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Research Article

Column Chromatography Fractionation and Antioxidant Activity of *Passiflora foetida* Leaves

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Abstract

Available synthetic antioxidants have been reported to have mutagenic and toxic effects. On the other hand, natural antioxidants show their superiority as they are not or less toxic. *Passiflora foetida* has the potential as an antioxidant, but the investigation of the antioxidant activity of the *P. foetida* chromatography column fraction has not been reported. This studied aims to investigate the antioxidant activity of the column chromatographic fractions of *P. foetida* leaves. An antioxidant assay using the DPPH and FRAP methods. The extraction was carried out by graded maceration, then fractionation using column chromatography. The antioxidant activity test was carried out using the DPPH and FRAP methods. Thin layer chromatography analysis was performed to determine the chromatogram pattern. The EC_{50} using DPPH method from *n*-hexane extract: 129.035 µg/mL, ethyl acetate extract: 206.398 µg/mL, methanol extract: 97.453 µg/mL; while the EC_{50} using FRAP method from *n*-hexane extract: 67.851 µg/mL, ethyl acetate extract : 68.981 µg/mL, and methanol extract: 58.787 µg/mL. Column chromatography fractions have antioxidant activity, with FMetPF6 as the fraction with the best activity, with percent inhibition 41.85±1.96 at concentration 25 µg/mL (DPPH), and with percent antioxidant activity 26.03±0.84 at concentration 9 µg/mL (FRAP). *Passiflora foetida* leaves have great potential as an antioxidant; both the extract and its fractions have antioxidant activity. The FMetPF6 has the best activity compare to other extracts and fractions. Further analysis to determine the various compounds in FMetPF6 using LC-MS/MS will facilitate the active compound's isolation.

Keywords:
Antioxidant
DPPH
Fractionation
FRAP
Passiflora foetida

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INTRODUCTION

Oxidative stress is represented by elevated levels of reactive oxygen species (ROS), which impairs the balance of intracellular reduction-oxidation, and this is seen in a wide variety of diseases[1]. Reactive oxygen species (ROS) such as hydroxyl radicals (OH), singlet oxygen (O_2), hydrogen peroxide free radicals (H_2O_2), ozone (O_3), and organic peroxides (ROOH) are thought to induce oxidative stress[2]. Oxidative stress occurs due to a lack of balance between reactive oxygen species (ROS) and antioxidant defenses[3]. This imbalance is a major cause of various diseases today, including cardiovascular disease and diabetes. It is also known to exacerbate DNA mutation and damage, cell damage, aging, neurodegenerative diseases, play a role in oncogenesis, and many other digestive diseases, including ulcers and gastrointestinal disorders[4].

Antioxidants are believed to be important in dealing with oxidative stress caused by ROS. Various synthetic antioxidants are available primarily in the food and beverage industry to preserve and prolong product stability, but these are often

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associated with nutrient loss and quality degradation[5]. Available synthetic antioxidants have been reported to have mutagenic and toxic effects. On the other hand, natural antioxidants show their superiority as they are not or less toxic[6]. Natural antioxidants are highly recommended for oxidative stress because they are less toxic than synthetic antioxidants[7]. The natural product offers a superb source of varied phytochemicals like alkaloids, phenols, flavonoids, vitamins, and many secondary metabolites which have antioxidant activities and thus are often explored in drug discovery and the development of functional foods[8].

Borneo (Kalimantan in Indonesian) is a fantastic source of medicinal plants, and various medicinal plant extracts are used against various diseases in systems of drugs. Only a few of Borneo's medicinal plants are scientifically explored. *Passiflora foetida* is a medicinal plant used for generations in Kalimantan, known as *kelubut* or *kemot* (**Figure 1**). *Passiflora foetida* has been used in various traditional medicines in Kalimantan; the root decoction for diabetes treatment, whole plant decoction for hypertension and anti-inflammatory, the fruit is for pain relief, and all parts of the plant are used as a sedative and diuretic[9,10]. Several activities of *P. foetida* have been reported: analgesic, anti-inflammatory, anti-diarrheal, cytotoxic, antimicrobial and antibacterial, antidiabetic, anti-cholesterol, gastroprotective, and anti-cancer[11-16].

Figure 1. *Passiflora foetida*

Scientific data about *P. foetida* concerning antioxidants is still limited; some antioxidant activity of *P. foetida* extracts has been reported, which explains that *P. foetida* has the potential to be developed as an antioxidant[17-22]. However, investigations of the antioxidant activity of the column chromatography fraction of *P. foetida* are still scarce. Research by Sisin *et al*.[23] reported the antioxidant activity of the *P. foetida* whole-plant fraction, which used isocratic elution: petroleum ether and ethyl acetate in this study. Research has yet to be carried out to reveal the antioxidant activity of *P. foetida* leaves column chromatography fractions with gradient elution. Gradient elution has essential advantages for separation[24]. The fractionation separates the active fractions from the inactive fractions, so the active fraction is expected to have better activity than the extract. Therefore, this study aims to investigate the antioxidant activity of gradient elution-column chromatographic fractions of *P. foetida* leaves using the DPPH and FRAP methods.

