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Isolation of Flavonoid Compounds Derived from Buni Leaves (*Antidesma bunius (L) Spreng.*)

Tonel Barus*, Ria Agnes Adelina Manalu, Lamek Marpaung
Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, Medan, 20155, Indonesia
*Corresponding Author: tonel_barus@yahoo.co.id

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ABSTRACT
The flavonoid compounds from the leaves of buni (*A. bunius (L) Spreng.*) have been isolated through a process of maceration utilizing methanol as the solvent. The concentrated methanol extract was combined with ethyl acetate. The concentrated ethyl acetate extract was subsequently dissolved in methanol and subjected to partition extraction using n-hexane. The concentrated methanol extract was acidified with 6% HCl, followed by partition extraction using chloroform. The concentrated chloroform extract was isolated using column chromatography, employing an eluent mixture of n-hexane and ethyl acetate in the following ratios: 90:10, 80:20, 70:30, and 60:40 (v/v). The compounds underwent purification via TLC preparative, resulting in a tawny paste with a mass of 30 mg and an Rf value of 0.31. The compound underwent additional identification through the application of Ultraviolet Visible (UV-Vis) spectroscopy, Fourier Transform-Infrared Spectroscopy (FT-IR), and Proton Nuclear Magnetic Resonance Spectroscopy (^{1}H-NMR), leading to the conclusion that the flavonoid in question is isoflavone.

Keywords: Buni Plant Leaves, Extraction, Isoflavones, Maceration

ABSTRAK
Senyawa flavonoid dari daun buni (*A. bunius (L) Spreng.*) telah diisolasi melalui proses maserasi dengan menggunakan pelarut metanol. Ekstrak metanol pekat digabungkan dengan etil asetat. Ekstrak etil asetat pekat kemudian dilarutkan dalam metanol dan dilakukan ekstraksi partisi menggunakan n-heksana. Ekstrak metanol pekat diasamkan dengan HCl 6%, diikuti dengan ekstraksi partisi menggunakan kloroform. Ekstrak kloroform pekat diisolasi menggunakan kromatografi kolom, menggunakan campuran eluen n-heksana dan etil asetat dengan perbandingan sebagai berikut: 90:10, 80:20, 70:30, dan 60:40 (v/v). Senyawa tersebut mengalami pemurnian melalui KLT preparatif, menghasilkan pasta berwarna kecoklatan dengan massa 30 mg dan nilai Rf 0,31. Senyawa tersebut mengalami identifikasi tambahan melalui aplikasi spektroskopi Ultraviolet Visibel (UV-Vis), Spektroskopi Fourier Transform-Inframerah (FT-IR), dan Spektroskopi Resonansi Magnetik Nuklir Proton (1H-NMR), yang mengarah pada kesimpulan bahwa flavonoid yang dimaksud adalah isoflavon..

Kata Kunci : Daun Tumbuhan Buni, Ekstraksi, Isoflavon, Maserasi

1 Introduction

Flavonoids are naturally occurring chemicals characterized by the presence of two aromatic hydroxyl rings, designated A and B, linked by three carbon atoms [1]. Estimates indicate that roughly 2% of the carbon assimilated by plants through photosynthesis is transformed into flavonoids or similarly related chemicals. Flavonoid chemicals are present in all plant components, including leaves, roots, wood, bark, pollen, nectar, flowers, fruit, and seeds [2]. Flavonoids present in plants can safeguard the human body against free radicals and diminish the risk of cancer and inflammation [3].

The *Antidesma bunius (L) Spreng* plant is found in many areas in the Philippines and mostly grows in mountainous areas with a tropical season. It belongs to the Euphorbiaceae family. This plant has fruit shaped in groups, whereas there are 30-40 small fruits on one stem. The fruit is green, then red and finally black,

indicating the fruit is ripe. The fruit tastes sweet and sour when red, and when it is ripe, it can be used in making jams and drinks [4].

