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Isolation, Identification, and Antioxidant Activity of Flavonoid Compounds in the Ethanol Extract in Bandotan Leaves (*Ageratum conyzoides*)

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Abstract

Isolation of flavonoid compounds from the ethanol extract of Bandotan leaves (*Ageratum conyzoides* L.) has been successfully conducted. The structure of flavonoid isolates was identified using UV-Vis spectrophotometry with the addition of shear reagents and FTIR. In addition, the antioxidant activity of the isolates was determined and studied using the DPPH (2,2-Diphenyl-1-picrylhydrazyl) method. This study aimed to isolate, identify, and test the antioxidant activity of flavonoid compounds from Bandotan leaves. An antioxidant activity test was carried out on ethanol extract, and flavonoid isolates using the DPPH method. Based on this research, Bandotan leaf ethanol extract yielded 13.964%. The results of the phytochemical screening test revealed that the leaf powder and ethanol extract of Bandotan leaves contained alkaloids, flavonoids, saponins, tannins, quinones, steroids, and triterpenoids. The total flavonoid content of the ethanol extract of Bandotan leaves was 129.27 mg EQ/g extract. Identification of flavonoid isolates using a UV-Vis spectrophotometer showed that flavonoid isolates belong to the flavanone compound class with maximum absorption at a wavelength of 315 nm (band I) and 280 nm (band II). FTIR analysis showed that the flavonoid isolates had functional groups O-H, C-H aromatics, C-H alkanes, C=O, C=C aromatics, C-O ethers, C-O alcohols, and substituted aromatic rings. Identification of the structure of flavonoid isolates with a spectrophotometer with the addition of shear reagent and FTIR, presumably that the isolate was a 4'-hydroxy flavanone compound. The antioxidant activity of ethanol extract showed moderate antioxidant activity (IC_{50} 118.19 µg/mL) and was classified as very weak (IC_{50} 1185.5 µg/mL).

1. Introduction

Free radicals are molecules containing unpaired, unstable, and highly reactive electrons. Free radicals are produced in the human body from the body's metabolic processes. However, external factors such as exposure to x-rays, ozone, cigarette smoke, air pollutants, and industrial chemicals are also sources of free radicals [1]. Excessive free radical reactivity in the human body will cause various disorders that can trigger the emergence of various diseases. The body naturally creates antioxidant molecules to lessen the activity of free radicals; however, the amount is insufficient to compete with the free radicals produced daily. Therefore, it takes the intake of antioxidants from outside the body. Many natural ingredients can be a source of natural antioxidants.

Bandotan is a wild-growing plant in Indonesia that has not yet gained widespread recognition as a medicinal plant. Only a few studies examine this plant, so its benefits are underutilized. Studies on the phytochemical investigations of Bandotan extract show high concentrations of phenolic and flavonoid compounds that can protect against disorders associated with excess free radicals or reactive oxygen species [2]. Based on a

review by Janarthanan *et al.* [3], Bandotan leaves are traditionally used as a wound healer, anti-inflammatory, analgesic, antipyretic, antispasmodic, gastroprotective, antimicrobial, antidiabetic, anticancer, antiulcer, antioxidant, hematopoietic, larvicidal, mosquito repellent, insecticide, and anthelmintic.

Phytochemical analysis of Bandotan leaf extract showed the presence of alkaloids, flavonoids, tannins, saponins, reducing sugars, steroids, phenols, cardiac glycosides, and anthraquinones [4]. Yusnawan and Inayati [5] reported that the flavonoids in leaf and flower extracts were four times higher than in stem and root extracts. Hassan *et al.* [6] stated that the total flavonoids in the ethanol extract of Bandotan leaves were 218.88 mg EQ/g extract. The ethanol extract showed a higher concentration of flavonoids than the n-hexane and ethyl acetate extracts. The ethanol extract of the leaves had the highest DPPH radical scavenging capacity with an IC_{50} of 9.18 to 15.88 µg/mL [7].

Research conducted by Hossain *et al.* [8] measured the antioxidant activity of the ethanol extract of Bandotan leaves from the Northeastern region of Bangladesh, which was obtained by maceration using 80% ethanol, showing a reasonably high DPPH radical scavenging capacity, resulting in an IC_{50} value of 18.91 µg/mL compared to standard ascorbic acid (IC_{50} 2.937 µg/mL) and butylated hydroxyanisole (BHA) (IC_{50} 5.10 µg/mL). In this study, the antioxidant activity test will be carried out on the ethanol extract of Bandotan leaves obtained by maceration using 96% ethanol. Research by Maulana *et al.* [9] showed that an increase in ethanol concentration was related to the amount of extracted flavonoid content, as evidenced by the total flavonoid content in 96% ethanol which was more than other solvents (90% ethanol, 80% ethanol).

In nature, most flavonoid compounds are known as glycosides, and relatively few are in the form of aglycones [9]. Munikishore *et al.* [10] reported that the ethanol extract of the Bandotan plant contains flavonoid glycoside compounds, namely 5,2'-dihydroxy-7-methoxy flavone 2'-O-β-D-glucopyranoside, and kaempferol-3-O-α-L-rhamnopyranoside.

Based on this description, a study was conducted on the content of secondary metabolites in Bandotan leaves, followed by the isolation of flavonoid compounds in the ethanol extract of Bandotan leaves accompanied by an acid hydrolysis process to obtain flavonoid aglycones and identification of flavonoid isolates using UV-Vis spectrophotometry with the addition of shear reagents, and FTIR. The antioxidant activities of the ethanolic extract and flavonoid isolate from Bandotan leaves (*Ageratum conyzoides* L.) were investigated using the DPPH method.

2. Methods

2.1. Materials

Bandotan leaves, 96% ethanol, distilled water, technical hexane (Brataco), technical ethyl acetate (Brataco), HCl 2 N, Dragendorff reagent, Mayer reagent, 25% ammonia (Merck), Mg powder, concentrated HCl, 1% $FeCl_3$, 1 N NaOH, acetic acid anhydride, concentrated H_2SO_4, $AlCl_3$ 10%, CH_3COOK 1 M, NaOH 2 M, $AlCl_3$ 5%, NaOAc, H_3BO_3, 1,1-diphenyl-2 -picrylhydrazyl (DPPH) (Sigma-Aldrich), quercetin standard (Aldrich). Amyl alcohol (Merck), methanol (Merck), ethanol (Merck), 1-butanol (Merck), glacial acetic acid (Merck), ethyl acetate (Merck), chloroform (Merck), n-hexane (Merck) were analytical grade.

