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Research Article

Antioxidant activity and identification of flavonoid compounds in Patat leaves (*Phrynium capitatum*) ethyl acetate extract

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Keywords

Antioxidant
Flavonoids
Patat
Total flavonoids
UHPLC MS/MS

Abstract

Flavonoids are the biggest compound from the phenolic group that has the function of antioxidants. One of the plants with flavonoids is patat (*Phrynium capitatum*), which is usually used as food wrapping material. This study aims to quantify total flavonoid content, antioxidant activity and identify flavonoids from patat leaves ethyl acetate extract. Patat leaves sample cleaned, dried, and grinded. Grinded patat leaves were macerated gradually for 2 × 24 hours with n-hexane, ethyl acetate, and methanol solvent. The extract undergoing total flavonoids test guided with AlCl3 and CH3COONa. Extract with the biggest flavonoids content proceed to antioxidant activity test by SOD (superoxide dismutase) method and analyzed for the flavonoid structure by UHPLC MS/MS (ORBITRAP HRMS). The biggest total flavonoids are ethyl acetate extract with 8.678 mg QE/g; then methanol extract with 5.296 mg QE/g; and n-hexane extract was not tested because of negative results in the qualitative test. The antioxidant activity of the ethyl acetate extract of patat leaves is classified as inactive, with an Ic50 value of 488.299 ppm. The low antioxidant activity is due to the sample, which is still a matrix containing many compounds. The flavonoid compounds identified by its fragmentation pattern were kaempferide, formononetin, and pinostrobin.

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Introduction

One of the uniqueness of the Indonesian people is the use of leaves as food wrapping material. An example is patat leaves (*Phrynium capitatum*) as rice wrappers (Obet et al. 2020). Patat leaves are traditionally used as traditional medicine (Noviadji, 2014; Wijaya et al. 2014; Lestari & Christina, 2018). Another function of patat leaves was documented by Perme et al. (2015). Traditionally, patat leaves are believed to be used as anti-diabetic, analgesic, and anti-hyperglycemic. Information about the content of patat leaves is still limited. Badar (2006) researched the Physico-chemical properties and nutritional content of patat leaves. Fathoni (2021) examined this further by conducting multilevel maceration. The phytochemical tests indicated the presence of alkaloids, triterpenoids, saponins, steroids, phenolic hydroquinones, flavonoids, and tannins which varied depending on the polarity of the solvent. Based on research by Obet et al. (2020), the ethanol extract of patat leaves (*Phrynium capitatum*) contains flavonoids, phenolics, and steroids. Apart from the general function of patat leaves, there has not been much research on the content and functions of patat leaves (*Phrynium capitatum*).

This study aims to quantify total flavonoid content, antioxidant activity and identify flavonoids from patat leaves ethyl acetate extract. Patat leaves sample is prepared to be macerated gradually for 2 × 24 hours with n-hexane, ethyl acetate, and methanol solvent. The extract undergoing total flavonoids test guided with AlCl3 and CH3COONa. Extract with the biggest flavonoid content then proceeded to antioxidant activity test by

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SOD (superoxide dismutase) method and analyzed for the flavonoid structure by UHPLC MS/MS (ORBITRAP HRMS).

Method

Materials and tools

The materials used were patat leaves which were ready to be used as wrappers (aged about 3 months) from Bogor, technical grade methanol, technical grade ethyl acetate, technical grade n-hexane, CH3COONa (merck), 5% AlCl3 in methanol, filter paper, folin-ciocalteu, Na2CO3, and a set of materials for testing enzyme activity was 0.5 mM riboflavin; 10 mM NBT (Nitroblue Tetrazolium); 1 M Phosphate Buffer (pH 7.4); and 0.1 M TEMED (Tetramethylethylenediamine).

The tools used were maceration containers, analytical balances, various standard chemical laboratory glassware, rotary evaporators, vacuum, UV-Vis Spectrophotometer (Agilent), UHPLC MS/MS (ORBITRAP HRMS) Instrument (ThermoScientific).

