Flavonoid Compound from Dichloromethane Extract Of Crinum amabile Donn Leaves

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Abstract
Flavonoid compound was isolated from Crinum amabile Donn. leaves. The separation of flavonoids was carried out by using chromatographic techniques to purify CH2Cl2 extract of Crinum amabile Donn. leaves. Column chromatography with silica gel and sephadex as stationary phases, thin-layer chromatography, and preparative thin-layer chromatography were the chromatographic techniques used in this research. The pure compound obtained in the form of white-yellow solid with the Rf value is 0.44. Compound identification using [1]*H-NMR spectrometer showed the presence of protons from the CH=CH ortho with the appearance of a doublet of doublet (dd) each having j values of 8.5 Hz and 8.1 Hz with chemical shifts in the range of 6.5 ppm and 7 ppm. The CH=CH ortho group with the appearance of doublet of doublet of doublet (ddd) each having j values 8.4 Hz and 9 Hz with chemical shifts in the range of 7.2 ppm to 7.4 ppm. The methoxy group (-OCH$_3$) was detected on chemical shift 3.8 ppm. Spectrometries of analysis showed that flavonoid compound obtained was 4-Hydroxy-7-Methoxyflavan.*
Keywords: ***Crinum amabile* Donn, chromatographic techniques, 4-Hydroxy-7-Methoxyflavan**

Introduction

Natural products (NPs) have been widely used worldwide for thousands of years to treat human diseases. The NPs are complex mixtures of hundreds of chemical constituents. As a result, it is difficult to evaluate and identify the active components responsible for their efficacy (Fu et al., 2019). (Atun, 2014) stated that natural product research had progressed more rapidly with the discovery of chromatographic separation techniques and spectroscopic molecular structure determination in the mid 20th century. This method is quite effective in finding several structures of bioactive compounds, for example, the discovery of alkaloids such as vinblastine and vincristine from *Catharanthus roseus* plants as a cancer drug or the discovery of taxol from the *Taxus brevifolia* plants as a drug of uterine cancer. This can encourage pharmaceutical companies to explore plant bioactive compounds as lead compounds for new drug discoveries. Nowadays, more than 30% of drug reagents on the market are natural products compound. Thus, there will be more new drugs that come from nature, both from plants and animals or organisms.

A hadith narrated by Imam Bukhari in his Sahih from the companions of Abu Hurairah that the Prophet Muhammad said, *"It is not Allah who sends down a disease, but He also sends down a medicine for its disease."* Another hadith narrated from Imam Muslim that the Prophet Muhammad said, *"Every disease must have medicine. If the medicine is suitable for the disease, he will recover with the permission of Allah."*

This hadith explains that the medicines will be determined with some research. In another hadith, the Prophet emphasized the need for medical science to study and seek medicine. This hadith invites to conduct medical research, as stated in his saying. In addition, the Prophet stated that the drug exists, but it takes people who are serious about doing research and finding out the drugs. Therefore, research related to natural products is essential because through this research can be found bioactive compounds that allow them to develop as medicinal compounds or drug reagents.

Crinum plants are one of the exciting types of plants used as an object in natural products research. The genus *Crinum* belongs to the *Amaryllidaceae* family and comprises approximately 160 species dis-

tributed throughout the tropics and warm temperate regions, mainly in Asia, Australia, Africa, and America(Tram et al., 2002).

Crinum, a plant commonly used in the medical sector, has many chemical constituents spread from the bulbs, stems, leaves, and flowers. Based on the review of the pharmacology of *Crinum* species, this genus has yielded more than 170 different compounds, most of which are alkaloids(Fennel & Van Staden, 2001). (Refaat et al., 2013) reviewed non-alkaloidal constituents contained in *Crinum* plants. The non-alkaloidal constituents contained in *Crinum* are flavonoids,chromones, coumarins, ionones, sterols, triterpenes, hydrocarbons, alcohols, aldehydes, ketones, esters, ethers, and carbohydrates, and the most common compound found in non-alkaloidal groups are flavonoids. (Zuhra et al., 2008) stated that flavonoids are part of phenolic or polyphenolic compounds that function as antioxidants.

