JNPDD
Journal of Natural Product for Degenerative Diseases
https://journal.univpancasila.ac.id/index.php/Natural

Identification of Ethanol Extract Butterfly Pea Flower (*Clitoria ternatea*) with LC-HRMS and Antioxidant Activity Testing

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Received: 31 January 2025 / Accepted: 19 March 2025

ABSTRACT: Butterfly Pea Flower (*Clitoria ternatea*) is a plant rich in bioactive compounds, such as flavonoids, anthocyanins, and phenolic compounds, which have the potential to provide health benefits. This study aims to identify active compounds in butterfly pea flower extract and evaluate their antioxidant activity. Compound identification was performed using LC-HRMS is an analytical technique that combines liquid chromatography (LC) with high-resolution mass spectrometry (HRMS), while antioxidant activity was tested using radical scavenging DPPH (2,2-diphenyl-1-picrylhydrazyl). Analytical results showed that butterfly pea flower contain major flavonoid compounds, Butterfly Pea Flower contain compounds glycitein, quercetin, apigenin, kaempferol, laserpitin. The antioxidant activity test showed that Butterfly Pea Flower extract has a weak antioxidant capacity, which has the potential to counteract free radicals and reduce oxidative stress. These findings confirm that butterfly pea flower have potential as a natural source of antioxidants that can be developed for pharmaceutical and functional food applications.

KEYWORDS: Butterfly Pea Flower; LC-HRMS; Antioxidant; DPPH; Sidomulyo.

1. INTRODUCTION

The Butterfly Pea Flower (*Clitoria ternatea*) is a plant widely known for its distinctive blue color and health benefits. The plant contains various bioactive compounds, such as flavonoids, anthocyanins, phenolics, and cyclic peptides, which contribute to its pharmacological properties. Based on many studies that have been conducted in various countries, the flowers are proven to contain secondary metabolites of alkaloids, tannins, glycosides, resins, steroids, saponins, flavonoids and phenols [1]. The research results obtained are quite diverse, total phenols from 1.9 mg / g GAE - 102.4 mg / g GAE etc. [2,3]. Total flavonoids 35.7 mg QE/g - 187.05 ± 3.18 mg quercetin/g TFC and total anthocyanins 28.60 ± 0.04 mg/L [4,5,6]. The pharmacological activity of *Clitoria ternatea* extract (CTE) has been demonstrated to inhibiting various disease and aging processes. Besides its phytochemical content, polyacylated anthocyanins and flavonol glycosides of quercetin exert protective effects against hydrogen peroxide-induced cytotoxicity and ultraviolet (UV)-induced mitochondrial DNA (mtDNA) damage in keratinocytes [7].

Identification of compounds in Butterfly Pea Flower is important to understand the active components responsible for its biological activity. Techniques such as high-performance liquid chromatography (HPLC), mass spectrometry (MS), and nuclear magnetic resonance (NMR) are often used for the analysis of bioactive compound content in Butterfly Pea Flower extracts [8]

One of the main benefits of Butterfly Pea Flower is its antioxidant activity. Antioxidants play a role in counteracting free radicals that can cause oxidative stress and contribute to various degenerative diseases, such as cancer, diabetes, and cardiovascular disease. The anthocyanins and flavonoids in Butterfly Pea Flower s are known to have strong antioxidant effects, which can be tested using methods such as DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS, and FRAP [8,9]

With more and more studies highlighting the health benefits of Butterfly Pea Flower, the identification of bioactive compounds and evaluation of their antioxidant activities are important steps in the development of health products, herbal medicines, and supplements based on this plant.

How to cite this article: Ninik S, Syamsudin S, Deni R, Nenden L. Identification of ethanol extract butterfly pea flower (*Clitoria ternatea*) with LC-HRMS and antioxidant activity testing. JNPDD. 2025; 2(2): 56-62.

https://doi.org/10.58511/jnpdd.v2i2.8283
ISSN: 3031-6847

2. MATERIALS AND METHODS

2.1. Materials

The research sample was Butterfly Pea Flower from Sidomulyo village, Bambanglipuro sub-district, Bantul, Yogyakarta. H_2O, 0.1% formic acid, and acetonitrile, ethanol, 2,2-Diphenyl-1- picrylhydrazyl (DPPH) (Merck)

