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Analysis of active compounds in kawista leaf (*Limonia acidissima* L.) fraction and its antioxidant activity using DPPH method

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Abstract

Background: Kawista (*Limonia acidissima* L.) is a plant that has been studied for its potential as a natural antioxidant. Research regarding kawista leaves has not been carried out to a more specific stage, so further fraction separation is carried out to determine compounds that have potential as antioxidant agents.

Objective: This study aimed to determine the antioxidant activity of kawista leaf fractions using the DPPH method and to determine the active compounds in the highest antioxidant agents of kawista leaf fracton.

Method: Kawista leaves were extracted by re-maceration using methanol, which was then purified by the liquid-liquid extraction method with the solvents n-hexane, ethyl acetate, and water. Phytochemical studies were carried out on the fractions using TLC on flavonoids, steroids, tannins, saponins, and alkaloids. The fraction was separated by the vacuum liquid chromatography method with 11 eluents, which were then tested for antioxidants and characterization of the most active compounds in the kawista leaf fraction. Then the highest results as antioxidant agents from the fraction were characterized for their active compounds using a spectrophotometer and FTIR.

Results: The results of the study showed that the most active purified extract in antioxidant activity was ethyl acetate, which indicates strong antioxidant activity. The combined fraction 2 (F2) indicates flavonoid compounds that played an active role as very strong antioxidants with an AAI value of 3.3808, which is not significantly different from the vitamin C as standard. Flavonoid compounds that have been characterized using a spectrophotometer interpret the wavelength shift data that possible F2 belongs to the flavanon group.

Conclusion: Active compounds from kawista leaf fraction (F2) have the highest antioxidant agents, with the possibility that flavonoid compound from F2 belong to the flavanon group.

Keywords: Antioxidant, DPPH, fraction kawista leaf, *Limonia acidissima*

1. Introduction

Free radicals or body oxidants are very dangerous. In fact, free radicals are simply compounds or molecules with one or two unpaired electrons in the outermost level of their atomic structure. These compounds are highly reactive because they have many unpaired electrons. Cell damage or uncontrolled cell development can arise from the binding or attack of molecular electrons by free radicals on nearby molecules such as lipids, proteins, or DNA (the carrier) (Sayuti & Yenrina, 2015).

There are two types of antioxidants: those derived from natural sources and those obtained through chemical synthesis. However, concerns about the side effects of synthetic antioxidants have prompted a shift towards natural antioxidants. Kawista (*Limonia acidissima* L.) is a plant that has been studied for its potential as a natural antioxidant. Kawista is known as one of the most important medicinal plants in India, with one part of the plant, namely the leaves, being used as an antibacterial (Panda *et al*., 2013), diuretic (Parial *et al*., 2009), and for hypoglycemia (Joshi *et al*., 2009).

The Kawista fruit's leaves, stems, fruit skin, and flesh all contain substances that have antioxidant activity, including tannins, steroids, alkaloids, flavonoids, and glycosides. This information is based on the study "Phytochemical screening, antibacterial and antioxidant activity of *L. acidissima* L." (Patil *et al*., 2012). The DPPH technique is used to measure antioxidant activity. Research related to the activities of the kawista plant (*L. acidissima* L.) that has been conducted includes antidiabetic activity, antitumor activity (Eluru *et al*., 2015), antibacterial activity (Anebaracy et al., 2015), and antioxidant activity (Ilango & Chitra, 2010). Research on the antioxidant activity of the kawista plant has been conducted year after year. Antioxidants in kawista fruit were studied by Ilango and Chitra (2010) using methanol extracts of kawista fruit with FRAP and DPPH methods. Meanwhile, Mandade *et al*. (2013) used the DPPH and ABTS methods.

According to Patil *et al*. (2012), antioxidant activity was also found in the leaves, roots, and skin of the kawista fruit. According to Tjahjandarie *et al*. (2017), the compound with antioxidant activity is coumarin found in kawista roots. Maceration with 96% ethanol was used to extract the leaves and peels of the kawista fruit (Rahmi & Rahmadewi, 2020), and the resulting IC_{50} value was 134.56 ppm. The highest antioxidant activity in the aqueous extract of kawista leaves was 66.12%; 89.51%; and 94.41% (Nachimuthu *et al.*, 2014).

