Suhesti et al (2024)
Vol 12(2): 12636
https://doi.org/10.20884/1.api.2024.12.2.12636

Acta Pharmaciae Indonesia:
Acta Pharm Indo
E-ISSN 2621-4520

RESEARCH ARTICLE Open Access

Antioxidant activity of ethanol extract and ethyl acetate fraction of blue pea flower (*Clitoria ternatea* L.)

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ABSTRACT

Background: The imbalance of free radicals can be mitigated by antioxidants. Natural sources, such as the blue pea flower (*Clitoria ternatea* L.), have gained attention as potential antioxidant alternatives.

Objective: This study aimed to evaluate the antioxidant potential of the blue pea flower by extracting its fractions using n-hexane and ethyl acetate.

Methods: The study employed an experimental design involving maceration for extraction, followed by fractionation using n-hexane and ethyl acetate solvents. Antioxidant activity was assessed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. Data from both qualitative identification and antioxidant tests were analyzed descriptively and quantitatively to determine the IC_{50} values.

Results: The analysis revealed that both the extracts and fractions of the blue pea flower contain bioactive compounds with antioxidant potential, including flavonoids, tannins, steroids, and terpenoids. The IC_{50} values for antioxidant activity were as follows: n-hexane fraction, 85.28 µg/mL; ethyl acetate fraction, 82.94 µg/mL; and ethanol fraction, 78.78 µg/mL.

Conclusion: Flavonoids, steroids, and terpenoids were identified in both the blue pea flower extract and ethyl acetate fraction, while tannins were present only in the ethanol extract. Strong antioxidant activity was observed in all fractions, including the ethanol extract, n-hexane, and ethyl acetate fractions.

Introduction

Currently, changes in lifestyle and unhealthy habits have led to an imbalance between free radical molecules and endogenous antioxidants in the body. Antioxidants are essential for neutralizing free radicals and preventing oxidative damage. These compounds, despite their low molecular weight, can inhibit the formation of free radicals, thereby reducing cell damage [1,2]. Antioxidants function by donating electrons to unpaired free radicals, mitigating their oxidative effects [3]. Additionally, antioxidants play a role in preventing and eliminating cancer cell growth through apoptotic mechanisms [4].

Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butyl hydroquinone (TBHQ), and propyl gallate (PG), are widely used. However, prolonged consumption of BHA and BHT has been linked to tumor formation in animals and may cause liver damage if consumed in excess. Therefore, alternative sources of antioxidants, particularly from natural ingredients, are needed [5]. One such natural antioxidant source is the blue pea flower (*Clitoria ternatea* L.), also known as telang. Blue pea flowers contain phenolic compounds, flavonoids, anthocyanins, and flavonol glycosides, all of which exhibit antioxidant properties [6,7,8]. The antioxidant potential of blue pea flowers is attributed to their high anthocyanin and flavonoid content [7,9]. Additionally, studies have shown that the flavonoid compounds in blue pea flowers exhibit significant free

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radical inhibition compared to standard antioxidants such as gallic acid and quercetin [2,4].

Fractionation is the second stage of the compound separation process following extraction. It is a technique used to separate and classify chemical compounds in extracts based on their polarity [10]. Fractionation involves isolating compounds using two immiscible solvents. Research on the antioxidant activity of blue pea flowers has primarily focused on ethanol extracts, with limited studies on fractionated extracts. Therefore, further research is needed to investigate the fractionation process and determine the antioxidant potential of the *n*-hexane and ethyl acetate fractions of blue pea flowers. Based on this background, this study aims to identify the compound groups present in these fractions and evaluate their antioxidant activity.

Method

Extraction and fractinations

The blue pea flowers used in this study were obtained from the Banyumas region, Central Java. The extraction process was carried out using the maceration method. Dried blue pea flowers were pulverized, and 500 g of the powder was macerated with 96% ethanol for 3 × 24 hours. The solvent was replaced every 24 hours with fresh ethanol. The resulting macerate was then evaporated at 50°C using a vacuum pump until a concentrated extract was obtained.