A. bunius (L) Spreng is one of the plants that has the potential to be developed as an anticancer drug. The leaves and bark of this plant contain alkaloids, saponins, tannins and flavonoids. In contrast, the roots contain saponin and tannin compounds. It is known as the buni plant, which is widely used by people as a traditional medicine to treat high blood pressure, heart palpitations, anemia and cancer [5]

Buni fruit is a wild plant belonging to the Euphorbiaceae family. In Indonesia, buni fruit can be used as a medicinal plant, which has been practised previously. Buni contains vitamins, anthocyanins, flavonoids and phenolic compounds [6].

Several previous studies have been carried out on this buni plant. From prior research, Haripyaree, Guneswhor and Damayanti reported that methanol extract from *A. bunius (L) Spreng* fruit had high antioxidant activity with an average IC¬¬50 value of 100.08 µg/ml compared to other fruits [7].

2 Materials and Methods

2.1 Equipment

The tools used including rotary evaporator (Bűchi R-114), rotary flask evaporator (Schoot/ Duran), distillator, Analytical balance (Mettler AE 200), UV lamp 254 nm/356 nm (UVGL 58), ^{1}H-NMR spectrophotometer (Jeol/Delta2NMR 500MHz), FT-IR spectrophotometer (Shimadzu), UV-Vis spectrophotometer (Shimadzu).

2.2 Materials

The materials used including Buni leaf, methanol, ethyl acetate, aquadest, n-hexane, silica gel 40 (70-230 mesh) ASTM. KgA, $FeCl_3$ 5%, NaOH 10%, Mg powder, HCl, H_2SO_4, Benedict's reagent, HCl 6%, Cotton, chloroform, silica gel 60 F_{254} KLT plate, preparative KLT plate 60 F_{254}, benzene, and acetone were purchased by E-Merck.

2.3 Method

2.3.1Preparation of Buni Plant Leaf Extracts

The examined sample consisted of Buni leaves sourced from Jalan Pelajar Timur Ujung No.51, Medan Denai sub-district, North Sumatra. Buni leaves were air-dried and subsequently ground to get 1200 g of Buni leaf powder. The identification of Buni leaf powder was conducted by the Phytochemical Screening technique. A qualitative preliminary test was conducted via a color reaction to confirm the presence of flavonoid components in Buni leaves. Ten grams of dried Buni leaf powder were placed into two Erlenmeyer flasks, followed by the addition of 100 mL of methanol to each flask. The mixture was allowed to sit overnight and subsequently filtered. The obtained sample extract was partitioned into four test tubes, with each tube receiving distinct reagents. In the tube, the addition of 5% $FeCl_3$ resulted in a black solution. Upon the addition of magnesium powder and strong hydrochloric acid, Tube II yielded a pink solution. Tube III, upon the addition of 10% NaOH, yielded a yellowish-green solution. The introduction of concentrated H_2SO_4 in Tube IV results in a yellowish-orange solution..

2.3.2Extraction of Buni Plant Leaves

1200 g of buni leaf powder was weighed and subsequently macerated with about 5 L of methanol until all samples were fully submerged, then permitted to stand for 24 hours. The maceration was gathered and condensed with a rotary evaporator to yield a concentrated methanol extract. Subsequently, the methanol solvent was completely evaporated. Tannin was isolated by dissolving the methanol-concentrated fraction in ethyl acetate and subsequently filtering. The filtrate is thereafter placed in the rotary evaporator and subjected to evaporation until all ethyl acetate solvent is removed. The concentrated ethyl acetate fraction was diluted in methanol and subjected to repeated partitioning with n-hexane until the n-hexane layer became nearly transparent. The methanol layer was isolated from the n-hexane layer, thereafter condensed using a rotary evaporator, and further evaporated to yield the concentrated extract of the methanol layer. The methanol fraction was analyzed for sugar concentration using Benedict's reagent and subsequently hydrolyzed with 6% HCl while heated in a water bath for approximately 45 minutes. The filtrate was subjected to filtration and partition extraction with chloroform three times. The chloroform extract was concentrated using a rotary evaporator, resulting in a final concentrated yield of 0.65 g.