One of the *Crinum* species that can contain flavonoid compounds is *Crinum* amabile Donn., the flower of the family of *Amaryllidaceae* and has the common name Giant Spider Lily. (Tram et al., 2002) said that in Vietnamese folk medicine, *Crinum amabile* Donn. was used as an emetic, rheumatism, and earache. Besides that, the pharmacological effects are efficacious as neutralizing toxins (antidotes), worm medicine (anthelmintic), stimulating the boils, eliminating swelling (anti-swelling), and relieving pain (analgesics). This research works on the isolation process of these extracts to get the pure compound of flavonoids as a natural product that can be used for medical drugs. The process is carried out using chromatography techniques, and the compound is characterized by Nuclear Magnetic Resonance (NMR).

Research Methodology

Materials and Chemicals

The fractions used in this research were PL-3-F18 and PL-3-F19. The solvents used were acetone (C_3H_6O), hexane (C_6H_{14}), ethyl acetate ($C_4H_8O_2$), methanol (CH_3OH), dichloromethane (CH_2Cl_2), ceric ammonium molybdate ($CeH_8Mo_3N_2O_{12}$), deuterated chloroform ($CDCl_3$), and petroleum ether. Column chromatography stationary phases were silica gel and sephedex LH-20. Chemical instrumentations used were column chromatography, thin-layer chromatography (TLC), preparative thin-layer chromatography (PTLC), UV (Ultra Violet) lamp, and nuclear magnetic resonance (NMR).

Separation Process and Analysis

a) Separation process by using column chromatography based silica gel

Combine the isolated samples and mix with silica gel. The samples and silica gel mixture is inserted into the column chromatography over silica gel 60 (230-400 mesh ASTM). Run the column with combination of acetone in hexane in various concentartion as mobile phases and separate it continuously until 100% acetone as mobile phase. A test tube was used to put the samples. Evaporate the sample by using a rotary evaporator and check the chromatogram with TLC. A chromatogram test is done to determine the separated sample type that needs to separate further to get the pure compound. Combine all the fractions that have similar chromatograms and put them into a vial bottle. Weight it with the balance sheet analysis and check the physical properties of each fraction.

b) Separation process by using column chromatography based sephadex

Combine the isolated samples and mix with silica gel. The samples and silica gel mixture is inserted into the column chromatography over Sephadex LH-20. Run the column with combination of methanol in hexane in various concentartion as mobile phases and separate it continuously until 100% of methanol as mobile phase. A test tube was used to put the samples. Evaporate the sample using a rotary evaporator and check the chromatogram with TLC. A chromatogram test is done to determine the separated sample type that needs to separate further to get the pure compound. Combine all the fractions with similar chromatograms and put them into a vial bottle. Weight it with the analyst balance sheet and check the physical properties of each fraction.

c) Separation process by using plate thin-layer chromatography

The sample was diluted with dichloromethane and spotted the sample in the PTLC surface. The PTLC checked with the solvent system and then checked the chromatogram under a UV lamp and moved the silica gel into erlenmeyer. The acetone:hexane with 10% concentration was added to the erlenmeyer and stirred for around 10 minutes. Filter the sample with a buchner and put the filtrate into a flask. Evaporate the filtrate using a rotary evaporator and move the sample into a vial bottle. Weight it with balance sheet analysis.

d) TLC analysis

The TLC testing should conduct as the illustration below (Figure 1a&1b).These measurements are the distance traveled by the solvent and the distance traveled by individual spots. When the solvent front gets

close to the top of the plate, the plate is removed from the beaker, and the position of the solvent is marked with another lie before it has a chance to evaporate. The measurement of TLC is shown on Figure 1c.

The Rf value for each dye is then worked out using the formula:

$$R_f = \frac{distance\ traveled\ by\ component}{distance\ traveled\ by\ solvent} \qquad (1)$$

(Kumar et al., 2013)

e) ^{1}H-NMR analysis

NMR explained the amount of hydrogen in each type and the environmental properties of each hydrogen atom. Analysis of a sample produces a ^{1}H-NMR spectrum which consists of a horizontal axis that represents the delta (δ) scale with values from 0 (on the right) to 10 (on the left), and a vertical axis representing the intensity of the resonance signal. Coupling constant (j) can be count to analyse the NMR spectrum.

$$j = \Delta\delta\,(chemical\ shift) \times (NMR\ Frequency) \qquad (2)$$

(Sastrohamidjojo, 2001).

Result and Discussion

Separation Process

The isolation process of chemical constituents in *Crinum amabile* Donn. leaves are carried out by the separation process using chromatographic techniques, which is column chromatography with the separation process depending on the polarity of the compound and continued with the thin layer chromatography for further verification. The column used is gravitational.The use of vacuum columns can increase the possibility of mixing fragmentations and reduce the effectiveness of its separation process.