2.2. Extraction of Flavonoid Compounds

Bandotan leaves were obtained from the corn garden in the Mertoyudan, Magelang. Bandotan leaves weighing 5,500 g are washed, air-dried, and then crushed into powder. Bandotan leaf powder (1000 g) was macerated using 96% ethanol, changing the solvent daily until the color became clear. The obtained ethanol extract was concentrated using a rotary evaporator to obtain a viscous extract and then weighed.

2.3. Phytochemical Screening

2.3.1. Test for Alkaloid

2.5 g of Bandotan leaf powder was added to 1 mL of 2 N HCl and 9 mL of distilled water and then heated and filtered. The resulting filtrate was divided in half and subjected to tests using the Mayer and Dragendorff reagents. The filtrate dropped by Dragendroff's reagent formed an orange color as a positive test. On the other hand, Mayer's reagent caused the formation of a white mist until a white precipitate in the filtrate indicated the presence of alkaloids [11]. The same procedure was also conducted on the ethanol extract.

2.3.2. Test for Flavonoid

Bandotan leaf powder (2.5 g) was boiled with 100 mL of distilled water and then filtered in a hot condition. The filtrate was added with Mg powder, 1 mL of concentrated HCl, and 2 mL of amyl alcohol. The mixture was shaken gently and allowed to form an amyl alcohol layer. A positive result was indicated by a red, yellow, or orange precipitate on the amyl alcohol layer [12]. The ethanol extract was also treated in the same procedure as the filtrate.

2.3.3. Test for Saponin

The hot filtrate was filtered by heating Bandotan leaf powder in distilled water is shaken vigorously. Positive results were indicated by the appearance of foam and remained stable with the addition of 1% HCl [11]. The same conducted was conducted on the ethanol extract.

2.3.4. Test for Tannin

The results of heating the Bandotan leaf powder in distilled water were left to cool and filtered. Subsequently, the filtrate was added with 1% $FeCl_3$. A positive test produced a dark green solution. The ethanol extract was subjected to the same procedure.

2.3.5. Test for Quinone

Bandotan leaf powder was boiled in distilled water for 5 minutes, allowed to stand, and filtered. The filtrate

was then added with 1 N NaOH. A positive test produced a red color solution. The same procedure was performed on the ethanol extract.

2.3.6. Test for Steroid/Triterpenoid

Bandotan leaf powder (1 g) was macerated in ether for 2 hours, then filtered. The filtrate obtained was placed in an evaporating cup and then evaporated to dryness. The residue on the evaporating cup was added two drops of acetic acid anhydride and one drop of H_2SO_4. The formation of a blue or purple color indicated the presence of steroids. Meanwhile, the presence of triterpenoids was shown by a red color [13]. The same treatment was carried out on the ethanol extract.

2.4. Determination of Total Flavonoid Content in Ethanol Extract

The aluminum chloride colorimetric method was employed to determine the total flavonoid concentration of the ethanol extract [14]. A 0.2 mL stock solution was added to 10 mL of methanol to obtain a concentration of 200 ppm. A 1 mL of 200 ppm extract solution was added with 3 mL methanol, 0.2 mL 10% $AlCl_3$, 0.2 mL CH_3COOK 1 M, and distilled water to a total volume of 10 mL. The mixture was left for 30 minutes at room temperature, and the absorbance was measured at 430 nm using a UV-Vis spectrophotometer (GENESYS). The total flavonoid content analysis was determined as mg quercetin equivalents per gram using the standard quercetin calibration curve equation.

2.5. Isolation of Flavonoid Compounds

The concentrated ethanol extract was dissolved in ethanol, added with distilled water (1:1), soaked for 24 hours, and filtered to remove chlorophyll. The filtrate obtained was concentrated and weighed. The condensed extract was then redissolved in ethanol and fractionated using n-hexane with a separatory funnel to form n-hexane and ethanol fractions. The ethanol fraction was then concentrated and weighed. The ethanol fraction was hydrolyzed using 2 N HCl (1:1) for 2 hours at 65°C. The hydrolysis results were fractionated using ethyl acetate so that two layers were formed: the upper layer was the ethyl acetate fraction, and the bottom layer was the ethanol fraction. The ethyl acetate fraction was evaporated to obtain a viscous ethyl acetate fraction which was then weighed.

2.6. Separation of Flavonoid Compounds

The ethyl acetate viscous fraction was dissolved in methanol and analyzed using the thin layer chromatography (TLC) method with the stationary phase of silica gel 60 F254, which was previously activated at 100°C for 10 minutes to remove water present on the plate. The mobile phases used were various single eluents (methanol, ethanol, n-butanol, acetic acid, ethyl acetate, chloroform, and n-hexane) and mixed eluents to obtain the best eluents. After obtaining the best eluent composition, then the components of the compound were separated using gravity column chromatography.

Silica gel 60% of the stationary phase (0.2-0.5 mm) was employed for separation by gravity column chromatography. The mobile phase was the best eluent. The fraction of the separation results were accommodated in vials every 15 mL. Each fraction was analyzed using TLC and observed under a 254 and 366 nm UV lamp. Fractions with the same stain are combined into large fractions. The large fraction obtained was tested for flavonoids by the TLC method, sprayed with 5% $AlCl_3$ spotting, and Rf stains obtained from flavonoid compounds.

Separation was continued by the preparative TLC method using the mobile phase, the best eluent for separating the positive fraction of flavonoids, and using silica gel 60F254 stationary phase. TLC stains were observed in 254 nm and 366 nm UV lamps. The flavonoid compound's positive stain was scraped, dissolved in methanol, and filtered. The filtrate was then dried, and flavonoid isolates were obtained.

2.7. Test for the purity of flavonoid isolates

Flavonoid isolates were tested for purity by TLC methods with various eluents and two-dimensional TLC. The single stain formed indicated that the flavonoids obtained were pure.

