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Exploration of Total Flavonoid Content, Toxicity, and Antibacterial Activity of *Acorus calamus* L. Rhizome Isolates

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ABSTRACT

Acorus calamus L. is a medicinal plant with rhizomes commonly used in traditional medicine. This study aimed to determine the total flavonoid content, toxicity, and antibacterial activity of isolates from the rhizome of *Acorus calamus* L. The rhizome extract was sonicated using 70% ethanol, yielding 18.73% (w/w), and partitioned using ethyl acetate, yielding 10.80% (w/w). Subsequently, the ethyl acetate fraction was further separated using column chromatography. FTIR analysis of the ethanol extract, ethyl acetate fraction, and column isolates confirmed the presence of O–H functional groups and other vibrations, indicating the presence of flavonoid compounds. UV analysis revealed absorption at the band I (302, 302, 383 nm) and band II (253, 254, 258 nm). The total flavonoid content of the isolates, determined using $AlCl_3$, was 32.95 mg QE/g. Liquid Chromatography-Mass Spectrometry (LC-MS) analysis of the column chromatography isolates identified the presence of isorhamnetin, quercetin, genistein, and glycitein. Furthermore, toxicity and antibacterial activity tests on the ethanol extract and ethyl acetate fraction showed LC50 values of 143.53 ppm and 41.16 ppm, respectively, and inhibition zone diameters ranging from 2.30-4.54 mm and 2.63-5.29 mm, respectively.

Keywords: Toxicity, Antibacterial activity, Sonicated, *Acorus calamus* L., Flavonoids

INTRODUCTION

Acorus calamus L. is a member of the Acoracea family whose rhizome is often used as a traditional medicine. The rhizome of *A. calamus* L. contains active compounds, including flavonoids, alkaloids, tannins, saponins, phenolic compounds, terpenoids, and steroids [1]. These active compounds support various pharmacological activities in the rhizome of Acorus calamus L., including flavonoids, which are known to be used as anti-inflammatory [2], antidiabetic [3], antifungal [4], and antiallergic [5], and tannins are known to have pharmacological activity as antioxidants [6].

Chandra & Prasad explained that *A. calamus* L. has been used in Asia and Europe as a traditional medicine to reduce appetite, gastrointestinal disorders, diarrhea, nervous disorders, chest pain, and bronchitis [7]. In Indonesia, the Dayak tribe harnesses the rhizome of Acorus calamus L. as an herbal remedy for typhoid and dengue fever [8], reflecting its deep-rooted role in cultural healing practices. Modern studies further validate its therapeutic potential, highlighting its effectiveness in combating oxidative stress [9,10], inflammation [11], and even cancer [12], making it a remarkable bridge between ancient wisdom and contemporary medicine.

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Utilization of the rhizome of *A. calamus* L. as an herbal medicine can also be used to treat bacteria that can cause infection [13–15]. Thus, this study was conducted to validate the pharmacological activity of Acorus calamus L. rhizome originating from Semarang, Indonesia, as an antibacterial and to determine its level of toxicity. It is important to test the toxic properties of the rhizome of Acorus calamus L. to determine the safety and dosage limits needed in the treatment process. In addition, secondary metabolites were identified to assess their role in supporting the pharmacological activity produced by the rhizome of Acorus calamus L. as a medicinal ingredient. The secondary metabolites of Acorus calamus L. Rhizomes exhibit varying compositions depending on the region where the plant grows, which in turn influences its pharmacological activities [15,16].

The toxicity test was conducted on Artemia salina leach shrimp larvae, while the antibacterial activity was on Staphylococcus aureus bacteria. Judging from the extraction method of *A. calamus* L. rhizome, most of them applied the maceration method, so in this study, the sonication method was used as an update. In addition, determining total flavonoid content in *A. calamus* L. rhizome isolates have not been widely carried out.

EXPERIMENT

Chemicals and instrumentation

The materials used in this study included the rhizome of *A. calamus* L. from Semarang, Central Java. Additionally, high-purity chemicals of pro-analysis quality, supplied by Merck, were ethanol, ethyl acetate, n-hexane, methanol, aluminum chloride ($AlCl_3$), sodium acetate, sodium bicarbonate, magnesium powder, ferric chloride ($FeCl_3$), anhydrous acetate, concentrated sulfuric acid (H_2SO_4), chloroform, dimethyl sulfoxide (DMSO), potassium bromide (KBr), and hydrochloric acid (HCl). Silica gel, Dragendroff reagent, Mayer reagent, nutrient agar (NA), nutrient broth (NB), quercetin standard (Sigma), aquades (Hydrobat), seawater (Banyu Mili, Malang), shrimp larvae *Artemia salina* L. (Supreme Plus), baker's yeast (Saf-instant), pure bacterial culture of *Staphylococcus aureus*, and Whatman filter paper No. 42.

The primary instruments utilized in this study are the UV-Vis spectrophotometer (Varian Cary50), Fourier Transform Infrared (FTIR) spectrometer (Varian 1000 Scimitar Series), and the mass spectrometer (TSQ Quantum Access Max) coupled with an LC-Accella 1250 system.

