

Phenolic Assay and Antioxidant Activity of Ethyl Acetate and Water Fractions of Ethanol Extract of Butterfly Pea Flower by DPPH and FRAP Methods

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ABSTRACT

Antioxidants play an important role in human life. Many studies have been conducted to discover the potential of antioxidants sourced from nature. Several methods can be used to test antioxidants. These methods include FRAP, ABTS, and DPPH. All three methods are widely used because they are stable and relatively easy to use. Several studies on TAC (Total Antioxidant Capacity) have reported different results regarding antioxidant testing using different methods on the same material. This study aims to test the ethyl acetate and water fractions of butterfly pea flower extract using the DPPH and FRAP methods and to compare the results with the findings of previous researchers who tested using the ABTS method. The research method began with the extraction of butterfly pea flowers using 70% ethanol solvent, followed by a total phenolic test using the Folin Ciocalteu method. Next, fractionation was carried out, and the ethyl acetate and water fractions were then tested for antioxidant activity using the DPPH method, while FRAP was performed for the ethyl acetate fraction. Quercetin was used as a reference standard. The results of the antioxidant activity test of butterfly pea flowers using the DPPH method showed an IC₅₀ value of 27.63 ppm $\pm$ 4.11 in the ethyl acetate fraction and 54.70 ppm $\pm$ 2.22 in the water fraction, while the IC₅₀ in the FRAP method was 97.47 ppm $\pm$ 3.2 in the ethyl acetate fraction. DPPH and ABTS did not show significant differences in the antioxidant activity test results, but showed different results in FRAP.

Keyword: Antioxidant, Butterfly pea flower, DPPH, FRAP

ABSTRAK

Antioksidan memiliki peran penting pada kehidupan manusia. Banyak penelitian dilakukan untuk menemukan potensi antioksidan yang bersumber dari alam. Beberapa metode dapat digunakan untuk melakukan pengujian antioksidan. Metode tersebut diantaranya, FRAP, ABTS, DPPH. Ketiga metode tersebut banyak digunakan, karena stabil dan relatif mudah digunakan. Beberapa penelitian mengenai TAC (Total Antioxidant Capacity) melaporkan hasil yang berbeda mengenai pengujian antioksidan dengan metode berbeda, pada bahan yang sama. Penelitian ini bertujuan untuk menguji fraksi etil asetat dan air ekstrak bunga telang menggunakan metode DPPH dan FRAP, serta membandingkan hasilnya dengan temuan peneliti sebelumnya yang menguji dengan metode ABTS. Metode penelitian diawali dengan melakukan ekstraksi bunga telang menggunakan pelarut etanol 70%, dilanjutkan uji total fenolik dengan metode Folin ciocalteu. Selanjutnya dilakukan fraksinasi, hasil fraksi etil asetat dan air selanjutnya diuji aktivitas antioksidan dengan metode DPPH, sedang FRAP dilakukan untuk fraksi etil asetat. Baku pembanding digunakan kuersetin. Hasil uji aktivitas antioksidan bunga telang menggunakan metode DPPH menunjukkan nilai IC₅₀ sebesar 27,63 ppm $\pm$ 4,11 pada fraksi etil asetat dan 54,70 ppm $\pm$ 2,22 pada fraksi air, sedang IC₅₀ pada metode FRAP, yakni 97,47 ppm $\pm$ 3,2 pada fraksi etil asetat. DPPH dan ABTS tidak menunjukkan perbedaan signifikan terhadap hasil uji aktivitas antioksidan, namun menunjukkan hasil berbeda pada FRAP.

Kata Kunci: Antioksidan, Bunga telang, DPPH, FRAP

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Introduction:

Antioxidants are substances that can inhibit the oxidation process, able to protect cells from the dangers of free radicals generated from the body's metabolism (Sadeer et al., 2020) or those derived from external factors (Rifqi, 2021). Free radicals have been classified into three main categories, namely ROS (Reactive Oxygen Species), RNS (Reactive Nitrogen Species) and RSS (Reactive Sulfur Species) (Wang *et al.*, 2018). Various types of antioxidants are involved to maintain ROS, including (i) endogenous antioxidants, such as albumin, bilirubin, glutathione, uric acid (ii) antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase, heme oxygenase-1, NAD (iii) dietary antioxidants such as vitamin C, vitamin E, carotenoids and various polyphenols (Chedea and Pop, 2019).

