

# Isolation and Characterization of Flavonoids from Black Glutinous Rice (*Oryza sativa* L. Var *Glutinosa*)

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**Abstract.** This study aims to isolate and characterize the isolated flavonoid compounds from glutinous rice (*Oryza Sativa* L. Var *Glutinosa*). The method that used was maserasi with methanol solvent, multilevel fractionation with n-hexane and ethyl acetate. The separation of chemical components using column chromatography and tested its purity by melting point test and TLC. Characterization of isolated flavonoids using color reagents ( $H_2SO_4$ , NaOH, Mg-HCl), Kkt-2A, UV-Vis and FT-IR. The powder (amorphous) flavonoid isolation results have a melting point of 236.5-237.8 °C. Flavonoid powder added  $H_2SO_4$  produces a yellow color, with NaOH producing a violet color, and Mg-HCl produces a red color. Kkt-2A with a BAA developer has  $R_f$  0.49 and 15% acetic acid having  $R_f$  0.09. The UV-Vis spectrum shows maximum absorption at 532 nm wavelength. The results of powder analysis (amorphous) flavonoid using FT-IR showed absorption at wave number 3,330,40  $cm^{-1}$ , 2838,96  $cm^{-1}$ , 1,656,23  $cm^{-1}$ , 1,421,85  $cm^{-1}$ , 1,112,25  $cm^{-1}$ , 1,019,27  $cm^{-1}$  and 697,76  $cm^{-1}$ . According to those data it can be concluded that flavonoids that isolated have the form of powder (amorphous) which belong to anthocyanin group that have o-diOH on ring B and have a conjugated double bond. Keywords- Black Sticky Rice, Flavonoid, Fractionation, FT-IR, Maserasi, UV-Vis

**Keywords:** Black Glutinous Rice, Flavonoid., Fractionation., FT-IR.

## 1. Introduction

Indonesia has abundant natural wealth. Almost all types of plants can grow in Indonesia. Herbs are widely used as medicines by the community. This happens because there are compounds or substances contained in these plants that function as drugs, these compounds are known as secondary metabolites. Secondary metabolite compounds are chemical compounds that have bioactivity values and are biogenesis of primary metabolites, these compounds are usually produced by high-level plants. Primary metabolite compounds are metabolic products used for survival such as amino acids, proteins and carbohydrates [1].

Secondary metabolite compounds function as self-defense for these plants, such as flavonoids function to attract insects that help plants in pollination and flavonoids also function to protect plants from herbivores and diseases, pathogens, insects, bacteria, fungi and viruses [2]. Secondary metabolite compounds include alkaloids, flavonoids, steroids, terpenoids, tannins and others. These secondary metabolites are very much functional in our body. For example,

flavonoid compounds found in mangosteen peel [3] and jengkol fruit skin which is 1,2 diylpropane (isoflavones) which are estimated to have 6,7-dihydroxy isoflavones or 7,8-dihydroxy isoflavones function as antidiabetic [4].

Flavonoids include natural phenolic compounds which are found in many stems, leaves, flowers and fruit in a plant. Flavonoids are generally polar compounds because they have non-substituted hydroxyl groups. Polar solvents such as ethanol, methanol, ethyl acetate or mixtures of these solvents can be used to extract flavonoids from plant tissues [5].

Flavonoids in the human body function as antioxidants so it is very good for cancer prevention, flavonoids can also protect cell structure, increase the effectiveness of vitamin C, anti-inflammatory, prevent bone loss, antidiabetic and as antibiotics [6]. Extraction of active compounds from plants can be carried out by extraction. In the extraction process the active compound will dissolve by the solvent in accordance with the polarity properties [5].

Flavonoids provide protection to the body against cardiovascular disease, diabetes mellitus, antioxidants, anti-inflammatory and anticancer. The mechanism of action of flavonoids as antioxidants by donating hydrogen atoms from the aromatic hydroxyl (-OH) group of polyphenols to bind free radicals and remove them from the body through the excretion system. The role of polyphenols as antioxidants is also able to protect pancreatic  $\beta$  cells from the toxic effects of free radicals produced under conditions of chronic hyperglycemia [7].

This flavonoid is thought to reduce blood glucose levels, namely by inhibiting the action of the  $\alpha$ -glucosidase enzyme found in the small intestine. This  $\alpha$ -glucosidase enzyme is used to hydrolyze oligosaccharides into glucose in the small intestine. These flavonoid glycoside compounds can also increase blood glucose solubility so that they are easily excreted in the urine [8].

