+	+	+
Tanin	+	+	+
Polyphenol	+	+	+
Quinone	+	+	+
Saponin	-	-	-
Monoterpenoid-seskuiterpenoid	+	+	+
Steroid-triterpenoid	+	+	+

Separation of the extract was carried out based on its polarity with the liquid-liquid extraction method using (1) non-polar n-hexane solvents, (2) the withdrawal of semipolar compounds using ethyl acetate solvent, and (3) the more polar compounds in the water fraction. The purpose of liquid-liquid extraction was to separate compounds based on the compared distribution of compounds in two solvents that are not intermixed, and the separation ends after equilibrium occurred. The water fraction obtained was then hydrolyzed using 2N hydrochloric acid: water (1:1) by heating for 30 minutes, then extracting liquid-liquid with chloroform (1:1) (Anonymous, 2017). This hydrolysis aimed at separating the glycone from the aglycone to result in free and easy-to-isolate aglycone.

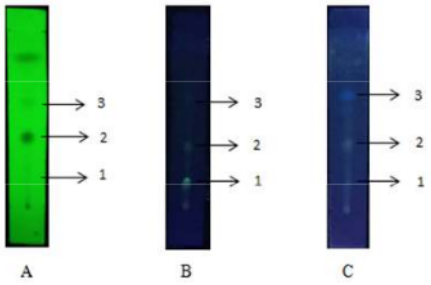


Figure 1. Thin layer chromatography fraction of chloroform pattern monitoring results. Stationary phase = silica gel 60 GF 254 plate; mobile phase = chloroform : ethyl acetate (6:4); A : Observed under 254 nm UV Light before spraying the visible spot 5% KOH with Rf 0.8; B : Observed under 365 nm UV Light before being prayed with visible spots 5% KOH with Rf 0.8; C : Observed under 254 nm UV light after sprayed with visible spots 5% KOH with Rf 0.8.

The chloroform fraction from hydrolysis was monitored by thin-layer chromatography (TLC) using chloroform: ethyl acetate (6:4) mobile phase and the results showed a yellow fluorescent spot at Rf 0.13; 0.35 and 0.62 under U.V. light 365 nm and 254 nm. The spraying of 5% KOH spots showed a blue spot at Rf 0.62 (see the result in Figure 1).

Isolation

The isolation stages consisted of an analysis of thin-layer chromatography, compound propagation by preparative thin-layer chromatography, and purification stages isolated by thin-layer chromatography with three different polarity levels and two-dimensional thin-layer chromatography.

Thin Layer Chromatography Preparative (TLCP) was used to separate the target compounds. The results of TLCP fraction of chloroform with silica GF_{254} stationary phase and chloroform: ethyl acetate (6:4) mobile phase showed one band having yellow fluorescent at the bottom and black at the top at UV 365 nm and extinguish at UV 254 nm. Band two fluoresced black at UV 365 nm and extinguish in UV 254 nm, band 3 fluoresced blue on U.V. light at 365 nm and extinguish in U.V. light 254 nm, while band4 fluoresced black on UV 365 nm and extinguished in UV 254 nm (see the result in Figure 2).

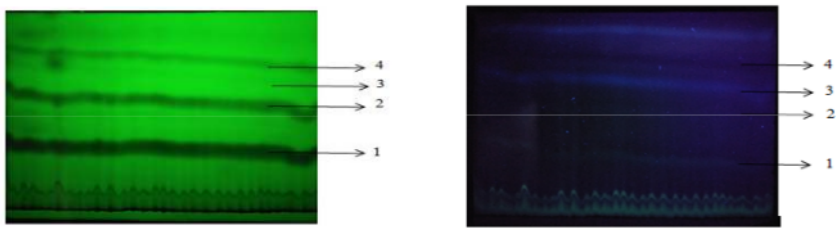


Figure 2. Preparative thin-layer chromatography pattern of chloroform fraction *Homalomena rostrata* Griff. Mobile phase: chloroform: ethyl acetate (6: 4) Stationary phase: silica gel 60 GF 254.

Liquid chromatography monitoring TLCP results on bands 1, 2, 3 and 4 were carried out using the mobile phase of chloroform: ethyl acetate (6:4). It only displayed the spot in band 2 by showing the reduction in Rf 0.8 under Uv 254 nm; while after spraying the appearance of 5% KOH, the spots showed the Uv 365 nm blue fluorescence results; in meaning that the spot was suspected to be coumarin group (see the results in Figure 3).

On contrary, the freshly-made coumarin in alkaline solutions stored in a dark place did not show fluorescence. However, if the solution was irradiated with ultraviolet light, it would quickly show blue fluorescence. The same can be done by leaving it under the sun for a long time. In this process, photo transformation occurred in the form of cis-hydroxynamic (III) acid, which does not fluoresce to fluorescence in the form of trans-hydroxynamic (IV) acid (Erniwati, 2005).