Extraction, partition, and phytochemical screening of *A. calamus* L. rhizome

Briefly, 30 g of sample was extracted with 300 mL of 70% ethanol for 30 min at room temperature. The resulting filtrate was then concentrated using a rotary evaporator, and the crude extract yield was determined. Hydrolysis was subsequently performed using HCl at a sample-to-HCl ratio of 1:2, followed by stirring for 1 hour. Sodium bicarbonate was then added to adjust the solution to a neutral pH. The hydrolysate was transferred to a separating funnel with 25 mL of ethyl acetate, shaken, and allowed to stand until two distinct layers formed. The organic phase was separated and concentrated using a rotary evaporator, and the yield was calculated. Phytochemical screening was conducted on the extract and the partitioned fractions, testing for flavonoids, alkaloids, tannins, saponins, steroids, and terpenoids.

Isolation of flavonoid compound

Isolation using eluent gradient column chromatography was carried out with a stationary phase in the form of silica gel and a mobile phase consisting of 100% n-hexane, n-hexane: ethyl acetate (7:3), n-hexane: ethyl acetate (6:4), ethyl acetate 100%, ethyl acetate: methanol

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(7:3), ethyl acetate: methanol (6:4), ethyl acetate: methanol (4:6), and 100% methanol. The elution process was performed at a 5 mL/min flow rate. The isolates obtained were then monitored by ATLC using a stationary phase in the form of a silica gel plate F254 with a size of 10 × 10 cm, which has been activated, and the mobile phase in the form of n-hexane: ethyl acetate (7:3). Furthermore, the separated stains were observed under UV light 254 and 366 nm before and after being sprayed with ammonia. The eluent that gave the best separation pattern in the gradient column chromatography process was re-analyzed using ATLC to determine the best eluent that could separate flavonoid compounds in the ethyl acetate fraction of *A. calamus* L. rhizome and used as an eluent in the separation process with non-gradient eluent column chromatography.

The isolation process was continued with eluent non-gradient column chromatography using a mobile phase of the best eluent from ATLC. Elution was carried out at a flow rate of 2 mL/min. The isolates obtained were monitored by ATLC using the stationary phase as a silica gel plate F254. Next, the isolate was spotted on a silica gel plate, eluted to the upper limit mark, and observed for the stains resulting from the separation under UV light.

Identification was carried out using FTIR and UV spectrometry to determine the main groups and functional groups in the ethanol extract, ethyl acetate fraction, and column isolates of *A. calamus* L. rhizome. The types of flavonoids present in the column isolates of *A. calamus* L. rhizomes were analyzed using LC-MS instruments.

Determination of flavonoid content in isolate

Determination of flavonoid content begins with making a standard quercetin solution in ethanol p.a with a concentration of 9 – 24 g/mL at intervals of 3. Then, 2 mL of each solution and 0.10 mL of 10% $AlCl_3$, 0.10 mL of 1 M sodium acetate, and 2.80 mL of distilled water were added. Next, it was incubated for 30 minutes, and the absorbance of the solution was measured at the maximum wavelength of the quercetin standard. Then, a calibration curve was made for the relationship between quercetin concentration and absorbance. Determination of flavonoid levels was done by dissolving the isolate in ethanol p.a. Then 10% $AlCl_3$, 1 M sodium acetate, and distilled water were added according to the preparation of a standard quercetin solution. The absorbance of the solution was measured at the maximum wavelength of the quercetin standard. After that, the flavonoid content of each isolate was determined using equation (a).

$$\text{Flavonoid content (mg QE/ g)} = \frac{\text{C x V}}{\text{M}} \qquad \text{(a)}$$

Where, C = quercetin equivalence level (mg/L)
V = sample dilution volume (L)
M = weight of isolate (g)

Toxicity and antibacterial activity test of *A. calamus* L. rhizome

The toxicity test was carried out using the Brine Shrimp Lethality Test (BSLT) method. The test begins with making a standard solution of ethanol extract with a concentration of 5–150 ppm and ethyl acetate fraction with a concentration of 5–50 ppm. Then, 2 mL of each solution was taken and put into a vial. The solvent was evaporated and added with 0.1 mL DMSO, 2 mL seawater, and one drop of baker's yeast solution. After that, 50 shrimp larvae of *A. salina* L. and seawater were added to the vial until the volume was 10 mL. Observations were made for 24 hours on the mortality of *A. salina* L.

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Sterilization is carried out on all tools and materials to be used. Furthermore, *S. aureus* bacteria were regenerated using the agar tilt and incubated for 18 hours. The test bacteria were prepared by taking 2–4 ose culture bacteria, put in a sterile vial containing NB media, and incubated for 18 hours. The inoculum's OD (Optical Density) was measured using UV-Vis spectroscopy at 600 nm. The disk diffusion method (paper disk) carried out an antibacterial activity test. Tests were carried out on the extract and the ethyl acetate fraction with 60, 50, 40, 30, and 20% concentrations in 100% DMSO solvent. Paper discs with a diameter of 6 mm were immersed in each sample for ±30 minutes. Put 100 µL of the test bacterial suspension in a petri dish. Added sufficient NA media and homogenized by pour plate. Disc paper was attached to the surface of the media and incubated for 24 hours, then the zone of inhibition was calculated. All treatments were carried out aseptically. Toxicity and antibacterial activity tests were verified with several repetitions.