PL-A3 (535.7 mg) was the combination of isolated *Crinum amabile* Donn. number PL-3-F18 (511.4 mg) and PL-3-F19 (24.3 mg). The PL-3-F18 and PL-3-F19 were combined and mixed with silica gel. It combined because the TLC checking shows similar chromatograms.

Based on the TLC result. The fractions developed in a column with 10% acetone in hexane as a mobile phase, increasing the polarity with acetone until 100% acetone and finally increasing the polarity with methanol until 100% methanol. The polar chromatograms that are possible for flavonoid compounds contained will continue to separate further until they produce two interesting fractions for further verification.

Further verification found an overlapping chromatogram with some samples spotted inside it. PTLC was used because of the characteristic of the fraction that can not separate by column chromatography even though with silica gel or sephadex LH-20 as a column stationary phases. The PTLC was developed with 8% acetone in hexane, that shown in TLC checking in Figure 2b. Figure 2b shows the TLC checking for determining the solvent systems of the fraction, but the separation result is not good enough. Ceric ammonium molybdate is used to verify the possibility of some compounds that can not see under UV light. It tells that the fraction has overlapped with the big spot and is hard to separate.

The fraction with this overlapping spotted continues with further treatment using column chromatography with Sephadex LH-20 stationary phase and PTLC. The TLC result shows the possibility of a pure compound in it. The possibility of the pure compound is seen from the chromatogram spots that appear. The spot in the middle has a different color density from the three spots when illuminated under UV light. The spot size shows the possibility of a higher composition if compared to the other spots, which are assumed to be impurities. The fraction is crystallized for removing the impurities and verifying the structure using ^{1}H-NMR.

In the TLC result, the uncrystallized fraction shows several chromatogram spots under UV radiation, and it is contrasted to the crystallized fraction, which only shows one chromatogram spot. This explains that the crystallization process is very effective for purifying substances and removing the impurities in the sample. Based on the results of the TLC test, the crystallized sample had the same chromatogram at R$_f$ 0.44 and was a pure compound.

Structure Elucidation of a Pure Compound

Analysis of ^{1}H-NMR compounds was conducted to describe various hydrogen atoms in molecules. The ^{1}H-NMR spectrum can inform the chemical environment, the number of hydrogen atoms in each environment,