2.3.3Thin Layer Chromatography Analysis

Thin layer chromatography (TLC) analysis was conducted on the chloroform extract with silica gel 60F254 Merck as the stationary phase. This investigation aimed to identify the suitable solvent system and ratio for column chromatography. The mobile phase employed was a solvent mixture of n-hexane and ethyl acetate in the ratios of 90:10, 80:20, 70:30, and 60:40 (v/v).

Introduce 10 ml of a 90:10 (v/v) n-hexane:ethyl acetate mobile phase solution into the chromatographic vessel, then saturate. Applied the concentrated chloroform extract onto the activated TLC plate. Place the plate into a vessel containing a saturated mixture of solvents, then seal and elute. The plate that was eluted and extracted from the vessel. Additionally, it was subsequently desiccated.

Detected the stain under ultraviolet light, thereafter treated with a 5% FeCl3 reagent. The hue of the emerging spots was observed, and the Rf value was determined. The identical procedure was applied to n-hexane with solvent ratios of ethyl acetate at 80:20, 70:30, and 60:40 (v/v).

2.3.4Isolation of Flavonoid Compounds by Column Chromatography

The isolation of flavonoid components using column chromatography was performed on the chloroform-concentrated extract that had been obtained. The stationary phase employed is silica gel 40 (70-230 mesh) ASTM, while the mobile phase consists of 100% n-hexane and a mixture of n-hexane and ethyl acetate in the ratios of 90:10, 80:20, 70:30, and 60:40 (v/v).

The apparatus for column chromatography was assembled. Dissolved silica gel 40 (70-230 mesh) ASTM was mixed with n-hexane until homogenous and thereafter introduced into the chromatography column. Subsequently, it was eluted with 100% n-hexane until the silica gel became firm and uniform. Dissolved 0.65 g of concentrated chloroform extract in chloroform solvent with silica gel, subsequently introduced it into a chromatographic column containing silica gel slurry. Then, the mobile phase of n-hexane and ethyl acetate in a 90:10 (v/v) ratio was gradually added, ensuring that the effluent flow from the column matched the influx of the mobile phase from above. The polarity was enhanced by including n-hexane: ethyl acetate mobile phases in the ratios of 80:20 (v/v), 70:30 (v/v), and 60:40 (v/v). The results were collected in vial bottles at approximately 10 mL intervals, thereafter subjected to thin-layer chromatography (TLC), and merged into fractions exhibiting identical Rf values, followed by testing with 5% FeCl3. Subsequently, it was evaporated until a paste was produced.

2.3.5Purification

The paste isolated via column chromatography is reconstituted with chloroform and subsequently evaluated using TLC to assess the purity of the recovered compounds and to identify appropriate mobile phases for preparative TLC. The mobile phase of chloroform and methanol at a 90:10 (v/v) ratio demonstrates optimal separation for saturating the preparative TLC vessel. Simultaneously, the previously dissolved paste was gradually and uniformly dispensed down the lower edge of the active TLC plate. The plate is placed into a sealed vessel containing a saturated mixture of solvents. Subsequent to elution, the plate is extracted from the vessel and desiccated, after which the results are analyzed under ultraviolet light. Each zone was delineated, abraded, and eluted using a methanol:ethyl acetate (1:1) solution. The elution results were evaporated until a brownish-yellow residue was achieved.