RESULT AND DISCUSSION

Extraction and phytochemical screening of *A. calamus* L. rhizome

Extraction using the sonication method with 70% ethanol as a solvent yielded 18.73% from 30 g of sample. The partition process resulted in a yield value of 10.80% from 5 g of sample. The phytochemical screening results for the ethanol extract and ethyl acetate fraction of *A. calamus* L. rhizome have been previously reported by Amalia et al. [17]. The phytochemical screening of the ethanol extract showed positive results, containing flavonoids, alkaloids, tannins, and terpenoids. In the ethyl acetate fraction, positive results were only shown in the flavonoid and tannin groups. Isolation of *Acorus calamus* L. rhizome from column chromatography only contains flavonoid compounds. The conclusion was based on phytochemical tests of isolated obtained through column chromatography using the Wilstatter method and 10% NaOH addition [17], supported by total flavonoid content analysis and mass spectrophotometry of isolates.

The ultrasonic method was chosen due to several advantages, including a simpler extraction process [18], shorter extraction time [19], more efficient use of solvent, make it environmental friendly [20], and the ability to minimize damage to the target compound through the use of low temperature [21]. Rita et al. extracted the rhizome of *A. calamus* L. by maceration method, and ethanol solvent yielded 18.6% from the 750 g sample [14]. This proves that the ultrasonic extraction method can increase the sample yield due to cavitation, which increases the solvent penetration effect so that the target compound can be extracted optimally. Moreover, in recent years, the ultrasonic method has been used to extract flavonoids from different plant species [22].

Isolation of flavonoid compound

The separation of flavonoid compounds in the ethyl acetate fraction of *A. calamus* L. rhizome was further analyzed using analytical thin-layer chromatography (ATLC) to determine the optimal eluent ratio. The separation was conducted with varying ratios of n-hexane-ethyl acetate (8:2, 7:3, 6:4, and 4:6) through gradient elution column chromatography. The eluents with n-hexane-ethyl acetate ratios of 8:2, 6:4, and 4:6 resulted in separation patterns with irregular spot distances and tailed spots. In contrast, the n-hexane-ethyl acetate ratio of 7:3 produced the highest number of spots, suspected to be flavonoids, with the most effective separation pattern. The spots were well-defined, with clear distances and no tailing for those alleged to be flavonoids. The process yielded 43 vials, 8 to 20 potentially containing flavonoid compounds.

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The n-hexane: ethyl acetate (7:3) eluent was utilized for the separation process via non-gradient column chromatography. This method was applied to 67 mg of the ethyl acetate fraction from the *A. calamus* L. rhizome, using 270 mL of n-hexane: ethyl acetate (7:3) solvent at a flow rate of 2 mL/min, resulting in 107 vials of eluate. ATLC monitoring of the isolates from the column chromatography revealed that 13 vials displayed the same stain color and Rf value, suggesting that the compounds in these isolates shared similar characteristics. Further ATLC monitoring of the isolates from non-gradient column chromatography showed spots with Rf values of 0.66 and 0.25, indicative of flavonoid compounds, likely belonging to the flavonol or flavone groups [23]. Compounds in these groups typically exhibit purple spots under a UV lamp at 366 nm and do not change color when exposed to NH_3. Various flavonol compounds demonstrated Rf values of 0.64, 0.66, and 0.28, while some flavones produced an Rf value of 0.66.

Identification using FTIR and UV-Vis spectrophotometry

Fourier Transform Infrared (FTIR) and Ultraviolet-Visible (UV-Vis) spectrophotometry are effective techniques for detecting impurities in samples, with their effectiveness varying based on the type of impurity and the sample matrix. In this study, samples analyzed using FTIR and UV-Vis, including crude ethanol extract, ethyl acetate fraction, and column isolate from the rhizome *A. calamus* L., are expected to exhibit progressively increasing levels of purity. Based on the FTIR result shown in Figure 1, the three samples contained –OH and C–O groups of secondary alcohols, indicating the presence of alcohol OH groups attached to carbon atoms in secondary bond positions. In addition, the presence of an aromatic C=C group, a chromophore group in a conjugated bond system, and a C=O ketone originating from a carbonyl group indicates a distinctive functional group of flavonoid compounds. The presence of flavonoid groups in the ethanol extract, ethyl acetate fraction, and column isolates have a specific absorption in the area 3375–3324 cm^{-1} which indicates the presence of O–H stretching vibrations, the area 1660–1466 cm^{-1} indicates the presence of aromatic C=C bonds, the area 1261–1066 cm^{-1} indicates the presence stretching vibrations of the C–O–C groups, and in the region 1281–1278 cm^{-1} and 1071–1022 cm^{-1} indicates the presence of C–OH groups [24,25]. In addition, the phytochemical results showed the presence of tannin compounds in the ethanol extract and ethyl acetate fraction, as well as terpenoid compounds in the ethanol extract terpenoid compounds, are indicated by the presence of bending vibrations of the C–H group as a geminal dimethyl group typical of terpenoids [17]. The absorption of C–H stretching vibrations at 1386 and 1457 cm-1 indicates the presence of terpenoid compounds, characteristic of methyl and methylene or geminal groups [26]. Flavonoid compounds, such as tannins, emphasize vibrations of O–H and aromatic C=C groups, with less prominent aliphatic C–H signals than terpenoids. Weaker C–H signals, dominated by phenolic and aromatic functional groups. In this study, the geminal C–H group in the ethanol extract and ethyl acetate fraction in the form of a methyl group (CH_3) was present at wavelengths of 1460 and 1461 cm^{-1}, and the methylene group (CH_2) at wavelengths of 1411 and 1409 cm^{-1}.