Antioxidants are naturally found in plants, in the form of phenol-derived compounds such as flavonoids. The antioxidant work system is generally divided into two, namely enzymatic (superoxide dismutase, catalase, peroxidase, ascorbic acid oxidase, glutathione reductase and polyphenol oxidase and non-enzymatic such as vitamin C, carotene, phenolics, tocopherols (Christodoulou *et al.*, 2022). Phenolics are compounds that play a role in the presence of plant antioxidant activity, several reviews have been published on the role of phenolics as antioxidants (Zeb, 2020).

Several measurement methods can be used to determine antioxidant activity. Antioxidant capacity measurements are classified into two, namely chemical based assay and biochemical based assay. Chemical based assay consists of radical/ROS based scavenging assay, such as ABTS, DPPH, ORAC, TOSC and non radical/redox potential based assay, such as FRAP, cupric, TPC (Martins *et al.*, 2021). Some of these test methods have different advantages, disadvantages and mechanisms (Danet, 2021). Some assay methods work by evaluating hydrogen atoms (HAT), other methods test electron transfer capacity (ET).

Butterfly pea flower is one of the plants that has been widely studied, because it is known to have antioxidant activity (Rahayu, Vifta and

Susilo, 2021). The extraction process of butterfly pea flower using 70% ethanol solvent shows a very strong antioxidant value (41.36 ppm) (Andriani and Murtisiwi, 2020), compared to extraction using methanol or ethyl acetate solvents (Rajamanickam, Kalaivanan and Sivagnanam, 2015). In a study by (Yumni Gharsina, Sumantri; Nuraini da Nafis, 2021), they tested the antioxidant activity of the ethyl acetate and water fractions of butterfly pea flower ethanol extract using the ABTS method. The results showed that the ethyl acetate and water fractions had antioxidant activity of 27.018 ppm and 33.42 ppm. Several studies on TAC (Total Antioxidant Capacity) have reported different results regarding antioxidant activity on the same material, using different testing methods (Rohmah, 2022), so in this study antioxidant activity testing will be carried out using different methods, namely DPPH and FRAP. A search of previous studies revealed no tests of ethyl acetate and water fractions in butterfly pea flowers using the DPPH method, and no studies comparing the antioxidant activity of ethyl acetate and water fractions in butterfly pea flowers using different methods.

In this study, extraction was carried out using butterfly pea flower with the same amount, solvent and the same ratio as the method (Yumni Gharsina, Sumantri; Nuraini da Nafis, 2021). Furthermore, phytochemical screening and total phenolic test will be carried out on the extract. The extract was then fractionated using N hexanes, ethyl acetate and water solvents. After obtaining the ethyl acetate and water fractions, in the early stages of the study, we will compare the measurement results of the standard series of quercetin standards with the DPPH and FRAP methods, this is intended to test the reproducibility and sensitivity of the two methods. In the next stage, we will compare the results of measuring the antioxidant activity of ethyl acetate and water fractions using both methods, we will also compare the findings of (Yumni Gharsina, Sumantri; Nuraini da Nafis, 2021) as a reference for our findings. Statistical analysis was conducted for each test

Methods:

Equipment

The tools used in this research are glassware, grinder, digital balance (O'Haus-USA), waterbath (Faithful®), rotary evaporator (IKA RV 3V), silica gel GF254 nm plates, chamber, tweezers, spotting atomizer, pH meter (Hanna Instrument pH 210 Microprocessor-USA®), UV-VIS spectrophotometer (Shimadzu-Japan®).