2.2. Procedures

Preparartion sample

Butterfly Pea Flower (*Clitorea ternatea*) were obtained from Sidomulyo village, Bambanglipuro sub-district, Bantul district - Yogyakarta and then *C. ternatea* simplisia powder was macerated using 70% ethanol solvent as much as 10L. Simplisia with solvent ratio 1:10. Butterfly Pea flowers macerated with ethanol solvent were tightly covered with aluminum foil so that it was not translucent for 24 hours at room temperature. Then kinetic maceration or stirring is carried out for 4 hours with an rpm of 300-500, after kinetic maceration is carried out filtering with a cotton stage first then filter paper. The results of the filtration in the form of filtrate are then put into a rotary flask and then rotavaporized at a temperature of 50-55^{o}C, rpm 90 and pressure 100mbar with the position of the rotary flask until the water in the waterbath is slightly higher than the extract, so that the filtrate evaporates into a thick extract. Viscous extracts are stored in glass bottles, tightly closed and stored in the refrigerator. Before the extract is stored in a glass bottle, the bottle is weighed first so that the amount of extract obtained is known.

Examination of secondary metabolites of CTE using LC-HRMS

The initial step was sample preparation of 5 mg in 1 mL of methanol with a 0.2 µm PTFE membrane. Testing of compounds in CTE using UHPLC Vanquish Tandem Q Exactive Plus Orbitrap HRMS Thermo Scientific. With Accucore C18 column, 100x2.1 mm, 1.5 µm (Thermo Scientific, USA). The eluent or mobile phase used was: H_2O + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B), with a flow rate of 0.2 mL/min and an injection volume of 5 µl. The separation of the solute mixture or gradient starts with a weak elution solvent and gradually increases the proportion of strong elution solvent, starting from the first 0-1 minute at 5% B, 1-7 minutes at 5-95% B, then 7-8 minutes at 95% B and 8.01-13 minutes at 5% B. The temperature on the column is 30^{o}C, and the column temperature is 30^{o}C. The temperature on the column was 30^{o}C, using positive ionization mode and on full MS using a resolution of 70,000 with a mass range of 100-1500 m/z. Data analysis using confirmation compound discoverer software (Thermo Scientific, USA) integrating untargeted compound profiles [10,11]

Antioxidant activity testing with DPPH

The method used is free radical scavenging with DPPH solution. Variation of concentration 10, 50, 100, 150, and 200 ppm. Furthermore, a standard comparison solution was made with various concentrations 2, 4, 6, 8, and 10 ppm. Next, the antioxidant power of the blank was measured at a wavelength of 517 nm. Finally, the antioxidant activity of the extract was measured.

$$\%\text{Inhibition} = \frac{A_{DPPH} - A_U}{A\ DPPH} \text{ x } 100\%$$

3. RESULTS

Results of LC- HRMS Examination

This test was preceded by preparing a sample of 5 mg in 1 mL of methanol with a PTFE membrane of 0.2 µm, LC-MS used in determining the compound content in CTE samples using the UHPLC Vanquish Tandem Q Exactive Plus Orbitrap HRMS ThermoScientific method. The results of sample testing on LC-HRMS are shown in the chromatogram in the form of peak height and molecular weight of the identified compounds. The results of the analysis of Butterfly Pea Flower ethanol extract samples are shown in Figure 1