2.8. Identification of Flavonoid Compounds

Identification of flavonoid isolates to determine the type of flavonoid was conducted by measuring the wavelength of the flavonoid isolates in methanol using the UV-Vis spectrophotometer method while determining the location of the hydroxy groups in the flavonoid structure was carried out by observing the shift in their absorption peaks when adding shear reagents, including 2 M NaOH, $AlCl_3$ 5 %, HCl, NaOAc, and H_3BO_3 [12] as well as identification using FTIR (PerkinElmer).

2.9. Antioxidant Activity Test using DPPH Method

2.9.1. Antioxidant Activity Qualitative Test

Flavonoid isolates were spotted on the TLC plate, eluted using the best eluent, dried, and sprayed using 0.1 mM DPPH solution. A positive test resulted in a color change of the stain from purple to yellow.

2.9.2. Antioxidant Activity Quantitative Test

The 0.1 mM DPPH solution was prepared by dissolving 1.97 mg of DPPH in 50 mL of methanol. The maximum wavelength was determined with a control solution prepared by adding 2 mL of 0.1 mM DPPH solution to 2 mL of methanol. The absorbance was measured using a UV-Vis spectrophotometer with a 400-600 nm range.

The ethanol extract was made in various concentrations of 5, 10, 20, 40, 80, and 100 ppm. Flavonoid isolates were made with various concentrations of 17, 40, 100, 136, 150, and 170 ppm in methanol. Meanwhile, the quercetin standard was made with a concentration variation of 0.5, 1, 1.5, 2, 2.5, and 3 ppm in methanol. Antioxidant activity was tested by adding 2 mL of 0.1 mM DPPH solution to 2 mL of samples (ethanol extract, flavonoid isolates, and quercetin

standard) at various concentrations. The mixture was homogenized and incubated for 30 minutes in the dark. Absorbance was measured using a UV-Vis spectrophotometer at the maximum wavelength of the measurement results in the control solution.

The amount of antioxidant activity was determined by the IC_{50} value, calculated from the percentage of absorption inhibition of various sample concentrations using the Equation obtained from the linear regression curve. The ability to scavenge DPPH radicals (inhibition) was calculated using Equation (1).

$$\%\ Inhibition = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100\% \quad (1)$$

The calculation of IC_{50}, which is the sample concentration to reduce 50% of DPPH radical activity, is obtained from the line intersection between 50% inhibition and the sample concentration [15].

3. Results and Discussion

3.1. Sample Preparation and Extraction of Active Compounds in Bandotan Leaves

Bandotan leaves that were washed were dried by aerating to avoid damage to the active substance by heat and reduce the water content to prevent decay by fungi. Dried Bandotan leaves were ground into powder to increase the surface area to maximize the contact process between the sample and the solvent during extraction. The drying and grinding process produced 1,075 g of Bandotan leaves.

Bandotan leaf powder was macerated with 96% ethanol, and the solvent was changed every 24 hours. Ethanol 96% was employed because it is selective, antifungal, non-toxic, has good absorption, and requires low heat for concentration, thereby minimizing the risk of degradation of active compounds due to heating [16]. The solvent replacement was carried out until the solvent turned clear, indicating no more extraction results were obtained. This process aimed to maximize the number of active chemicals extracted from Bandotan leaves into the solvent.

The maceration filtrate was concentrated using a rotary evaporator at 60-65°C to evaporate the solvent to obtain a thick extract. This process was stopped when the solvent was no longer dripping on the round bottom flask. Concentration using a rotary evaporator produced a reusable solvent and obtained a viscous green-black ethanol extract of 139.64 g, yielding 13.964%.

3.2. Phytochemical Screening

Phytochemical screening was performed on the powder and ethanol extract of Bandotan leaves. This is the first step in research regarding qualitatively identifying the active compound content in Bandotan leaves. Phytochemical screening tests included testing for flavonoids, saponins, alkaloids, quinones, tannins, triterpenoids, and steroids [11, 13]. The results of the phytochemical screening test for powder and ethanol extract of Bandotan leaves can be seen in Table 2.

Table 2 indicates that secondary metabolites such as alkaloids, flavonoids, saponins, tannins, quinones, steroids, and triterpenoids are present in Bandotan leaves. These results are consistent with the results of the phytochemical screening of Osuntokun *et al.* [4], who stated that *A. Conyzoides* leaves contained alkaloids, flavonoids, tannins, saponins, steroids, phenols, and anthraquinones.

Table 2. Results of phytochemical screening test of powder and ethanol extract of Bandotan leaves

Test	Leaf powder	Ethanol extract
Alkaloids	+	+
Flavonoids	+	+
Saponins	+	+
tannins	+	+
Quinone	+	+
Steroids	+	+
Triterpenoids	+	+

3.3. Determination of Total Flavonoid Content in Ethanol Extract

The total flavonoid content was determined using a colorimetric method with $AlCl_3$ reagent with the principle that $AlCl_3$ would form a complex with the C-4 keto group and then with the neighboring C-3 or C-5 hydroxyl groups. In addition, $AlCl_3$ also forms acid complexes with ortho-dihydroxy groups on rings A or B of flavonoids, resulting in a shift in wavelength towards visible, as seen from the yellow color in the solution [14]. The reaction between flavonoids and the $AlCl_3$ reagent can be seen in Figure 1.

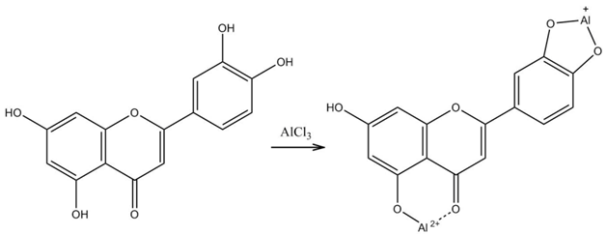

Figure 1. The reaction of flavonoid with $AlCl_3$ reagent

Flavonoids are one of the secondary metabolites produced by plants included in the large group of polyphenols [12], which can scavenge free radicals. This antioxidant activity can be proven due to a relatively large amount of flavonoids in the ethanol extract of Bandotan leaves, equal to 129.27 mg EQ/g extract.