Materials

The materials used in this study were butterfly pea flower powder (B2P2TOOT), FeCl₃ (Smart Lab-Indonesia), Mg powder, HCl (p) (Smart Lab-Indonesia), HCL (Smart Lab-Indonesia), NaOH, amyl alcohol (Merck-Germany), gelatin (Brataco-Indonesia), Dragendorff reagent, Mayer reagent, Bourchardat reagent, anhydrous acetic acid (Merck-Germany), H₂SO₄ (p) (Panreac Quimica-Barcelona), anisaldehyde, methanol p. a (Smart Lab-Indonesia), vanillin, methanol, toluene, ethyl acetate, formic acid, butanol, chloroform, n-hexane, ethyl acetate (Smart Lab-Indonesia), DPPH (Sigma-Japan), quercetin (Sigma-Japan), 96% ethanol, K₃Fe(CN)₆, TCA (Smart Lab-Indonesia).

Extraction of Butterfly Pea Flower

Butterfly pea flower powder was extracted by maceration method using 70% ethanol solvent as much as 4500 mL (1: 7.5). A total of 600 g of butterfly pea flower simplisia plus 70% ethanol as much as 4500 mL and macerated and allowed to stand for 3x24 hours, occasionally stirring. After the maceration process, the extract was filtered with kola cloth. The macerate that has been obtained is concentrated with a 45° C rotary evaporator, then evaporated on a waterbath at 45°C until a thick extract of butterfly pea flower is obtained.

Fractionation

Fractionation was carried out using distilled water, n-hexane and ethyl acetate. The thick extract was dissolved with distilled water then placed in a separatory funnel and n-hexane solvent was added. The mixture was whipped and allowed to stand until it separated into two

phases, then the two phases were separated. The aqueous phase was put back into the separatory funnel and added back n-hexan solvent, the mixture was fractionated again which was obtained clear. The remaining water phase of the n-hexan fraction was then fractionated again by adding ethyl acetate solvent and whipped again and allowed to stand until separation into two phases. This process is carried out until the ethyl acetate phase obtained is clear, then the ethyl acetate solvent and water are separated, then the yield of the fraction obtained is calculated.

Phytochemical Screening

Phytochemical screening tests were carried out on ethanol extracts of butterfly pea flowers including flavonoids, tannins, alkaloids, and anthocyanins.

Total phenolic test of butterfly pea flower ethanol extract

Determination of total phenolic was carried out by Folin Ciocalteu test, as described in (Martins *et al.*, 2021). For analysis, 0.1 mL of sample solution (2 mg extract in 1 mL DMSO) was pipetted into a 10 mL volumetric flask, then 0.5 mL FC reagent and about 7 mL aq dest were added. One minute after adding FC reagent, 1 mL of sodium carbonate solution was added, and sufficed with aq dest. This measuring solution was incubated for 30 min at 40°C before measuring the absorbance at 750 nm, as a standard using gallic acid.

Preparation of quercetin standard

Preparation of quercetin standard solution for DPPH method, calibration curve was made with five concentrations of 1, 3, 5, 7 and 9 ppm. DPPH solution was prepared in a concentration of 0.07 mM. Weighed carefully 2.8 mg DPPH, put into a 100.0 mL measuring flask, dissolved with methanol p.a until dissolved, enough to the limit mark of 100.0 mL. The sample was read at a wavelength of 515 nm, with an operating time of 30 minutes.

Preparation of quercetin standard solution for testing by FRAP method. Made in 6 concentrations of 20 ppm, 24 ppm, 28 ppm, 32 ppm, 36 ppm and 40 ppm. The 1000 ppm

quercetin standard solution was made by dissolving 50 mg of quercetin into 50 mL of 96% ethanol. Each concentration series was added with 1 mL of 0.2 M phosphate buffer (pH 6.6) and 1 mL of $K_3Fe(CN)_6$ 1%, then incubated for 20 minutes at 50°C. After incubation, 1 mL of TCA was added and centrifuged at 3000 rpm for 10 minutes. After centrifugation, 1 mL of the top layer was pipetted into a test tube, 1 mL of distilled water and 0.5 mL of $FeCl_3$ 0.1% were added. The solution was allowed to stand for 10 minutes, then measured the absorbance at a maximum wavelength of 715 nm, with an operating time of 11 minutes.

Antioxidant Activity Test DPPH Method Preparation of Test Sample Solution

The fraction of butterfly pea flower was weighed 10 mg, dissolved in a 10 mL flask with methanol p.a sufficient to the limit mark (1000 ppm), then made a sample solution of butterfly pea flower fraction. Taken 0.4; 0.5; 0.6; 0.7 and 0.8 mL of the parent sample solution (1000 ppm), put in a 5 mL flask and sufficed with methanol p.a until the limit mark.