Sample preparation

The cleaned sample were air-dried at room temperature and then grinded. Extraction was carried out by multiple maceration method. 284 g sample was macerated with 3 L of n-hexane. After 24 hours, the filtrate was taken and then the solvent replaced with the same amount of n-hexane. The residue was macerated alternately with ethyl acetate and methanol using the same method. The total filtrate obtained for each solvent was 6 L. Crude extract was obtained by concentrating the filtrate at 40°C in vacum condition with a rotary evaporator.

Flavonoid content assay

Sample for qualitative test was prepared by dissolving the concentrated sample to a concentration of 100 ppm in methanol. The test was carried out by putting 0.2 mL of the sample in a drop plate. A change in color to red, orange or green after adding 0.05 mL of Mg and 0.1 mL of concentrated HCl indicates that the sample contains flavonoid compounds (Kristanti et al. 2008; Wijaya et al. 2014).

The quantitative test of flavonoids using a UV-Vis spectrophotometer with quercetin as a standard solution. The quercetin solution was prepared with a concentration series of 50 ppm; 25 ppm; 12.5 ppm; 6.25 ppm; 3.125 ppm; and 0 ppm. The samples were prepared by adding 0.5 mL of 1000 ppm sample with 1,5 mL metanol. Samples and standards were reacted with 0,1 mL AlCl3 10%, 0,1 mL CH3COONa 1M and 2.8 mL aquades. Absorbance readings were conducted at a wavelength of 421 nm after incubation for 30 minutes on the sample. The data obtained from the standard solution is processed in the form of linear regression and will be used as a calibration curve (Aryal et al. 2019; Chotimah, 2019).

Antioxidant activity test

Antioxidant testing using the SOD (Superoxide Dismutase) method (Kostyuk et al. 2007). The test reagent is made into working solutions 1 and 2. Working solution 1 contains 191.1 μL of aquabides; 3.2 µL phosphate buffer; 1.7 µL NBT (Nitroblue Tetrazolium); 1.6 µL TEMED (Tetramethylethylenediamine); and 2.4 µL riboflavin. Working solution 2 was prepared with the same composition as riboflavin replaced with distilled water. Working solution 1 was used for blank 1 and sample, while working solution 2 was used for blanks 2 and 3. The concentration series used is 625 ppm; 312.5 ppm; 156.25 ppm; and 78.125 ppm. Irradiation with a fluorescent lamp (20 w, 20 cm) was carried out for 10 minutes and measured with a microplate reader spectrophotometer at 560 nm. Percent inhibition is calculated through the following formula. Percent inhibition is calculated through the following formula (Sigma-Aldrich, 2018):

$$\%inhibition = \frac{(blank\ 1 - blank\ 3) - (sample - blank\ 2)}{(blank\ 1 - blank\ 3)}\ x\ 100$$

Structure elucidation

Structural elucidation with the UHPLC MS/MS (ORBITRAP HRMS) Instrument (ThermoScientific). Separation column using C18 (100 x 2.1 mm, 1.5 μm). The eluent uses H2O + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B) flowing in a gradient system [0-1 minute (5% B), 1-25 minutes (5-95% B), 25- 28 minutes (95%B), 28-30 minutes (5%B)]. Ionization systems used are positive and negative ESI (Electrospray Ionization). Compound analysis was carried out in the range of 100-1500 m/z. The compound structure was elucidated by analysis of fragmentation patterns and compared with the reference fragment.

Results and Discussion

The extraction yields of n-hexane, ethyl acetate, and methanol were 0.21%, 2.04% and 0.77%, respectively. The yield was obtained by calculating the percentage of concentrated extract compared to the initial sample mass. Ethyl acetate extract produced the highest yield, consistent with the results of previous studies (Fathoni, 2021).