Table 3. The results of measuring the total flavonoid content in the ethanol extract of Bandotan leaves

Parameter identity	Measurement results
Absorbance	0.2013
Concentration (ppm)	2.5854
Dilution factor	500
Flavonoid concentration	1292.7
Volume(L)	0.01
Extract weight (grams)	0.1
Total flavonoids*	129.27

*mg quercetin equivalent per gram of extract

3.4. Isolation of Flavonoid Compounds from Bandotan Leaves

Dissolving the viscous ethanol extract in ethanol and distilled water (1:1) aimed to bind and separate the chlorophyll present in the sample. This process produced a brown aqueous ethanol extract of 60.6 g (49.43%). The water-ethanol extract was extracted with n-hexane to remove nonpolar compounds such as carotenoids, fats, and waxes from the sample to obtain a polar layer and concentrated into 56.75 g of ethanol extract (93.65%).

As plant flavonoids are usually attached to sugars, acid hydrolysis is required to break the glycoside bonds in the flavonoid structure to release free flavonoids (aglycones). Extraction of the flavonoid aglycones from the mixture was carried out by liquid-liquid extraction using ethyl acetate solvent in order to separate the more polar glycosides and the semipolar flavonoid aglycones. From the results of the liquid-liquid extraction, a lower layer (water fraction) was formed, possibly containing glycosides. In contrast, the upper layer (ethyl acetate fraction), which contained aglycone flavonoids, was separated. The ethyl acetate fraction was concentrated again to obtain an ethyl acetate fraction of 5.87 grams (10.34%). Further compound separation could be carried out.

3.5. Separation of Compounds in the Ethyl Acetate Fraction of Bandotan Leaves

3.5.1. Separation of Compounds in the Ethyl Fraction by Thin Layer Chromatography (TLC)

Separating compounds in the ethyl acetate fraction using the TLC method obtained the best eluent in a mixture of chloroform: ethyl acetate: methanol (9:1:1) which gave a reasonably good separation. The results of the TLC were sprayed using a flavonoid compound spot marker, namely $AlCl_3$ 5%, which can be seen in Figure 2.

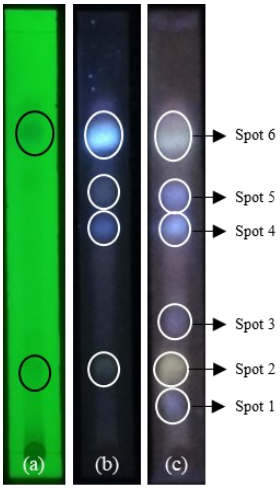

Figure 2. TLC results of the ethyl acetate fraction with chloroform: ethyl acetate: methanol (9:1:1) as the eluent (a) UV 254 nm, (b) UV 366 nm, (c) sprayed with 5% $AlCl_3$

The TLC results after spraying with 5% $AlCl_3$ showed a total of 6 spots, namely spot 1 (Rf = 0.13), spot 2 (Rf = 0.22), spot 3 (Rf = 0.31), spot 4 (Rf = 0.55), spot 5 (Rf = 0.61), and spot 6 (Rf = 0.75). Spraying with 5% AlCl3 resulted in the appearance of positive flavonoid stains at spots 2 (from dim yellow to clear yellow) and 6 (from blue to yellow). Thus the eluent chloroform: ethyl acetate: methanol (9:1:1) was used to separate flavonoid compounds by gravity column chromatography.

3.5.2. Separation of Compounds in the Ethyl Acetate Fraction by Gravity Column Chromatography

After obtaining the best eluent, chloroform: ethyl acetate: methanol (9:1:1), the components were separated in the ethyl acetate fraction using gravity column chromatography. A total of 3 g of the ethyl acetate fraction was carried out by a separation process using 120 g of silica gel 60 (0.2-0.5 mm) stationary phase using a column with a length of 50 cm and a diameter of 3 cm and using an eluent of chloroform: ethyl acetate: methanol (9:1:1).

Gravity column chromatography resulted in 102 vials (15 mL per vial). The results of gravity column chromatography were monitored using the TLC method using chloroform: ethyl acetate: methanol (9:1:1) as the eluent so that the spot pattern for each fraction was obtained according to Figure 3.

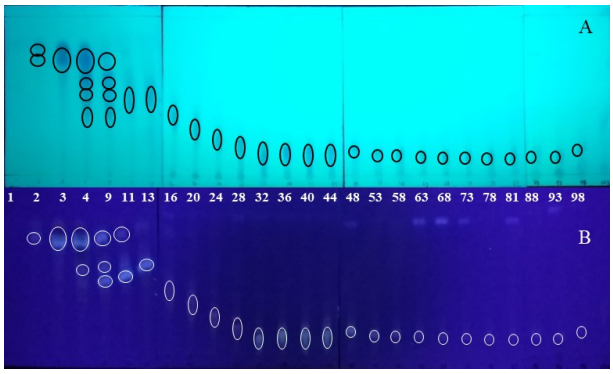

Figure 3. The spot pattern of each fraction of the column results with chloroform: ethyl acetate: methanol (9:1:1) as eluent was seen under UV light at (A) 254 nm and (B) 366 nm

The results of the TLC combined fractions based on the same spot pattern to produce six large fraction groups, namely fraction A (1-3), fraction B (4-8), fraction C (9-15), fraction D (16-28), fraction E (29-46), and fraction F(47-102). Gravity column chromatography still causes spots after separation; therefore, more research into the optimal eluent is required for additional separation. Separating the six fractions using the TLC method obtained the best eluent for each fraction.

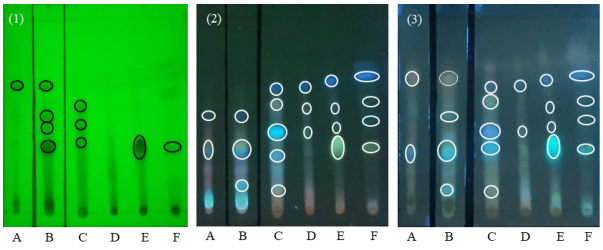

Figure 4. TLC results of fractions A and B with hexane: ethyl acetate (7:3) eluent and C, D, E, and F fractions with n-hexane: ethyl acetate (4:6) eluent as seen below

(1) UV 254 nm, (2) UV 366 nm, and (3) sprayed with 5% $AlCl_3$

The search results for the best eluent showed that fractions A and B could be separated using n-hexane: ethyl acetate (7:3) eluent, while fractions C, D, E, and F could be separated quite well using n-hexane: ethyl acetate (4:6) (Figure 4). Based on the flavonoid test by spraying with 5% $AlCl_3$, a positive test for flavonoids was shown in fraction C, namely in the spot with Rf = 0.44, which produced a color change from bright blue to purplish-blue after being sprayed with 5% $AlCl_3$ which was observed under UV light at 366 nm.