Fractionation of the extract was performed using the liquid-liquid extraction method. A total of 1.42 g of the extract was dissolved in a methanol-water mixture (1:1) and placed in a separatory funnel. The *n*-hexane fraction was obtained by adding *n*-hexane in the same ratio (1:1), shaking the mixture carefully, and allowing it to stand for approximately 30 minutes until two distinct phases formed. The *n*-hexane-soluble phase was then separated, concentrated using an evaporator, labeled, and stored. The remaining residue was further fractionated using ethyl acetate following the same procedure. The ethyl acetate fraction was evaporated to obtain a concentrated fraction [10].

Identification of compounds using thin-layer chromatography (TLC)

Metabolites in the extract and fractions were identified using thin-layer chromatography (TLC). The stationary phase consisted of a silica gel 60 GF254 TLC plate, while the mobile phase was a chloroform-methanol mixture (9:1). TLC results were observed under UV light at 254 nm and 366 nm. To identify the chemical compounds present, the TLC plates were sprayed with specific reagents, including citroborate, Dragendorff, $FeCl_3$, Liebermann-Burchard, and 2,2-diphenyl-1-picrylhydrazyl (DPPH). The resulting color changes were then examined under UV light at 254 nm and 366 nm.

Antioxidant activity testing using the DPPH method

The free radical scavenging activity of the samples was evaluated using the DPPH assay. Test solutions were prepared by dissolving the blue pea flower extracts and fractions in ethanol. Each sample was mixed with a 0.2 mM DPPH working solution, shaken until homogeneous, and incubated for 30 minutes. The absorbance of the samples was measured using a UV-Vis spectrophotometer.

Data analysis

The TLC chromatogram profiles were analyzed descriptively and qualitatively by comparing the chromatographic profiles of the ethanol extract, *n*-hexane fraction, and ethyl acetate fraction. Antioxidant activity was analyzed using linear regression to determine the IC_{50} (inhibitory concentration) values, and the results were classified based on their antioxidant strength. The Kruskal-Wallis test was performed to assess the statistical significance of the antioxidant activity among the fractions, following normality and homogeneity tests.

Results

Extraction and fractionation

The extraction process yielded 70 g of extract from 500 g of *simplisia* powder, resulting in an extract yield of 14%. The blue pea flower extract was then sequentially fractionated using *n*-hexane and ethyl acetate solvents. The initial weight used for fractionation was 1.42 g, with a final weight of 0.111 g, yielding 7.81% (Table 1).

Phytochemical screening

The phytochemical screening revealed differences in the metabolite content of the ethanol extract and the ethyl acetate fraction of blue pea flowers. The ethanol extract contained a broader range of metabolites than

Table 1. Fractionation yield of ethanol extract of blue pea flower

Sample	Initial weight (gram)	Weight (gram)	Yield (%)
Ethanol extract	500	70	14
N-hexane fraction	1.42	0.11	7.81
Ethyl acetate fraction	1.42	0.84	58.94

Table 2. Phytochemical screening of blue pea flower extracts and fractions

Compound group	Ethanol extract	Fractions	
		***n*-Hexane**	**Ethyl acetate**
Flavonoids	+	-	+
Alkaloids	-	-	-
Tanins	+	-	-
Terpenoids	+	+	+
Steroids	+	+	+

Note: (+) indicates the presence of the compound; (-) indicates the absence of the compound.