The qualitative test of the flavonoids showed positive results for the ethyl acetate and methanol extracts (Fig. 1). Flavonoids in plants are generally in the form of glycosides; the addition of HCl is intended to hydrolyze flavonoid glycosides. In hydrolysis products, the addition of Mg reduces flavonoids and creates a complex compound that is red, orange, or green (Ikalinus et al. 2015; Panche et al. 2016). The reaction is shown in Fig. 2.

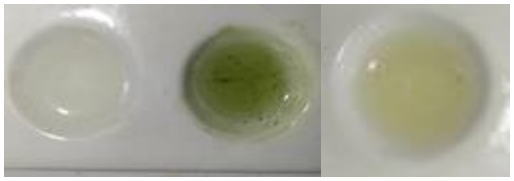

Figure 1. Qualitative phytochemical test of flavonoids (a) n-hexane extract, (b) ethyl acetate extract, (c) methanol extract.

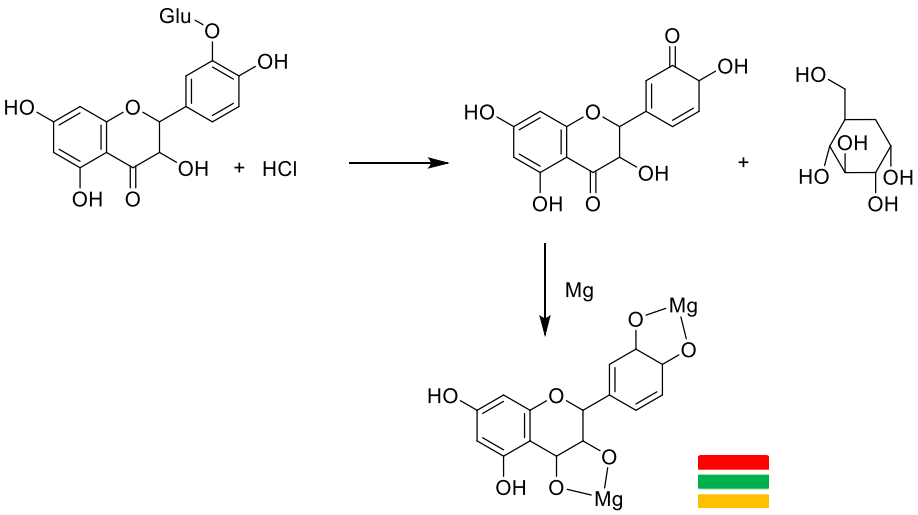

Figure 2. The reaction of flavonoids with Willstater reagent (Nugrahani et al. 2016).

The total flavonoid test was carried out on ethyl acetate extract and methanol extract. The total flavonoid content in the ethyl acetate extract and methanol extract were 8.678 mg QE/g and 5.296 mg QE/g, respectively (Table 1). The highest total flavonoid content in the ethyl acetate extract can be attributed to the polarity character of the flavonoids (Wikanta et al. 2012; Simorangkir et al. 2019; Juwitaningsih et al. 2022). Flavonoids with polar properties are usually flavonoid glycoside compounds, while the aglycone forms are less polar, such as isoflavones and flavonones (Andersen & Markham, 2006; Wang et al. 2018). As a comparison, Rachmi (2016) extracted Jati Belanda (*Guazuma ulmifolia* Lam.) leaves using the same method. The results showed the highest total flavonoid content in the ethyl acetate extract.

Table 1. The result of flavonoid test

Ekstrak	Qualitative test	Total flavonoid content (Mg QE/g)
Metanol	+	5.296
Etil asetat	+	8.678
N-heksana	-	N/A

An antioxidant activity test using the SOD (Superoxide dismutase) method was carried out on ethyl acetate extract. The principle of antioxidant activity test using the SOD (Superoxide dismutase) method is the formation of superoxide anions which will reduce NBT (Nitroblue Tetrazolium) to NBT-diformazan, which is purple. The superoxide anion comes from riboflavin which undergoes photoreduction in the presence of TEMED (Tetramethylethylenediamine) to become semiquinone. This semiquinone will later donate its electrons to oxygen so that a superoxide anion is formed. The value of the antioxidant activity of SOD

(Superoxide dismutase) will be inversely proportional to the amount of formazan formed (Deawati et al. 2017). The regression equation resulting from the standard measurement is y = 0.128x – 11.428 with R2 = 0.9507. Based on sample absorbance measurements and calculations, an IC50 value of 488.299 ppm was obtained, which was categorized as inactive (Saputra, 2020). The low antioxidant activity is due to the sample, which is still a matrix containing many compounds.