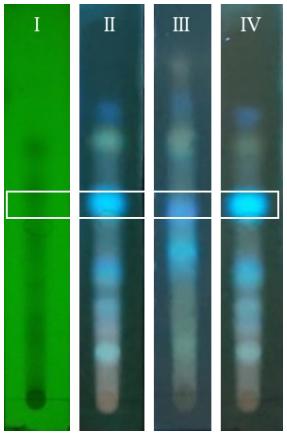

Figure 5. TLC results of fraction C with n-hexane: ethyl acetate (4:6) as eluent (I) UV 254 nm, (II) UV 366 nm, (III) sprayed with $AlCl_3$, and (IV) steamed with NH_3 with Rf = 0.44 for all samples

Based on Figure 5, the flavonoid test using 5% $AlCl_3$ spotting produced a positive test on the spot with Rf = 0.44. A further test was carried out for the flavonoid with NH_3 steam, which changed the spot's color from light blue to greenish blue, indicating a positive flavonoid stain (Rf = 0.44) from the flavanone group, which did not contain 5-OH [12].

3.5.3. Separation of Fraction C by Preparative Thin Layer Chromatography

Separation using the gravity column chromatography method produced a positive fraction of flavonoids, fraction C. Five spots were separated by TLC using n-hexane: ethyl acetate (4:6) as eluent. The positive flavonoid spot (blue color) was separated using the preparative TLC method with the same eluent, namely n-hexane: ethyl acetate (4:6), and using a silica gel 60 F254 TLC glass plate as the stationary phase.

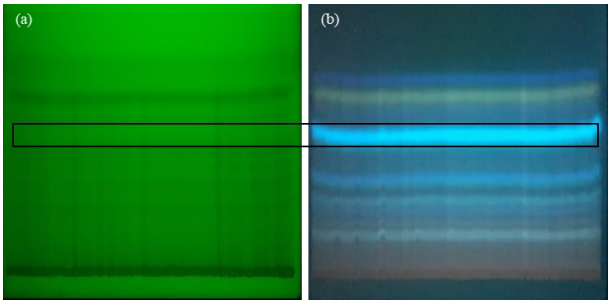

Figure 6. The results of preparative TLC for fraction C with n-hexane: ethyl acetate (4:6) as eluent were observed under UV light (a) 254 nm and (b) 366 nm with Rf = 0.48

The positive band of flavonoids (Rf = 0.48) adsorbed on the silica gel was then peeled off and dissolved in methanol so that the compound may be separated from the silica gel by filtration. The filtered filtrate was then dried and produced a residue which was a flavonoid isolate in the form of a yellowish-white solid. The flavonoid isolates obtained can then be tested for purity.

3.6. Purity Test for flavonoid isolate

The flavonoid isolates obtained were tested for purity using the TLC method employing various single or mixed eluents. The eluent used for the purity test has different levels of polarity, from polar to nonpolar. The single eluents used were methanol, n-butanol, ethyl acetate, chloroform, and n-hexane. Toluene: ethyl acetate: acetic acid (6:3:1) was used for mixed eluents. The results of the purity test of flavonoid isolates can be seen in Figure 7.

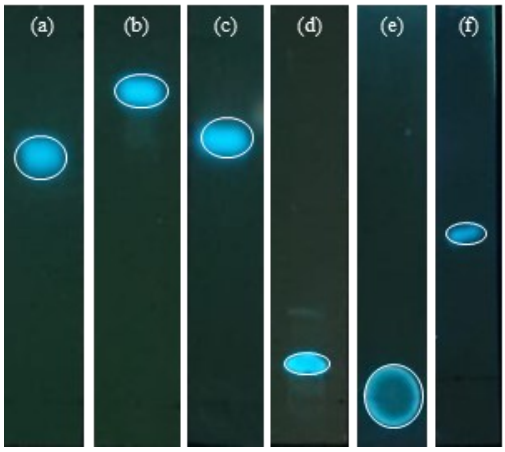

Figure 7. TLC results of purity test of flavonoid isolates with eluents (a) methanol, (b) n-butanol, (c) ethyl acetate, (d) chloroform, (e) n-hexane, and (f) a mixture of toluene: ethyl acetate: acetic acid (6:3:1) observed under UV light at 366 nm

The purity test results showed that the flavonoid isolates were pure because there was only one spot. The purity test was then carried out by two-dimensional TLC using two mixed eluent systems. The mixed eluent used for the first elution (E1) was chloroform: methanol (3:1), whereas the mixed eluent for the second elution (E2) was n-hexane: ethyl acetate (2:8). The results of a two-dimensional TLC can be seen in Figure 8.

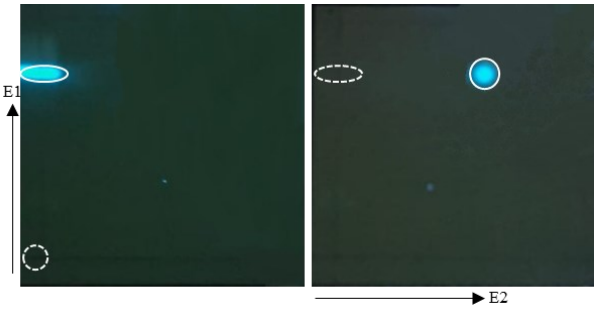

Figure 8. Two-dimensional TLC results of flavonoid isolates were observed under UV light at 366 nm

The first elution using chloroform: methanol (3:1) produced one spot with Rf = 0.78. The second elution using n-hexane: ethyl acetate (2:8) also produced one spot with Rf= 0.62. Figure 8 showed one spot so that the flavonoid isolates obtained were pure. The next step was to identify the structure of the flavonoid isolates.

3.7. Identification of Flavonoid Compounds

3.7.1. Identification of Flavonoid Isolates Using a UV-Vis Spectrophotometer

The flavonoid isolates obtained were identified using a UV-Vis spectrophotometer by dissolving them in methanol and then measuring them at a wavelength range of 200–400 nm so that the spectrum of flavonoid isolates in methanol was obtained according to Figure 9.