Tannins are a phenolic group, so they have an absorption similar to that of flavonoids, consisting of absorption of –OH groups, C–O stretching alcohols, C=C aromatics, and C–OH. Typical absorption of tannin compounds is shown in wave numbers 2970-2929 cm^{-1}, the C–H stretching (methylene) group of condensed and hydrolyzed tannins. In addition, the presence of absorption in the range 1198-1025 cm^{-1} also indicates a typical absorption of the C–O–C stretching ether groups of tannin compounds, which are symmetrical conjugations of C-O-C groups in condensed tannin pyran rings [27]. In this study, the C–H stretching group

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(methylene) in the ethanol extract and ethyl acetate fraction were located at 2939 and 2931 cm^{-1}, respectively. Meanwhile, the C–O–C ether stretching groups are in the 1076 and 1038 cm^{-1} regions. In the *A. calamus* L. isolate obtained from column chromatography, the typical absorption of tannin compounds was not observed in this region.

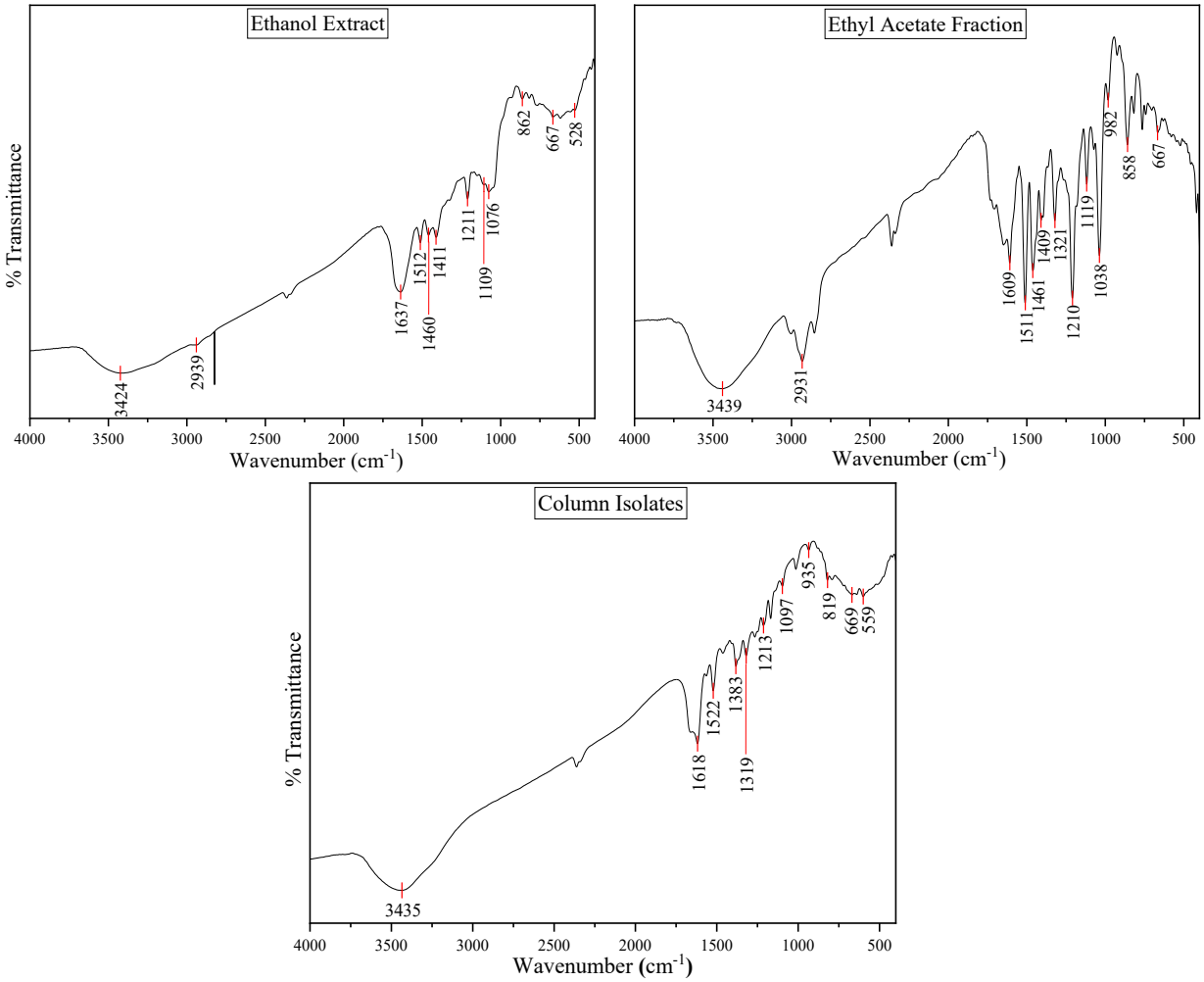

Figure 1. FTIR spectra of ethanol extract, ethyl acetate fraction, and column isolates of *A. calamus* L. rhizomes