Mass spectroscopic analysis was performed on ethyl acetate extract with a UHPLC MS/MS (ORBITRAP HRMS) Instrument (ThermoScientific). Reverse chromatography system was applied to separate the compound components in the extract. Polar compounds will elute first, followed by less polar compounds (Nagy & Vékey, 2008). The identified flavonoid compounds can be seen in Table 2. Identification of compounds are based on fragmentation patterns and compared with a database of compound spectra. Fragments are generated with a collision energy of 18, 35, and 53 eV.

Table 2. The identified flavonoid compounds

Retention Time (minutes)	Molecular Weigh (Da)	MS1 (m/z)	MS2 (m/z)	Similarity (%)	Compound name
12.96	300.0634	299.05585 [M-H]	299.05585; 284.03259; 256.03279	91.3	Kaempferide
14.05	268.0736	269.08035 [M+H]	269.08035; 254.05664; 226.06169	92.3	Formononetin
19.12	270.0892	271.09570 [M+H]	271.09570; 173.05945; 167.03374; 131.04906	94.8	Pinostrobin

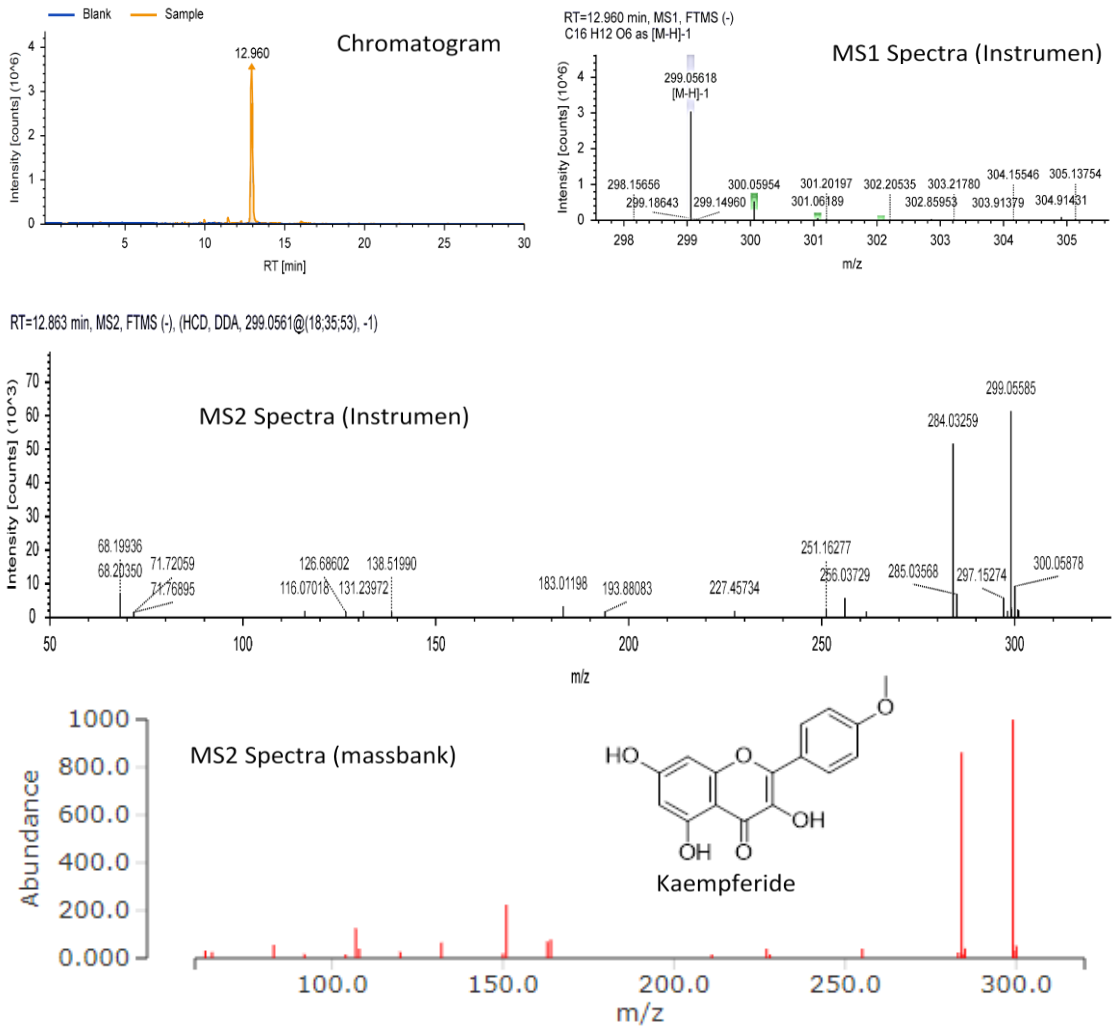

Figure 3. Chromatogram and spectra of kaempferide.