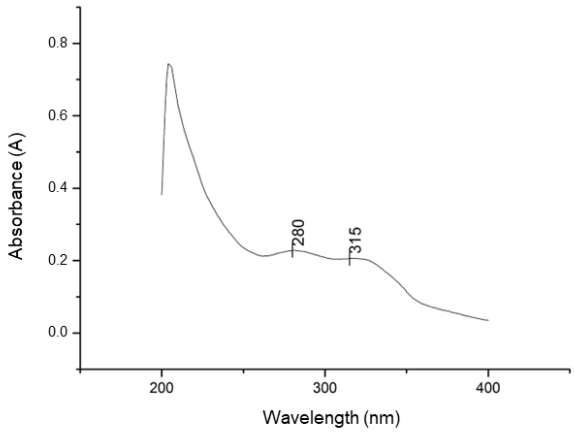

Figure 9. UV-Vis spectrum of flavonoid isolates in methanol

Based on Figure 9 shows that there are two absorption wavelengths from band I (315 nm) and band II (280 nm). According to Markham [12], the flavonoid isolate belongs to the flavanone group based on its maximum absorption range of band I between 300-330 nm and band II between 275-295 nm.

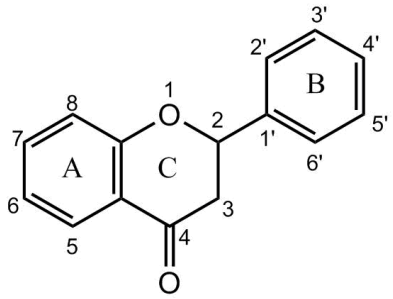

Figure 10. Flavanone structure

The oxygenation pattern of the flavonoid core can be determined by adding a shear reagent to the test solution so that a shift in the absorption peak is produced. The shear reagents used in this study were 2 N NaOH, 5% $AlCl_3$, HCl, NaOAc, and H_3BO_3. According to Markham [12], the addition of NaOH is intended to detect hydroxyl groups that are acidic and unsubstituted. The results of the analysis of flavonoid isolates using a UV-Vis spectrophotometer with the addition of NaOH shear reagent are shown in Figure 11 and Table 4.

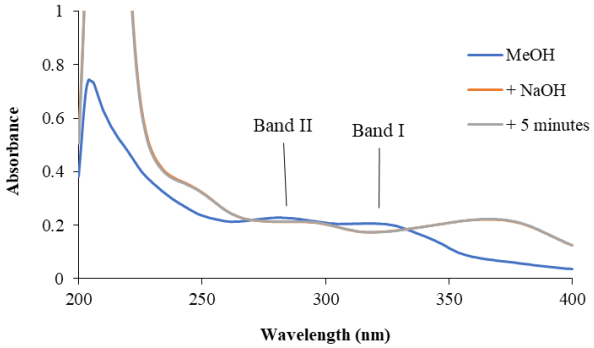

Figure 11. UV-Vis spectrum of flavonoid isolates with the addition of NaOH shear reagent

Table 4. Absorption and absorbance peaks of flavonoid isolates with the addition of NaOH shear reagent

	Wavelength (nm) (Absorbance (A))		
	Methanol	+NaOH	+5 minutes
Ribbon I	315 (0.206)	368 (0.221)	368 (0.223)
Ribbon II	280 (0.228)	291 (0.212)	291 (0.214)

The change in the absorption peak of the first flavonoid isolate was caused by adding 2 N NaOH, causing a shift in the absorption peak of band I by 53 nm and band II by 11 nm. This indicates that there is oxygenation in ring B at position 4'. According to Markham [12], changes in ring A tend to be reflected in absorption band II, whereas changes in displacement in rings B and C tend to be more clearly reflected in band I absorption. After allowing it to stand for 5 minutes, there was no shift in the absorption peaks in band I and band II. This shows that no decomposition occurred [12].

The addition of sodium acetate was used primarily to detect the presence of free 7-hydroxyl groups or equivalents because the addition of sodium acetate causes ionization of the hydroxyl groups of the most acidic flavonoids. While the addition of boric acid to detect the presence of o-dihydroxy groups with the formation of boric acid complexes with flavonoids [12]. The results of the analysis of flavonoid isolates using a UV-Vis spectrophotometer with the addition of NaOAc and H_3BO_3 are shown in Figure 12 and Table 5.

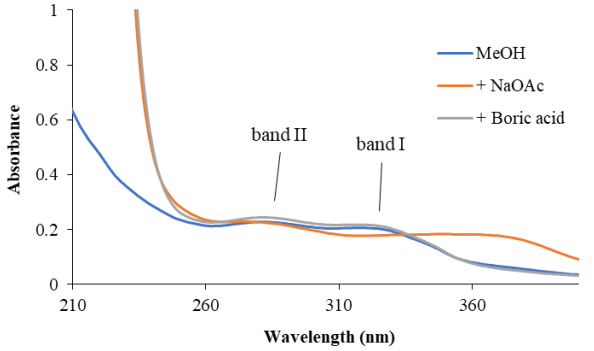

Figure 12. UV-Vis spectrum of flavonoid isolates with the addition of NaOAc and H_3BO_3 shear reagents

Table 5. Absorption and absorbance peaks of flavonoid isolates with the addition of NaOAc and H_3BO_3 shear reagents

	Absorption peak (Absorbance)		
	Methanol	+NaOAc	+ H_3BO_3
Ribbon I	315 nm (0.206 A)	351 nm (0.182 A)	319 nm (0.218 A)
Ribbon II	280 nm (0.228 A)	275 nm (0.228 A)	282 nm (0.245 A)

The addition of NaOAc causes a peak shift in the absorption band I of 36 nm and a hypochromic shift in band II of 5 nm. This indicates the presence of a hydroxy group in ring B at position 4' [12]. H_3BO_3 did not cause a significant peak shift, indicating the absence of ortho-dihydroxy in the flavanoid core.

$AlCl_3$ and HCl were used to detect the presence of neighboring hydroxy and ketone groups and ortho-dihydroxy groups [12]. The results of the analysis of flavonoid isolates using a UV-Vis spectrophotometer with the addition of $AlCl_3$ and HCl shear reagents can be seen in Figure 13 and Table 6.