The results of identification using a UV spectrophotometer at a wavelength of 200-800 nm are shown in Figure 2. Based on the identification results, it is known that the ethanol extract, ethyl acetate fraction, and isolates from column chromatography have maximum wavelengths in band I of 302, 302, and 383 nm and in band II of 253, 254, and 258 nm, respectively. The ethanol extract spectrum reflected a complex mixture with minimal chromophore conjugation. The ethyl acetate fraction showed improved purity with a slight shift in band II, indicating better compound separation. The column isolate of *A. calamus* L. rhizomes exhibited the highest purity, marked by a significant bathochromic shift in the band I, suggesting the presence of highly conjugated compounds (likely flavonols). These differences in maximum wavelength highlight the progressive increase in purity from the crude extract to the column isolate, aligning with the separation of active compounds. Bands I and II,

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it is indicated that there are C=C and C=O chromophore groups from conjugated aromatic groups, causing electron transitions π→π* and n→π*. Both types of electronic transitions align with the findings of Jhonson et al. [28], which demonstrate that the absorption wavelength is influenced by the chemical structure surrounding the electron during UV light absorption. Arora & Itankari [29] explained that flavones have band II transitions in the 250-290 nm region, while flavonols have an absorption region at 270-290 nm wavelength. In the absorption band I, flavones are at a wavelength of 310-350 nm, while flavonols have an absorption area of 350-385 nm. The adjacent wavelength ranges make distinguishing the two types of flavonoids difficult. The electron transition at n→π* indicates the presence of a single C=O chromophore group.

Meanwhile, band, I show the excitation of π→π* electrons, indicating the presence of a conjugated C=C chromophore group. In a study by Mustarichie & Runadi [30], the absorption area of the UV-Vis spectrum in band II with a range of 250 – 280 nm and band I with 350 – 385 nm belongs to the flavonol group (3–OH free). The flavonol type with a free 3–OH is quercetin, while kaempferol has two free groups [31].

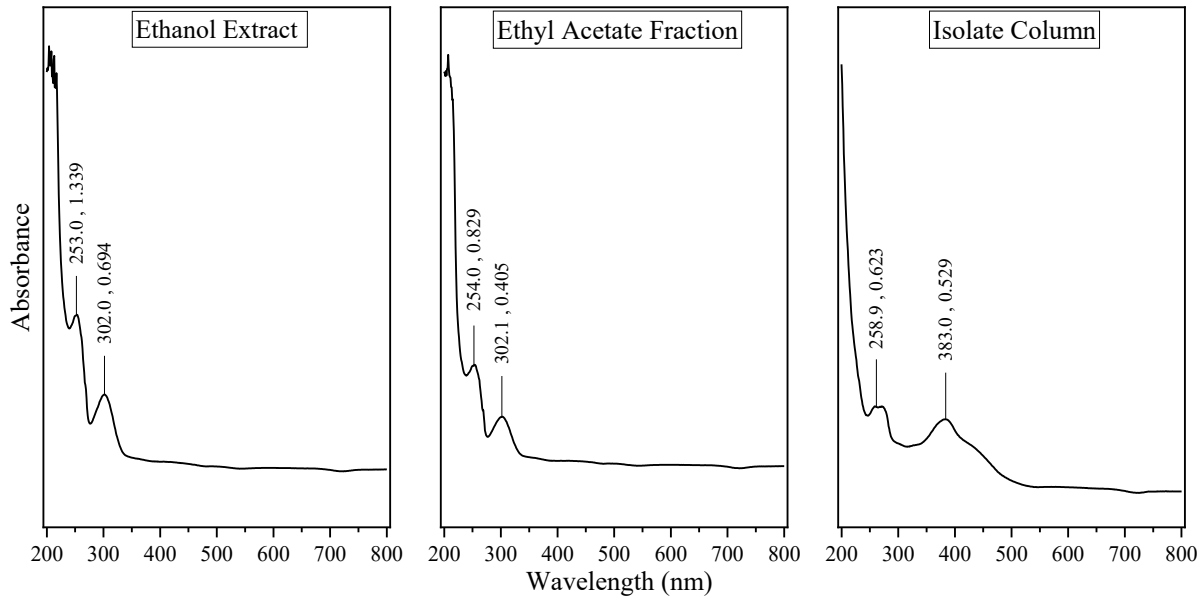

Figure 2. UV-Vis spectra of ethanol extract, ethyl acetate fraction, and column isolate of *A. calamus* L. rhizomes

Determination of flavonoid content in isolate

Determining total flavonoid content begins with measuring the maximum wavelength of the quercetin standard in the 400–600 nm range using a UV-Vis spectrophotometer, which yielded a maximum wavelength of 440.90 nm. The maximum wavelength obtained was used to determine the standard curve of quercetin and flavonoid content in isolates from *A. calamus* L. rhizome column chromatography. The results of the measurement of the standard curve of quercetin obtained the value of y = 0.03195x-0.02173 and R^2 = 0.9978. After obtaining the standard quercetin curve, the absorbance measurement was continued on the isolates resulting from the *A. calamus* L. rhizome column chromatography, which aims to determine the level of equivalence of the quercetin standard solution with the sample. The absorbance measurement of the sample was repeated three times, and the results were obtained as shown in Table 1.