Kaempferide ($C_{16}H_{12}O_6$) was detected at a retention time of 12.96 minutes (Fig. 3). This compound has a molecular weight of 300.05585 Da, ionizes in the negative ESI mode, and loses one H+ atom to give a parent ion m/z 299.05585 [M-H]. Fragmentation of parent ions in MS2 produces fragment ions m/z 284 (loss of CH_3) and m/z 256 (loss of CH_3 and CO), as shown in Fig. 4. Analysis of the spectra shows a similarity of 91.3% to the reference. Proposed fragmentation of kaempferide is shown in Fig. 5. Kaempferide has previously been identified in Alpinia officinarum Hance (Hongrui et al. 2016) and Chromolaena odorata Linn (Nath et al. 2015; Kumkarnjana et al. 2019). Kaempferide has the potential as an anticancer and is proven to induce apoptosis in cervical cancer cells (Nath et al. 2015).

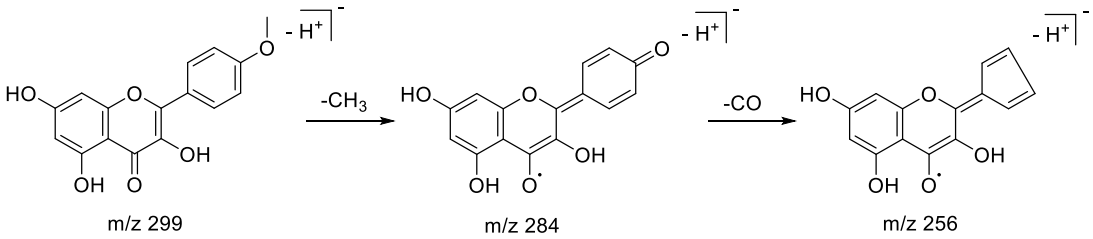

Figure 4. Proposed fragmentation of kaempferide

Formononetin ($C_{16}H_{12}O_4$) was detected at a retention time of 14,05 minutes (Fig. 5). It was previously identified in red propolis (das Neves et al. 2016) and Astragalus membranaceus (Nie et al. 2018). This compound has adipocyte thermogenesis activity, which is related to the ability to mitigate obesity (Nie et al. 2018). Another activity is as a fungicide against Candida Sp. (das Neves et al. 2016).