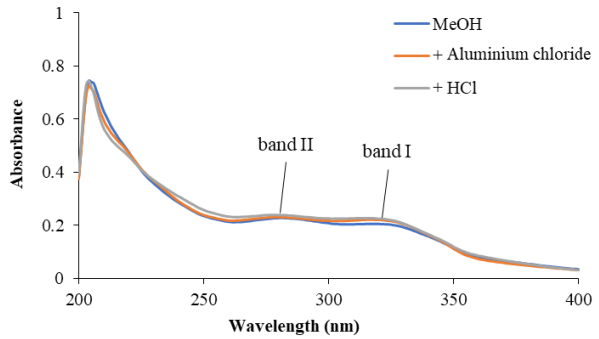

Figure 13. UV-Vis spectrum of flavonoid isolates with the addition of $AlCl_3$ and HCl shear reagents

Table 6. Absorption and absorbance peaks of flavonoid isolates with the addition of $AlCl_3$ and HCl shear reagents

	Absorption peak (nm) (Absorbance (A))		
	Methanol	+$AlCl_3$	+HCl
Ribbon I	315(0.206)	317 (0.220)	317 (0.226)
Ribbon II	280 (0.228)	280 (0.230)	280 (0.238)

There is no hydroxy group at position 5 and no ortho-dihydroxy group, as evidenced by the lack of a significant change in the absorption peaks in bands I and II with the addition of $AlCl_3$ and HCl. The results of the absorption peak shift due to the addition of shear reagents can be seen in Table 7.

Based on the identification using a UV-Vis spectrophotometer with the addition of the shear reagent, it can be concluded that the flavonoid isolates are thought to belong to the flavanone group with oxygenation in ring B at position 4, or 4'-hydroxy flavanone. The structure can be seen in Figure 14.

Table 7. Changes in the absorption peaks of flavonoid isolates with the addition of shear reagents

Shear reagent	Wavelength (nm)		Wavelength Shift (nm)		Interpretation
	Ribbon II	Ribbon I	Ribbon II	Ribbon I	
MeOH	280	315	-	-	Flavanone
MeOH + NaOH	291	368	11	53	oxygenation of ring B
MeOH+ NaOH+ 5 minutes	291	368	11	53	oxygenation of ring B
MeOH+NaOAc	275	351	-5	36	4'-OH
MeOH+ NaOAc+ H_3BO_3	282	319	2	4	without o-diOH
MeOH+ $AlCl_3$	280	317	0	2	without 5-OH
MeOH+ $AlCl_3$+ HCl	280	317	0	2	without o-diOH ring B

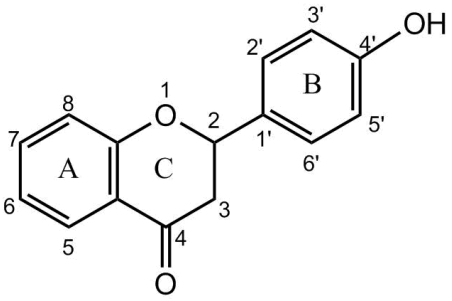

Figure 14. The suspected structure of the flavonoid isolate as 4'-hydroxy flavanone

3.7.2. Identification Using FTIR

The identification stage using FTIR was used to strengthen the suspected results of identification using a UV-Vis spectrophotometer, which from this identification, showed functional groups in the structure of flavonoids. Identification results are shown in Figure 15.

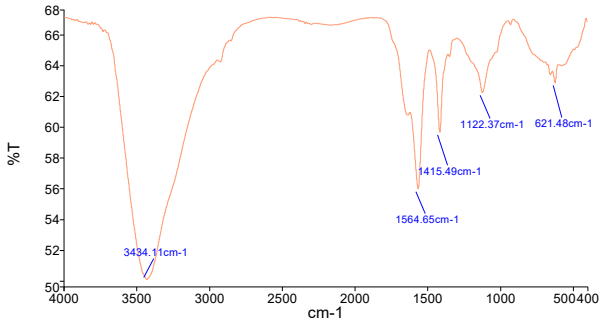

Figure 15. FTIR spectrum of flavonoid isolates

Figure 15 shows absorption at 3434.11 cm^{-1}, indicating the presence of vibrations from the OH group. The bands at 1564.65 and 1415.49 cm^{-1} indicate an aromatic C=C group. Meanwhile, the absorption at 1122.37 cm^{-1} is associated with alcohol CO vibrations. The absorption of the substituted aromatic ring appears at 621.48 cm^{-1}. The absorption band at 3100–3000 cm^{-1} is the area for aromatic CH vibrations. However, in the IR spectrum of the flavonoid isolates, absorption peaks did not appear in that area, so an IR spectrum deconvolution

was performed to determine the absorption that might have accumulated. Deconvolution was carried out at wave numbers 3150–2800 cm^{-1}, the results can be seen in Figure 16.

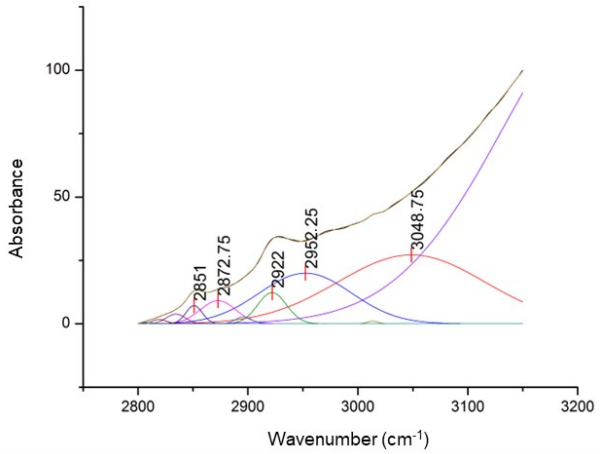

Figure 16. The deconvolution results of the IR spectrum at 3150–2800 cm^{-1} region

The deconvolution result shows aromatic CH absorption at 3048.75 cm^{-1}. Absorptions at 2952.25, 2922, 2872.75, and 2851 cm^{-1} indicate the presence of a CH alkane group. It is necessary to deconvolute again in the 1800-1550 cm^{-1} region to determine the presence of the C=O group. The deconvolution results can be seen in Figure 17.

