THE PARTICLE SIZE OF BUTTERFLY PEA (*Clitoria ternatea*) POWDER IMPROVED THE PHENOLIC, FLAVONOID, AND ANTIOXIDANT ACTIVITY USING ETHANOL ULTRASOUND-ASSISTED EXTRACTION

S Samiyarsih[1*], Juwarno[1], W Herawati[1], N Fitrianto[2]

[1] Faculty of Biology, Jenderal Soedirman University, Purwokerto, Central Java, Indonesia, 53123
[2] Research Center for Food Technology and Processing, National Research and Innovation Agency, Gunungkidul, Yogyakarta, Indonesia 55861

Email : siti.samiyarsih@unsoed.ac.id

Abstract. Extraction of phytochemical compounds is closely related to particle size and solvent. This research evaluates the effect of particle size of butterfly pea (*Clitorea ternatea*) powder and percentage of ethanol (EtOH) solvent (0%, 40%, and 80%) using ultrasound-assisted extraction on total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity using 1,1- difenil-2-pikrilhidrazil (DPPH) method. The crude EtOH extract was extracted from dried powders of *C. ternatea* with various particle sizes (crude,> 30 mesh, > 50 mesh, > 70 mesh, and < 90 mesh). The abundance of phytochemicals showed results at smaller particle sizes. The particle sizes decreased as the TPC, TFC, and antioxidant activity significantly increased ($P<0.05$). *C. ternatea* at the smallest particle size (< 90 mesh) in 40% EtOH have the highest abundance of TPC and TFC, 62.70±1.83 mg GAE/g dry samples, and 167.23±0.73 mg quercetin/g dry samples, respectively. Furthermore, the antioxidant activity under the same treatment conditions and smaller particle size significantly increases the IC50 value. The particle size in < 90 mesh;40% EtOH has the highest DPPH radical scavenging activity at 68.83±0.95 %. The findings from this research stated that the particle size and solvent percentage could improve the extraction process for phytochemical compounds. Antioxidant activity had a strong correlation with total phenolic and flavonoid content.

Keywords: Antioxidant, *Clitorea ternatea*, flavonoid, particle size, phenolic

1. Introduction

The herbaceous plant *Clitoria ternatea* is a species belong to Fabaceae family, which has several health advantages. It has drawn attention recently due to its possible medical uses and ability to provide natural food coloring and antioxidants that can be added to food to make it look prettier. Locals refer to the commercially available *C. ternatea* flower as "Bunga telang" and utilize it extensively as a food coloring. To access its bioactive components, which are what give it its antioxidant qualities, extraction is essential [1]. Given its various advantages, research on *C. ternatea* should be done to find the best conditions for extraction and, as a result, to extract the most bioactive components for use in health applications [2]. Selecting an extraction method that will produce anthocyanins with a low cost, high purity, and stability is, therefore, essential. The extraction of anthocyanins from dried blue pea flower petals using organic solvents has

been the subject of several investigations [3]. *C. ternatea* is a good source of naturally occurring antioxidants, as evidenced by the extractable phenolic, flavonoid, and anthocyanin components. The most widely used solvents for extracting plant antioxidants include ethyl acetate, acetone, methanol, and ethanol. However, different species of plants have varying solvent efficaciousness when removing bioactive chemicals from them [4]. Despite the need for more agreement regarding the optimal extraction solvent, organic aqueous mixtures rather than a single solvent achieved higher extraction efficiency. A solvent's capacity to extract specific compounds depends on the material it pulls, including variations at the variety or cultivar level [5]. This study aimed to examine the active compounds of *C. ternatea* flower extracts, including total flavonoids, phenolic compounds, and antioxidants in various particle sizes using ethanol solvent.

2. Methods

2.1. Sample Collection and Preparation

Fresh samples of *C. ternatea* were collected from Central Java in various geographical stratifications. The voucher specimen number of *C. ternatea* herbarium was found in the Jenderal Soedirman University's Faculty of Biology. Following its removal from the tree, the petal was frozen for a whole night in the refrigerator. It was then placed in a freeze drier set at -20ºC for three days to eliminate moisture and produce a powder that could be extracted. Dried powders of *C. ternatea* with different particle sizes (crude, > 30 mesh, > 50 mesh, > 70 mesh, and < 90 mesh) were used to extract the crude ethanol extract. Following total drying, the materials were crushed into a powder and placed in vacuum-sealed bags within a desiccator to await additional examination. The percentage of ethanol (EtOH) solvent was 0%, 40%, and 80%.