Formononetin has a molecular weight of 268,0736 Da, ionizes in the positive ESI mode, and binds one H+ atom to give a parent ion m/z 269,08035 [M+H]. Fragmentation of parent ions in MS2 produces fragment ions m/z 254 (loss of CH_3) and m/z 226 (loss of CH_3 and CO). Other fragments arise from further fragmentation. Analysis of the spectra shows a similarity of 92.3% to the reference. The proposed fragmentation pattern formed can be seen in Fig. 6.

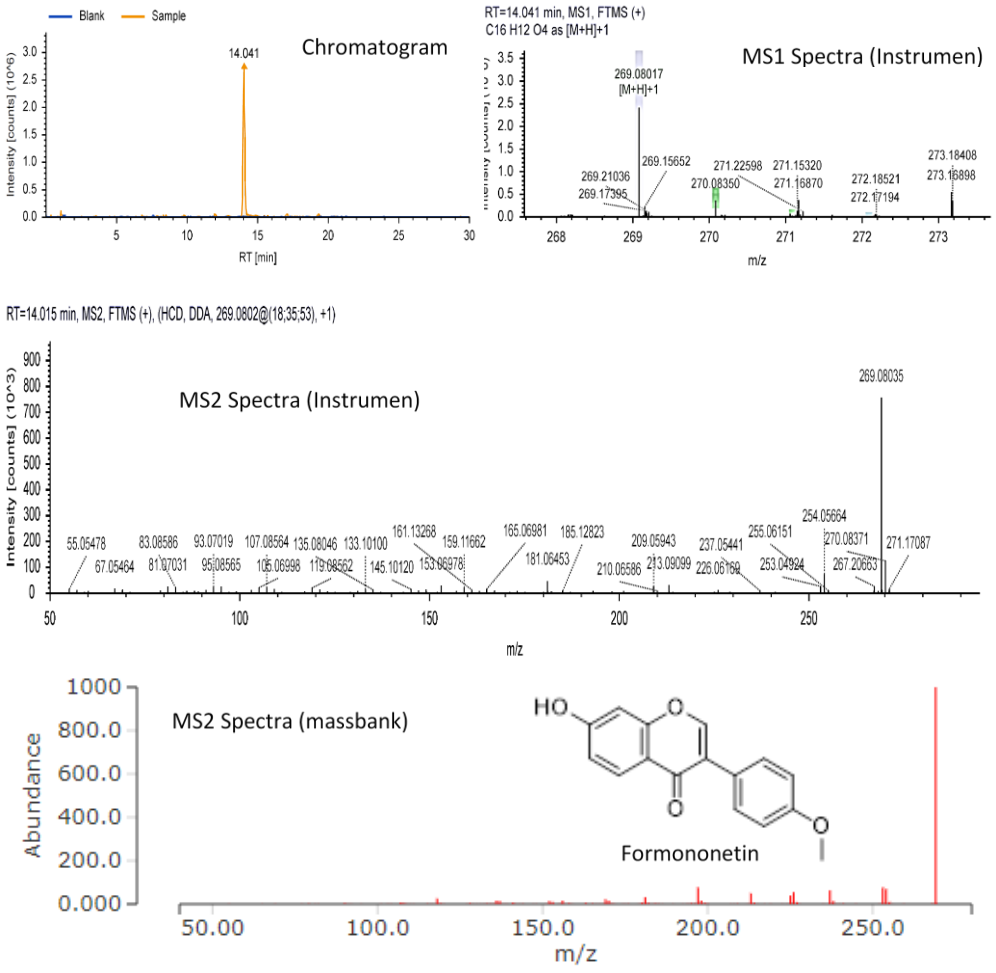

Figure 5. Chromatogram and spectra of formononetin.