Plants that can reduce blood glucose levels such as avocado seeds, crown gods, dragon fruit, guava, bitter melon, saledri plants [9] and black glutinous rice [10]. Ethnobotany black glutinous rice is used by people in West Sumatra to reduce blood glucose levels by soaking it with boiling water. Preliminary identification results (phytochemical test) of black glutinous rice (*Oryza sativa* L. Var *glutinosa*) found that black glutinous rice was positive containing alkaloids and flavonoids.

Iryani [10] has conducted research on the bioactivity test of ethyl acetate fraction and aqueous fraction of black glutinous rice as antidiabetic against white mice. The results showed that black glutinous rice ethyl acetate fraction and aqueous fraction can reduce blood glucose levels in mice with diabetes. The controls used in the study were negative control of NaCMC 1% and positive control of glibenclamide. To find out the types of flavonoids found in black glutinous rice, the authors have conducted a study entitled "Isolation and Characterization of Flavonoids from Black Glutinous Rice (*Oryza sativa* L. Var *glutinosa*)".

This research was carried out by maceration extraction method using methanol solvent, fractionation with n-hexane and ethyl acetate. The separation of the constituents of chemical components was carried out by chromatography, as well as the characterization of compounds with qualitative and spectrophotometric IR and UV-Vis examination. This study aims to determine the characteristics and classify flavonoids isolated from black glutinous rice. Other paragraphs are indented (BodytextIndented style).

## 2. Experimental Section

### 2.1. a Tools and Materials

The equipment used in this study is a set of distillation apparatus, test tubes, beaker, drop pipette, separating funnel, maceration jar, oven, a set of rotary evaporator, mortar and pestle tools, test tube rack, filter paper, funnel, column, stative, clamp, chamber, UV lamp, UV Agilent 8453 spectroscopy, IR Jasco FT-IR 460 plus spectroscopy, gallenkamp melting point apparatus. The materials used include samples of black glutinous rice,  $\text{CH}_3\text{OH}$ ,  $\text{CH}_3(\text{CH}_2)_4\text{CH}_3$ ,  $\text{CH}_3\text{OC}(\text{O})\text{CH}_3$ , HCl pa, powder Mg,  $\text{CHCl}_3$ ,  $\text{NH}_3$ ,  $\text{H}_2\text{SO}_4$ ,  $\text{CH}_3\text{COOH}$ , NaOH,  $\text{AlCl}_3$ , metal Na,  $\text{CH}_3\text{COONa}$ ,  $\text{H}_3\text{BO}_3$ , cotton, aquades, silica gel, cotton, whatman paper and capillary pipes.

### 2.2. b Equipment and Materials

The equipment used in this study is a set of distillation apparatus, test tubes, beaker, drop pipette, separating funnel, maceration jar, oven, a set of rotary evaporator, mortar and pestle tools, test tube rack, filter paper, funnel, column, stative, clamp, chamber, UV lamp, UV Agilent 8453 spectroscopy, IR Jasco FT-IR 460 plus spectroscopy, gallenkamp melting point apparatus.

The materials used include samples of black glutinous rice,  $\text{CH}_3\text{OH}$ ,  $\text{CH}_3(\text{CH}_2)_4\text{CH}_3$ ,  $\text{CH}_3\text{OC}(\text{O})\text{CH}_3$ , HCl pa, powder Mg,  $\text{CHCl}_3$ ,  $\text{NH}_3$ ,  $\text{H}_2\text{SO}_4$ ,  $\text{CH}_3\text{COOH}$ , NaOH,  $\text{AlCl}_3$ , metal Na,  $\text{CH}_3\text{COONa}$ ,  $\text{H}_3\text{BO}_3$ , cotton, aquades, silica gel, cotton, whatman paper and capillary pipes.

### 2.2. Research Procedures

The study began with a preliminary test of alkaloid content (Culvenor-Fitzgeraid method), flavonoids (Shinoda Test method), saponin (foam test), terpenoids and steroids (Lieberman-Burchead method). Samples in the form of positive black glutinous rice contain alkaloids and flavonoids. The flavonoid compounds contained in the sample were more than other compounds, so flavonoid isolation was carried out.