Based on study of Patil *et al.* (2012), 200 ppm extract concentration, the antioxidant activity was measured in the following ways: leaves (57.34 g/mL), stems (55.94 g/mL), fruit skins (55.94 g/mL), and fruit flesh (40.55 g/mL); at 400 ppm extract concentration, the values were as follows: leaves (67.10 g/mL), stems (79.02 g/mL), fruit skins (67.83 g/mL), and fruit flesh (51.04 g/mL); at 600 ppm extract concentrations, the values were as follows: leaves (95.80 g/mL), stems (82.51 g/mL), and fruit skins (67.83 g/mL).

Based on the article obtained in the study “Phytochemical investigation and *in vitro* antioxidant activity of extracts from leaves of *L. acidissima* Linn. (Rutaceae),” compounds that have antioxidant activity found in kawista leaves are flavonoids and flavonols (Attarde *et al*., 2011). Based on the principle of "like dissolves like," secondary metabolite compounds can be separated from plant parts using solvents with appropriate polarity levels to achieve higher biochemical activity. The separation carried out by previous researchers reached the extraction stage, so a more specific separation of kawista leaves has never been conducted. Therefore, a separation process is necessary until active compounds that provide the most optimal antioxidant activity are identified. A fractionation process is needed to obtain active compounds as antioxidants from kawista leaves. This study aimed to determine the antioxidant activity of kawista leaf fractions using DPPH method, and to determine the active compounds in the highest antioxidant agents of kawista leaves fracton.

2. Method

2.1. Tools and materials

The tools used in this research are maceration equipment consisting of a maceration vessel and a beaker glass (Herma). Another tool used in this research is a UV lamp (Recent RCC), a set of UV-VIS spectrophotometry equipment from Shimadzu 1700, an analytical balance (Fujitsu), an oven (Binder ED), a mesh sieve 40, a blender (Phillips), a rotary evaporator (B ONE RE 2000), and silica GF 254 nm. (Merck).

The material used in this research is kawista leaf powder produced from Dresi Kulon Village, Kaliori District, Rembang Regency. The chemical materials used in this research were 96% ethanol p.a (Sigma), DPPH (Sigma), methanol p.a (Sigma), aquadest (Bratachem), n-hexane (Bratachem), ethyl acetate (Bratachem), n-butanol (Bratachem), vitamin C (Sigma), ether (Bratachem), acetic acid (Bratachem), sulfanilic acid (Nitrokimia), formaldehyde (Bratachem), ammonia (Bratachem), chloroform (Bratachem), amyl alcohol (Bratachem), NaOH (Sigma), $NaNO_2$ (Bratachem), HCl (Bratachem), sulfuric acid (Bratachem), vanillin (Bratachem), anisaldehyde (Bratachem), Dragendorff's reagent (Bratachem), Meyer's reagent (Bratachem), and $FeCl_3$ (Bratachem).

2.2. Method and result analysis

The selected extract that continued the purifying process was the methanol extract, which will henceforth be referred to as the concentrated extract of kawista leaves. It has the best AAI value among the three extracts that have been tested for DPPH antioxidant activity using a spectrophotometer UV-Vis (Azizah *et al*., 2024).

2.2.1. LLE Method

Methanol extract of kawista leaves was re-extracted on a large scale until a selected concentrated extract was obtained for liquid-liquid extraction process, which includes the separation of the concentrated extract from the leaves first, then partitioned into n-hexane, ethyl acetate, and water. The extract was dissolved in a 1:1 mixture of n-hexane and distilled water. After shaking for 10–15 minutes in the separating funnel, the water will settle at the bottom and n-hexane will rise to the top.