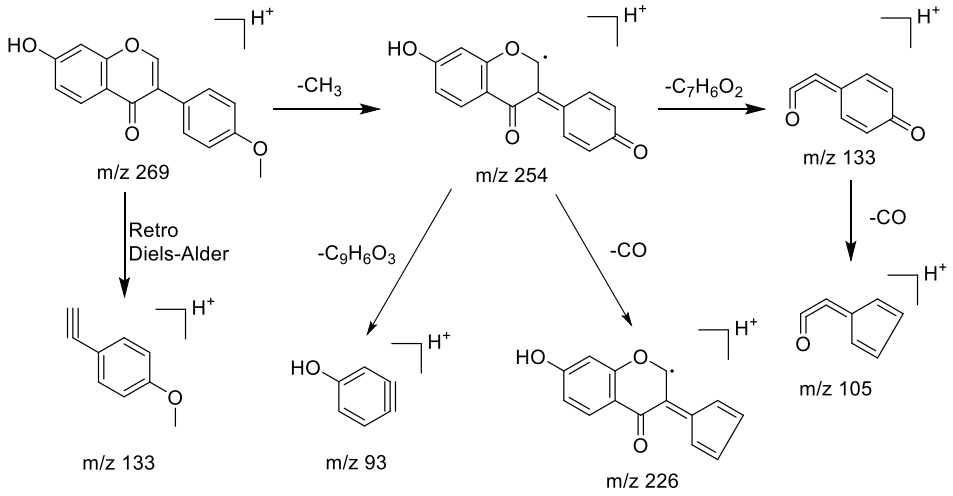

Figure 6. Proposed fragmentation of formononetin.

Pinostrobin ($C_{16}H_{14}O_4$) was detected at a retention time of 19.12 minutes (Fig. 7). Pinostrobin has been identified in Artocarpus odoratissimus (Nyokat et al. 2017). This compound has a molecular weight of 270.0892 Da, ionizes in the positive ESI mode, and binds one H+ atom to give a parent ion m/z 271.09570 [M+H]. Fragmentation of parent ions in MS2 produces fragment ions m/z 173; 167; 131. A similar pattern was reported by (Sun et al. 2020) in a study studying the effects of pinostrobin in rats. The proposed fragmentation pattern formed can be seen in Fig. 8. Analysis of the spectra shows a similarity of 94.8% to the reference.