#### 2.2. a. Extraction

The extraction process of black glutinous rice was carried out by multilevel maceration, as much as 10.00 kg of black glutinous rice were mashed using mortar and pestle. Black black glutinous rice has been macerated using methanol in stages (1/7 days) 11 times. Maseration is done until the flavonoid test is negative. Extraction results were collected and evaporated using a rotary evaporator at 50oC, until the solvent had evaporated, so that the concentrated extract of black glutinous rice was obtained. The concentrated extract obtained was dissolved with hot water of 50oC as much as 300 mL.

#### 2.2. b. Fractionation

Fractionation of black glutinous rice extract was carried out by partitioning method, extracts which had been obtained were then fractionated successively with n-hexane and ethyl acetate. The n-hexane fraction was tested for its flavonoid content with the shinoda test method showing a negative result, while the ethyl acetate fraction showed positive results containing flavonoids. The ethyl acetate fraction was then evaporated using a rotary evaporator at 50oC. So that obtained thick viscous ethyl acetate.

#### 2.2. c. Separation

Ethyl acetate extract was first monitored by thin layer chromatography (TLC). This is done to get suitable eluents to be used in column chromatography. Eluent used is a single compound and a mixture of several single compounds. The ethyl acetate fraction was eluted with eluent ethyl acetate, n-hexane and mixed the two compounds with several comparisons. Stains that arise on the plate (chromatogram) are observed using UV lamps at a wavelength of 366 nm.

The component separation from ethyl acetate fraction is then carried out by column chromatography. The stationary phase used is silica gel 60 (70-230 mesh), while the mobile phase used is ethyl acetate: methanol SGP. Packing columns is done by inserting glass wool first, then slurry silica gel. The silica gel in the column must be compacted before use so that the obtained separation is good, the method is to pass the solvent repeatedly into the column and silence the column for 5 days.

The concentrated ethyl acetate extract was dissolved with a little methanol and added with silica gel then put into the packing column. The extract is then eluted with eluent. Eluent used is ethyl acetate: methanol with a ratio of 10: 0 (100 mL), 9: 1 (100 mL), 8: 2 (100mL), 7: 3 (100 mL), 6: 4 (100 mL), 5: 5 (100 mL), 4: 6 (100 mL), 3: 7 (100 mL), 2: 8 (200 mL), 1: 9 (200 mL), and 0:10 (200 mL). The resulting eluate is collected in vials that have been given numbers. Vials obtained were 102 vials. Eluat in the vial is monitored by TLC, eluat which has the same Rf are combined in one vial. The results of the merger were obtained by 7 groups.

#### 2.2. d. Purification and Purity Test

Flavonoid (amorphous) powder was purified by TLC and melting point test. TLC plates used first are given the lower limit and upper limit with a distance of 1-2 cm. Eluent used in this TLC with a ratio of 10: 0, 8: 2, 4: 6, 2: 8 with a volume of 20 mL. TLC is done by bottling the isolation solution on the TLC plate using a capillary tube. Then, the plate is inserted into the vessel containing the eluent and left the plate eluted to the upper limit, after reaching the upper limit the plate is removed and dried at room temperature. Isolated stains obtained have been singular where the Rf value for the eluent ratio of 10: 0 is 0.1639, 8: 2 is 0.66, 4: 6 is 0.73, 2: 8 is 0.816

The melting point is used to ensure the powder is pure. The melting point of the crystal is tested with a melting point of the gallenkamp apparatus brand. Observations are made when the powder begins to melt until all the powder in the capillary tube melts completely. Isolated powder is pure if it has a melting point range  $<2^{\circ}\text{C}$ . The pure powder was then tested for Mg-HCl before it was characterized to ensure that the powder still contained flavonoid compounds.

#### 2.2. e. Characterization of Isolation Results

Characterization of pure flavonoid powder is carried out with color reagents, Kkt-2A, UV-Vis and FT-IR. Isolated flavonoids were identified using color reagents with 10% NaOH, concentrated  $\text{H}_2\text{SO}_4$  and Mg-HCl. Flavonoid crystals obtained were dissolved in a little methanol, then the solution was divided by three and added with color reagents. The first tube when added with 10% NaOH changed its color to violet, the second tube when added with concentrated  $\text{H}_2\text{SO}_4$  changed its color to yellow, and the third tube when added with Mg-HCl changed its color to red.