MATERIALS AND METHODS

Materials

Passiflora foetida leaves were obtained from Barito Kuala, South Kalimantan, Indonesia, and the authenticity of the plant has been determined at the Laboratory of Mathematics and Natural Sciences Department, Universitas Lambung Mangkurat (022c/LB.Labdasar/I/2019). Chemicals used were methanol pro-analysis, ethyl acetate, acetone, chloroform, *n*-hexane, dimethyl sulfoxide (PT. Smart Lab), silica gel 60 F254 (Merck), silica gel 70-230 mesh (Merck); 2,4,6-tripyridyl-s-triazine (TPTZ) (Sigma Aldrich); $FeCl_3$ pro-analysis, HCl, acetic acid (glacial), sodium acetate (Merck), quercetin (Sigma Aldrich), and DPPH (1,1-diphenyl-2-picrylhydrazyl) (Sigma Aldrich). At the same time, the instruments used were a UV-visible spectrophotometer (Thermo Scientific) and UV light (Camag).

Methods

Extraction of P. foetida leaves extract

The fresh *P. foetida* leaves were sorted, then cleaned under running water, placed at a temperature of 45°C in a drying oven, and after drying, they were pollinated using a grinder. *Passiflora foetida* leaves powder was stored in a cold room until analyzed. *Passiflora foetida* leaves powder maceration was carried out using *n*-hexane, ethyl acetate, and methanol. Maceration was carried out in stages, starting with *n*-hexane, ethyl acetate, and then methanol. The macerated filtrate was then evaporated until all the solvent disappeared, and the extract obtained was weighed. This maceration produces *n*-hexane, ethyl acetate, and methanol extract.

Fractionation of P. foetida leaves extract

Fractionation was carried out on the most active extract using liquid-solid chromatography : column chromatography with a gradient system. Silica gel 70-230 mesh was used as the stationary phase, while the mobile phase/eluent uses a combination of organic solvents, ranging from low to high polarity eluents: *n*-hexane and ethyl acetate from a ratio of 9 : 1; 8 : 2; and further until 0 : 10, followed by ethyl acetate and methanol from a ratio of 10 : 0, 9 : 1, 8 : 2; and further until 0 : 10. The elution results were analyzed by thin-layer chromatography (TLC) to determine the fraction. The fraction obtained was tested for its antioxidant activity, both by the DPPH and FRAP methods.

Analytical TLC

Thin layer chromatography analysis was performed to determine the chromatogram pattern for fraction determination. Besides that, it was also used to identify the flavonoid content in the most active fraction. In TLC analysis, the sample was applied to the TLC plate, then inserted into the chamber containing the saturated mobile phase. Elution was carried out until the eluent reached the plate's upper limit. After the plates dried, the chromatogram pattern was seen under UV 254 or 366 nm. The most active fraction as an antioxidant was identified for its flavonoid content using a spray reagent of 1% ethanolic solution of $AlCl_3$, while the yellowish color under UV 366 light indicates the presence of flavonoids[25].

DPPH assay

The extract and fraction obtained were tested for antioxidant activity using the DPPH method. Fractions and extracts of *P. foetida* leaves were prepared to obtain the desired concentration series. The 1 mL test sample solution was pipette, added with 2 mL methanol and 1 mL DPPH 100 µg/mL, and incubated for 30 minutes at 37°C, and then the absorption was measured at λ 517 nm. The test was done in triplication. The DPPH test method uses an adopted method[26] with slight modifications. **Equation 1** was used to obtain the DPPH scavenging percentage (% inhibition). The antioxidant activity of the sample is based on the test using the DPPH method, expressed by the effective concentration value of 50% (EC_{50}), which shows the sample concentration that can reduce 50% of DPPH radicals.

$$\%DPPH\ scavenging = (1 - \frac{Abs\ sample - Abs\ blank}{Abs\ control - Abs\ blank}) \times 100\% \qquad [1]$$

FRAP assay

The extract and fraction obtained were also tested for antioxidant activity using the FRAP method. Fractions and extracts of *P. foetida* leaves were prepared to obtain the desired concentration series. The 0.1 mL of sample solution was pipetted, added with 3 mL FRAP solution and 0.3 mL of distilled water, incubated for 30 minutes, and the absorbance of the sample was

measured at λ 593 nm. The test was carried out in triplicate. The FRAP test method uses an adopted method[26] with slight modifications. **Equation 2** was used to obtain the percentage of antioxidant activity. The antioxidant activity of the sample was based on the test using the FRAP method, expressed by EC_{50} (concentration of the sample to reduce 50% ferric ion).

