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Research Article

Comparison of Antioxidant Activity Testing of Rosemary Leaves Using the DPPH Method

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ABSTRACT

The ability of rosemary leaf extract to scavenge free radicals was tested using the DPPH method in this research. Rosemary leaves were selected based on their natural richness in antioxidant compounds such as polyphenols and flavonoids. The test results showed that the ethanol extract, ethyl acetate fraction, and n-hexane fraction all exhibited very strong antioxidant potential, with IC_{50} values below 50 ppm. The ethanol extract was the most potent with an IC_{50} of 23.98 ppm, followed by the ethyl acetate fraction with an IC_{50} of 26.60 ppm and the n-hexane fraction with an IC_{50} of 27.95 ppm. The superiority of the ethanol extract is attributed to its ability as an optimal polar solvent for dissolving phenolic compounds. These compounds play a crucial role in stabilizing free radicals through hydrogen/electron donation, as well as resonance mechanisms.

Keywords: Rosemary Leaves, DPPH, Antioxidants

1. Introduction

Free radicals are naturally produced in the body as a result of metabolic processes. However, external factors such as pollution, UV exposure, and poor lifestyle choices can trigger excessive free radical production [1]. The uncontrolled accumulation of free radicals can lead to oxidative stress, a condition that causes damage to cells, proteins, and DNA within the body. This cellular damage contributes to the onset of various degenerative diseases, including heart disease, cancer, and premature ageing.

Antioxidants are known to play a crucial role in neutralising the damaging effects of free radicals [2]. Although the body is capable of producing its antioxidants, additional intake from external sources, such as herbal plants, is still necessary to strengthen the body's defences [3]. One potential natural source is rosemary (Rosmarinus officinalis L.), a herbal plant commonly used as a spice and in traditional medicine [4]. The rosemary leaves used in this study contain high levels of potent and relatively stable antioxidants, particularly carnosic acid, carnosol, and rosmarinic acid, which remain effective even under processing conditions such as heat and light exposure [5] It is rich in secondary metabolites such as polyphenols, flavonoids, and phenolic acids, which are the primary sources of its natural antioxidant activity [6], [7], [8]

The DPPH (2,2-diphenyl-1-picrylhydrazyl) method is an efficient colorimetric test for determining the antioxidant potential of a substance. Antioxidant activity testing using the DPPH method is based on a rapid and straightforward colour change reaction. This method utilizes DPPH, a stable free radical whose solution is a deep purple colour. When an antioxidant, such as rosemary leaf extract, is added, it donates a hydrogen atom to neutralize the DPPH radical. This hydrogen donation neutralizes the DPPH radical, which is visually indicated by the fading of the purple colour to yellow. The intensity of this colour change is then measured using a UV-Vis spectrophotometer to calculate the IC_{50} value, which is the concentration required to inhibit 50% of the DPPH radicals. The lower the IC_{50} value, the stronger the antioxidant activity of the sample [9].

This research was designed to measure the antioxidant activity of rosemary leaf extract using the DPPH method. This research is expected to produce quantitative data on the effectiveness of rosemary leaves as antioxidants. Additionally, this

research can serve as a basis for selecting raw materials for various industries, including cosmetics, chemicals, and pharmaceuticals.

2. Materials and Methods

2.1 *Materials*

They materials used in this research were rosemary leaves, absolute ethanol (Merck), distilled water, ethyl acetate (Smartlab), 96% ethanol (Smartlab), and n-hexane (Smartlab).

2.2 *Method*

2.2.1 *Production of Rosemary Leaf Powder*

Fresh rosemary leaves are washed with running water, then chopped and dried by airing them indoors. The sample is pulverised and sieved using a No. 40 mesh sieve.

2.2.2 *Production of Rosemary Leaf Ethanol Extract*

Dried rosemary leaves that have been ground into powder (100 g) are soaked in 650 mL of 96% ethanol for 7 x 24 hours. This soaking or maceration process aims to extract the active compounds from the rosemary leaves into the ethanol solvent. After the maceration process, the solution is filtered to separate the crude extract from the residue. The crude extract is then evaporated using a rotary evaporator to remove the solvent. To obtain a dry extract ready for testing, the concentrated extract from the previous evaporation is slowly reheated using a water bath at a low temperature until all remaining solvent has evaporated.