This paper chromatography uses whatman paper 3 MM (20 × 20 cm). The powder (amorphous) is bottled using a capillary tube from the edge of the paper and 3 cm from the final fold. The paper was put into a chromatographic vessel which had been filled with the first

development, where the first development used was the developer BAA (n-BuOH: HOAc: H<sub>2</sub>O) with a ratio of 4: 1: 5. If the developer used has risen to the upper limit of the paper, the paper is removed and dried in a fume hood. When the paper is dry it is detected using a 366 nm UV lamp and the chromatogram on the first developer has a R<sub>f</sub> of 0.49. Then the position of the paper is rotated 90° from the initial position and inserted into the vessel which already contains 15% acetic acid developer, eluted until the developer moves to the upper limit specified. Then the paper is dried in a fume hood. The R<sub>f</sub> generated in this second developer is 0.09.

A small amount of flavonoid powder was dissolved in methanol p.a then tested by Ultra Violet (UV) Agilent 8453 spectroscopy which was standardized with methanol p.a. The spectrum is then recorded by adding shear reagents. The shear reagent used is Sodium Metoxide (NaOMe), AlCl<sub>3</sub>/HCl, Sodium Acetate/Boric Acid.

The functional group of isolated compounds is known by using an infrared spectrophotometer. Isolated powder is taken a little using a spatula then put into the sample hole in the tool (the sample container on the appliance is cleaned with alcohol before entering the sample). The intensity of uptake of isolated compounds was measured at wave numbers 4,000 cm<sup>-1</sup> to 600 cm<sup>-1</sup>, from the measurement results obtained specific peaks of the functional groups contained from the isolated compounds.

### 3. Results and Discussion

#### 3.1. Extraction and Fractionation

Samples of black glutinous rice used in this study as much as 10 kg were macerated with methanol distillate. The maceration filtrate was then concentrated using a rotary evaporator to obtain 211 grams of concentrated red methanol extract. The concentrated extract is then added with 45 °C of hot water as much as 300 mL to produce aqueous extract. The aqueous extract was then fractionated using n-hexane solvent so that the n-hexane fraction of 4400 mL was obtained and the negative n-hexane fraction contained flavonoids when tested with shinoda test so that it was not continued to the next stage. The aqueous fraction was then fractionated again with ethyl acetate to obtain 8000 mL ethyl acetate fraction. The ethyl acetate fraction and aqueous fraction were tested for their flavonoid content using shinoda test and the results showed that the two positive fractions contained flavonoids. The ethyl acetate fraction was then concentrated so that a concentrated extract of 44.0597 grams was obtained.

#### 3.2. Isolation

Component separation was carried out by column chromatography, before the eluent monitoring was carried out using KLT. The best eluent for separation is the ratio of ethyl acetate: methanol (2: 8). Column chromatography was carried out using the Step Gradient Polarity (SGP) method with an eluent ratio which can be seen in Table 1. The SGP method was carried out by changing the polarity of the eluent used gradually, with gradual changes in the polarity level of the components to the solvent and their respective attachments. each component of the stationary phase will vary according to the nature of each component.

Table I. comparison Of Ethyl Acetate With Methanol By Step Gradient Polarity (Sgp)

No	Ethyl acetate	Methanol	Total Volume
1	10	0	100
2	9	1	100
3	8	2	100
4	7	3	100
5	6	4	100
6	5	5	100
7	4	6	100
8	3	7	100
9	2	8	200
10	1	9	200
11	0	10	200

The sample extracts separated from column chromatography were 5,0076 grams of ethyl acetate extract Eluet in column chromatography is accommodated in vial bottles. Column chromatography carried out produced 102 vials. Vials that have the same Rf are then combined so that 7 large groups can be seen in Table 2. The groups are allowed to dry the solvent at room temperature.

Table 2. Group Obtained From Colomn Chromatography

Group	Vial	Rf	Color	Form
1	1 – 15	0,6	Yellow	Clot
2	16 – 34	0,78	Red	Clot
3	35 – 50	0,71	Dep Red	Clot
4	51 – 77	0,66	Red Brown	Amorphous
5	78 – 85	0,32	Light Brown	Oil
6	86 – 94	0,1	Brown	Oil
7	95 - 102	0	Brown	Evaporated

### 3.3. Purity test

Pure flavonoid powder was tested for its purity by melting point and TLC test. The melting point test was carried out using the gallenkamp melting point apparatus, the melting point obtained had a range of 236.5-237.8 °C with a difference of 1.3 °C. TLC uses 4 eluent comparisons namely ethyl acetate: methanol (10: 0, 8: 2, 4: 6, 2: 8). The chromatogram obtained is in the form of a single stain where the value of Rf is 0.1639, 0.66, 0.73 and 0.816. The melting point test results have a close range and the results of TLC have a single stain which indicates that the flavonoid powder from isolation is pure. This is in accordance with the opinion of [10] which states that if the compound tested by TLC gives a single stain and the range of melting points is < 2 °C then the isolated compound is pure. Pure powder was tested with shinoda test to

ensure that the powder still contained flavonoids. The result is a positive powder containing flavonoids and it can be concluded that the powder obtained is pure flavonoid powder.