Determination of Antioxidant Activity

A total of 1.0 mL of each sample, namely butterfly pea flower fraction sample, was put into a vial that had been covered with aluminum foil and black plastic and then added 4 mL of 0.07 mM DPPH solution. The mixture was homogenized and waited according to the operating time for 30 minutes in a dark place. The absorbance of the solution was read with a UV-Vis spectrophotometer at the maximum wavelength (Zhang, Yang and Zhou, 2018). Antioxidant activity was calculated using the equation below:

$$\% \text{ Absorbance} = (\text{control absorbance} - \text{sample absorbance}) / (\text{control absorbance}) \times 100\%$$

Antioxidant Activity Test FRAP Method

Butterfly pea flower fraction was carefully weighed as much as 10 mg and dissolved with 96% ethanol in a 10 mL volumetric flask, so that a concentration of 1000 ppm was obtained. The sample solution was pipetted 400 μ L, 500 μ L, 600 μ L, 700 μ L and 800 μ L, respectively, into a 5 mL volumetric flask, until a concentration of 80, 100, 120, 140 and 160 ppm was obtained, then pipet 1 mL of each concentration, put in a test tube. Add 1 mL of 0.2 M phosphate daphar (pH 6.6) and 1 mL of $K_3Fe(CN)_6$, and incubate for 20 minutes at 50°C. 1 mL of 10% TCA solution was added to each reaction tube, then centrifuged at 3000 rpm for 10 minutes. After centrifugation was complete, pipet 1 mL into a test tube, add 0.5 mL of 0.1% $FeCl_3$ and 1 mL of distilled water. The solution was allowed to stand for 10 minutes and read the absorbance at a wavelength of 715 nm with an operating time of 11 minutes. Calculation of antioxidant activity test FRAP method using the equation:

$$\% \text{ Inhibition} = (\text{sample absorbance}) / (\text{control absorbance}) \times 100\%$$

Results:

The results of ethyl acetate fractionation of butterfly pea flower extract with 70% ethanol obtained a yield of 3.45% and an aqueous fraction of 74.88%.

Phytochemical screening

The results of phytochemical screening of ethanol extracts of butterfly pea flowers show that ethanol extracts are positive for flavonoids, tannins, alkaloids, and anthocyanins.

Total phenolic test results of ethanol extract of butterfly pea flower.

The results of the total phenolic test on the ethanol extract of butterfly pea flower were obtained at $1.3 \pm \text{mg QE/g extract}$.

Standard reading results

Table I. Comparison of Standard Solution Test Results.

	DPPH	ABTS (*)	FRAP
a	0.7698	8.098	0.0759
B	-0.0396	2.3668	0.0149
r	0.9947	0.9958	0.9989
R²	0.9895	0.9918	0.9978
CV	0.632	0.527	0.249

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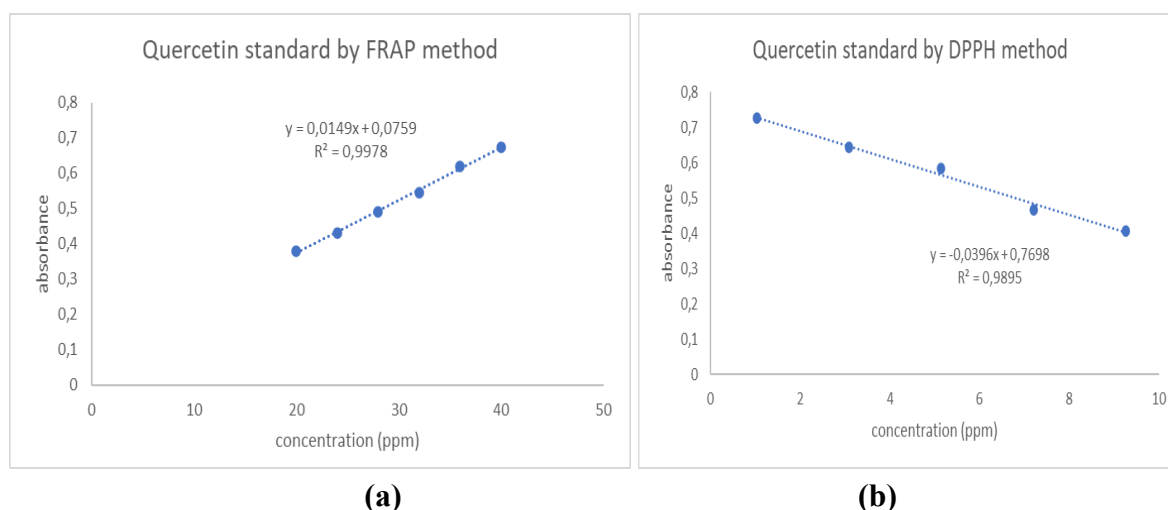