2.2.3. Vacuum liquid chromatography method

As much as 5 grams of the selected sample kawista leaf was based on the activity test above, then add it to approximately 5 grams of the stationary phase of the silica gel column and stir until it forms a powder. Then, it was re-fractionated using vacuum column chromatography with silica gel as the stationary phase and a solvent mixture as the mobile phase. This was done to look at the

fractions that separated the best in the TLC analysis, starting with 50 mL (Gritter *et al*., 1991). From non-polar (n-hexane) to polar (ethyl acetate) to non-polar (methanol), the elution solvent increases in polarity. To ensure maximum density, the sample was deposited at the top of the column and evenly spread before the filter paper was placed on top and the vacuum device is activated. The column was completely left blank after each collection.

2.2.4. Study of antioxidant activity

DPPH solution was prepared by adding 50 mg of DPPH powder into a 50.0 mL volumetric flask, p.a. methanol, resulting in a concentration of 1000 ppm. The sample was diluted 10 times using a 5.0 mL pipette and p.a. methanol to reach a concentration of 100 ppm up to the mark of a 50.0 mL volumetric flask. The determination of antioxidant activity was carried out by adding 2 mL of 100 ppm DPPH solution into a test tube, followed by the addition of 2 mL of each sample solution: each concentration of 60, 80, 100, 120, and 140 ppm. Next, the mixture was homogenized with a vortex for 10 seconds and allowed to stand according to the operating time of each test solution in a dark place. The solution's absorbance was measured using a UV-Vis spectrophotometer at the maximum wavelength. Measurements of the absorbance of the control solution (DPPH) and the standard of vitamin C were also conducted (Erika *et al*., 2014). The absorbance value of kawista leaves was then used to calculate the percentage of free radical scavenging activity using the formula (inhibition percentage):

$$\frac{(\text{Abs control} - \text{Abs sample})}{\text{Abs control}}\ x\ 100\%$$

After obtaining the inhibition percentage from each replication, a linear regression calculation was performed between the sample concentration (ppm) (X) and the DPPH inhibition percentage (%) (Y), resulting in the equation: Y = BX + A. The IC_{50} value was obtained from the x value after substituting y with 50 by inserting the values of B and A. The same calculation was also performed on the vitamin C standard. Once the IC_{50} value was obtained, it was then converted into the AAI (Antioxidant Activity Index) value, which was used to determine the antioxidant activity index using the formula (AAI):

$$\frac{\text{DPPH concentration (ppm)}}{\text{IC50 (ppm)}}$$

2.2.5. Study of phytochemicals

The identification of compounds conducted includes qualitative antioxidant tests, flavonoid TLC tests, alkaloid TLC tests, saponin TLC tests, and tannin TLC tests. The stationary phase used is silica gel GF254. The mobile phase for the flavonoid test was n-butanol:acetic acid:water (4:1:5), and

the reagent used was ammonia vapor. The mobile phase for the alkaloid test was methanol:chloroform (0.5:9.5), and the reagent used was Dragendorff's reagent. The mobile phase for the saponin test was chloroform:methanol:water (6:3:1), and the reagent used was H_2SO_4 – anisaldehyde. The mobile phase for the tannin test was ethyl acetate:methanol:water (100:13.5:10), and the resulting chromatogram was detected with visible light, UV254 light, and UV 366 light, then marked and the Rf values were calculated (Harborne, 1987). Then the highest fraction had identified of compounds using FTIR aims to determine the functional groups of a compound that produce characteristic bands of that compound, if the compounds had characterized be flavonoids so was carried out through analysis using spectrophotometer UV-Vis.

3. Result and discussion

The three solvents used in this study were n-hexane, which has a polarity index of 0, meaning it is non-polar, ethyl acetate, which has a polarity index of 4.4; indicating that ethyl acetate could dissolve semi-polar compounds; and water, which had a polarity index of 9.0; meaning it was very polar and could attract the polar compounds. The highest yield resulted from the water fraction, followed by the n-hexane and ethyl acetate fractions.