### 3.4. Characterization

Characterization of isolation flavonoid powder using color reagent, KKt-2A, UV-Vis spectrophotometer and FT-IR. Characterization with color reagents gives violet color when added to NaOH, yellow when H<sub>2</sub>SO<sub>4</sub> is added and red when added with Mg-HCl. The results of the color reagent above can be said that the flavonoids from isolation belong to the anthocyanin group Flavonoids with the addition of NaOH will experience basic decomposition into simpler molecules. flavonoids with the addition of concentrated H<sub>2</sub>SO<sub>4</sub> resulted in an electrophilic substitution reaction, whereas with the addition of Mg-HCl reducing flavonoid compounds, the addition of HCl resulted in a reduction oxidation reaction between Mg metal as a reducing agent and flavonoids [11]

Two-way paper chromatography was carried out with 2 eluents, the first eluent was BAA and the second was acetic acid 15%. The first developer has an R<sub>f</sub> value of 0.49 and the second developer has a value of R<sub>f</sub> 0.09. KKt-2A results in a single pink stain. Single stains found in chromatography show the presence of anthocyanin in O-glycosides from the sample.

Characterization with UV-Vis spectrophotometer to determine the presence of conjugated double bonds, the results of the characterization show absorption at a wavelength of 532 nm with absorbance 0.75966 Å which can be seen in Figure 1. The absorption band is in conjugated diene absorption in the transition  $\pi \rightarrow \pi^*$  in the area  $\geq 270$  nm. This shows the double bond in the conjugated flavonoid powder [12].

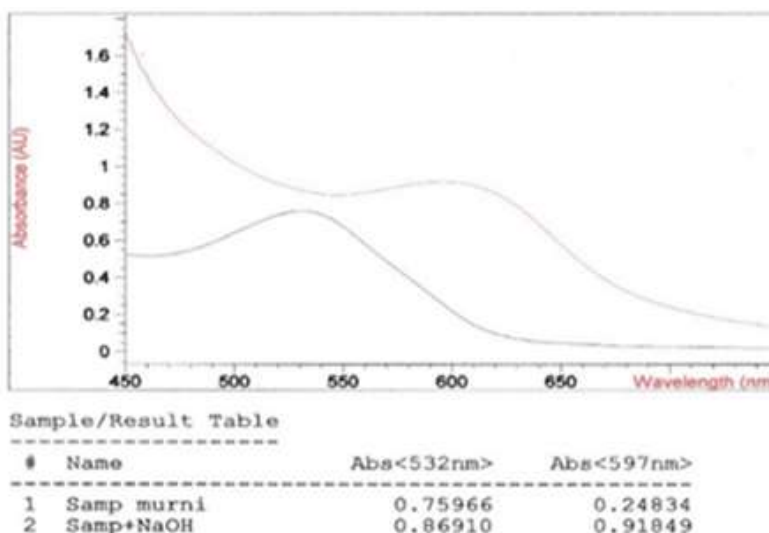


Figure 1. UV-Vis Flavonoid Spectrum Results of Isolation with Methanol Solvents and Addition of NaOH Shear Reagent.

The addition of NaOH shear reactor causes a shift in wavelength to show the presence of OH on ring B [14] anthocyanin and anthocyanidin compounds will decompose with the addition of NaOH. The UV-Vis spectrum can be seen in Figure 1.

The second shear reagent, AlCl<sub>3</sub>, was carried out to see whether or not there was a bathochromic shift, which indicates the presence or absence of orthohydroxyl groups. The addition of AlCl<sub>3</sub> shear reagent caused a 28 nm bathochromic shift, from 532 nm to 560 nm, according to o-di OH in anthocyanins [13]. The spectrum can be seen in Figure 2.