2.2. Quantification of total phenolic

The Folin-Ciocalteu technique Barbosa et al. 2020 was modified to assess the total phenolic content by utilizing gallic acid (GA) as a standard. GA was weighed at 1.3 mg, and 1.3 mL of methanol was added to yield a 1000 ppm concentration GA solution. GA preparation solutions were prepared at concentrations of 210, 180, 150, 120, 90, 60, 30, and 0 ppm to generate a GA standard curve. Subsequently, a 96-well plate was designed and loaded thrice with 20 uL of GA solution. A micropipette was used to add 100 uL of Folin-Ciocalteu reagent to the 96-well plate, which was then homogenized and incubated for five minutes. It was set for two hours after adding 80 uL of 20% Na_2CO_3 solution to the 96-well plate. Subsequently, a 96-well plate was prepared and loaded with 20 uL of GA solution three times. A micropipette was used to add 100 uL of Folin-Ciocalteu reagent to the 96-well plate, which was then homogenized and incubated for five minutes. It was set for two hours after adding 80 uL of 20% Na_2CO_3 solution to the 96-well plate. For each treatment, repeat the process using a 20 ul sample of TGF kombucha. An Elisa Reader was used to measure the absorbance at 765 nm after two hours of incubation. A calibration curve was constructed using the relationship between GA content and absorbance at each concentration. The gallic acid concentration relationship and the absorbance of the gallic acid reaction with the Ciocalteu Folin Reagent are displayed on the x-axis. The gallic acid equivalent (GAEq) of the total phenolic content was determined in milliliters (mL) as mg dry extract [6].

2.3. Quantification of total flavonoid

Quercetin standards were created to calculate the total flavonoid levels. Weigh one milligram of quercetin and add one milliliter of methanol to get a quercetin solution with a concentration of one thousand parts per million. Quercetin preparation solutions containing 160, 140, 120, 100, 80, 60, 40, 20, and 0 ppm were prepared to establish a quercetin standard curve. Then, using a micropipette, 100 uL of 10% AlCl3 and 1 M KCH3COO solutions were added to the 96-well plate and incubated for 30 minutes. Afterward, a 96-well plate was created and filled with 10 uL of quercetin solution thrice. For each treatment, repeat the process using a 10 uL sample of flower extract. Using the correlation between quercetin content and absorbance at each concentration, an Elisa Reader was used to detect absorbance at 415 nm after incubation. This allowed for the creation of a calibration curve. One way to express the total flavonoid concentration is as mg of quercetin acid equivalents (QAEq) per milliliter.

2.4.Antioxidant activity of 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) assay

Using a modified procedure [7], a DPPH control solution was created as the initial step of antioxidant activity [6]. 0.2 mM DPPH was produced by dissolving 0.78 mg of DPPH powder in 10 mL of ethanol. Next, methanol was used to dilute the 100% kombucha sample to 10, 20, 30, 40, and 50%. A 96-well plate was filled with a 40 uL sample, and 160 uL of DPPH 0.2 mM was added. The absorbance was measured at a wavelength of 517 nm using an Elisa Reader after 30 minutes in the dark. A standard curve for ascorbic acid at a 1000 ppm concentration was created by weighing 1 milligram of ascorbic acid and adding 1 mL of methanol. Serial 10, 20, 30, 40, and 50 ppm diluted ascorbic acid preparation solutions were employed. The next step involved building a 96-well plate, filling it with 40 uL of ascorbic acid solution three times, and then using a micropipette to add 160 uL of 0.2 mM DPPH solution to the plate and incubating it for 30 minutes. Following incubation, the absorbance was determined at 517 nm using an Elisa Reader and a microplate reader (Multiskan® Go, Thermo Scientific, Vantaa, Finland). The formula calculated DPPH radicals at each concentration of the sample solution:

$$\% \text{ Antioxidant activity} = (Ak - As)/Ak \times 100\%$$

Wheres: (Ak)=the absorbance of DPPH, (As)= the absorbance of the sample

2.5. Statistical analysis

Linear equations are calculated using Microsoft Excel 2010. Mean data values are presented with standard error. To determine the significance of the variables investigated, analysis of variance (ANOVA) was performed on the responses in particle size and percentage of ethanol. A one-sample t-test based on IBM SPSS statistic 23 examined the significant level of the anticipated and experimental values of phenolic, flavonoid, and DPPH.