2.3.6Purity Test of Isolation Results with Thin Layer Chromatography

The purity assessment of the paste was conducted via thin layer chromatography employing a silica gel 60 F254 stationary phase, utilizing an n-hexane:ethyl acetate 70:30 (v/v) mobile phase and a chloroform:methanol 70:30 (v/v) system. Introduce 10 mL of mobile phase solution into the thin layer chromatography vessel, then saturate. The paste that was previously dissolved in chloroform was imaged on the TLC plate. Insert the TLC plate into a saturated thin layer chromatography vessel. Upon the mobile phase solvent reaching the designated limit, the TLC plate was extracted from the vial, dried, examined under UV light, and subsequently treated with a 5% FeCl3 reagent in methanol to provide black spots indicative of flavonoid chemicals..

2.4 Identification of Isolated Compounds

Analysis with a UV-visible spectrophotometer was obtained from the Pharmaceutical Research Laboratory, Faculty of Pharmacy, USU. Analysis with FT-IR Spectrophotometer obtained from the Laboratory of Chemical Research Center - LIPI, PUSPITEK Serpong Area, Tangerang using KBr. Analysis with ^{1}H-NMR

Spectrometer obtained from the Laboratory of Chemical Research Center - LIPI, PUSPITEK Serpong Area, Tangerang using Acetone as a solvent.

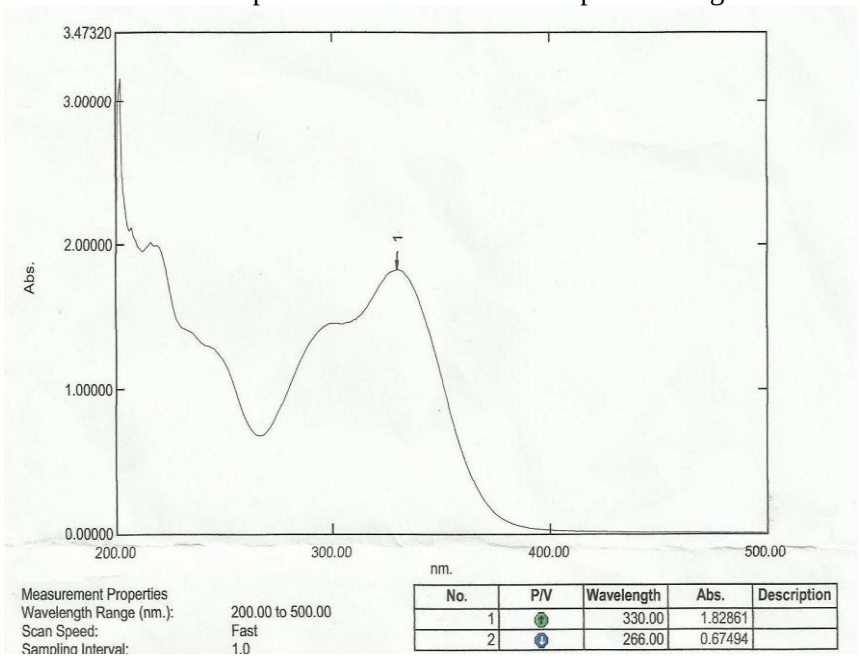
3 Results and Discussion

The early screening results of methanol and ethyl acetate extracts from the leaves of buni plants (*A. bunius (L) Spreng*.) indicated the presence of flavonoids when tested with color reagents. The elution results of the solvent ratio n-hexane:ethyl acetate 70:30 (v/v) in fractions 47-71 were subjected to preparative TLC using an eluent of chloroform:methanol 90:10 (v/v) to isolate pure chemicals. The pure chemical is acquired as a brownish-yellow paste, weighing 30 mg, with an Rf value of 0.31.