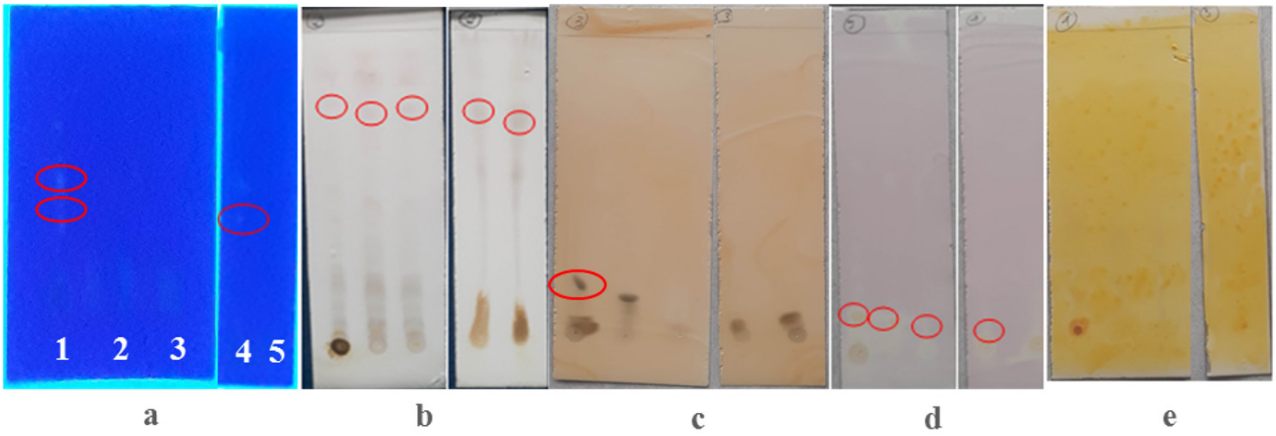

Figure 1. The TLC profiles of the extracts and fractions were analyzed after being sprayed with different reagents: (a) citroborate, (b) Liebermann-Burchard, (c) $FeCl_3$, (d) DPPH, and (e) Dragendorff. The samples included (1) ethanol extract, (2) *n*-hexane fraction, (3) chloroform fraction, (4) ethyl acetate fraction, and (5) residue.

the fractions (Table 2). Bioactive compounds with antioxidant properties include flavonoids, tannins, terpenoids, and steroids [11].

Phytochemical screening was conducted to determine the classes of compounds present in the test samples. Thin-layer chromatography (TLC) was used due to its efficiency in rapidly separating compounds. When the separated compounds were sprayed with staining reagents, direct interactions occurred between the reagents and the compounds, producing distinct color changes. These changes were more specific and less likely to be influenced by overlapping colors from other compound classes that might react with the same reagent [12]. The stationary phase used was silica gel 60 GF254, and the mobile phase consisted of chloroform:methanol (9:1). The results of the phytochemical screening of blue pea flower extracts and fractions were visualized based on the colors produced by each compound.

DPPH analysis and IC_{50} determination

The maximum absorbance wavelength of DPPH was determined to be 516.0 nm, with an absorbance value of 0.697 (Figure 2). This result aligns with previous literature, which states that DPPH radicals exhibit maximum absorbance within the 515–520 nm range [12,13].