Tabel 1. Yield value of kawista fraction

Methanol extract (gram)	Solvent	Result (gram)	Yield (%)
38.1000	n-hexane	10.1320	26.59
	Ehtyl Acetate	5.8033	15.23
	Water	12.9670	34.03

The results in **Table 1** show that the yield of ethyl acetate solvent is lower compared to other solvents, due to the high levels of bioactive compounds present in the ethyl acetate sample. The antioxidant activity quantitatively of the kawista leaf purified extract using the methods above (spectrophotometer UV-Vis), maximum wavelength obtained is 515.9 nm with an absorbance of 0.733 (within the range of 0.2 - 0.8).

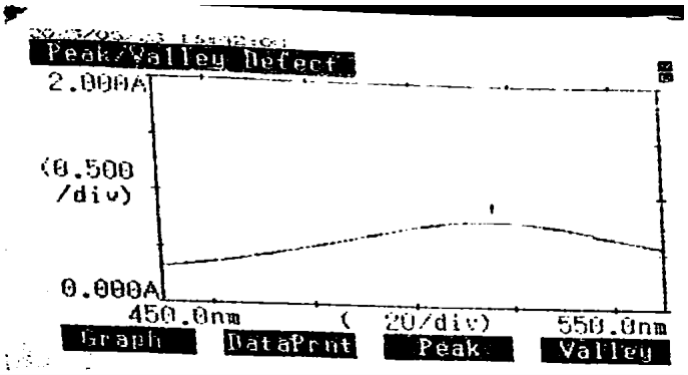

Figure 1. Maximum wavelength of DPPH solution

Furthermore, the operating time obtained from the vitamin C standard is at the 30th minute, and for the n-hexane, ethyl acetate, and water fractions, the operating times obtained are at the 28th, 26th, and 29th minutes, respectively. The AAI value data for the kawista leaf purified extract can be seen at **Figure 2**.

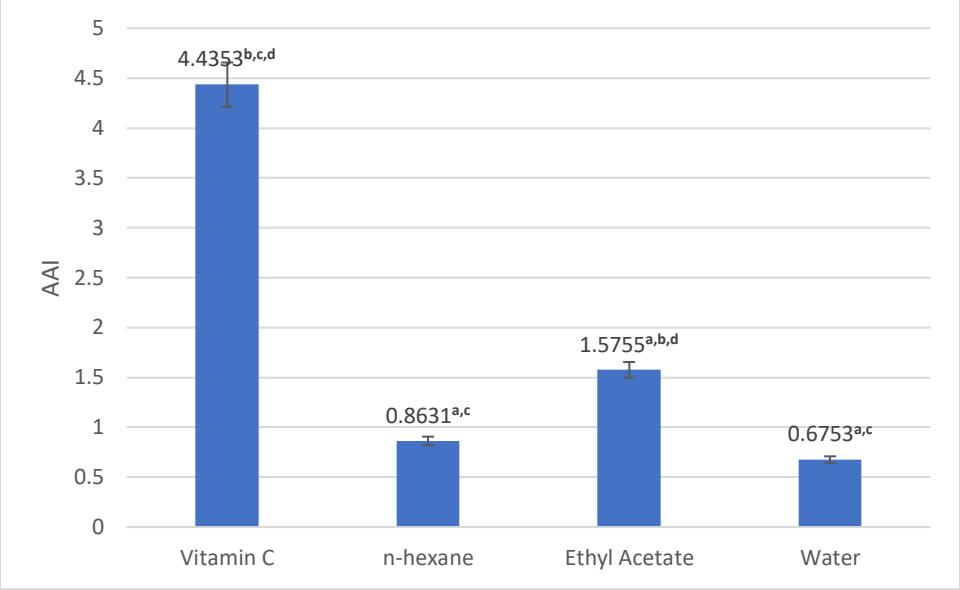

Figure 2. Test antioxidant activity of purified extract

Description:
a. significantly different ($p < 0.05$) from vitamin C
b. significantly different ($p < 0.05$) from the n-hexane sample
c. significantly different ($p < 0.05$) from the ethyl acetate sample
d. significantly different ($p < 0.05$) from the water sample

The selection of the sample that will continue with the separation using VLC is based on the best AAI values. As seen from the statistical test, the aqueous sample and the n-hexane sample did not show significant differences from each other. Therefore, if only one solvent was chosen to proceed with the VLC separation, it was highly likely that there will be no difference in antioxidant activity.