3. Results And Discussion

The ultrasonic-assisted extraction (UAE) method uses sound energy at extremely high frequencies (more than 20 kHz) to break all plant cells and increase the surface area so that the solvent may pierce them. Changes in total phenolic, flavonoid, and antioxidant activity affect particle size. The standard curve for each parameter of total phenols, flavonoid, and antioxidant levels is y=0.0201x+1.0088, y=-0.0007x+0.1407, and y=-0.0032x+0.4139 (Figure 1-3).

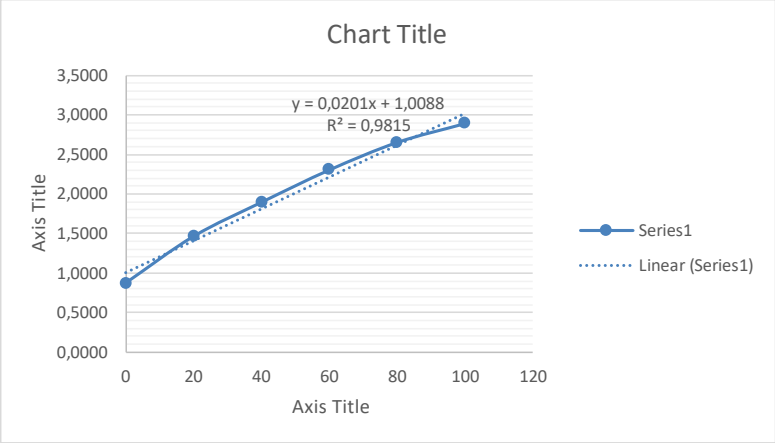

Figure 1. The Phenolic standard curve

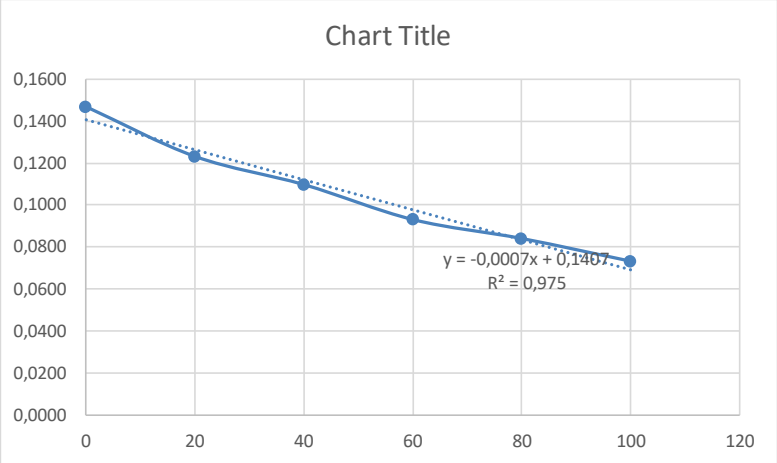

Figure 2. The Flavonoid standard curva

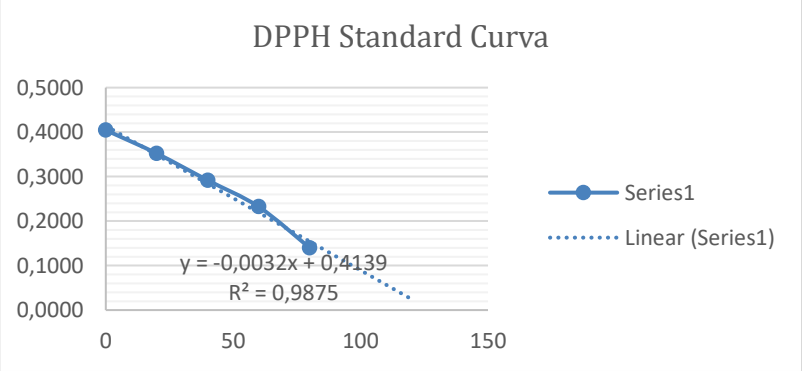

Figure 3. The DPPH standard curve

The abundance of phytochemicals showed results in smaller particle sizes. The particle sizes decreased as the total phenolic, flavonoid and antioxidant activity significantly increased ($P<0.05$). *C. ternatea* at the smallest particle size (< 90 mesh) in 40% EtOH have the highest abundance of TPC and TFC, 62.70±1.83 mg GAE/g dry samples, and 167.23±0.73 mg quercetin/g dry samples, respectively (Table 1.).