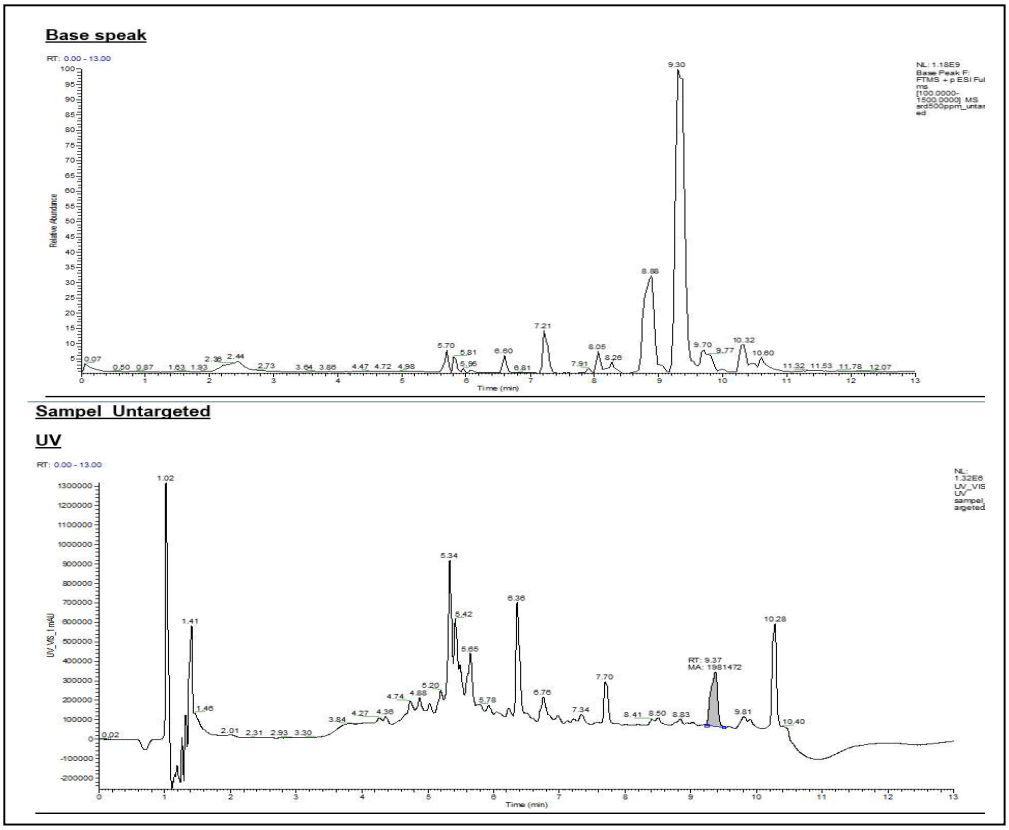

Figure 1. Chromatogram of *Clitoria ternatea* Flower Extract (CTE)

LC-HRMS testing is needed to obtain information on the content of chemical compounds in CTE test samples [10] . Based on the results of LC-HRMS examination on CTE samples, 63 secondary metabolite compounds were found. The flavonoids, phenols, terpenoids, alkaloids and vitamins found in CTE are shown in Figure 1 the results of the content is closely related to the test results of antioxidants, total phenols and total flavonoids because of the test results CTE contains more secondary metabolites that are not the group above. Flavonoids, polyphenols, sesquiterpenoids and vitamins have direct or indirect biological antioxidant properties by inhibiting several proinflammatory enzymes and cytoprotection [12,13]. The presence of other active compounds and fatty acids in synergy will provide better benefits [14-17] Based on several studies compiled by Jeyaraj et al. (2020), Butterfly Pea flowers contain bioactive compounds including: kaempferol, quercetin, and mirisetin [18].

https://doi.org/10.58511/jnpdd.v2i2.8283
JNPDD 2025; 2(2): 56-62

Table 1 LC-HRMS Examination Results.

No	Chemical name	Group	Formula	Calc. MW	RT (min)	Area Sampel
1	Glycitein	Flavonoid	C16H12O5	284.06746	7.772	4016073302
2	Quercetin	Flavonoid	C15H10O7	302.04167	4.641	2562862144
3	Nictoflorin (antiinflamasi)	Flavonoid	C27H30O15	594.15648	5.409	1916933229
4	Quercetin-3β-D-glucoside	Flavonoid	C21H20O12	464.09416	5.496	3993868121
5	Apigenin	Flavonoid	C15H10O5	270.05195	6.783	682008315.9
6	Apigenin-7-4'alloside	Flavonoid	C29H32O16	636.16655	5.723	658037186.6
7	Trifolin (kaempferol-3-O-galactoside)	Flavonoid	C21H20O11	448.09941	5.672	541625789.1
8	2',4',7-Trihydroxy-5-methoxyisoflavone	Flavonoid	C16H12O6	300.06236	6.931	519566306.8
9	Pseudobaptigenin	Flavonoid	C16H10O5	282.05199	6.998	363362411.8
10	5-Hydroxy-6-methoxy-3-(4-methoxyphenyl)-4-oxo-4H-chromen-7-yl beta-D-glucopyranosiduronic acid	Flavonoid	C23H22O12	490.10964	5.726	321186034.6
11	4',5-Dihidroksi-6,7-dimetoksiflavon	Flavonoid	C17H14O6	296.06743	7.990	311984683.9
12	Hexadecanamide P	Fenol	C16H33NO	255.25536	8.87	1383365126
13	Kaempferol	Fenol	C15H10O6	286.04689	6.849	971920332.5
14	Trans-beta-D-glucosyl-2-hydroxycinnamic acid	Fenol	C15H18O8	343.12575	4.789	578967638.4
15	4-Aminophenol	Fenol	C6H7NO	109.05283	1.097	372889241.8
16	2,6-di-tert-butyl-4-ethylphenol	Fenol	C16H26O	234.19673	7.927	357883406
17	N-feruloylglycine	Fenol	C12H13NO5	251.078	5.522	320798896
18	Pentalenolactone	Terpenoid	C15H16O5	298.08327	7.286	889757553.8
19	Laserpitin	Terpenoid	C25H38O7	450.25997	7.628	371929983.3
20	Olean-12-ene-3,16,21,22,23,28-hexol	Terpenoid	C30H50O6	506.35979	6.785	2039404803.9
21	2-Formylglycine	Alkaloid	C6H5NO2	107.03725	1.084	166389991.8
22	Nicotinamide	Vitamin	C6H6N2O	122.04793	1.426	2498448387
23	2-O-Ethyl Ascorbic Acid	Vitamin	C8H12O6	204.06294	1.146	1190084382

Antioxidant Activity

The standard of comparison in the CTE antioxidant test uses Ascorbic acid. Results of CTE antioxidant activity testing.