The selected ethyl acetate sample was further processed using VLC to separate the active compounds from kawista leaves that have the best antioxidant activity. The choice of separation method using VLC was due to the fact that the sample can migrate quickly in both the stationary and mobile phases under vacuum conditions, thus enabling the production of compounds more effectively (Mutmainnah *et al.*, 2017). The ethyl acetate sample was first impregnated by carefully weighing 5 grams of the ethyl acetate fraction from the kawista leaves, then adding it to approximately 5 grams of the stationary phase of silica gel column and the mobile phase of n-hexane, ethyl acetate, and methanol (Rahimah *et al*., 2013).

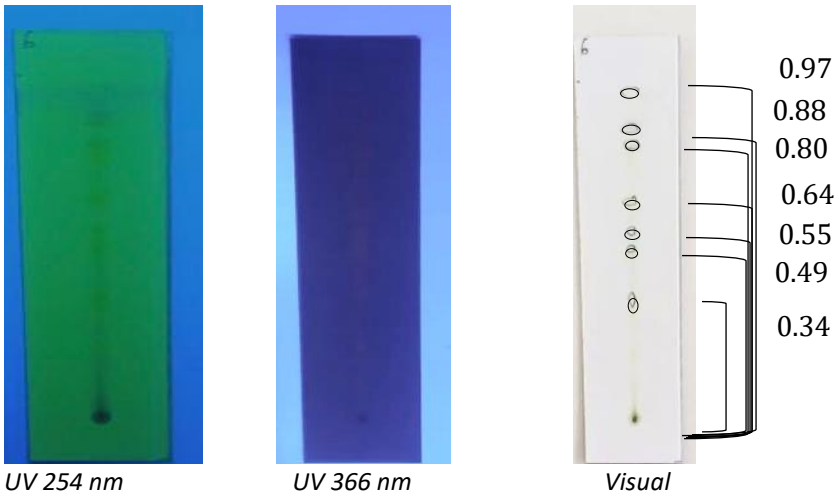

Figure 3. Chromatogram profile of methanol extract optimization
[the stationary phase: silica gel GF 254; mobile phase: n-hexane:ethyl acetate (v/v) as (7:3)]

The result from the VLC separation was collected in glass bottles (11 eluates). The separation of several fractions could be observed from the appearance of color differences in the compounds that elute along with the mobile phase, where the colors range from the darkest to nearly clear, and the clear color resembling that of the mobile phase is considered to not contain significant active compounds. Re-analyze using TLC with a stationary phase of silica gel GF254 and an optimal mobile phase ratio of n-hexane:ethyl acetate:methanol on the methanol extract. Among the various mobile phases used, the sixth mobile phase, which was n-hexane:ethyl acetate (v/v) (7:3), was the optimal mobile phase for TLC results from VLC. This was because the spots provide a fairly good separation pattern on the methanol extract, as seen in **Figure 3**.

Then, 11 eluates were eluted using TLC with the optimal mobile phase of n-hexane:ethyl acetate (v/v) (7:3). If the same spot pattern is observed under UV light, they can be combined to obtain a simpler fraction result. The results of the chromatogram pattern of the fraction can be seen in **Table 2**.

Table 2. Result of chromatogram pattern of kawista fraction

Eluates	Eluents	Total	Total of spots	Rf	Fraction code
1st	n-hexane	100% (v/v) 100 mL	0	-	F1
2nd	n- hexane : ethyl acetate	8 : 2 (v/v) 100 mL	0	-	F1
3rd	n- hexane : ethyl acetate	6 : 4 (v/v) 100 mL	0	-	F1
4th	n- hexane : ethyl acetate	4 : 6 (v/v) 100 mL	4	0.1 0.2 0.38 0.61	F2
5th	n- hexane : ethyl acetate	2 : 8 (v/v) 100 mL	4	0.1 0.2 0.38 0.61	F2
6th	ethyl acetate	100% (v/v) 100 mL	4	0.1	F2