Table 1. Total phenolic content, flavonoid content, and antioxidant activity

Concentration of ethanol (%)	Particle size	Total phenolic (mg GAE/g dry sample)	Total flavonoid (mg QAE/g dry sample)	Antioxidant activity (%)
0%	Crude	49.73±0.74	145.35±0.73	48.40±0.91
	> 30 mesh	50.55±1.67	145.55±0.34	49.07±0.87
	> 50 mesh	51.05±1.83	148.50±0.87	51.10±0.59
	> 70 mesh	52.40±1.34	150.75±1.12	51.70±0.55
	> 90 mesh	52.90±1.67	155.40±1.67	54.85±0.40
40%	Crude	54.37±0.33	160.80±1.34	62.70±0.07
	> 30 mesh	55.40±1.67	163.40±0.97	65.70±0.01
	> 50 mesh	60.10±0.76	163.20±0.52	67.05±0.03
	> 70 mesh	60.65±1.67	165.73±0.34	67.11±0.07
	> 90 mesh	62.70±1.83	167.23±0.73	68.83±0.95
80%	Crude	53.90±1.34	164.67±1.12	58.70±0.97
	> 30 mesh	54.07±1.67	164.90±0.27	61.10±0.50
	> 50 mesh	55.20±0.33	164.95±0.32	62.05±0.45
	> 70 mesh	55.85±1.67	165.32±0.80	62.90±0.80
	> 90 mesh	56.01±1.83	165.91±0.38	63.05±0.74

The analysis of total phenolic contents was selected from the highest oxidant activity from the extraction to analyze the flower. The percentage of fresh weight/dried weight of *C. ternatea*. The antioxidant activity analysis by DPPH method found a statistically significant difference between 0%, 40%, and 80% ethanol-macerated *C. ternatea* flowers and standard Trolox equivalent. At > 90, mesh gave the highest antioxidant activity using macerated 80% ethanol *C. ternatea*. Because a wide range of parameters affect the extraction yield of plants, optimization is crucial in large-scale industrial activities involving the enrichment of their products with antioxidant chemicals. Therefore, to optimize extraction further, we should investigate additional factors, including pH, different solvents, and solvent-to-sample ratio, that impact the extraction yield of phenolic content and antioxidant components. To more thoroughly analyze the impact of each variable (such as temperature, duration, and ethanol concentration) on the extraction of phenolic content and antioxidant activity, a broad range of independent variables should be selected for this experiment. The extracted polyphenol and flavonoid components are best done with a high solvent ratio. This finding is consistent with another investigation that showed the yields of Pegaga (*Centella asiatica*) compounds increased progressively with the maximum polyphenol and flavonoid concentration attained by increasing the solid-to-solvent ratio [8].

The phenolic chemicals in these everyday foods are the main antioxidant constituents. Heart disease and several types of cancer can be avoided with a diet high in fruits, vegetables, grains, and olive oil [9]. It is common knowledge that phenolic chemicals aid in delivering health advantages, function as defense systems against reactive oxygen species (ROS), and improve nutritional value and quality by altering the color, flavor, taste, and scent. Results for total phenolic content are still unable to indicate the amount of higher chemicals, particularly in the obtained extract [10]. Complete flavonoid analysis is still required as a prerequisite for an additional specific collection of chemicals [11]. These were higher than ethanol with other concentrations. However, various things need to be considered when using this solvent, given the highwater content in the solvent [12].

In contrast, the most effective solvent extract (50% ethanol) was found to have an extraction efficacy comparable to heat-assisted extraction at 50°C for one hour in the water extraction

method. This suggests that future research exploring other possible bioactivities of C. ternatea flowers may not need to use solvents at all. It is advised to employ water extraction rather than solvent extraction because it is more economical, ecologically benign, and similarly effective [12].

The total phenolic content still cannot reveal the presence of more specialized chemicals in the extracted material. Testing for total flavonoids is still required as a precaution for more precise chemical group compounds. These findings demonstrated a relationship between the total amount of extracted flavonoids and the rise in ethanol concentration. According to the study's solvent polarity characteristics, 80% of the ethanol had more complex flavonoids than other solvent concentrations.

4. Conclusion

Ultrasonic can be successfully applied to extract bioactive compounds with high bioactivity from butterfly pea flowers. The particle size in < 90 mesh;40% EtOH has the highest phenolic content, flavonoid content, and DPPH radical scavenging activity at 62.70±1.83 mg GAE/g dry samples, 167.23±0.73 mg quercetin/g dry samples, and 68.83±0.95 % respectively. As a result, the particle size and solvent percentage could improve the extraction process for phytochemical compounds. Antioxidant activity had a strong correlation with total phenolic and flavonoid content.