$$\%Antioxidant\ activity = (Abs\ sample - Abs\ blank) \times 100\% \quad [2]$$

RESULTS AND DISCUSSION

Antioxidant activity of the extracts of P. foetida leaves

This study investigated the antioxidant activity from extracts (*n*-hexane extract, ethyl acetate extract, methanol extract) and fractions from column chromatography. The antioxidant activity of *P. foetida* leaves compared to quercetin by DPPH and FRAP methods is shown in **Table I**. *Passiflora foetida* leaves methanol extract showed the best potential for antioxidant activity compared to *n*-hexane extract and ethyl acetate extract with EC_{50} of 97.453 µg/mL, using DPPH method and EC_{50} of 58.787 µg/mL, using FRAP method. Quercetin has better activity than the most active extracts because quercetin is a single compound that is active as an antioxidant, while the extract is still a mixture of compounds, where the active compounds are still mixed with less active compounds. Several study reports show that *P. foetida* contains phytoconstituents, including alkaloids, flavonoids, saponins, tannins, glycosides, anthraquinone glycosides, terpenoids, steroids, phenolic compounds, and saponins[27,28]. Flavonoids and phenolic compounds have a role in antioxidant activity[29,30]. This causes *P. foetida* extract to have antioxidant activity, whereas methanol extract has the best activity.

Table I. Antioxidant activity of *P. foetida* leaves extracts and quercetin

No.	Sample	EC_{50} (µg/mL) with DPPH Method	EC_{50} (µg/mL) with FRAP Method
1.	*n*-hexane extract	129.035	67.851
2.	Ethyl acetate extract	206.398	68.981
3.	Methanol extract	97.453	58.787
4.	Quercetin	7.669	5.759

Fractionation yield and antioxidant activity of the column chromatography fractions

The methanol extract was then fractionated to separate the fractions in the extract based on their polarity. Methanol extract fractionation was carried out using column chromatography, where this column chromatography technique has been used for the fractionation process of several extracts of natural products such as the fractionation of *Garcinia lateriflora*, *Pluchea odorata*, *G. kydia*, *Senna ocidentalis*, and *G. hombroniana*[31-34]. The fractionation process using column chromatography on methanol extract as the most active extract as an antioxidant resulted in eight fractions, as shown in **Table II**. All fractions produced showed antioxidant activity, where the best activity was owned by fraction 6, as shown in **Tables III**.

Table II. Fractions of *P. foetida* leaves methanol extract

Fraction	Eluent of column
FMetPF1	*H* / *E* = 9:1 – 7:3
FMetPF2	*H* / *E* = 7:3 – 6:4
FMetPF3	*H* / *E* = 6:4 – 4:6
FMetPF4	*H* / *E* = 4:6 – 3:7
FMetPF5	*H* / *E* = 3:7 – 0:10
FMetPF6	E / M = 9:1
FMetPF7	E / M = 8:2 – 5:5
FMetPF8	E / M = 5:5 – 0:10

Description= FMetPF 1-8: fractions from *P. foetida* leaves methanol extract; H: *n*-hexane, E: ethyl acetate, M: methanol.

Table III. Antioxidant activity of *P. foetida* leaves fractions

No.	Sample	% Inhibition ± SD of 25 µg/mL (DPPH)	% Antioxidant activity ± SD of 9 µg/mL (FRAP)
1	FMetPF1	27.22 ± 0.49	7.50 ± 1.15
2	FMetPF2	30.00 ± 0.19	7.17 ± 1.05
3	FMetPF3	27.10 ± 1.72	6.80 ± 0.53
4	FMetPF4	26.91 ± 0.11	9.23 ± 1.10
5	FMetPF5	30.56 ± 0.37	6.20 ± 0.66
6	FMetPF6	41.85 ± 1.96	26.03 ± 0.84
7	FMetPF7	40.31 ± 0.77	18.60 ± 0.56
8	FMetPF8	35.43 ±1.12	12.20 ± 0.26

Note: Data in triplicates

FMetPF6 shows the best potential as an antioxidant compared to other fractions. Overall, the fractions obtained from the fractionation of *P. foetida* leaf methanol extract had an antioxidant activity with a range of activity (% inhibition) between 26.91±0.11 to 41.85±1.96 when tested with the DPPH method at a test concentration of 25 µg/mL, and an activity range (% antioxidant activity) of 6.2±0.66 µg/mL to 26.03±0.84 µg/mL when tested by the FRAP method with a test concentration of 9 µg/mL. FMetPF6 has good activity as an antioxidant, both tested by the DPPH and FRAP methods. This shows the ability of the compounds contained in FMetPF6 both as hydrogen donors and in transferring single electrons. Compounds that act as hydrogen donors and can transfer electrons are compounds capable of acting as antioxidants[35,36]. FMetPF6 is the most active fraction as an antioxidant, with EC_{50} of 38.607 µg/mL using the DPPH method and EC_{50} of 46.502 µg/mL using the FRAP method. FMetPF6, as the most active fraction, has better activity than methanol extract, probably because the less and inactive fractions have been separated, but quercetin has better activity than the most active fraction (**Table IV**).