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The flavonoid content of the isolates from the column chromatography exhibited different values across each vial. The variation in flavonoid levels was caused by differences in the weight of the isolates collected in each vial, reflecting the successful separation of different fractions during column chromatography. Vials 13-14 are initial vials that are positive for flavonoid compounds so that they can be assumed as the starting point for the decline of flavonoid compounds with a total weight of isolates from both vials of 5.5 mg. Furthermore, in vials 15-16, 17-18, and 19-20, the weight of the successfully accommodated isolates increased by 6.7, respectively, 6.8 and 6.7 mg. However, in vials 21-23 and 24-25, the weight of the isolates decreased by 5.2 and 4.8 mg, respectively, which is suspected that the reduced flavonoid compounds were residual and had decreased maximally in the previous vial so that in vials 26 and so on the isolates that were successfully accommodated showed negative results for flavonoid compounds. Isolates positive for flavonoid compounds were diluted with the same amount of solvent. The more successfully accommodated isolates, the higher the concentration of flavonoids in the sample. The total flavonoid content of the isolates obtained from column chromatography was 32.95 mg QE/g of the total isolates of 35.7 mg.

Table 1. Flavonoid content in isolates from column chromatography

No.	Sample	Average Flavonoid Content ± SD (mg QE/g)
1.	Isolate 13-14	4.9037 ± 0.2661
2.	Isolate 15-16	6.1883 ± 0.0260
3.	Isolate 17-18	6.4379 ± 0.0122
4.	Isolate 19-20	6.1572 ± 0.2258
5.	Isolate 21-23	4.9298 ± 0.0603
6.	Isolate 24-25	4.3299 ± 0.0868

The Total Ionic Chromatogram (TIC) data, obtained from LC-MS analysis of *A. calamus* L. rhizomes isolated through column chromatography, provides an overview of the quantity and distribution of compounds in the sample, facilitating their identification and quantification. The TIC shows the highest peak at a retention time of 6.18 minutes (Figure 3). The TIC was further analyzed using an Extracted Ion Chromatogram (EIC). The EIC provides a more detailed view of specific compounds within the column isolate of *A. calamus* L. rhizomes. This method allows for highly accurate identification and quantification of individual compounds, aiding in understanding their chemical composition, such as flavonoids. Figure 4(a) displays an EIC chromatogram with a peak at a retention time of 5.93 minutes, corresponding to an M-H (M-1) value of 301, indicating a molecular weight of 302 g/mol. The compound exhibits a Neutral Loss (NL) value of 2.06×10^4, consistent with quercetin. Figures 4(b–d) present peaks at retention times of 6.17, 6.16, and 6.19 minutes, with M-H (M-1) values of 269, 283, and 315, corresponding to molecular weights of 270, 284, and 316 g/mol, and NL values of 1.39×10^3, 1.72×10^4, and 3.36×10^4, respectively. These values from EIC Figure 4(b-d) are consistent with (b) genistein, (c) glycitein, and (d) isorhamnetin.

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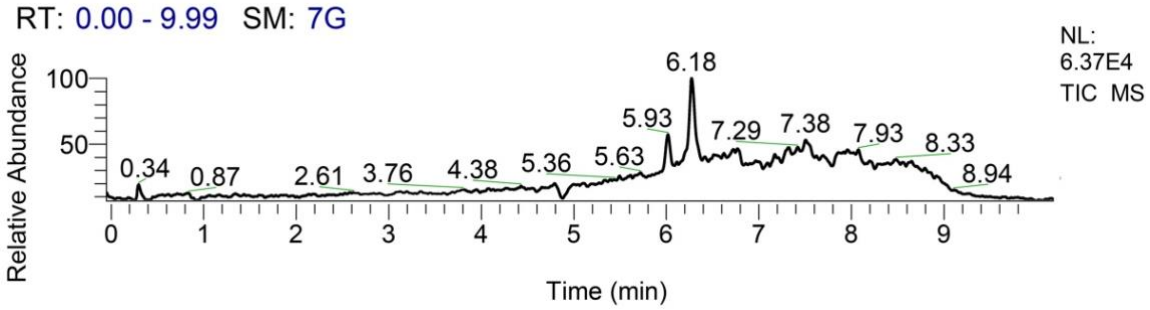

Figure 3. The Total Ionic Chromatogram (TIC) from LC-MS for Isolate column chromatography of *A. calamus* L. rhizomes