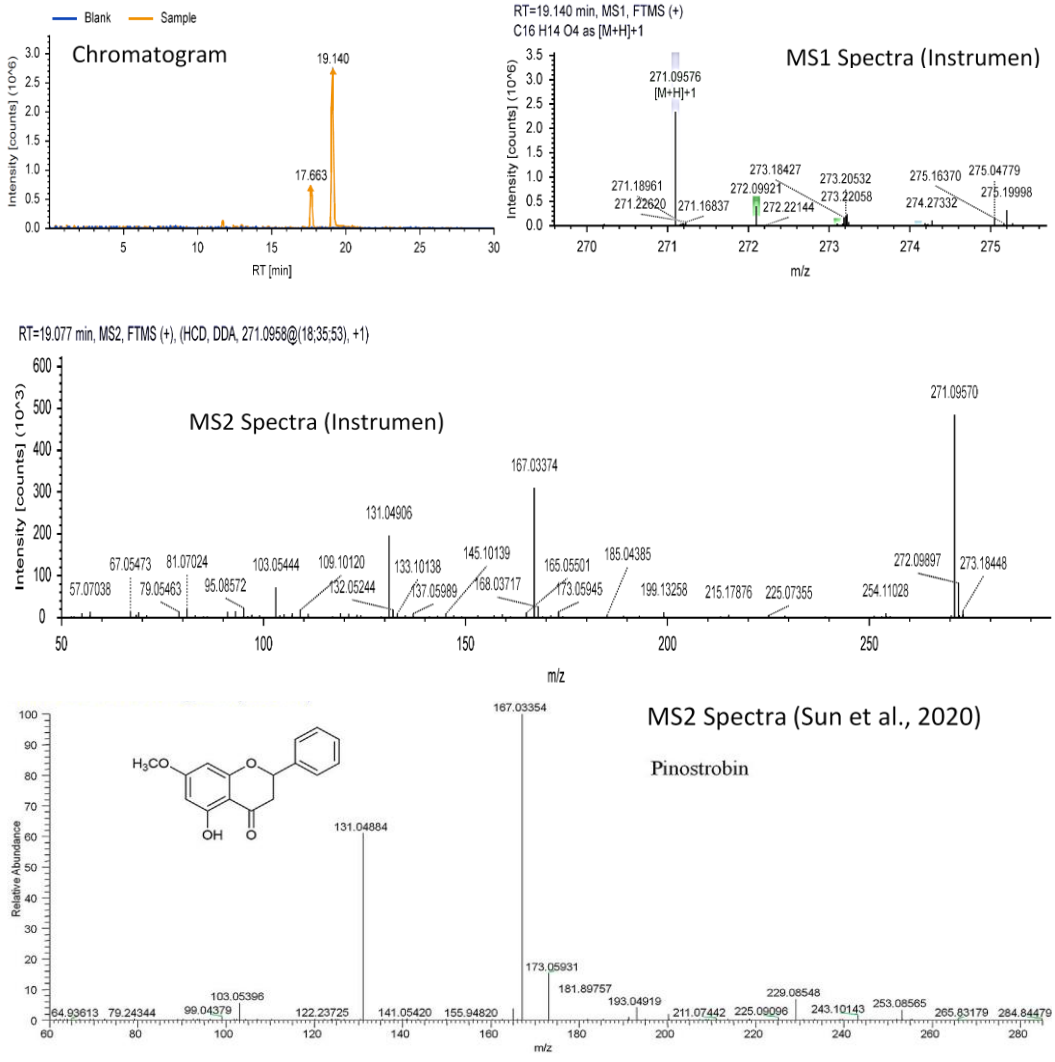

Figure 7. Chromatogram and spectra of pinostrobin.

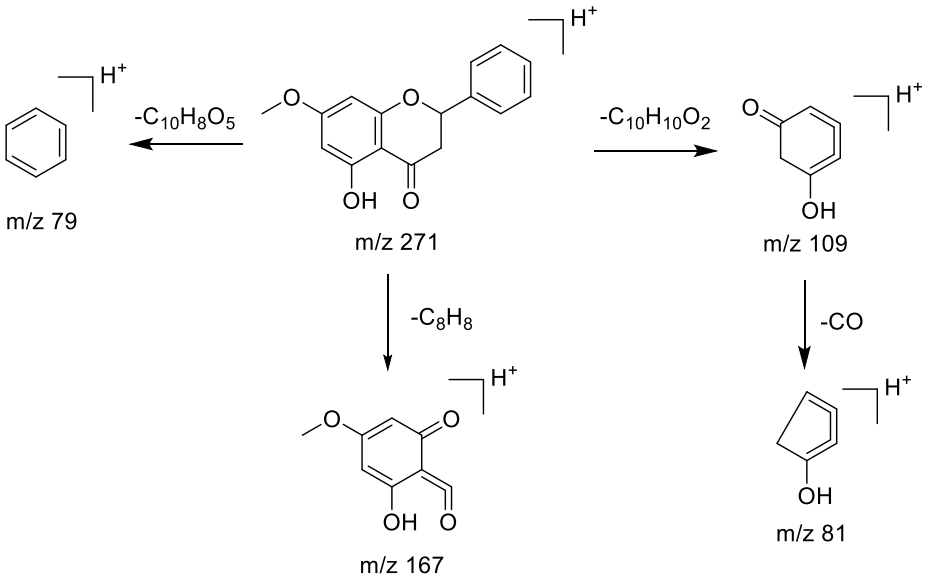

Figure 8. Proposed fragmentation of pinostrobin.

Conclusion

The biggest total flavonoids are in ethyl acetate extract with 8.678 mg QE/g; The antioxidant activity of the ethyl acetate extract of patat leaves is classified as very weak with an Ic50 value of 488.299 ppm. The flavonoid compounds identified by its fragmentation pattern were kaempferide, formononetin, and pinostrobin.

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