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In Vitro Antioxidant Activity of *Chrysanthemum indicum* Flowers Extract and Its Fractiont

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Abstract

Chrysanthemum indicum L., commonly known as Chrysanthemum flower, is an herbaceous plant that has a long-established history of medicinal usage. There has been extensive research about C. indicium L, especially about its antioxidant activities, but not much has been done on its fraction. This study aimed to explore the efficacy of the ethanol extract and its fraction derived from Chrysanthemum flowers in scavenging free radicals. The antioxidant potential of the ethanol extract, as well as its aqueous and n-hexane fractions, was evaluated using the 2,2-diphenyl-1-picrilhidrazine (DPPH) method in vitro. The degree of antioxidant activity was quantified by determining the IC_{50} value, which corresponds to the concentration of the extract or fractions required to inhibit 50% of DPPH free radicals. The results obtained from this investigation provide strong evidence that the ethanolic extract, as well as its aqueous and n-hexane fractions, exhibited significant antioxidant activity. The measured IC_{50} values for the ethanolic extract, aqueous fraction, and n-hexane fraction were 1.350 µg/mL, 1.109 µg/mL, and 7.588 µg/mL, respectively.

1. Introduction

Indonesia, situated in the tropical region, possesses abundant biodiversity and a vast array of plant species [1, 2]. Indonesian communities extensively utilize numerous plants for their medicinal properties in treating various ailments [3, 4]. The empirical use of these medicinal plants varies across different regions. Consequently, research endeavors have been undertaken to investigate and validate their efficacy. These studies strongly contribute to the advancement of utilizing plants that were originally employed empirically, leading to the development of diverse forms of medicinal preparations [5].

Chrysanthemum flowers are among the plants that have not yet been utilized for medicinal preparations. These flowers exhibit a wide range of color variants including white, red, yellow, purple, green, orange, and various other shades. In addition to their diverse colors,

chrysanthemums also possess a gentle, distinct fragrance, and are often associated with symbols of admiration or praise. However, in Indonesia, the utilization of chrysanthemum flowers is primarily limited to their use as cut flowers, with little to no development towards their potential as food or medicinal products [6].

Previous studies have elucidated the chemical composition of *chrysanthemum indicum* L, revealing the presence of saponins, steroids, flavonoids, tannins, terpenoids, and alkaloids [7]. The characteristic fragrance of chrysanthemum flowers has led to the development of preparations in the form of tea. They conducted research highlighting the antioxidant properties of chrysanthemum flowers, which can serve as a relaxing agent, enhance visual acuity, alleviate heartburn, combat fatigue, detoxify the body, and improve blood circulation.

Antioxidants are bioactive compounds that possess the ability to impede oxidation reactions by binding to free radicals and reactive molecules. Antioxidant treatment has been suggested as efficacious treatment modalities in multiple diseases. These free radicals have the potential to inflict damage upon macromolecules responsible for cellular formation, thereby contributing to the development of degenerative diseases. While endogenous antioxidants exist within the human body to scavenge free radicals, an insufficiency of antioxidants relative to the abundance of free radicals necessitates the inclusion of exogenous antioxidants to mitigate the adverse effects of free radicals [8]. The assessment of antioxidant activity is commonly conducted using the DPPH assay, a method that is characterized by its simplicity, rapidity, and the minimized requirement for reagents compared to other techniques [9]. Therefore, this research aimed to analyze the antioxidant content of extracts and its aqueous and n-hexane fractions derived from Chrysanthemum flowers.

2. Materials and Methods

2.1. Sample Preparation

Chrysanthemum flowers (*Chrysanthemum indicum* L.) were acquired from the city of Tomohon, located in North Sulawesi (Indonesia) on February 11, 2023. Subsequently, the flowers were cleansed by thorough washing with running aquadest to eliminate any dust or impurities present on the samples. The obtained samples were subsequently subjected to a drying process in an oven maintained at a temperature of 40 °C for 48 hours.

2.2. Extraction

The dried samples were pulverized using a specialized blender for dry ingredients. Subsequently, extraction was conducted using the maceration method employing 95% pro analysis grade ethanol as the solvent. A quantity of 500 grams of the pulverized samples was macerated with 2000 mL of the solvent in a 3000 mL jar. The maceration process spanned a duration of 3 days, with the sample solution being stirred daily. The initial maceration results were filtered using Whatman filter paper no. 1, and the filtrate was subsequently dried at 40 °C. The residue from the initial maceration was subjected to a second maceration process, involving the addition of 1000 mL of 95% ethanol. After a further 3 days of re-maceration, the obtained filtrate was combined with the results of the initial maceration, followed by drying in an oven at 40 °C for 48 hours. This process yielded a crude extract derived from the *chrysanthemum indicum* flower sample.

