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JCNaR
Journal of Chemical Natural Resources

Isolation of Flavonoids Compounds from Kesumba Keling Leaves (*Bixa orellana* L.)

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Abstract. Isolation of flavonoid compounds from leaves of Kesumba Keling (Bixa orellana L.). Extraction has been done with maceration by methanol solvent. The concentrated extract of methanol was added with ethyl acetate. The concentrated ethyl acetate extract was then dissolved with methanol and partitioned with n-hexane. The concentrated methanol extract was acidified by 6% HCl, then partition extracted with chloroform. The concentrated chloroform extract was separated by column chromatography with eluent n-hexane: ethyl acetate 90:10; 80:20; 70:30; 60:40; 50:50 (v/ v). The compounds were purified with TLC preparative yielding yellow gum weighing 29.7 mg with Rf=0.51. The compound was further identified by Ultraviolet-Visible spectroscopy (UV-Vis), Fourier Transform Infra Red Spectroscopy (FT-IR), and Proton Nuclear Magnetic Resonance Spectroscopy (^{1}H-NMR). Spectroscopic data show that the compound was isoflavone.

Keywords: Isolation, Flavonoids, Kesumba Keling Leaves.

Received [18 May 2022] | Revised [15 July 2022] | Accepted [10 August 2022]

1 Introduction

Flavonoids are natural compounds with the characteristic structure of two aromatic hydroxyl rings, A and B, connected by three carbon atoms. Flavonoids are found in all parts of plants, including roots, wood, bark, pollen, nectar, flowers, fruits, and seeds. Only a few have reported the presence of flavonoids in animals (Torssell, 1981; Markham, 1988).

Flavonoids are generally found in plants as glycosides. The sugar group is bonded to one or more phenolic hydroxyl groups. The hydroxyl group is always on carbon atoms 5 and 7 in ring A. The hydroxyl or alkoxyl group is in carbon numbers 3 and 4 in ring B (Sirait, 2007). Flavonoids are the most extensive polyphenolic compounds in nature found in different parts of plants in free form and as glycosides.

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Journal Homepage: http://jcnar.usu.ac.id

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The flavonoid word comes from the Latin word "flavus," which means yellow. Most of the flavonoids are yellow. It is used as a plant pigment or co-pigment. Flavonoids can also protect the human body from free radicals and reduce the risk of cancer and inflammation (Bhat, 2005; Nessa, 2003).

Flavonoids are the largest group of natural product compounds widespread in higher plants and lower plants, such as algae. Anthocyanins are abundant in coloured flowers in the angiosperm group, but in colourless flowers, they are also widely distributed and abundant (Connolly, 1991).

Kesumba Keling (*Bixa orellana*. L.) originates from tropical America. This plant is widely planted in parks, fence edges, and near fences and sometimes grows wild in forests and other places from 1-1200 m above sea level. The community uses Kesumba Keling leaves as a medicine for dysentery, dia, flatulence, jaundice, and bleeding to increase appetite (Dalimartha, 2009).

The seeds of this plant, known as annatto ("achiote" in Mexico), have a high reddish-orange dye content and are widely used as natural dyes in food and beverage ingredients. Recently, B. orellana L. has been used as the world's second most important producer of natural dyes, after caramel, found in the seed coat (Narvaez, 2001).

Several previous studies have been conducted on this Kesumba rivet plant. Marie-Claire Castello (2002) researched anti-microbial activity tests on crude extracts from parts of the Kesumba rivet plant. The study results showed that the maximum activity was found in the Kesumba rivet leaves. The extracts of the leaves and seeds of the Kesumba Keling plant have been subjected to phytochemical screening, which states that the extract contains tannins, saponins, flavonoids, alkaloids, proteins, and steroids (Selvi et al., 2011). The preliminary tests that the researchers carried out, namely the phytochemical screening test with Mg-HCl, 5% $FeCl_3$, and 10% NaOH, showed that the methanol and ethyl acetate extracts of the leaves of the Kesumba Keling plant (*B. orellana* L.) contained flavonoid compounds.