Acknowledgments

The author would like to thank the Chair of the Institute for Research and Community Service and BLU, Jenderal Soedirman University, who provided funds so that this research could be carried out well.

References

[1]. Jaafar, N. F., Ramli, M. E., & Salleh, R. M. (2020). Optimum extraction condition of *Clitorea ternatea* flower on antioxidant activities, total phenolic, total flavonoid, and total anthocyanin contents. *Tropical life sciences research*, *31*(2), 1.

[2]. Vidana Gamage, G. C., Lim, Y. Y., & Choo, W. S. (2021). Anthocyanins from Clitoria ternatea flower: Biosynthesis, extraction, stability, antioxidant activity, and applications. Frontiers in Plant Science, 12, 792303.

[3]. Ha, V. T., & Le, N. T. (2022). Extraction of anthocyanins from Clitoria ternatea L. petals in Vietnam and determination of its antioxidant and antimicrobial activities. Jordan Journal of Pharmaceutical Sciences, 15(2), 145-157.

[4]. Borrás-Linares I, Fernández-Arroyo S, Arráez-Roman D, Palmeros-Suárez P, Val-Díaz R D, Andrade-Gonzáles I, Gonzáles A and Segura-Carretero A. (2015). Characterization of phenolic compounds, anthocyanidin, antioxidant and antimicrobial activity of 25 varieties of Mexican Roselle (Hibiscus sabdariffa). Industrial Crops and Product 69: 385–394

[5]. Samiyarsih S, Fitrianto N, Proklamasiningsih E, Juwarno, Muljowati JS. 2020. Phytochemical diversity and antimicrobial properties of methanol extracts of several cultivars of Catharanthus roseus using GC-MS. Biodiversitas 21(4): 1332-1344

[6]. Barbosa, E. L., Netto, M. C., Junior, L. B., de Moura, L. F., Brasil, G. A., Bertolazi, A. A., ... & Vasconcelos, C. M. 2022. Kombucha fermentation in blueberry (Vaccinium myrtillus) beverage and its in vivo gastroprotective effect: Preliminary study. Future Foods, 5, 100129.

[7]. Prasedya, E. S., Frediansyah, A., Martyasari, N. W. R., Ilhami, B. K., Abidin, A. S., Padmi, H., ... & Sunarwidhi, A. L. 2021. Effect of particle size on phytochemical composition and antioxidant properties of Sargassum cristaefolium ethanol extract. Scientific reports, 11(1), 17876.

[8]. Liu, S. C., Lin, J. T., Wang, C. K., Chen, H. Y., & Yang, D. J. (2009). Antioxidant properties of various solvent extracts from lychee (Litchi chinenesis Sonn.) flowers. Food Chemistry, 114(2), 577-581.

[9]. Bendary, E., Francis, R. R., Ali, H. M. G., Sarwat, M. I., & El Hady, S. (2013). Antioxidant and structure–activity relationships (SARs) of some phenolic and aniline compounds. Annals of Agricultural Sciences, 58(2), 173-181.

[10]. Sengul, M., Yildiz, H., Gungor, N., Cetin, B., Eser, Z., & Ercisli, S. (2009). Total phenolic content, antioxidant, and antimicrobial activities of some medicinal plants. Pakistan Journal of Pharmaceutical Sciences, 22(1).

[11]. Maulana, T. I., Falah, S., & Andrianto, D. (2019, July). Total phenolic content, total flavonoid content, and antioxidant activity of water and ethanol extract from Surian (Toona sinensis) leaves. In IOP Conference Series: Earth and Environmental Science (Vol. 299, No. 1, p. 012021). IOP Publishing.

[12]. Hikmawanti, N. P. E., Fatmawati, S., & Asri, A. W. (2021). The effect of ethanol concentrations as the extraction solvent on antioxidant activity of Katuk (Sauropus androgynus (L.) Merr.) leaves extracts. In IOP conference series: Earth and environmental science (Vol. 755, No. 1, p. 012060). IOP Publishing.

[13]. Jeyaraj, E. J., Lim, Y. Y., & Choo, W. S. (2021). Effect of organic solvents and water extraction on the phytochemical profile and antioxidant activity of Clitoria ternatea flowers. ACS Food Science & Technology, 1(9), 1567-1577.