2.3. Fractionation

A quantity of 25 grams of crude extract obtained from Chrysanthemum flowers was weighed and subsequently dissolved in 100 ml of distilled water. The resulting solution was then transferred into a separatory funnel. Next, 100 ml of n-hexane solvent was added to the separatory funnel. The mixture was vigorously shaken and allowed to settle until two distinct layers were formed. Subsequently, the n-hexane layer was separated from the separating funnel and subjected to evaporation in an oven until complete solvent evaporation occurred. This process resulted in the production of the n-hexane fraction (non-polar fraction) as well as the aqueous fraction (polar fraction).

2.4. Preparation of DPPH Stock Solution

The stock solution of the DPPH radical, prepared by dissolving 4 mg DPPH in 100 mL of 96% ethanol was thoroughly mixed to ensure homogeneity. The solution was kept in a refrigerator until further use. The working solution of the radical was prepared by diluting the DPPH stock solution with 96% ethanol to obtain an absorbance of about 0.98 (±0.02) at 517 nm [10].

2.4.1. Preparation of Test Solution

A stock solution of the samples was prepared at a concentration of 1000 ppm by weighing 10 mg of each sample and dissolving it in 10 mL of 96% ethanol. The antioxidant activity of each sample was evaluated using varying concentrations (2, 4, 6, 8, and 10 µg/mL). To achieve these concentrations, sequential additions of the samples were made, specifically (0.01, 0.02, 0.03, 0.04, and 0.05 mL), with a final volume of 5 mL for each concentration.

2.4.2. Antioxidant Activity Test with the DPPH Method

Antioxidant activity of the extracts and fraction of Chrysanthemum flowers was tested by DPPH radical scavenging assay as described by Blois [11] and Brand-Williams et al. [12] with slight modifications. The hydrogen atom donating ability of the plant extractives was determined by the decolorization of methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). DPPH produces violet/purple color in ethanol solution and fades to shades of yellow color in the presence of antioxidants. To each of the prepared test solutions, 2 mL of DPPH solution was added, followed by the addition of 96% pro analysis grade ethanol to reach a final volume of 5 mL. The reaction mixture was vortexed thoroughly and left in the dark for 30 min. The absorbance of the solutions was measured at a wavelength of 517 nm using a UV-Vis spectrophotometer, with the measurement taken in the last five minutes before the completion of the 30-minute reaction time. The antioxidant activity test was conducted on both the extract samples and the fractions derived from chrysanthemum flowers. The equation to calculate the free radical scavenging activity is presented in Equation 1.

$$\%\ \text{DPPH RSA} = \frac{A_{control} - A_{sample}}{A_{control}} x100\% \qquad (1)$$

where $A_{control}$ is the absorbance of the control, and A_{sample} is the absorbance of the extract/fraction. After the percentage of free radical scavengers (indicated by inhibition) has been obtained, the calculation of IC_{50} was performed using the linear regression equation: y = A + Bx, where x represents the concentration of the sample solution in µg/mL, and y represents the percentage of free radical scavengers (inhibition). The expression of antioxidant activity is done through the determination of the 50% Inhibition Concentration or IC_{50}, which is the concentration of the sample solution that can reduce DPPH radicals by 50% [13]. The IC_{50} value is obtained by substituting y with 50 in the equation, resulting in the determination of the x value. The experiment was repeated three times at each concentration

3. Results and Discussion

3.1. Extraction and Fractionation

The extraction process utilized 95% ethanol as the solvent due to its universal properties, enabling the dissolution of non-polar, semi-polar, and polar compounds. The maceration method was employed for the extraction, which involved a 3-day duration with one remaceration and daily stirring. This method was chosen to prevent the potential degradation of antioxidant-active secondary metabolite compounds such as phenolic compounds and flavonoids, which can occur when subjected to heat [14]. Prolonged soaking allowed for the disruption of cell walls and membranes, facilitated by pressure differentials inside and outside the cells. Consequently, the secondary metabolites present in the cytoplasm were able to dissolve in the solvent [15].

Table 1. Rendemen of the Chrysanthemum flowers extract and fraction

Sample	Ethanol Extract	n-Hexane Fraction	Aqueous Fraction
Sample weight (g)	500	20	20
Yield weight (g)	63.30	6.99	4.93
% Rendemen	12.66	34.95	24.65
Color	Deep green	Orange	Brownish green

Throughout the extraction process, the 95% ethanol extract solution underwent evaporation using an oven set at a temperature of 40 ºC. This was carried out with the objective of obtaining a high-quality crude extract, as the temperature utilized during evaporation has an impact on the extract's overall quality. This is because there is a possibility that excessively high temperatures can potentially weaken the antioxidant activity of the sample [16].