From the description above and some research literature conducted on Kesumba Keling plants, researchers are interested in examining the leaves of *B. orellana* L., a species of the Bixa Genus, especially regarding the flavonoid compounds contained in this plant.

2 Materials and Methods

2.1 Equipment

The tools used in this study were a measuring cup, beaker glass, Erlenmeyer glass, glass funnel, separating funnel, extractor, test tube, dropper pipette, capillary tube, spatula, Bÿchi R-114 rotary evaporator, distillation apparatus, bottom flask, column chromatography, 254/356 nm UV lamp,

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analytical balance, water bath, vials, chamber, stands and clamps, stir bar, UV Visible spectrophotometer, IR spectrophotometer, and ^{1}HNMR spectrometer.

2.2 Materials

The materials used were leaves, methanol, n-hexane, ethyl acetate, aquadest, chloroform, acetone, benzene, silica gel 40 (70-230 mesh) ASTM, 5% FeCl3, 10% NaOH, Mg powder, HCl(p)), 6% HCl, TLC Plantae, Benedict's reagent.

2.3 Provision of Samples

Samples studied were Kesumba Keling leaves obtained from Sentang, Kisaran Timur District, Asahan Regency, North Sumatra. Kesumba Keling leaves are dried in the open air and then pulverized until a fine dry powder of 1000 g of Kesumba Keling leaves is obtained.

2.4 Phytochemical Screening Test

A preliminary qualitative test was carried out to determine the flavonoid compounds from the Kesumba Keling plant. As much as 10 g of fine dry powder of dried Kesumba Keling leaves was put into two Erlenmeyer glasses. Add 100 mL of methanol to the Erlenmeyer glass, then set aside for one night. Next, filtered and divided each sample extract was placed into 3 test tubes. The plate is inserted into a saturated solvent mixture vessel, then closed and eluted. The dish that has been eluted is removed from the vessel and then dried. The stains formed were observed under UV light, then fixed with 5% $FeCl_3$ reagent. Observe the colour of the spots that appear and calculate the Rf values obtained. - Add each reactant: a. Tube I with $FeCl_3$ 5% to produce a black solution, b. Tube II with NaOH 10% produces a yellowish-green solution, and c. Tube III: with Mg-HCl to produce a pink solution

2.5 Extraction of Plant Leaves Kesumba Keling

The fine dry powder of the leaves of the Kesumba Keling plant was weighed as much as 980 g, and then it was macerated with ± 6 L of methanol until all samples were submerged and left for 24 hours. The maceration was accommodated and concentrated using a rotary evaporator to obtain a concentrated methanol extract and tested with $FeCl_3$ 5% and then evaporated until all the methanol solvent evaporated. Then the tannins were separated by dissolving the concentrated methanol extract with ethyl acetate until the flavonoids were negative and filtered. The filtrate was then put in a rotary evaporator and then evaporated until all the ethyl acetate solvent had evaporated. Then the concentrated ethyl acetate extract was tested with $FeCl_3$ 5%.

Ethyl acetate concentrated extract was dissolved with methanol and partitioned with n-hexane until the n-hexane layer was transparent. The methanol layer was separated from the n-hexane layer, then tested with $FeCl_3$ 5% and concentrated again with a rotary evaporator, and evaporated again until obtained 100 ml of methanol extract was tested for sugar content with Benedict's

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reagent, then hydrolyzed using HCl 6%while heated over a water bath for ± 60 minutes. Then filtered and the filtrate obtained was partitioned with chloroform extraction three times to a layer of flavonoid-negative chloroform. The chloroform extract was concentrated using a rotary evaporator and re-evaporated to obtain 1.7 g concentrated chloroform extract.

2.6 Thin Layer Chromatography (TLC) Analysis

Thin Layer Chromatography analysis was performed on the chloroform extract using Merck's silica gel 60F254 stationary phase. This analysis is intended to find the appropriate solvent system and ratio for column chromatography. The mobile phase used was a mixture of solvents n-hexane: ethyl acetate with a ratio of 90:10, 80:20, 70:30, 60:40, 50:50, and 40:60(v/v).