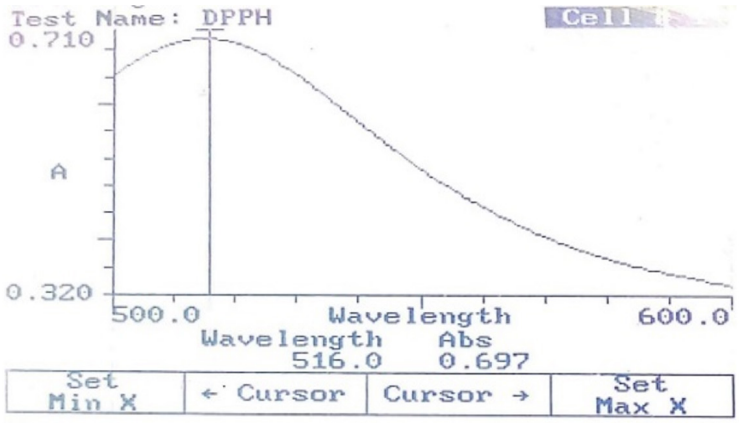

Figure 2. Maximum wavelength and absorbance of DPPH

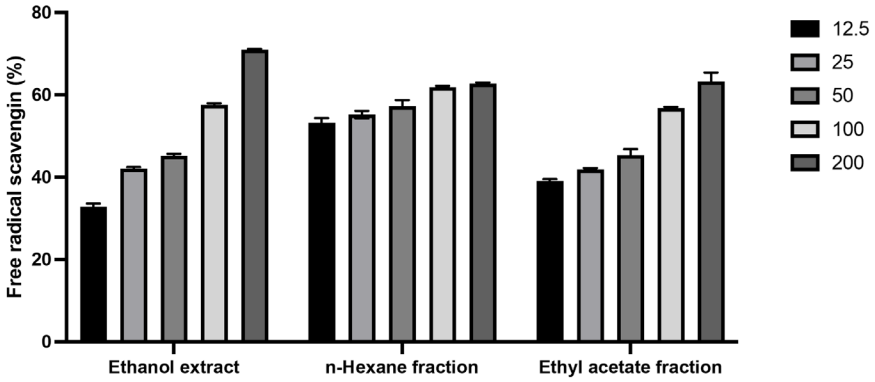

Figure 3. Percent free radical scavenging in blue pea flower extracts and fractions**.** Linear regressions. Ethanol extract: y = 0.189x + 35.111 (r^2 0.9510), n-hexane fraction**:** y = 0.0496x + 54.23 (r^2 0.8226), ethyl acetate fraction: y = 0.1305x + 39.176 (r^2 0.9325)

Antioxidant activity was evaluated by measuring absorbance using the DPPH reagent. Solutions of test samples at five different concentrations were mixed with a 0.05 mM DPPH standard solution. The absorbance of each test sample was measured using UV-Vis spectrophotometry at a wavelength of 516 nm. The absorbance values obtained for each concentration were used to calculate the percentage of DPPH radical inhibition (% inhibition) (Figure 3).

The percentage of radical scavenging activity was used to generate a linear regression curve, with concentration as the x-axis and % inhibition as the y-axis. The linear regression equation was then used to calculate the IC_{50} values. Based on these results, the ethanol extract, *n*-hexane fraction, and ethyl acetate fraction were classified as having strong antioxidant activity [20].

The Shapiro-Wilk normality test indicated that the data were not normally distributed (sig = 0.003). The homogeneity test further showed that the data

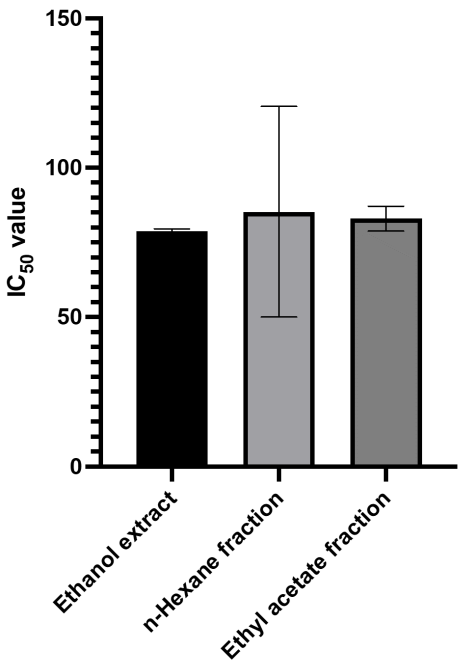

Figure 4. IC_{50} values of blue pea flower extracts and fractions

were not homogeneous (sig = 0.037). Therefore, statistical analysis was conducted using the Kruskal-Wallis test, which yielded a significant result (sig = 0.012). Consequently, post-hoc analysis using Duncan's Multiple Range Test (DMRT) was performed, revealing a significant difference in the average IC_{50} values between the ethanol extract, *n*-hexane fraction, and ethyl acetate fraction (Figure 4).

Discussion

The blue pea flower extract was prepared using the maceration method with 96% ethanol as the solvent. This method was chosen because it does not require heating, thereby minimizing the risk of degradation or decomposition of natural compounds. Ethanol is a universal solvent capable of extracting a wide range of compounds, from polar to non-polar substances [14].