2.2.3 *Preparation of N-Hexane and Ethyl Acetate Fractions from Rosemary Leaves*

The procedure begins by dissolving 5 grams of crude ethanol extract of rosemary leaves in 10 mL of distilled water. This solution is then transferred to a separating funnel, to which 20 mL of ethyl acetate is added. The mixture is shaken and left to stand for approximately 12 hours until it forms three layers. The top layer is the ethyl acetate layer, the middle layer is the ethanol extract residue, while the bottom layer is the water layer. Fractionation is performed three times by adding the same amount of ethyl acetate solution. All ethyl acetate layers are collected and combined, then subjected to evaporation until concentrated. The evaporation product is placed in a water bath to obtain a dry fraction for testing. Five grams of concentrated ethanol extract of rosemary leaves are taken, mixed with 10 mL of distilled water, and placed in a separatory funnel. Twenty millilitres of n-hexane are then added. The mixture is shaken and left to stand for approximately 12 hours until three layers form. The top layer is the n-hexane layer, the middle layer is the ethanol extract residue, while the bottom layer is the water layer. Fractionation is performed three times by adding the same amount of n-hexane solution. All ethyl acetate layers are collected and combined, then subjected to evaporation until concentrated. The evaporation product is placed in a water bath to obtain the dry fraction for testing [6].

2.2.4 *Preparation of DPPH Solution*

The DPPH solution was prepared using Smartlab brand DPPH crystals, weighing 0.004 grams (4 mg), which were then dissolved in methanol in a measuring flask lined with aluminium foil. Methanol was added to a volume of 100 mL, resulting in a concentration of 40 ppm, equivalent to 0.004%. To maintain its stability, the DPPH solution must be used immediately or stored in a cool, dark place, protected from exposure to sunlight to prevent degradation.

2.2.5 *Determination of Maximum λ*

A 100 ppm DPPH solution was pipetted into 1 mL and added with 3 mL of 96% ethanol, then incubated for 30 minutes and measured with a UV Vis spectrophotometer at a maximum wavelength of 400-800 nm. The λ max value of a compound can vary depending on the conditions and type of equipment used. In free radical scavenging assays using a UV-Vis spectrophotometer, λ max typically falls within the range of 514–519 nm. Thus, a maximum wavelength of 516 nm was selected as the standard reading wavelength to monitor the decrease in DPPH absorbance during reactions with ethanol, ethyl acetate, and n-hexane extracts of rosemary leaves.

2.2.6 *Preparation of Test Solution*

The crude extract and each fraction were weighed at 5 mg and dissolved in 50 mL of 96% ethanol. Then, they were homogenized to obtain a test solution concentration of 100 ppm. This was then diluted to several concentration variations, namely 10, 20, 30, 40, and 50 ppm.

2.2.7 *Measurement of Antioxidant Activity Using the DPPH Method*

In the antioxidant activity testing stage, using the DPPH method, test solutions were prepared at several different concentrations: 10, 20, 30, 40, and 50 ppm. From each concentration, 3 mL was pipetted into a test tube to test its antioxidant activity using the DPPH method. Then, 1 mL of 100 ppm DPPH was added to each concentration and the mixture was homogenised. The solution was then incubated at 37°C for 30 minutes, and its absorbance was measured using a visible spectrophotometer at a wavelength of 516 nm.

2.2.8 *Data Analysis*

Quantification of antioxidant activity using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method is expressed as a percentage of inhibition. The absorbance values obtained from the measurements are used to calculate the percentage of inhibition, which can be calculated using equation 1:

$$\%\ \text{Inhibition} = \frac{(\text{Abs control} - \text{Abs sample})}{\text{Abs control}} \times 100\% \qquad (1)$$

$$Y = aX + b \quad \Rightarrow \quad X_{IC_{50}} = \frac{50 - b}{a}$$

The IC_{50} value is calculated using the linear regression equation Y = aX + b where Y is 50 to obtain the IC_{50} value for the Materials.

3. Result and Discussion

3.1 *Determination of Maximum λ*

The maximum wavelength of 516 nm was selected as the standard reading wavelength to monitor the decrease in DPPH absorbance.

3.2 *Antioxidant Testing Using the DPPH Method*

The first step in the DPPH antioxidant testing method is to determine the maximum wavelength (λ) of the DPPH solution. Determining the maximum wavelength (λ) is important to ensure the accuracy of the sensitivity and reproducibility of the data obtained. The determination of the wavelength (λ) shows that the maximum wavelength (λ) absorption value of DPPH obtained is 516 nm. This result is consistent with the research by [10].