10 ml of n-hexane: ethyl acetate 90:10 (v/v) mixed mobile phase solution was saturated in the chromatography vessel. Dot the concentrated chloroform extract on the activated TLC plate. The plate is inserted into a saturated solvent mixture vessel, then closed and eluted. The dish that has been eluted is removed from the vessel and then dried. The stains formed were observed under UV light, then fixed with 5% FeCl3 reagent. Observe the colour of the spots that appear and calculate the Rf values obtained. The same treatment was carried out for the ratio of n-hexane: ethyl acetate with a percentage of 80:20, 70:30, 60:40, 50:50, and 40:60 (v/v).

2.7 Isolation of Flavonoid Compounds Using Column Chromatography

Isolation of flavonoid compounds by column chromatography was carried out on the concentrated chloroform extract that had been obtained. The stationary phase used was silica gel 40 (70-230 mesh) ASTM, and the mobile phase was n-hexane 100%, n-hexane: ethyl acetate solvent mixture with a ratio of 90:10, 80:20, 70:30, 60:40, 50:50 (v/v).

Column chromatography strung. First, silica gel 40 (70-230 mesh) ASTM is slurried using n-hexane, stirred until homogeneous, and then put into the chromatography column. They were then eluted using n-hexane 100% until the silica gel was solid and homogeneous. Pulverized 1.7 g chloroform concentrated extract with silica gel with chloroform solvent, then placed into column chromatography containing silica gel slurry, then added the mobile phase n-hexane: ethyl acetate 90:10 (v/v) slowly and adjusted so that the flowing phase out of the column as much as the addition of mobile phase from above. The polarity was increased by adding the mobile phase n-hexane: ethyl acetate in the ratio 80:20 (v/v), 70:30 (v/v), 60:40 (v/v), and 50:50 (v/v). The results were collected in vials every ± 10 mL, then in TLC, combined with fractions with the same Rf value, tested with $FeCl_3$ 5%, and then evaporated to form the gum.

2.8 Purification

The gum obtained from isolation by column chromatography was redissolved with ethyl acetate and then analyzed by TLC to determine whether the compound obtained was pure or not while at

the same time looking for a suitable mobile phase for preparative TLC. Benzene: acetone 70:30 (v/v) was the mobile phase, showing the best separation for further use to saturate the preparative TLC vessel. Meanwhile, the gum that dissolved earlier was dabbed slowly and evenly along the bottom edge of the activated TLC plate. The plate is inserted into a saturated solvent mixture vessel and then closed. After elution, the dish was removed from the vessel, dried, and the results were examined under UV light. Each zone was marked and dredged and then eluted with methanol: ethyl acetate (1:1). The elution results were evaporated to obtain yellow gum.

2.9 Test the Purity of Isolation Results by Thin Layer Chromatography (TLC)

Gum purity was tested by thin layer chromatography using silica gel 60 F_{254} stationary phase with n-hexane: ethyl acetate 50:50 (v/v) and benzene: acetone 70:30 (v/v) mobile phases. Put 10 mL of the mobile phase solution into a thin layer chromatography vessel, then saturate it. Gum previously dissolved with ethyl acetate was spotted on the TLC plate. Insert the TLC plate into the saturated thin-layer chromatography vessel. After the mobile phase solvent seeped to the mark limit, the TLC plate was removed from the vessel, dried, observed under UV light, and fixed using $FeCl_3$ 5% reagent in methanol to produce black spots indicating the presence of flavonoid compounds.