Figure 1. Calibration curves of quercetin by FRAP (a) and DPPH (b) methods.

Comparison Antioxidant Activity Testing Results of Ethyl Acetate and Water Fractions of Ethanol Extract of Butterfly Pea Flower.

Table II. Comparison of IC₅₀ Values

	DPPH		ABTS(*)		FRAP
	EA Fraction	Water fraction	EA Fraction	Water fraction	EA Fraction
A	5,88	-5,806	15,98	10,49	12,5
B	1,3887	0,9888	1,2588	1,1923	0,3849
R	0,9989	0,9913	0,9913	0,9946	0,9793
R²	0,9989	0,9827	0,9827	0,9892	0,9594
CV	0,148	0,320	0,527	0,527	0,184
IC₅₀ (%)	27,63±4,11	54,70±2,22	27,018	33,42	97,47±3,2

*Yumni Gharsina dkk

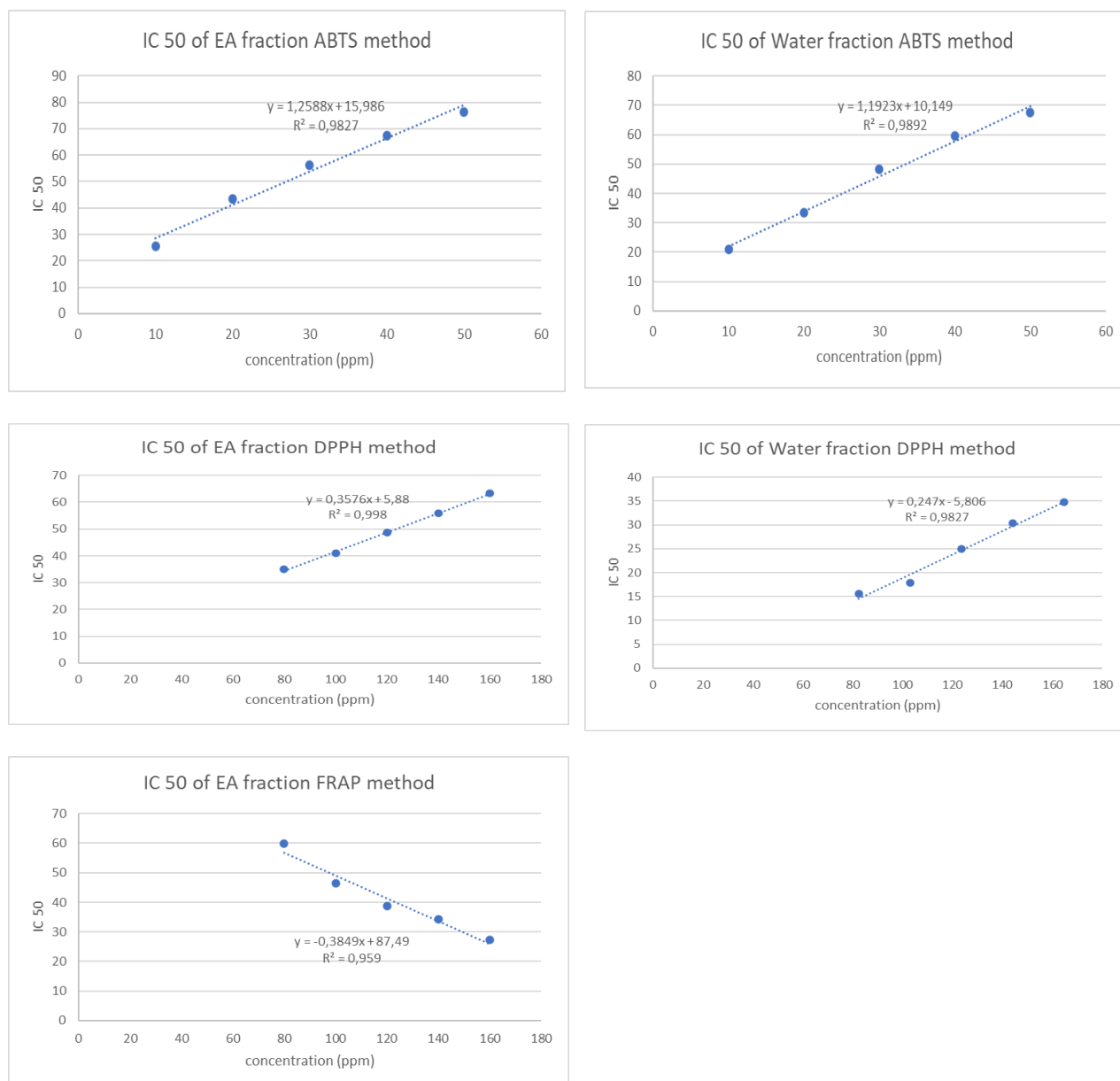


Figure 2 . IC₅₀ test results of fractions (a) ethyl acetate DPPH method (b) ethyl acetate ABTS method (c) ethyl acetate FRAP method (d) water DPPH method (e) water ABTS method

Discussion:

Phytochemical screening

The results of phytochemical screening of ethanol extracts of butterfly pea flowers show that ethanol extracts are positive for flavonoids, tannins, alkaloids, and anthocyanins. This is in accordance with the results obtained (Frisca, Lindawati and Murtisiwi, 2021).

Total phenolic test results of ethanol extract of butterfly pea flower.

Phenolic is the largest compound in plants. The results of the total phenolic test on the

ethanol extract of butterfly pea flower were obtained at $1.3 \pm \text{mg QE/g extract}$. This result is not far from the findings of researchers (Waruwu, Rawar and Kristiyani, 2023) who also calculated the total phenolic content of butterfly pea flower. Determination of polyphenol analysis can be done by TOC and spectroscopy. The structure of phenols is optically active, so UV Vis spectrophotometry at 280 nm, is used to evaluate phenol composition. Generally, the total phenol content test is carried out with Folic-ciocalteu (Martins *et al.*, 2021).