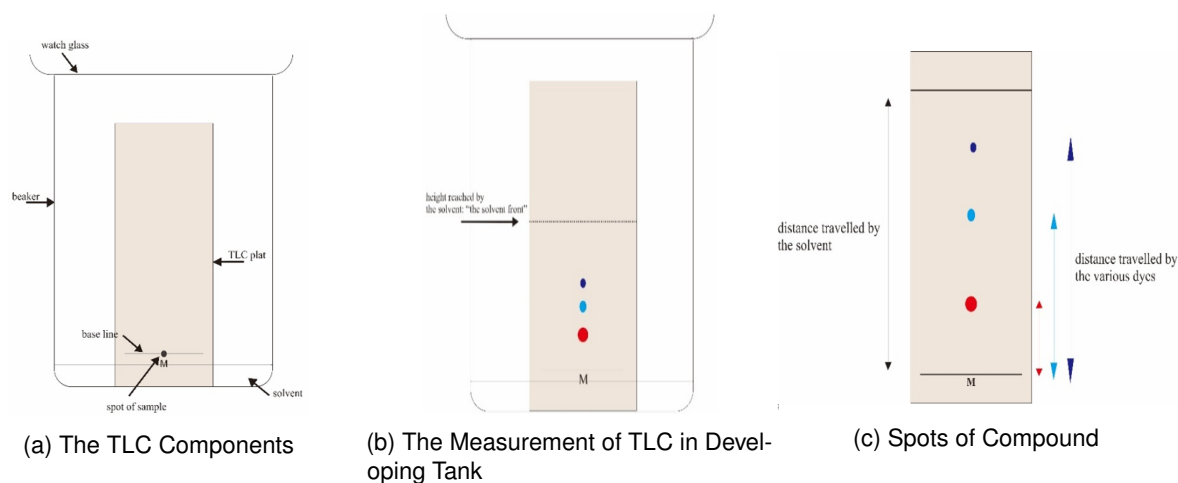(a) The TLC Components

(b) The Measurement of TLC in Developing Tank

(c) Spots of Compound

Figure 1: TLC analysis

(a) The TLC Checking to Determine the Solvent Systems

(b) An Overlapping Zone
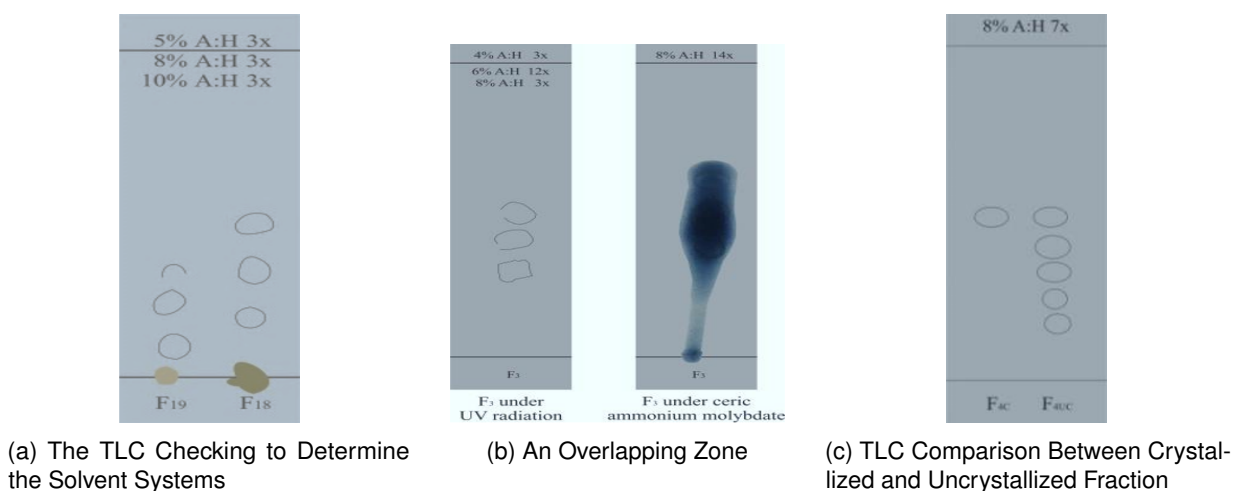
(c) TLC Comparison Between Crystallized and Uncrystallized Fraction

Figure 2: Separation Process

and the group structure adjacent to each hydrogen atom. The ^{1}H-NMR spectrum was tested at 300 MHz.The ^{1}H-NMR spectrum result of the pure compound is shown in the Figure 3. Based on the spectrum, shows several compound groups, such as aromatic, that have a characteristic in the ^{1}H-NMR test.

The ^{1}H-NMR spectrum will cut in several details to explain the position of each hydrogen atom. Figure 4a. above shows the position (a) and (d) are the signals for hydrogen that have coupling constant (j) around 8.4 with the appearances of doublet of doublet of doublet (ddd) as an ortho position in an aromatic ring. The signal shows in the chemical shift (δ) range of around 7.2 to 7.4 because of the electronegativity effect of oxygen in the hydroxy group. The (c) signal is for the ortho relation with the j = 8.1. The (e) is for two types of hydrogen—doublet of doublet (dd) found in this signal. The j = 8.5 signal shows the same range between j ortho in the spectrum (c). The other signal shows with j = 2.7 as an overlapping signal, ex-

plaining the meta condition. The (b) is not a signal for aromatic hydrogen. It is just a signal for chloroform-d as a solvent used that usually shown in the range of 7 ppm.

Figure 4b. shows the spectrum of methoxy compounds. The spectrum is shown with the singlet signal. This result has two singlet signals in the range of 1.6 ppm and 3.8 ppm. Methoxy compound would be shown in the range of 3.8 ppm with higher signals. Following research conducted by (Prabawati et al., 2017), methoxy groups from flavonoid synthesis were detected at a chemical shift of 3.99 ppm with a single appearance. That is because of the effect of oxygen in this compound. The characteristic of electronegativity made the reaction happen in with high energy and would produce a high chemical shift in the ^{1}H-NMR spectrum.