Coumarin and its derivatives were highly reactive compounds. The presence of a methyl group at position C-4 or C-6 makes the coumarin nucleus more reactive and can be caused the coumarin nucleus to undergo halogenation and condensation reactions with aldehydes. Carbon-6 in the aromatic ring can undergo electrophilic attacks, such as sulfonation or Friedel-Craft acylation reactions. A methyl substituent in the coumarin nucleus reacted differently, depending on the position of the attack. For example, a methyl group bound to C-6 or C-4 was more reactive than a methyl group in the C-3 or C-5 position (Rashamuse, 2008). The changes to the basic structure of coumarin were known to affect their biological activity. For example, coumarin with hydroxy groups can be used as an anti-inflammatory agent. Submarine synthesis by adding various pharmacophoric groups at positions C-3, C-4 and C-7 are intensively active as antimicrobial, anti-HIV, anticancer, antioxidant, and anticoagulant. Coumarin-3-sulfonamides and carboxamides have been reported to have toxic effects on cancer cells. In accordance, the substitution at position C-4 with carboxymethyl, arylaminomethyl, and dichloroacetamidomethyl groups showed potential as antimicrobial and anti-inflammatory (Dighe, et al., 2010; Loncar et al., 2020; Önder, 2020, Liu et al.,

2020; Mishra et al., 2020; Krüger et al., 2018; Keri et al., 2015; Ramírez et al., 2019).

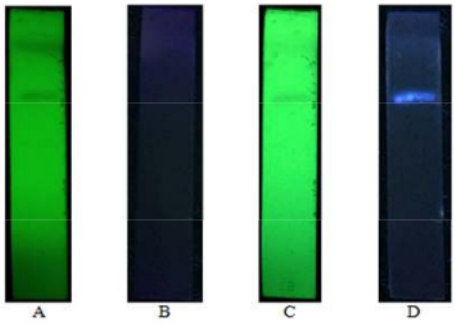


Figure 3. Thin-layer chromatography pattern monitoring TLCP results. Stationary phase = silica gel 60 GF 254 plate; mobile phase = chloroform:ethyl acetate (6:4); A= Observed under 254 nm UV Light before spraying the visible spot 5% KOH with Rf 0.8; B= Observed under 365 nm UV Light before being prayed with visible spots 5% KOH with Rf 0.8; C= Observed under 254 nm U light after sprayed with visible spots 5% KOH with Rf 0.8; D= observed under UV 365 nm light after being sprayed with visible spots 5% KOH with Rf 0.8

Lastly, the purity test was the TLC method with three mobile phases. The test isolates were developed using three different mobile phases, the first mobile phase used was n-hexane: ethyl acetate (6:4) produced a spot with an Rf value of 0.33; the second mobile phase used toluene: ethyl acetate (6:4) produced spots with Rf value of 0.55; and the third used methanol: ethyl acetate (1:9) with Rf value of 0.86 (Figure 4).

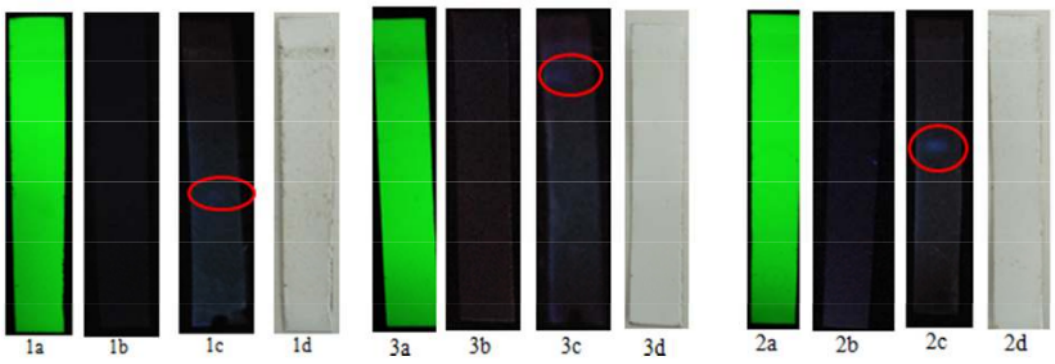


Figure 4. Thin Layer Chromatography Pattern isolates with Three different Mobil Phase. 1= Mobile phase of n-hexane: ethyl acetate (6:4) with Rf 0.33; 2 = Mobile phase of toluene: ethyl acetate (6: 4) with Rf 0.5; 3 =Mobile phase of ethyl acetate : methanol: (9:1) with Rf 0.86. a =

Under UV 254 nm; b = Under UV 365 nm; c = Under UV 365 nm after spraying 10% H_2SO_4 spotting appearance; d = after spraying 10% H_2SO_4 visually

Isolate Characterization

Identification of isolates was carried out using UV-Vis spectrophotometry and infrared spectrophotometry, which was carried out at a wavelength of 240-400 nm using methanol pro analysis. The results of the isolate examination obtained a maximum wavelength of 278.8 nm and 213.2 nm (see results in Figure 5).