Phytochemical identification was performed using specific reagents to detect the presence of various bioactive compounds. The presence of flavonoids was indicated by a yellow change upon the addition of citroborate reagent, which is highly sensitive to flavonoids and specifically detects ortho-dihydroxy groups [15]. Tannins were identified using a 1% $FeCl_3$ solution, where a blue-black or green-black change indicated their presence. The reaction likely occurs due to the interaction of $FeCl_3$ with hydroxyl groups in tannin compounds. $FeCl_3$ is commonly used for detecting phenolic compounds, including tannins [16].

Steroids and terpenoids were identified using the Liebermann-Burchard reagent, which produced a red-purple color. The reaction mechanism involves the acetylation of hydroxyl groups by acetic anhydride, leading to the formation of a double bond. The subsequent release of hydrogen atoms and their electrons results in resonance stabilization and electrophilic addition, ultimately producing the characteristic red-purple color [17].

The antioxidant test using the DPPH reagent resulted in a color change from purple to yellow. The purple color of DPPH is due to delocalized electrons, which shift to hydrazine yellow upon reduction by antioxidants. This reduction process occurs when hydrogen donors from the sample react with DPPH radicals, leading to a decrease in the purple color intensity [14].

Phytochemical screening confirmed the presence of flavonoids, tannins, steroids, and terpenoids in the ethanol extract of blue pea flowers (Table 2). These findings are consistent with previous studies [1,13,17] that reported similar phytochemical compositions in blue pea flower ethanol extracts. In addition, the ethyl acetate fraction also contained flavonoids, steroids, and terpenoids, aligning with prior research [18] that identified flavonoids in blue pea flower fractions. However, flavonoids were not detected in the *n*-hexane fraction.

Antioxidant activity was quantitatively assessed using the DPPH assay. The measurement principle is based on the color intensity change of the DPPH solution, which is proportional to the concentration of DPPH molecules. As DPPH radicals react with hydrogen atoms from the sample, the solution changes from purple to yellow, leading to a decrease in absorbance at the maximum wavelength of DPPH, as measured by UV-Vis spectrophotometry. The antioxidant activity is expressed as the inhibitory concentration (IC_{50}) value, which represents the concentration required to inhibit 50% of DPPH radicals [19].

In each test sample, the percentage of inhibition was directly proportional to the sample concentration, meaning that higher concentrations resulted in greater inhibition of DPPH radicals. This relationship suggests that at higher concentrations, the sample exhibits enhanced antioxidant activity [1].

The ethanol extract, *n*-hexane fraction, and ethyl acetate fraction were classified as having strong antioxidant activity, with IC_{50} values ranging from 50 to 100 µg/mL [20]. Among these, the ethanol extract exhibited the highest antioxidant activity, with the lowest IC_{50} value of 78.78 µg/mL. The ethyl acetate fraction demonstrated stronger antioxidant activity than the *n*-hexane fraction, likely due to its higher flavonoid content, which contributes to greater antioxidant potential [19].

Conclusion

Phytochemical screening confirmed the presence of flavonoids, steroids, and terpenoids in both the blue pea flower extract and the ethyl acetate fraction, while tannins were detected only in the ethanol extract. The *n*-hexane fraction did not contain tannins or flavonoids. Antioxidant activity analysis indicated that the ethanol extract, *n*-hexane fraction, and ethyl acetate fraction all exhibited strong antioxidant properties, with the ethanol extract showing the highest activity.

Acknowledgments

The authors express their gratitude to Universitas Jenderal Soedirman for providing financial support through the Risin 2024 Research Grant Program.

Conflict of interest

The authors declare that there are no conflicts of interest associated with the preparation of this article.

Author contributions

TSS played a key role in conceptualizing and designing the study. DSR was responsible for data collection. NY and WN contributed to data interpretation and analysis. VV was involved in manuscript writing and statistical analysis. All authors participated in data interpretation and approved the final version of the manuscript.

Received: July 17, 2024
Revised: October 23, 2024
Accepted: October 28, 2024
Published: February 18, 2025

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