Table III. Antioxidant activity of FMetPF6 and quercetin

Method	Sample	EC_{50} (µg/mL)
DPPH	FMetPF6	38.607
	Quercetin	7.669
FRAP	FMetPF6	46.502
	Quercetin	5.759

Identification of flavonoids by TLC analysis

The ability of flavonoids as antioxidants has been the subject of many studies in the past years, and the structure-activity relationships of the antioxidant ability have been established. Thin layer chromatography on FMetPF6 using the mobile phase ethyl acetate : acetone : acetic acid (2 : 3 : 0.1) shows the flavonoid content, which is indicated by a yellow color under UV 366 nm light after spraying with 1% ethanolic solution of $AlCl_3$[25], this can be seen in **Figure 2**. Flavonoids have a role in antioxidant activity because they reduce free radicals by donating hydrogen atoms or transferring single electrons[37]. Flavonoids can form chelate or bind metal ions to prevent oxidation, thus preventing the formation of free radicals. Another mechanism of flavonoids as antioxidants is by inhibiting enzymes that play a role in the formation of free radicals, such as lipoxygenase, cyclooxygenase, microsomal monooxygenase, NADPH oxidase, xanthine oxidase, mitochondrial succinoxidase, and protein kinase C, besides flavonoids also can induce internal antioxidant enzymes[38].

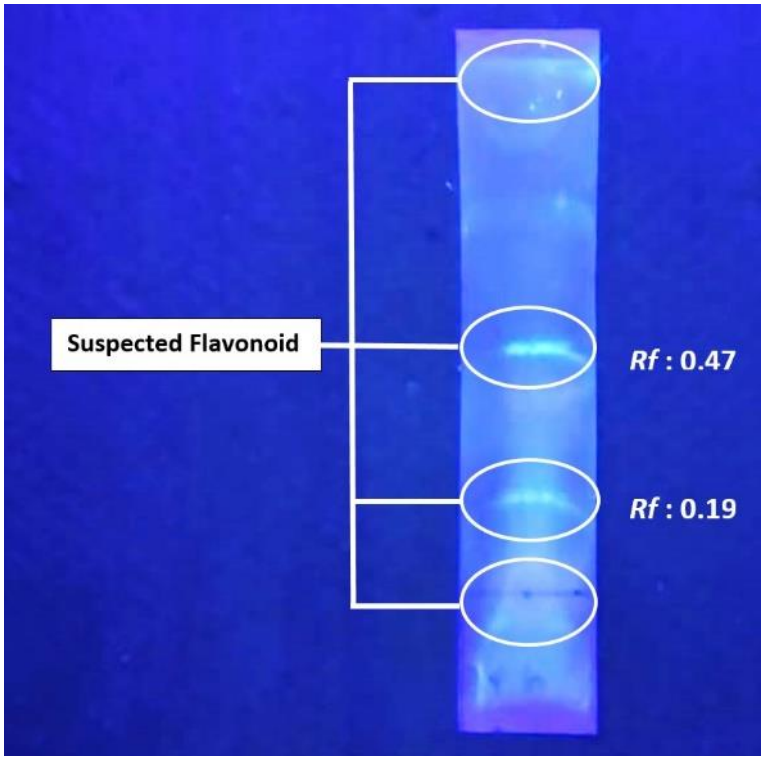

Figure 2. TLC Profile of FMetPF6

Quercetin was used as the standard in this antioxidant activity test. Quercetin is a class of flavonols in various natural sources, so it is easy to obtain and has acted as a strong antioxidant[39]. The antioxidant action of quercetin is related to the location and presence of the hydroxyl group (-OH) with the catechol type B ring, i.e., the presence of ortho hydroxy and hydroxyl substitution at positions 3 and 5[40,41].

CONCLUSION

Passiflora foetida leaves have the potential as antioxidants; both the extracts and their fractions show antioxidant activity. FMetPF6, successfully separated from other fractions, had the best activity compared to other extracts and fractions, with EC_{50} of 38.607 µg/mL (DPPH) and 46.502 µg/mL (FRAP). Further analysis to determine the various compounds in FMetPF6 using LC-MS/MS will facilitate the isolation of the active compound.

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AUTHORS' CONTRIBUTION

All authors have an equal contribution in carrying out this study.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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