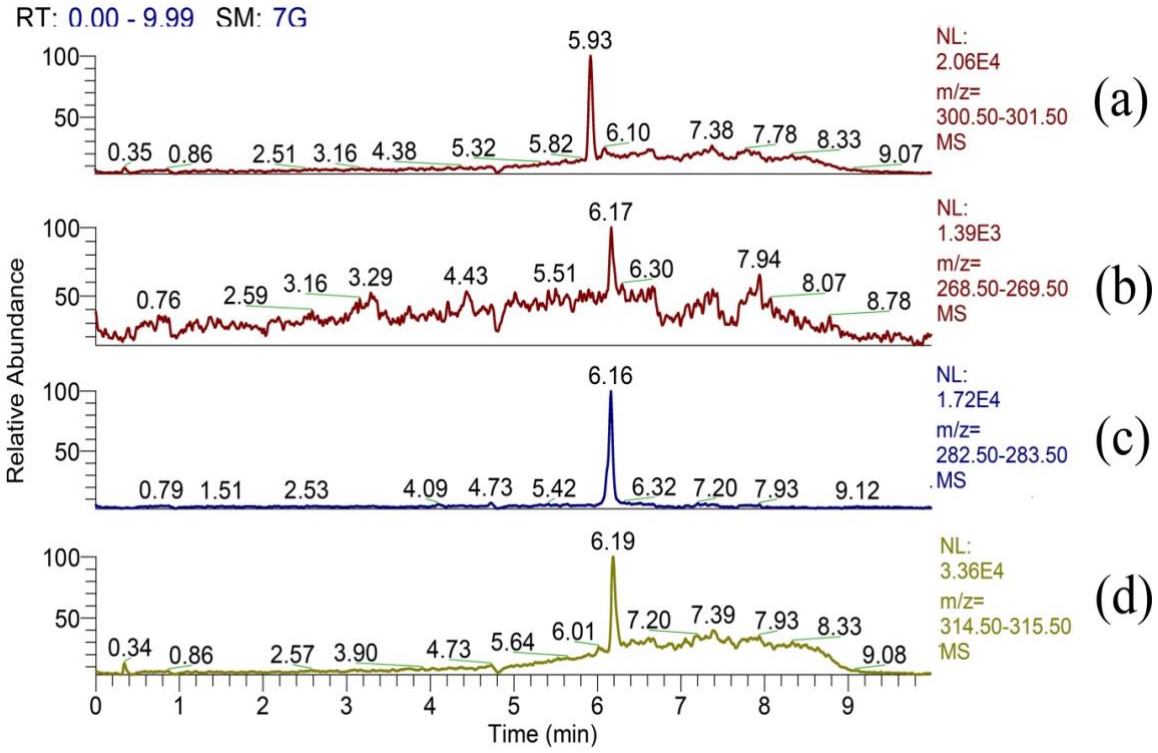

Figure 4. The Extracted Ionic Chromatogram (EIC) for Isolate column chromatography of *A. calamus* L. rhizomes

Based on EIC analysis, the abundance of flavonoid compounds in the column chromatography isolates of *A. calamus* L. extract includes isorhamnetin, quercetin, glycitein, and genistein. Subsequently, flavonoid functional groups were classified, revealing that isorhamnetin and quercetin belong to the flavonol group, while glycitein and genistein are classified as isoflavones [32]. This analysis indicates that flavonols contain more functional groups than other flavonoid types in the column chromatography isolates of Acorus calamus L. rhizomes. This conclusion is supported by phytochemical screening data from Amalia et al. [17], where the presence of flavonol functional groups was confirmed by the appearance of a yellow color under alkaline conditions (NaOH) in the phytochemical tests [33]. In contrast, the same screening test identified isoflavone functional groups, yielding colorless results.

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Toxicity and antibacterial activity test of *A. calamus* L. rhizome

Sample *A. calamus* L. rhizome extract will be directed toward its application as an herbal remedy; therefore, bioactivity tests will be conducted exclusively on crude extracts and partitioned fractions. A toxicity test is the initial stage to determine the toxic effect of the active compound of *A. calamus* L. rhizome. The level of toxicity was measured using the parameter LC_{50} (Lethal Concentration 50%), which is the dose value at which the compound can kill 50% of the test animals. The toxicity test used the Brine Shrimp Lethality Test (BSLT) method, which used the brine shrimp larvae death method. This method is used because it is fast, simple, and inexpensive to screen for cytotoxic activity. The toxicity test of *A. calamus* L. rhizome resulted in an LC_{50} value of ethanol extract of 143.53 ppm and ethyl acetate fraction of 41.16 ppm (Table 2). In this study, the concentration of the test solution used in the ethanol extract was greater than in the ethyl acetate fraction. The maximum concentration of the ethyl acetate fraction was 50 ppm and was able to kill more than 50% of the total *A. salina* L. shrimp larvae used as the test sample. However, at this concentration, the ethanol extract could not kill *A. salina* L. shrimp larvae, so the concentration was increased to 100 ppm to determine the level of toxicity of the ethanol extract to *A. salina* L. shrimp larvae. This indicates that the ethyl acetate fraction has more toxic properties than ethanol extract. In the ethanol extract, the secondary metabolite compounds are still in their glycoside bonds and contain various compounds. Meanwhile, in the ethyl acetate fraction, the glycoside bonds in the sample were broken, and the secondary metabolites contained in the ethyl acetate fraction were less by the similarity of polarity between the solvent and the secondary metabolites.

Based on the results of phytochemical screening, it is known that the ethanol extract contains several secondary metabolites, and the ethyl acetate fraction only contains flavonoids and tannins. Thus, the toxic nature of the *A. calamus* L. rhizome can be presumed to come from these two compounds. The level of toxicity can be categorized into three parts: the non-toxic category, which has an LC_{50} value of >1000 ppm; the toxic category, which has an LC_{50} value of 30-1000 ppm; and very toxic, with an LC_{50} value of <30 ppm. The LC_{50} value produced in the ethanol extract and ethyl acetate fraction is less than 1000 ppm so that it can be categorized as toxic. Meanwhile, the test sample that produces an LC50 value of 30-200 ppm has the potential to be an antibacterial [34,35]. Thus, the ethanol extract, the ethyl acetate fraction, which produced LC50 values of 143.53 and 41.16 ppm, were included in the toxic category with the potential to be antibacterial.