Antioxidant activity testing results of ethyl acetate and water fractions of ethanol extract of butterfly pea flower.

Antioxidant activity testing was carried out on the ethyl acetate and water fractions because they contained polyphenolic compounds, especially flavonoids and anthocyanins, which are semi-polar and polar, known to reduce free radicals. The aglycone form and the form bound to sugar as a glycoside will each be distributed into the ethyl acetate fraction and the water fraction according to their solubility and polarity, flavonoids in the form of aglycones will be distributed into the ethyl acetate fraction such as flavonol. Flavonoids in the form of glycosides will be distributed into the water fraction such as flavonoid glycosides with mirisetin, quercetin, and kaemferol aglycones. The n-hexane fraction contains the lowest flavonoid compounds but contains the most types of non-polar compounds such as fats, waxes, oils, steroids (Susiloningrum, Erliani and Sari, 2021).

In addition to the test method, sample pretreatment plays an important role when investigating the activity of plant extracts, as the extraction solvent affects the antioxidant capacity (Zengin *et al.*, 2022). To minimize this influence on the comparison of antioxidant activity assays by DPPH and FRAP methods, we extracted butterfly pea flowers with the same ratio and solvent as previous researchers. Other factors that affect the test are pH, temperature and operating time. In general, electron transfer occurs faster, while hydrogen transfer is slower. DPPH and ABTS are HET-based test methods, so the operating time is slower than FRAP whose mechanism of action is electron transfer (Schaich, Tian and Xie, 2015). The operating time in our test using DPPH found the same operating time as the ABTS method, while FRAP found the operating time at 11 minutes.