Eluates	Eluents	Total	Total of spots	Rf	Fraction code
				0.2 0.38 0.61	
7th	ethyl acetate : methanol	8 : 2 (v/v) 100 mL	2	0.1 0.2 0.38	F3
8th	ethyl acetate : methanol	6 : 4 (v/v) 100 mL	2	0.1 0.2 0.38	F3
9th	ethyl acetate : methanol	4 : 6 (v/v) 100 mL	2	0.1 0.2	F4
10th	ethyl acetate : methanol	2 : 8 (v/v) 100 mL	2	0.1 0.2	F4
11th	methanol	100% (v/v) 100 mL	2	0.1 0.2	F4

Furthermore, the four combined fractions were subjected to quantitative antioxidant activity testing using the same methods and procedures as the antioxidant tests of the kawista leaves. The maximum wavelength obtained is 514.8 nm with an absorbance of 0.785 (within the range of 0.2 - 0.8). Next, the operating times obtained from F1, F2, F3, and F4 were at the 25th, 27th, and 29th minute, respectively. The results can be seen at **Table 3**.

Based on the results of the statistical test, it can be concluded that the vitamin C group does not have a significant difference compared to the F2 group of kawista leaves, indicating that the activity of the F2 group is equivalent to that of the positive control. The identification results of the chemical compounds in the ethyl acetate fraction of the kawista leaves only positively indicated the presence of flavonoids and tannins. Therefore, the researchers concluded that the compounds likely present in subfraction F2 are these two compounds.

Table 3. Test antioxidant activity of kawista fraction

No	Sample	IC_{50} (ppm) ± SD	AAI ± SD
1	F1	66.6703 ± 4.7025	1.5049 ± 0.1068
2	F2	29.7671 ± 2.9362	3.3808 ± 0.3255
3	F3	48.3196 ± 5.4403	2.0877 ± 0.2426
4	F4	123.4360 ± 1.3663	0.8102 ± 0.0090
5	Vitamin C	22.6424 ± 1.7887	4.4353 ± 0.3576

Separation of the F2 fraction using preparative TLC with silica gel 60 F254 stationary phase, with the spots elongated along the TLC plate. Then, a positive band containing flavonoids was produced with the mobile phase of n-butanol: ethyl acetate: water, and the band was extracted in 3 mL of methanol solvent. After the solvent evaporated, the formed crystals were further analyzed using UV-Vis and FTIR spectrophotometers.

The FTIR spectrum produced from the active fraction of kawista leaves (F2) is shown in **Figure 4**.