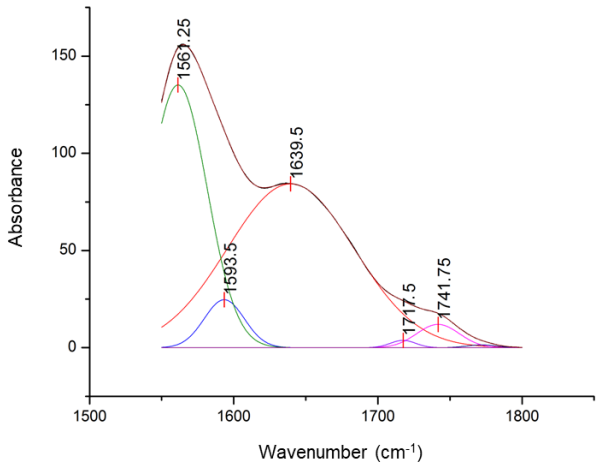

Figure 17. The deconvolution results of the IR spectrum at the 1550–1800 cm^{-1} region

Figure 17 shows the absorption of C=O vibrations at 1741.75 cm^{-1}. The absorptions peak at 1639.5, 1593.5, and 1561.25 cm^{-1} indicate the presence of C=C vibrations. Deconvolution was again carried out in the area of 1275-1020 cm^{-1} to determine the presence of vibrational absorption of the CO ether group. The deconvolution results can be seen in Figure 18.

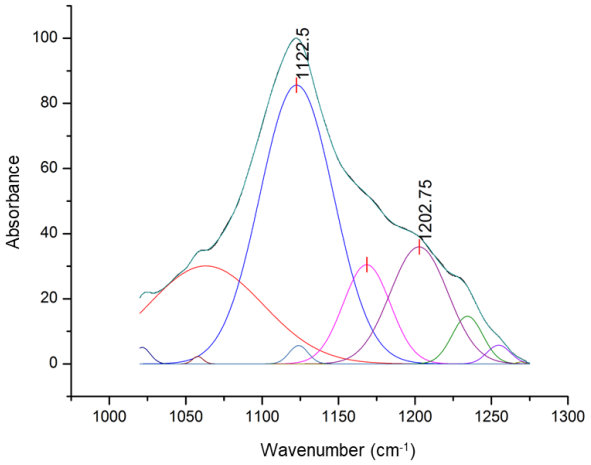

Figure 18. The results of deconvolution of the IR spectrum at 1275–1020 cm^{-1} region

The deconvolution result (Figure 18) shows the absorption of ether CO vibrations at 1202.75 cm^{-1}. The absorption peak at 1122.5 cm^{-1} indicates the presence of alcohol CO vibrations. The identified flavonoid isolates have OH groups, aromatic CH, alkane CH, C=O, aromatic C=C, ether CO, alcohol CO, and substituted aromatic rings. It was concluded that the structure of the flavonoid isolate was thought to be 4'-hydroxy flavanone.

3.8. Antioxidant Activity Test of Ethanol Extract and Flavonoid Isolates using the DPPH Method

The antioxidant activity tests using the DPPH method were determined on ethanol extracts and flavonoid isolates with the quercetin standard as a comparison. Flavonoids will neutralize DPPH free radicals with the ability to donate protons which will cause reduction to form non-radical DPPH. This ability can be seen from the reduced intensity of the purple color of the DPPH solution caused by the reaction of the DPPH radical molecule with one hydrogen released by the antioxidant (test substance) so that a yellow color is formed, which is a non-radical DPPH.

The parameter that interprets the results of the antioxidant activity test with the DPPH method is the Inhibition Concentration (IC_{50}), which is the concentration of a substance that causes 50% of the DPPH to lose its activity [15]. The IC_{50} value was obtained through the Equation of the linear line obtained from plotting the concentration with % inhibition, where % inhibition was obtained by comparing the absorbance of the test solution with the absorbance of the control. Ethanol extracts at various concentrations (5, 10, 20, 40, and 80 ppm) were tested for their antioxidant activity. The results of these tests can be seen in Figure 19.

Table 8. Result of measurement of antioxidant activity

Sample	IC_{50} value	Antioxidant activity
Leaf ethanol extract	118.19 µg/mL	Moderate
Flavonoid isolates	1185.5 µg/mL	Very weak
Quercetin standard	1.54 µg/mL	Very strong

Based on Table 8, it can be concluded that the ethanol extract of Bandotan leaves has moderate antioxidant activity, and flavonoid isolates are very weak antioxidants when compared to standard quercetin. Čanadanović-Brunet *et al.* [17] stated that samples with IC_{50} less than 50 µg/mL are considered to have very strong antioxidant activity, 50-100 µg/mL are strong antioxidants, 101-150 µg/mL are moderate antioxidants, and samples with IC_{50} greater than 150 µg/mL were a weak antioxidant. The antioxidant activity of the flavonoid isolate was suspected to be 4'-hydroxy flavanone (see the structure in Figure 19). It was classified as very weak compared to standard quercetin, which has very strong antioxidant activity.

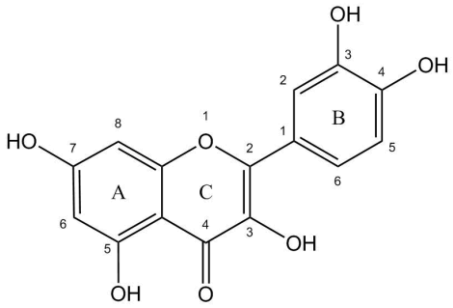

Figure 19. Quercetin Structure [12]

The antioxidant activity of flavonoids was determined by the number and position of the OH groups in rings A and B and the conjugation length between rings B and C [18]. The structure of quercetin had an o-dihydroxy group (3',4'-diOH) in ring B, which caused high stability of the phenoxyl radical due to hydrogen bonding or electron delocalization. The C2-C3 double in quercetin also participated in radical stabilization by delocalizing electrons in all three ring systems. Also, 3-OH and 5-OH maximize quercetin's capacity and strong radical absorption.

4. Conclusion

Isolation of flavonoids from Bandotan leaves (*Ageratum Conyzoides*) and determination of their antioxidant activity have been successfully carried out in this study. Bandotan leaf extraction produced an ethanol extract of 129.27 mg EQ/g. From FTIR analysis, the ethanol extract has a flavonoid compound which is suspected to be 4'-hydroxy flavanone. The antioxidant activity of the ethanol extract of Bandotan leaves was moderate (IC_{50} 118.19 µg/mL). In contrast, the flavonoid isolates were classified as very weak (IC_{50} 1185.5 µg/mL).

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