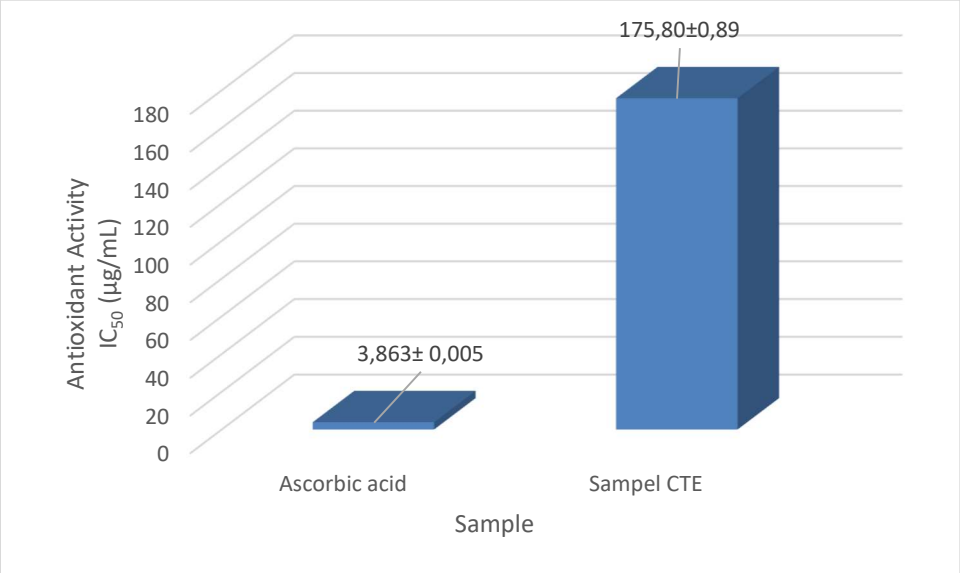

Figure 2. Antioxidant activity comparison for ascorbic acid and CTE

The results of the examination of antioxidant activity of CTE with three variations of extract weight using DPPH (1,1-diphenyl-2-picrylhydrazyl) method showed an average IC_{50} of 175.803 (µg/mL). Compared to the standard vitamin C, these results indicate CTE shows potential as an antioxidant. Based on the strength of antioxidant activity, the IC_{50} value: 150-200 µg/mL is classified as an antioxidant with weak activity. These results are in line with some previous studies where the antioxidant activity of CTE including the results obtained 1mg/mL, 12.47 ± 2.96 mg/mL, 95.30 ± 0.10 (mg/ml), 241.84 ± 7.84µL/mL and IC_{50} 480 ± 1.5 µg/ml [18,19] Similarly, one of the results of antioxidant testing of *C. ternatea* ethanol extract conducted by the Biochemistry Department of the Bogor Agricultural Institute (IPB) with the DPPH method showed IC_{50} results of 620.536 ± 50.427 µL/mL [20]. The results of antioxidant activity cannot be separated from several influencing factors because *C. ternatea* is very vulnerable to various factors. The place where the plant grows is influenced by temperature, water, salinity, pH, sunlight, fertilizer, aeration as well as when drying the simplisia, the type of extraction determines the phytochemical content of CTE. Stability of anthocyanins in CTE is also affected by factors such as temperature, pH, light, presence of enzymes, oxygen, metal ions, sulfur dioxide and phenolic acids[21,22,23]. CTE has activity in free radical inhibition, CTE is rich in other chemical content, so that the content can improve each other for better benefits.

With the proof that Butterfly Pea Flower extract contains antioxidants, Butterfly Pea Flower extract can be used as an antiaging preparation. During the skin aging process, both caused by intrinsic and extrinsic factors, it causes a reduction in structural integrity, damage to defense functions and loss of physiological functions of the skin due to the oxidation process. This condition can be counteracted by antioxidants because the counteraction mechanism of antioxidants usually occurs during the initiation or propagation of oxidation reactions of fats or other molecules in the body by absorbing and neutralizing free radicals or decomposing peroxides[25,26]

4. CONCLUSION

Butterfly pea flowers are rich in chemical compounds such as glycitein, Quercetin, Apigenin, kaemferol, laserpitin compounds including antioxidants, so that the content can improve each other for better benefits although classified antioxidant as weak.

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