Antioxidant activity testing using the DPPH method is expressed as the IC_{50} value, defined as the concentration of the sample required to inhibit 50% of DPPH free radicals. The IC_{50} value is determined from the curve showing the relationship between % inhibition and the test solution. The smaller the IC_{50} value, the better the antioxidant activity [11].

Table 1. Data on the antioxidant activity of rosemary leaf extract using the DPPH method

No.	Test Solution	Concentration	Absorbance	Blank Absorbance	% Inhibition
		10	0,066		-13,793
		20	0,034		41,379
1.	Extract ethanol	30	0,034	0,058	41,379
		40	0,027		53,448
		50	0,023		60,345
		10	0,040		31,034
		20	0,028		51,724
2.	Ethyl acetate fraction	30	0,028	0,058	51,724
		40	0,021		63,793
		50	0,020		65,517
		10	0,040		31,034
		20	0,028		51,724
3.	N-hexane fraction	30	0,028	0,058	51,724
		40	0,020		65,517
		50	0,016		72,414

Table 2. Shows the calculation steps for each sample, calculated from the regression equation of % inhibition against concentration

Sample	a	b	Y	(50 – b)	X (IC_{50}, ppm)
Extract ethanol	1,6034	11,552	50	38,448	23,98
Ethyl acetate fraction	0,8103	28,448	50	21,552	26,60
N-hexane fraction	0,6724	31,207	50	18,793	27,95

The IC_{50} value is determined by creating a linear regression equation from the available data. In this equation, concentration (ppm) is used as the X-axis (abscissa) and percentage inhibition as the Y-axis (ordinate). After obtaining the equation Y = aX + b, the IC_{50} value is calculated as the X value when Y is 50.

The DPPH method for measuring antioxidant activity showed that all three fractions of rosemary leaf extract had very strong antioxidant potential, with values of 23.98 ppm, 26.60 ppm, and 27.95 ppm, respectively. The ethanol extract was the most potent with an IC_{50} value of 23.98 ppm, meaning that at a concentration of 23.98 ppm, the ethanol extract of rosemary leaves could scavenge 50% of DPPH free radicals, indicating that the ethanol extract possesses very strong antioxidant properties. Meanwhile, the ethyl acetate fraction and n-hexane fraction also showed very strong activity with IC_{50} values of 26.60 ppm and 27.95 ppm, respectively. The IC_{50} calculation results indicate the superiority of the ethanol extract of rosemary leaves over the ethyl acetate and n-hexane fractions, as indicated by the lowest IC_{50} value. According to the parameters set by Guna et al. (2020), where very strong antioxidant activity is defined by an IC_{50} value of 50 ppm, the ethanol extract of rosemary leaves can be classified as having such potential [12].

The low antioxidant activity observed can be attributed to several factors, primarily the time and place of harvest. This is because the harvest time determines the concentration of active compounds, and the right harvest time is when the accumulation of these compounds is at its highest. Ideal harvesting is done when the concentration of active compounds is at its maximum level, which for secondary metabolites often occurs in the morning [13].

The order of antioxidant activity strength is ethanol extract > ethyl acetate fraction > n-hexane fraction. This is reflected in the lowest IC_{50} value in the ethanol extract. This trend underscores the crucial role of solvent polarity in extracting the dominant antioxidant compounds [14]. No fraction has an IC_{50} lower than the ethanol extract. The ethanol extract remains the most active due to its polar nature, enabling it to dissolve phenolic compounds, flavonoids, and tannins compounds known as effective hydrogen/electron donors in the DPPH assay. In line with [15]research, it was found that polar solvents (ethanol) are more efficient at extracting phenolic compounds than semi-polar solvents (ethyl acetate) and non-polar solvents (n-hexane).

4. Conclusion

The results of the research concluded that the evaluation of the antioxidant activity of Rosmarinus officinalis L. leaf ethanol extract was conducted. The order of antioxidant activity strength from highest to lowest is ethanol extract (IC_{50} 23.98 ppm), followed by ethyl acetate fraction (IC_{50} 26.60 ppm), and finally n-hexane fraction (IC_{50} 27.95 ppm). The intensity of antioxidant activity, in descending order, was ethanol extract, ethyl acetate fraction, and n-hexane fraction.

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