An antibacterial activity test was carried out on ethanol extract and ethyl acetate fraction against *S. aureus* bacteria. The diameter of the formation of a clear zone around the paper disc measured its activity against S. aureus. Negative control in the form of 100% DMSO and positive control using 20% chloramphenicol in DMSO. The size of the resulting inhibition zone is presented in Table 3 and Figure 5. The disc diffusion's method was taken placed for the antibacterial activity test with a bacterial density of 0.5. The suspension complies with the turbidity standard of McFarland I. A bacterial inoculum with a turbidity of 0.5 is equivalent to a population of 1-2x108 CFU/mL [36]. Based on the test results, the antibacterial activity of the ethanol extract increased with increasing concentration. The inhibition zone formed in the ethanol extract was 2.30-4.54 mm. The inhibition zone formed in the ethyl acetate fraction was 2.63-5.29 mm. According to Davis & Stout [37], the inhibition zone of 70% ethanol extract is still in the weak category (≥ 5 mm), while in the ethyl acetate fraction, the inhibition zone at a concentration of 60% is in the medium category (5-10 mm). The phytochemical results of the ethyl acetate fraction and the ethanol extract showed positive flavonoids. Flavonoids work as an antibacterial against *S. aureus*, which includes gram-positive bacteria. The lipophilicity of

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flavonoids makes flavonoids an inhibitor of the activity of gram-positive bacteria. The presumed mechanism of flavonoids in the lipid bilayer is between hydrophilic flavonoids and the polar heads of phospholipids or the partition of more hydrophilic flavonoids in the lipid bilayer. This interaction changes the nature of the membrane so that food inhibits the activity of gram-positive bacteria [38].

Table 2. LC_{50} value of the *A. calamus* L. rhizomes isolates

Sample	Concentration (ppm)	Number of Dead Nauplii						Mortality (%)	LC_{50} (ppm)
		I	II	III	IV	V	Mode		
Ethyl Acetate Fraction	0	0	0	0	0	0	0	0	41.16
	5	0	0	1	0	0	0	0	
	10	0	0	0	1	0	0	0	
	15	1	1	0	1	0	1	10	
	20	1	2	2	2	1	2	20	
	25	2	2	0	2	1	2	20	
	50	6	6	6	7	6	6	60	
Ethanol Extract	0	0	0	0	0	0	0	0	143.53
	5	0	0	0	0	0	0	0	
	25	0	1	1	2	1	1	10	
	50	1	2	2	1	2	2	20	
	100	1	2	2	2	2	2	20	
	150	5	6	5	5	5	5	50	
	200	10	7	8	7	7	7	70	
	250	10	8	10	10	10	10	100	

Table 3. Inhibition zone ethanol extract and ethyl acetate fraction

Concentration (%)	Sample	
	Ethanol Extract	Ethyl Acetate Fraction
60	4.54	5.29
50	3.82	4.85
40	3.68	4.38
30	2.65	2.93
20	2.30	2.63
Chloramphenicol	22	
DMSO	0	

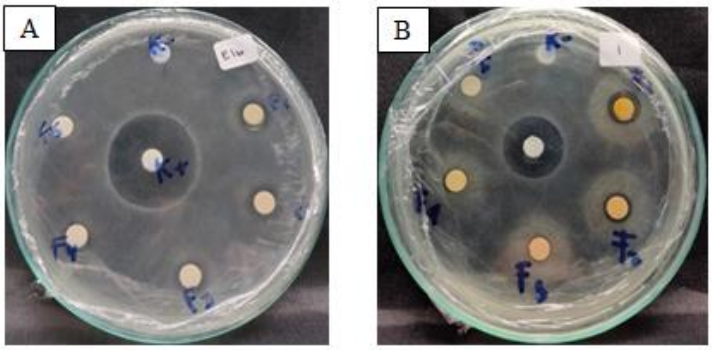

Figure 5. Inhibition zone on 70% ethanol extract (A) and ethyl acetate fraction (B)

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CONCLUSION

This study carried out an ultrasonic extraction to produce a yield of 18.73%. Isolation of flavonoid compounds was carried out in 2 ways, namely gradient and non-gradient isolation, with the best eluent from ATLC monitoring, namely n-hexane: ethyl acetate (7:3). A purple stain was obtained, which was suspected to be a flavonoid compound of the flavonol group by strengthening the results of UV-Vis and FTIR characterization. The isolate was then tested for the total flavonoid content, 32.95 mg QE/g, from the total isolates of 35.7 mg. Ethanol extract and ethyl acetate fraction were tested for toxicity and antibacterial. The LC_{50} toxicity value of the ethanol extract was 143.53 ppm, and the ethyl acetate fraction was 41.16 ppm. In the antibacterial ethanol extract, the weak category inhibited 2.30-4.54 mm, while the fraction was included in the medium category, which could inhibit 2.63-5.29 mm.

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