The obtained yield of the extract was 63.30 g, corresponding to a yield percentage of 12.66%, and the extracted sample exhibited a dark green color, as indicated in Table 1. The results from the extraction process suggest a relatively high yield percentage. This outcome is influenced by various factors, including the duration of sample immersion, the solvent type, the volume of solvent employed, and the size of the sample particles or their extensive surface area that interacts with the solvent [16].

Fractionation is employed as a means of segregating the constituents within the extract samples based on their polarity using diverse solvents. The liquid-liquid fractionation method, employing a separating funnel, is utilized in this process, where in two solvents, namely n-hexane and distilled water, are employed. The objective of using n-hexane is to attract non-polar compounds, while distilled water is employed to attract polar compounds. Upon dissolution of the extract samples in the two solvents, the formation of two distinct layers occurs, with the solvent possessing lower density occupying the upper layer, and the solvent with higher density settling in the lower layer. Subsequently, the fractionation solution obtained from the n-hexane solvent is subjected to evaporation, resulting in the production of a concentrated extract. The outcomes of the n-hexane fraction obtained from chrysanthemum flowers are detailed in Table 1.

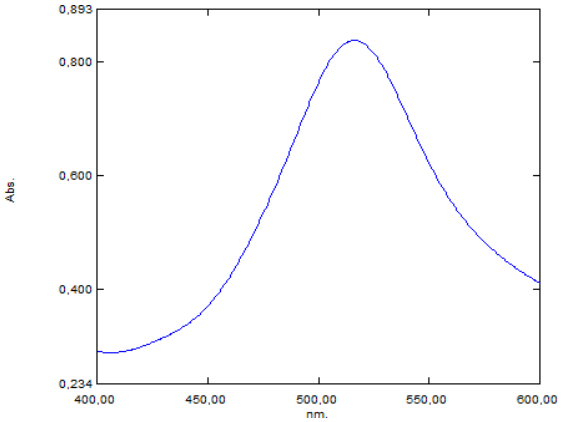

Figure 1. Maximum Wavelength Data from DPPH.

Table 2. Inhibition Activity of Chrysanthemum flower extract and Its Fraction.

Sample	Concentration (µg/mL)	Inhibition activity (%)	IC50 (µg/mL)
Ethanol extract	2	58.934	
	4	67.164	
	6	74.240	1.350
	8	82.053	
	10	87.900	
n-Hexane	2	61.257	
Fraction	4	73.592	
	6	85.549	1.109
	8	90.135	
	10	90.350	
Aqueous Fraction	2	39.439	
	4	43.508	
	6	47.470	7.588
	8	49.212	
	10	55.418	

3.2. Antioxidant Activity

The assessment of antioxidant activity was conducted employing the DPPH (1,1-diphenyl-2-picrylhydrazyl) method. This methodology relies on the computation of absorbance values obtained through spectrophotometric measurements. Specifically, the absorbance value is determined at the maximum wavelength of the DPPH control solution, where the wavelength utilized for the DPPH analysis in this study was 517 nm. The absorbance value recorded for the DPPH control solution was 0.838, as illustrated in Figure 1.

The assessment of antioxidant activity involves analyzing the absorbance value of the sample subsequent to its reaction with DPPH free radicals. The DPPH test is a simple, accurate and commonly used method for evaluating the potential free radical scavenger of an extract [17]. It is observed that a lower absorbance value corresponds to a higher level of antioxidant activity. This phenomenon is accompanied by a discernible color transformation of the solution from purple to yellow.

According to the findings presented in Table 2, the Chrysanthemum flower sample demonstrated the presence of compounds exhibiting remarkably potent antioxidant activity. This conclusion is drawn based on the IC_{50} value, which is less than 50 µg/mL [18]. The ethanol extract and n-hexane fraction exhibited similar IC_{50} values of 1.350 µg/mL and 1.109 µg/mL, respectively. It is noteworthy that higher IC_{50} values indicate lower levels of antioxidant activity [19].

There are many weaknesses of using DPPH for in vitro antioxidant research such as DPPH can only dissolved in organic solvents rather difficult to analyze hydrophilic compounds [20], the DPPH radical can interact with other radicals and the response time of the curve to reach a steady state is not linear with the difference in the ratio of antioxidants to DPPH [21]. This study also uses an oven for the extraction process to evaporate the solvent so that it takes a long time to obtain dry extracts, it is recommended to use a rotary evaporator.

4. Conclusions

This study focused on the extraction, fractionation, and evaluation of antioxidant activity of Chrysanthemum flowers (*Chrysanthemum indicum* L.). The extract and fractions obtained from *Chrysanthemum indicum* L. exhibited a classification of "very strong" antioxidant activity, as indicated by their respective IC_{50} values. However, further research is needed such as in silico and in vivo research for clinical use as antioxidant preparations aimed at mitigating degenerative diseases caused by free radicals.

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