The TLC results indicate that an optimal solvent ratio for isolating flavonoid components from buni plant leaves is n-hexane:ethyl acetate at 70:30 (v/v), demonstrating superior separation of the resultant stains. The TLC examination further demonstrates the presence of two stains with an increased separation distance between them. Following separation via column chromatography, TLC analysis was conducted to merge the fractions, resulting in three fractions. The first and second fractions, totaling 98 mg, were selected for further analysis using a chloroform-methanol solvent system at a ratio of 90:10 (v/v), subsequently undergoing Preparative Thin Layer Chromatography. Chromatography utilizing an appropriate solvent system of chloroform and methanol in a 90:10 (v/v) ratio is conducted under UV light observation, followed by scraping the silica gel and eluting it with a solvent mixture of methanol and ethyl acetate in a 10:10 (v/v) ratio. The compound was subsequently subjected to purity testing via TLC using n-hexane:ethyl acetate (70:30 v/v) and chloroform:methanol (90:10 v/v) as eluents, revealing a single spot for the resultant molecule.

Structural elucidation of flavonoid compounds in a brownish-yellow paste utilizing UV-Vis, FT-IR, and ^{1}H-NMR spectrophotometry. The UV-Vis spectrophotometer is employed to identify flavonoid classes, as each flavonoid type exhibits distinct maximum wavelength absorption (λ max). The FT-IR spectrophotometer is employed to examine functional groups, namely those of aromatics, including C=C, hydroxyl, carbonyl, and ether. The 1H-NMR spectrophotometer is employed to identify the types of protons from rings A, B, and C. A combination of Ultraviolet-Visible Spectrophotometry (UV-Vis), Fourier Transform Infrared Spectrophotometry (FT-IR), and Proton Nuclear Magnetic Resonance Spectrophotometry (^{1}H-NMR) data can be utilized to elucidate the structure of the isolated flavonoid compounds.

The UV-visible spectrum of the isolated compound using methanol solvent is shown in Figure 1 below:

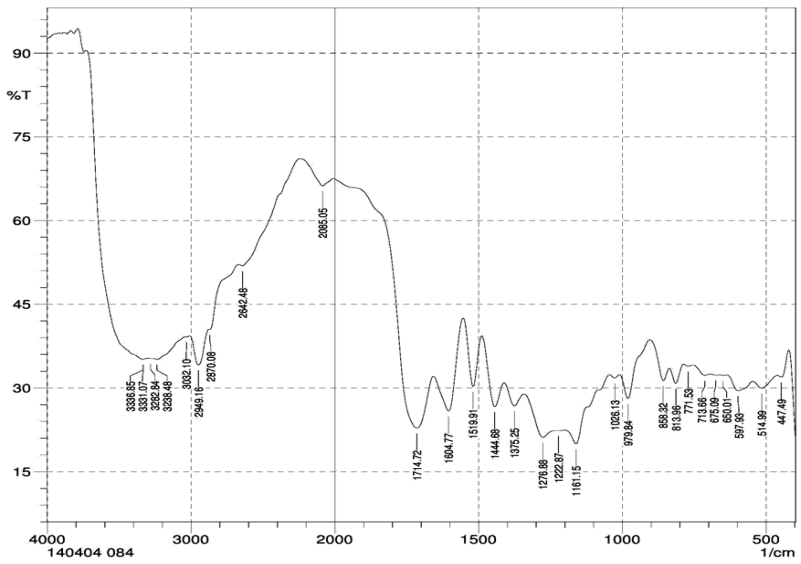


Band	Wavelength (nm)	Abs.
I	330.00	1.82861
II	266.00	0.67494

Figure 1 UV-visible spectrum of isolation result compound

The interpretation of the UV-visible spectrum with methanol solvent (Figure 1) gives a wavelength (λ max) of 330.0 nm for band I and 266.0 nm for band II. This shows that the isolated compound is in accordance with the UV-visible spectrum of flavonoid comparator compounds, namely Isoflavones.

FT-IR spectrophotometer analysis of the isolated paste produced absorption bands in the wave number region, and ^{1}H-NMR analysis gave chemical shifts in the (ppm) region, both of which can be seen in Figure 2 and Figure 3.