Figure 5. (a) shows the doublet signal and has the j = 2.4. Figure 5. (b) and Figure 5. (c) are a little bit complex because they show many close signals. The
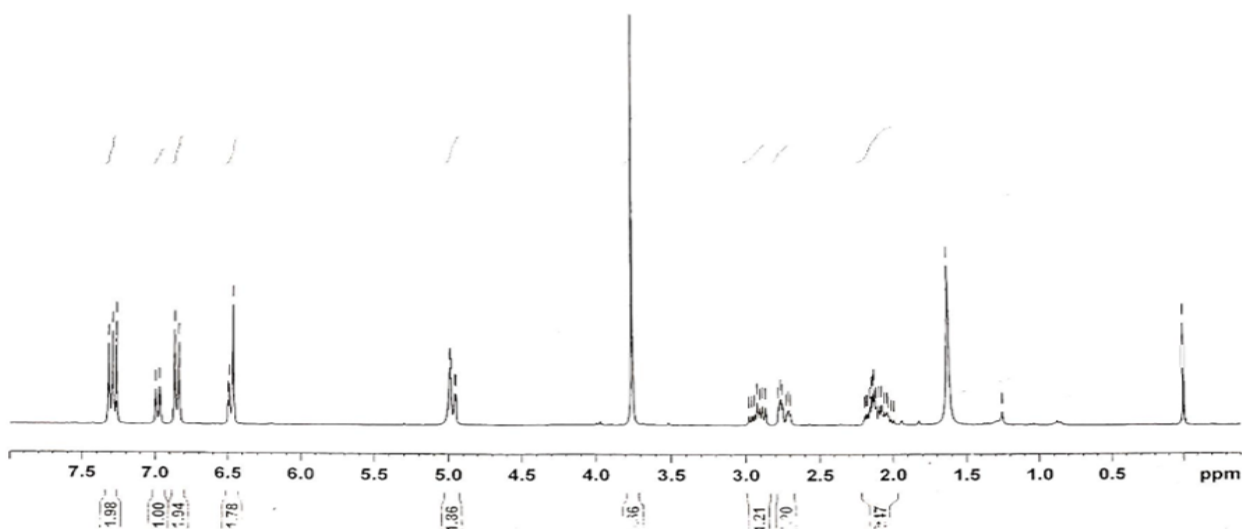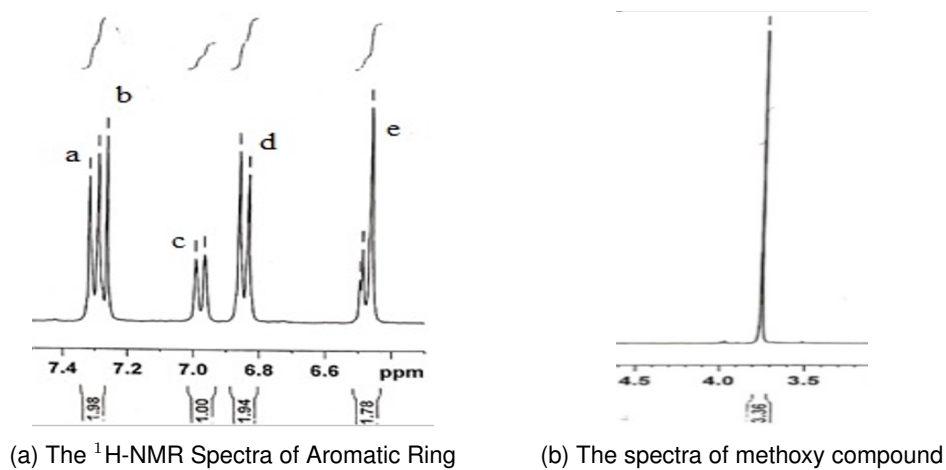
Figure 3: ^{1}H-NMR Spectra of Pure Compound

(a) The ^{1}H-NMR Spectra of Aromatic Ring

(b) The spectra of methoxy compound
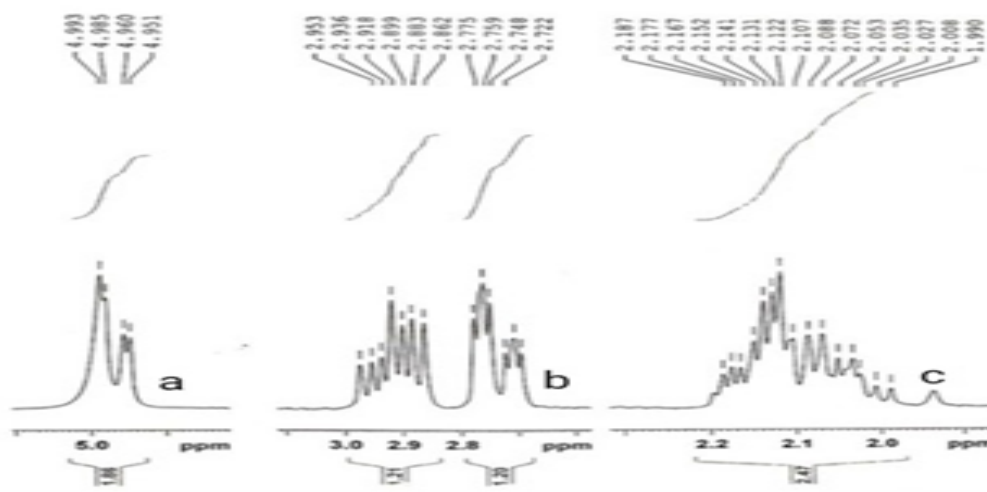
Figure 4: Structure Elucidation of a Pure Compound