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3 RESULT AND DISCUSSION

From the preliminary screening of the methanol and ethyl acetate extracts from the leaves of the Kesumba Keling plant (*B.orelana* L.) in the presence of the addition of colour reagents to determine the class of compounds. The chemical contained using a flavonoid reagent was a positive sample containing flavonoids. The elution results for fractions 43-58; preparative TLC was carried out with benzene: acetone 70:30 (v/v) to obtain a purer isolated compound. In order to get the isolated compound in the form of yellow gum, weighing 29.7 mg, and the Rf value which indicates the isoflavone group (Figure 1):

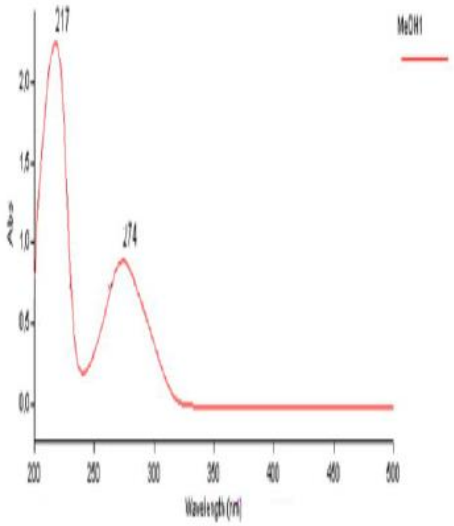

Figure 1. UV-Visible Spectrum of Isolation Compound Results

The results of the FT-IR spectrophotometer analysis of the isolated gum (Figure 2) produce absorption bands in the wave number region which can be seen in Table 1:

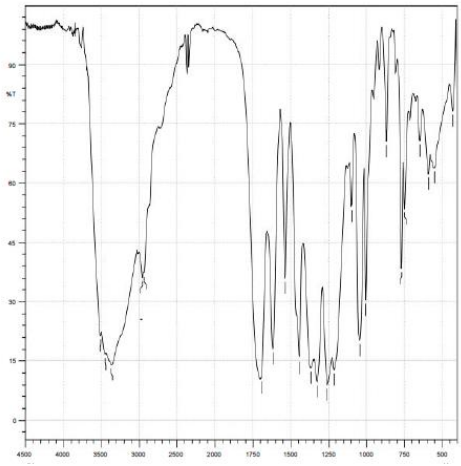

Figure 2. FTIR spectrum isolated compound

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Table 1. Interpretation of the FT-IR Spectrum of the Isolated Compound Intensity Wavenumber (cm-1)

No	Formula Molecule	Area (%)	Time Retention (Minute)	Massa Relative	Compound Allegedly
1	$C_6H_{10}S$	13.06	3.529	114	Allyl sulfide
2	C_4H_8S	11.87	4.686	120	Methyl allyl disulfide
3	$C_4H_8S_2$	0.72	5.255	120	Trans propenyl methyl disulfide
4	$C_2H_6S_3$	0.44	5.811	126	Dimethyl trisulfide
5	$C_6H_{10}S_2$	44.98	8.308	146	Diallyl disulfide
6	B_5H_9	1.18	8.492	113	Pentaborane
7	$C_6H_{10}S_2$	2.06	8.626	146	Diallyl disulfide
8	$C_6H_{10}S_2$	6.85	8.729	146	Diallyl disulfide
9	$C_4H_8S_3$	4.84	9.332	152	Methyl allyl trisulfide
10	$C_6H_{10}S_3$	13.63	12.120	178	1.3 Diallil tris sulfide
11	$C_6H_{10}S_2$	0.36		15.491	146 Diallyl disulfide

The Proton Nuclear Magnetic Resonance Spectrum (1HNMR) of the isolated compounds can be seen in Figure 3:

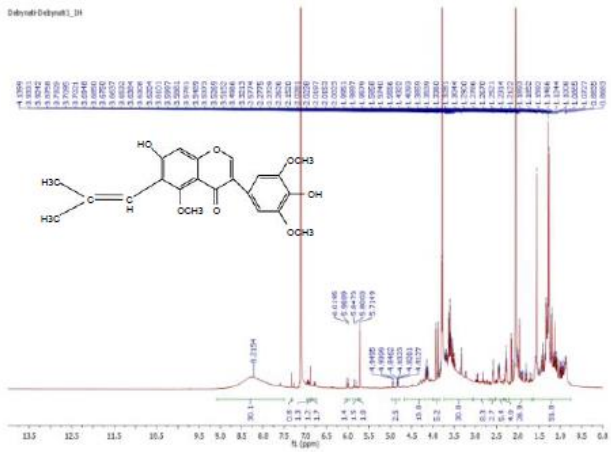

Figure 3. The ^{1}H-NMR spectrum of the resulting compound isolation

From the results of the interpretation of the UV Visible spectrum with methanol solvent (Figure 4), it gives a wavelength (ÿ max) of 274.00 nm for band II. It shows that the isolated compounds match the UV-Visible spectra of the flavonoid comparator compound, namely Isoflavones.