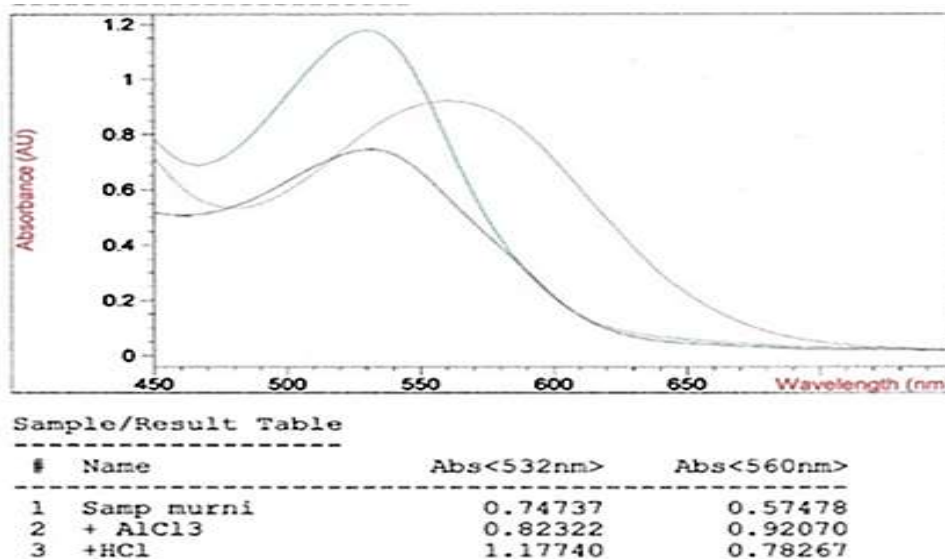


Figure 2. UV-Vis Flavonoid Spectrum Results of Isolation with methanol Solvent and addition of AlCl<sub>3</sub> Sliding Reagent.

Addition of the third shear reagent namely Sodium acetate and boric acid. Sodium acetate (NaOAc) can cause ionizing which means the hydroxyl group of flavonoids. NaOAc/H<sub>3</sub>BO<sub>3</sub> serves to detect o-dihydroxyl groups Because NaOAc/H<sub>3</sub>BO<sub>3</sub> can bridge the two hydroxyl groups found in flavonoids. The addition of NaOAc/H<sub>3</sub>BO<sub>3</sub> shear reagents gives rise to a bathochromic shift of 27 nm in band 1 which means there is o-di-OH in ring B. UV-Vis spectrum can be seen in Figure 3.



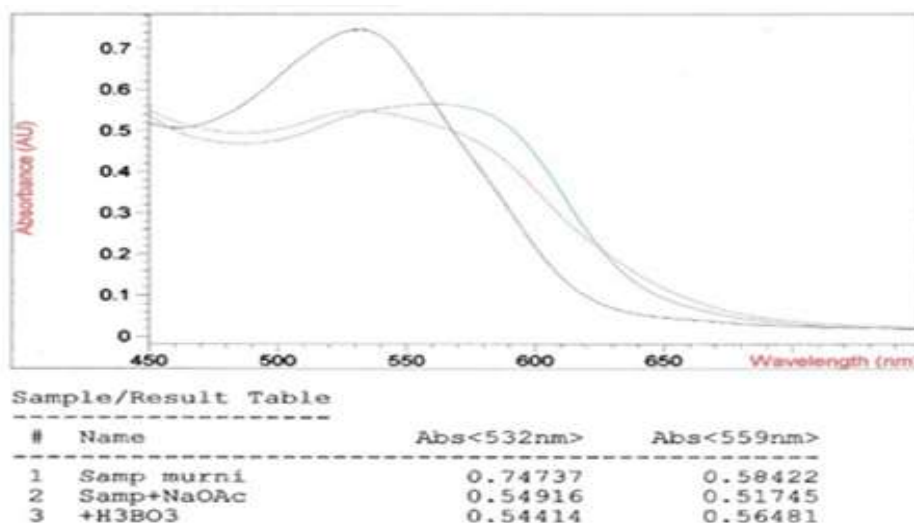


Figure 3. UV-Vis Flavonoid Spectrum Results of Isolation with Methanol Solvent and Addition of NaOAc/H<sub>3</sub>BO<sub>3</sub> Slide Reagent.