Figure 5: ^{1}H-NMR Spectra for Other Hydrogens

(a) Structure of 4-hydroxy-7methoxyflavan
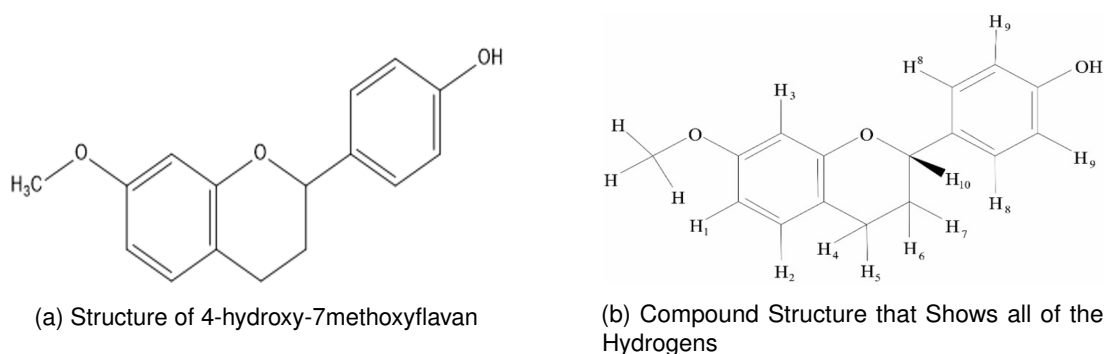

(b) Compound Structure that Shows all of the Hydrogens

Figure 6: The structure of a Pure Compound

Figure 5. (b) has the j = 9.5; 6.0; and 4.8 with the ddd interaction. At the same time, Figure 5. (c) shows the dddd interaction with the j = 3.0; 4.5; 4.8; 5.4.

Based on the ^{1}H-NMR analysis of the crystallized fraction sample, the flavonoid found in this research is 4-hydroxy-7-methoxyflavan. The structure of this compound is shown in the Figure 6a.

Figure 6b. shows all the hydrogen in the compound structure to explain the chemical environment in each hydrogen atom.

Figure 4a. describe hydrogens in the aromatic ring. Signal (a) refers to H_9, and signal (d) refers to H_8, the ortho position with ddd appearance. Signal (c) explained the ortho position between H_1 and H_2. Signal (e) refers to H_1 and H_3 with metha position. In contrast, signal (b) is a solvent chloroform-d. Figure 5. (a) shows the signal for H_{10} that doublet to H_6 and H_7. Otherwise, Figure 5. (b) explains the signal for H_4 and H_5, then Figure 5. (c) is for H_6 and H_7. The methoxy group in the left-hand side of the compound structure showed in Figure 4b.

Table 1: Hydrogens and Methoxy Appearances in 4-Hydroxy-7-Methoxyflavan

Compound	Mult
H_1	Dd
H_2	Dd
H_3	Dd
H_4	Ddd
H_5	Ddd
H_6	Dddd
H_7	Dddd
H_8	Ddd
H_9	Ddd
H_{10}	Dd
Methoxy	S

S = singlet; Dd = doublet of doublet;Ddd = doublet of doublet of doublet;Dddd = doublet of doublet of doublet of doublet.

Conclusion

Based on this research, it can be concluded that,

1. The method that can be used to separate the flavonoid compounds from *Crinum amabile* Donn. leaves is chromatographic techniques such as column chromatography, thin-layer chromatography, and preparative thin-layer chromatography.

2. Based on the NMR analysis, ten types of proton hydrogens are found in this compound' and one methoxy group that is correlated to 4-hydroxy-7-methoxyflavan.

Recommendation

Based on the development of research related to natural products, the author suggests that further research can be carried out to test the bioactivity of 4-hydroxy-7-methoxyflavan, so it can be applied in a few years from now in the medical world.

Acknowledgment

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