We tried to compare the results of our calibration curve generation using the DPPH and FRAP methods, with the test results conducted by Yumni *et al* using the ABTS method (we calculated the r , R^2 , CV values from the test data listed in the study). The results obtained show that DPPH and ABTS have higher r and R^2 values than FRAP.

The results of antioxidant testing of ethyl acetate fraction using DPPH and FRAP methods, showed that the IC₅₀ value in ethyl acetate fraction using DPPH method obtained IC₅₀ value of 27.63 ppm $\pm$ 4.11 in EA fraction and 54.70 ppm $\pm$ 2.22 in water fraction, while the results obtained using FRAP method obtained value of 97.47 ppm in EA fraction. We also compared with the results of the IC₅₀ test with the ABTS method by Yumni *et al*, found no significant difference between the IC₅₀ values in the ethyl acetate fraction, whether measured by DPPH or ABTS methods, but had different values when tested with FRAP. DPPH and ABTS have the same mechanism of action, namely HAT (Hydrogen Atom Transfer) while FRAP works with the ET (Electron Transfer) mechanism (Munteanu and Apetrei, 2021). It is important to note that there is a correlation between the phenolic and flavonoid content of plant species, which will affect the antioxidant profile (Kiss *et al.*, 2025). The results of total flavonoid determination in Garsina's study found that total flavonoids in the water fraction were higher than total flavonoids in the ethyl acetate fraction. Although the antioxidant activity test results were in the same category (very strong), the water fraction showed a smaller IC₅₀ value. The results of the IC₅₀ test on the three methods are presented in Table II.

The same test comparing DPPH, ABTS, FRAP methods to test the antioxidant activity of citrus fruits, obtained test results between the three methods resulted in a similar category of antioxidant activity which is very strong (Gironés-Vilaplana, Moreno and García-Viguera, 2014). According to (Zhang, Yang and Zhou, 2018) that the number of hydroxyls does not always correlate with the test value of antioxidant activity in the ABTS test. Hydroquinone, carechol, resorcinol have the same number of hydroxyls but there is a considerable difference in the antioxidant activity test, but asthma rosmariat, p-coumaric acid show the same test value despite the difference in hydroxyl groups four times. Other studies have found similar results regarding the reliability of test results between the DPPH and FRAP methods. Both methods show high sensitivity in detecting the antioxidant activity of

similar compounds, especially polyphenols in spices. The DPPH and FRAP methods show fairly strong predictions, but not higher than those of DPPH and TEAC (Kiss *et al.*, 2025). The results of the calibration curve of the ethyl acetate fraction assay using DPPH, FRAP and ABTS methods (Yumni Gharsina, Sumantri; Nuraini da Nafis, 2021) are shown in Figure 2.

FRAP is a simple, rapid, inexpensive, good reproducibility, high sensitivity and precision method. Reducing power is an indicator of the potency of an antioxidant test compound. In the FRAP method, reducing power is measured by the ability of an antioxidant to convert Fe^{3+} to Fe^{2+} (Shah, 2016). Compounds that have the ability to reduce can be used as antioxidants because they can neutralize the presence of free radicals by donating electrons or hydrogen atoms radical compounds can change into a more stable form. The FRAP test was conducted at pH 3.6 to prevent iron precipitation (Christodoulou *et al.*, 2022). The addition of FeCl_3 aims to form a green to blue complex (berlin blue). Testing antioxidant activity using the ABTS method has the advantage that this reagent is quite stable, the reagent can be stored at temperatures below 5°C to maintain its stability.

Conclusion:

The results of antioxidant testing of the ethyl acetate fraction using the DPPH and FRAP methods, showed that the IC_{50} value in the ethyl acetate fraction using the DPPH method obtained an IC_{50} value of $27.63 \text{ ppm} \pm 4.11$ in the EA fraction (very strong) and $54.70 \text{ ppm} \pm 2.22$ in the water fraction (strong), while the results obtained using the FRAP method obtained a value of 97.47 ppm in the EA fraction (medium).

Conflict of Interest:

The authors declare no conflicts of interest.

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