Characterization of the subsequent flavonoids from FT-IR. Characterization using FT-IR was carried out to determine the functional groups contained in the isolation powder. The FT-IR spectrum provides specific absorption at wave numbers 3,330.40 cm<sup>-1</sup>, 2838.96 cm<sup>-1</sup>, 2,040.79 cm<sup>-1</sup>, 1,656.23 cm<sup>-1</sup>, 1,421.85 cm<sup>-1</sup>, 1,112.25 cm<sup>-1</sup>, 1,019.27 cm<sup>-1</sup> and 697.76 cm<sup>-1</sup>. The FT-IR spectrum can be seen in Figure 4.

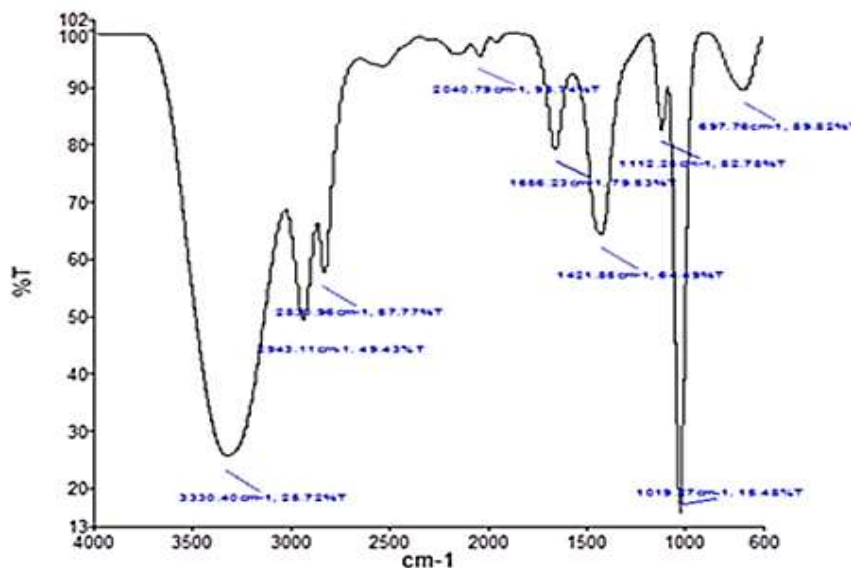


Figure 4. FT-IR spectrum of isolated flavonoid

Characterization using FT-IR (Figure 5) to find out the functional the presence of strain vibration absorption of -OH at 3,330.40 cm<sup>-1</sup> (3650-3,200 cm<sup>-1</sup>) wave spectra which is thought to be OH from the alcohol group. Vibration uptake appears at wave groups contained in the

sample. The FT-IR spectrum shows number  $1,019.27\text{ cm}^{-1}$  ( $1,300\text{-}900\text{ cm}^{-1}$ ) which is a strain vibration of C-O alcohol, this confirms that the isolated flavonoids have a hydroxy (OH) functional group [14]. C-O without OH at  $1112.25\text{ cm}^{-1}$  ( $1300\text{-}1000\text{ cm}^{-1}$ ).

Other absorption bands are seen at wave numbers  $1,656.23\text{ cm}^{-1}$  ( $1680\text{-}1600\text{ cm}^{-1}$ ) which is a vibration of the C = C group conjugated strain followed by the emergence of vibration absorption  $697.76\text{ cm}^{-1}$  ( $1,000\text{-}650\text{ cm}^{-1}$ ) which is a bending of = CH alkene and CH vibration appears at wave number  $2838.96\text{ cm}^{-1}$  ( $3,000\text{-}2,800\text{ cm}^{-1}$ ). Uptake at wave number  $1,421.85\text{ cm}^{-1}$  shows the presence of an aromatic C = C group. The data obtained is in accordance with the literature [15].

#### 4. CONCLUSIONS

Based on the results of the research that has been done, the following conclusions can be taken: Isolated flavonoids from black glutinous rice in the form of brownish red powder with a melting point of  $236.5\text{-}237.8\text{ }^{\circ}\text{C}$  Characterization of isolated flavonoid compounds with color reagents ( $\text{H}_2\text{SO}_4$  produces yellow, NaOH Violet, Mg-HCl red), Kkt-2A (BAA has Rf: 0.49, 15% acetic acid has Rf: 0.09) UV-Vis spectrophotometers have absorption at wavelengths of 532 nm and FT-IR (OH, CH, C = C, COC, CO, = CH). The characterization results show that the isolated flavonoids belong to the anthocyanin group that has o-diOH on ring B and has a conjugated double bond.

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