Wave number
3336.85 –3331.07 cm^{-1}
3282.84 –3238.48 cm^{-1}
2949.16 –2870.08 cm^{-1}
1714.72 cm^{-1}
1604.77 cm^{-1}
1375.25 cm^{-1}
1276.88 cm^{-1}
1161.15 cm^{-1}
979.84 cm^{-1}

Figure 2. FT-IR spectrum of isolated compounds

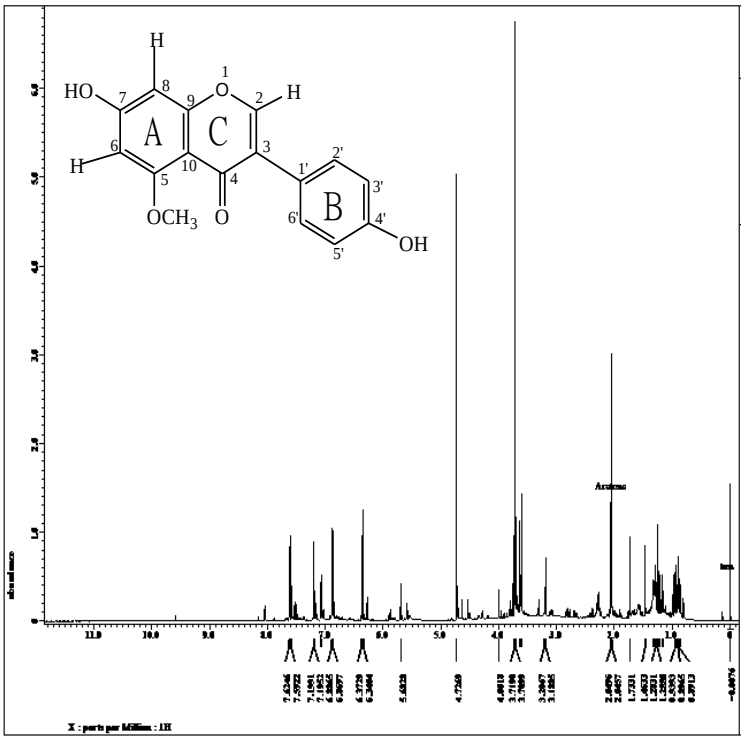


Figure 3. ^{1}H-NMR spectrum of isolated compounds

The results of the interpretation of the FT-IR spectrum (Figure 2) and ^{1}H-NMR spectrum (Figure 3) using acetone solvent in TMS standard obtained a chemical shift at δ = 3.7190 ppm singlet peak showing the proton of the methoxy group -OCH_3. This is supported by the infrared spectrum at wave numbers 2927.94-2872.01 cm-1 with a medium peak showing the presence of aliphatic -CH stretching vibrations and FT-IR spectrum at wave numbers 1367.53 cm^{-1} with a medium peak showing the bending vibrations of -CH_3. This is also supported at wave number 1116.78 cm^{-1} with a medium peak showing the presence of unsymmetrical C-O-C stretching vibrations. Furthermore, the chemical shift in the region δ = 6.3404-6.3729 ppm doublet peak shows the protons of H-6 and H-8 in ring A flavonoid structure. This is supported by the infrared spectrum at wave number 1604.77 cm^{-1} with a medium peak showing the vibrations of the C=C double bond of the aromatic

system and at wave number 979.84 cm-1 with a sharp peak showing the presence of -CH stretching vibrations on the aromatic ring.