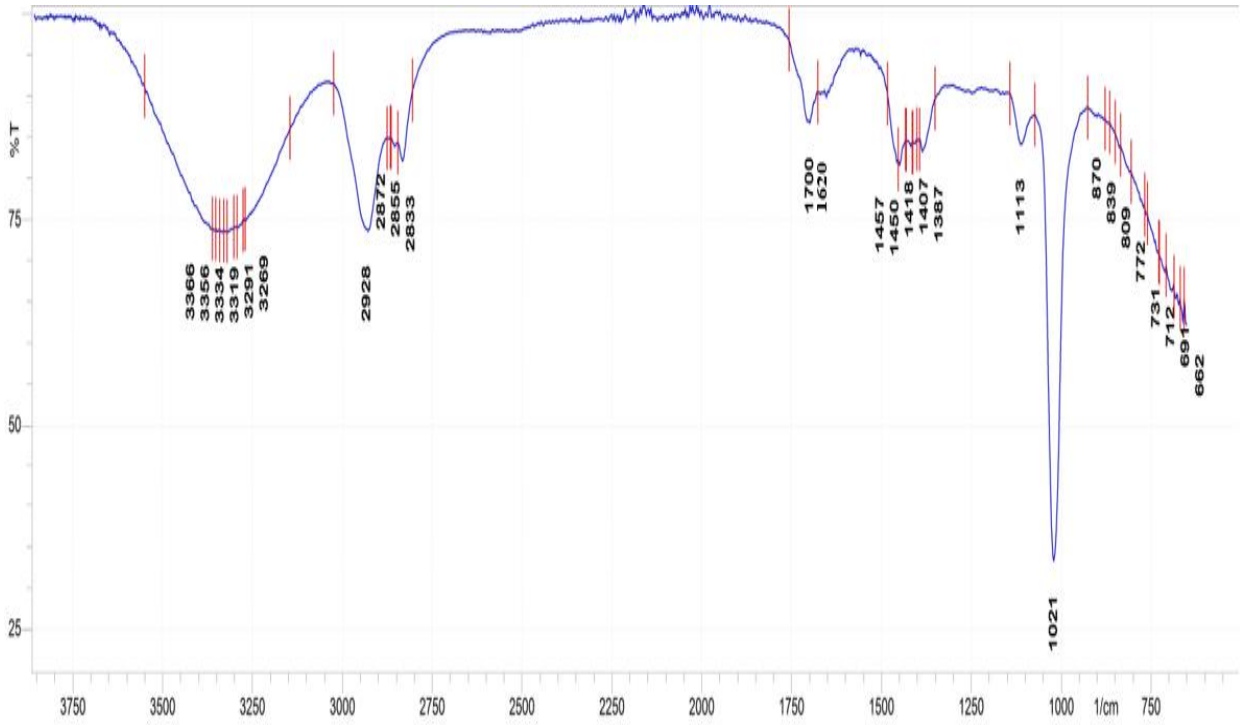

Figure 4. FTIR Spectrum of F2

The FTIR spectrum in **Figure 4** showed the presence of characteristic vibrational absorptions that identify several functional groups present in the structure of flavonoid bonds. The sharp absorption peak at the wave number 1021 cm^{-1} indicates the presence of the stretching vibration of the C–O ether bond.

The characteristics that support the presence of aromatic rings are indicated by several vibrational absorptions, including those that show the stretching vibration =C–H aromatic at wavenumbers to the left of 3000 cm^{-1} and the bending vibration =C–H aromatic at wavenumbers 870 – 691 cm^{-1}, as well as the vibrational absorptions in the wavenumber region of 1600 cm^{-1} and 1457 cm-1, which are the stretching vibrations C=C of the aromatic ring as a chromophore group typical of flavonoids in a conjugated bonding system. The absorption band at the wavenumber of 1700 cm^{-1} indicates the presence of C=O stretching vibrations of ketones, and it is close to the absorption band of C=C stretching vibrations of alkenes around the wavenumber of 1620 cm^{-1}.

Based on the interpretation of the wavelength shift data obtained, the flavonoid compound that may belong to subfraction F2 is classified as a flavonol, with the possibility of hydroxyl groups present at C-7 and C-4'. The wavelength shift in fraction F2 allows us to conclude the proposed structure of the flavanon compound as shown in **Figure 4**.

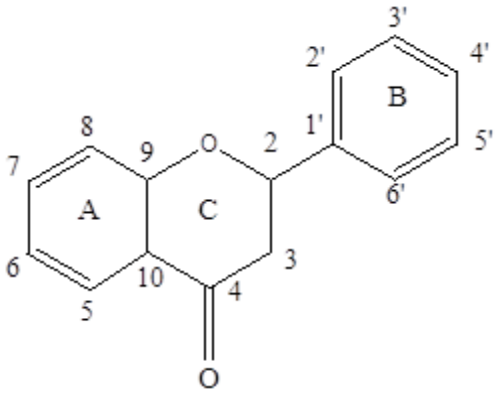

Figure 5. The structural hypothesis of flavonoid compounds in fraction F2 (flavanon)

4. Conclusion

Active compounds from kawista leaves fraction (F2) as the fraction using n-hexane and ethyl acetate solvents had the highest antioxidant agent (AAI = 3.3808 $\pm$ 0.3255) equivalent to the positive control (Vitamin C) with possibility flavonoid compound from F2 belong to the flavanon group.

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