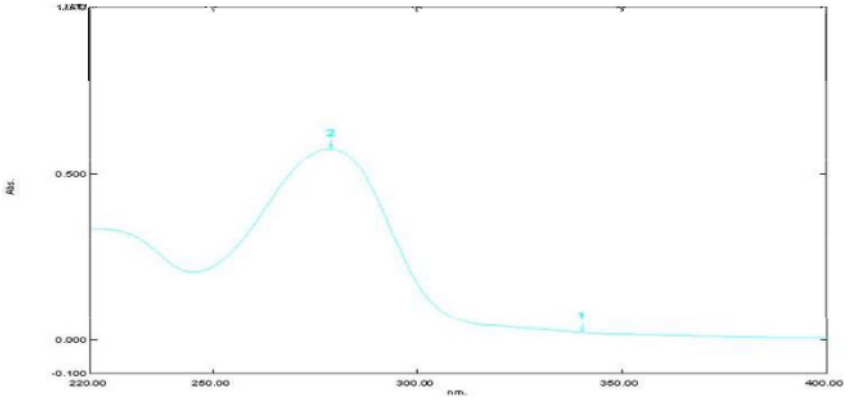


Figure 5. The spectrum of isolates in methanol solvent analysis

Identification by infrared spectrophotometry was employed to analyze the functional groups of a compound in the range 400-4000 cm^{-1}. The infrared spectrum of a molecule was the result of a transition between different levels of vibration energy. Infrared spectrophotometric examination of isolates showed the O-H strain at wavenumbers 3388.93 and 3419.79 (cm^{-1}) (3750 - 300 cm^{-1}), stretching aromatic C-H at wavenumbers 2922.16; 2953.02; 3115.04 (cm^{-1}) (3300-2900 cm^{-1}), stretch C = O in wave number 1672.28 ; 1735.93; 1867.09 (cm^{-1}) (1900-1650 cm^{-1}), stretch C = C aromatic at wavenumbers 1585.49 (cm^{-1}) (1675-1500 cm^{-1}) (Nandiyanto et al., 2019) (see results in Figure 6).

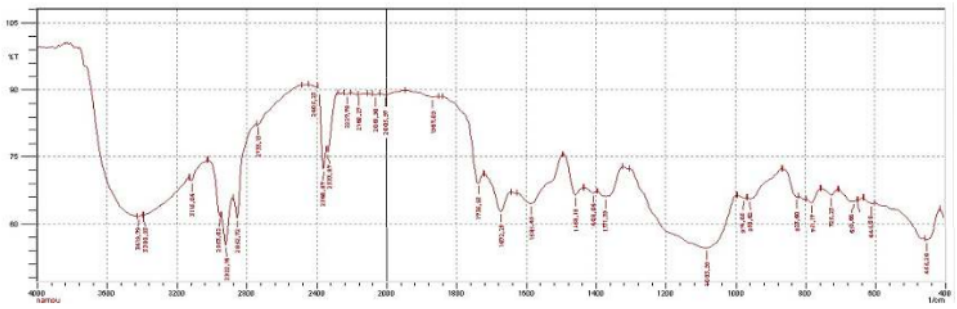


Figure 6. Infrared Spectroscopy of isolate

Mulia (2017) isolated coumarin from *Citrus nobilis* Lour and the results of its characterization using UV-Vis spectrophotometry showed that the isolate provided maximum absorption at a wavelength of 206 nm. The coumarin isolated from *Artemisia annua* (L) produced absorption at a wavelength of 210 nm (Isnawati et al., 2012; Umashankar et al., 2015). The results of the wavelength characterization in this research are 213.2 nm, which was similar to the results of previous studies conducted by Isnawati et al. (2012) and Umashankar et al., (2015).

The results of the characterization of coumarin isolates in previous studies using FT IR reported the presence of -OH stretch, C - O stretch, conjugated C = O stretch, and C = C benzene stretch (Isnawati et al., 2012). These results are in accordance with the results of the FTIR characterization of isolates suspected to be coumarins. Coumarin compounds have the potential as anti-cancer (Önder et al., 2020; Lončar et al., 2020), antimicrobial (Al-Majedy et al., 2017; and antioxidant (Robledo-O'Ryan et al., 2017; Pérez-Cruz et al., 2018).

Conclusion

The analysis of isolate characterization was employed using spectrophotometry, UV-Vis spectrophotometry, and infrared spectrophotometry of isolates suspected to be coumarin compounds.

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