Furthermore, the chemical shift at δ = 7.1991 ppm singlet peak shows the flavonoid structure's H-2 proton in ring C. This is supported by the infrared spectrum at wave number 1703.14 cm^{-1} sharp peak showing the C=O double bond stretching vibration of ketone and infrared spectrum at wave number 1165.00 cm^{-1} sharp peak showing the -C-C-O-C- stretching vibration of ketone, and at wave number 1033.85 cm^{-1} medium peak showing the symmetric -C-O-C- stretching vibration. Furthermore, the chemical shift in the region of δ = 6.8697-6.8865 ppm doublet peak shows the H-3 'and H-5' protons in ring B flavonoid structure. This is supported by the infrared spectrum at wave number 1604.77 cm^{-1} with a medium peak showing the vibrations of the C=C double bond of the aromatic system and at wave number 979.84 cm^{-1} with a sharp peak showing the presence of -CH stretching vibrations on the aromatic ring. The chemical shift in the region δ = 7.5922-7.6246 ppm doublet peak shows the protons of H-2' and H-6' in the B ring of flavonoid structure. This is supported by the infrared spectrum at wave number 1604.77 cm^{-1} with a medium peak showing the vibration of the C=C double bond of the aromatic system and at wave number 979.84 cm^{-1} with a sharp peak showing the presence of -CH stretching vibrations on the aromatic ring [8].

In addition, the chemical shift in the area δ = 4.7269 ppm sharp singlet peak which cannot be interpreted and determined the type and location of the substituent which is thought to show the proton of -CH3 due to the sharp peak intensity but cannot be confirmed because the -CH3 proton is generally located between δ = 1 - 2 ppm. The δ = 3 - 4 ppm region typically indicates the presence of -OH, but the peak intensity that appears for -OH is not sharp. In accordance with Pavia et al. (1979) literature on chemical shifts, no chemical shift shows atoms or groups bound to methyl located at δ = 4.7269 ppm [9].

^{1}H-NMR data of isolated compounds support that the isolated compounds are flavonoid compounds of the isoflavone group. This is evidenced that in the ^{1}H-NMR data, there is a single peak in the region of 7.1991 ppm for protons at C-2, and this chemical shift region is characteristic of isoflavone compounds, namely in the region around 7.0-8.0 ppm for protons at C-2 [2]. Based on data analysis and interpretation on UV-Vis, FT-IR, and ^{1}H-NMR spectra, it can be concluded or suspected that the isolated compounds from buni plant leaves are flavonoid compounds of the isoflavone group [10]. However, the author recognizes that the ^{1}H-NMR data is less pure because of the mixture of isolated compounds, and this mixture can be further seen using HPLC. The following is the structure of the isolated compound:

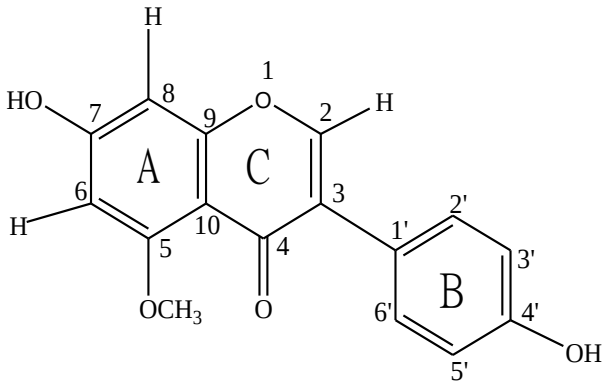


Figure 4. Compound structure of isoflavone isolated results

4 Conclusion

The isolation results obtained from 1200 g of buni plant leaves (A.bunius (L) Spreng.) is a brownish yellow paste, obtained as much as 30 mg, Rf = 0.31 with eluent n-hexan: ethyl acetate 70:30 (v/v). Based on the flavonoid phytochemical screening of pasta, isolation results from the leaves of buni plants (*A. bunius (L) Spreng*.) showed positive results for flavonoid compounds. Based on the number of isolation compounds obtained, namely 30 mg, it can be obtained that the percentage of flavonoid compounds contained in 1200 g of buni plant leaves (*A. bunius (L) Spreng*) is 0.025%. The results of analysis by UV-Vis, FT-IR, and ^{1}H-NMR spectrophotometry showed that the isolated compounds from the leaves of buni plants (*A. bunius (L) Spreng.*) are thought to be flavonoid compounds of the isoflavone group.

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6. Conflict of Interest

Authors declare no conflicts of interest

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