From the ^{1}H-NMR spectrum of the isolated compound, the range supports that the isolated compound is a flavonoid compound of the isoflavone group. It is evidenced by the presence of doublet peaks in the region of 7.2648-7.2686 ppm, indicating the presence of H-2' and H-6' protons in ring B. And the chemical shift of 8.2154 ppm indicates the presence of OH protons. A chemical shift of 6.8813 ppm indicates the presence of H-8 protons, and at 5.7149 ppm, the presence of H-Vinilik protons. The chemical shift of 7.3242 ppm indicates the presence of H-2

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protons. At 1.2670 ppm and 1.2900 ppm, indicate the presence of – CH_3 protons. The chemical shifts of 3.9242 ppm, 3.9331 ppm, and 3.7929 ppm indicate the presence of OCH_3 protons.
In addition, from the infrared spectrum of the isolated compounds obtained at wave numbers 3448.72-3367.71 cm-1, the broad peaks indicate the presence of –OH stretching vibrations. At wave numbers 2956.87-2935.66 cm^{-1}, the moderate peak indicates the presence of CH stretching vibrations. At wave number 1695.43 cm^{-1}, the medium peak indicates a stretching vibration of the C=O double bond of the ketone. At wave number 1616.35 cm^{-1}, the medium peak indicates a C=C stretching vibration of the alkene system. At wave number 1259.52 cm^{-1}, the moderate peak indicates CO stretching vibrations from the alcohol groups.

At wave number 1097.50 cm^{-1}, the sharp peak indicates C-CO C stretching vibrations from the ketone group. At wave number 1002.98 $cm^{-1,}$ a moderate peak indicates stretching vibrations of the symmetrical COC. At wave number 867.97 cm^{-1}, the moderate peak indicates the presence of aromatic CH bending vibrations. From the results of the discussion above, UV-Vis spectra, infrared, and ^{1}H NMR data, and based on phytochemical screening, it can be assumed that the compound isolated from the leaves of the Kesumba rivet (*B. Orellana* L.) plant is a flavonoid compound of the isoflavone group with a framework (Figure 4):

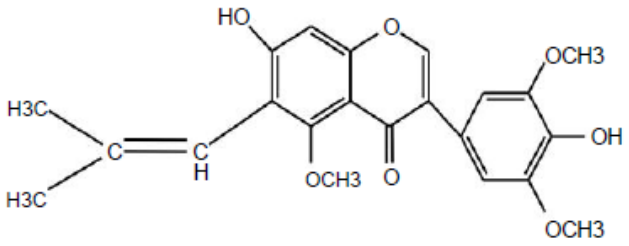

Figure 4. Isoflavone structure of isolated compounds

4 Conclusion

The isolation obtained from 980 g of the leaves of the Kesumba rivet (B.orellanaL.) plant was a yellow gum, obtained as much as 29.7 mg, Rf = 0.51 with the eluent n-hexane: ethyl acetate 50:50 (v/v). Based on the results of the phytochemical screening of flavonoids on gum isolated from the leaves of Kesumba Keling (*B. orellana* L.), the results showed positive results for flavonoid compounds. The results of analysis using UV-Visible Spectrophotometry, Infrared Spectrophotometry (FT-IR), and Proton Nuclear Magnetic Resonance Spectrometry (^{1}H-NMR) showed that the compounds isolated from the leaves of the Kesumba Keling plant (*B. orellana* L.) were suspected to be flavonoid compounds belonging to the isoflavone group.

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