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Activity of Superoxide Dismutase (SOD) in guava (*Psidium guajava L.*) leaf extracts using the Water Soluble Tetrazolium Salt-1 (WST-1) method

Mitha Oktavia Mandasari*, Ana Indrayati, Desi Purwaningsih

Faculty of Pharmacy, Universitas Setia Budi, Indonesia
*Corresponding author: 25195900a@mhs.setiabudi.ac.id

Abstract

Background: Antioxidants are compounds that inhibit cell damage caused by free radicals. Antioxidant enzymes include superoxide dismutase (SOD), glutathione, and catalase. SOD protects cells against free radicals implicated in various diseases. The guava plant (*Psidium guajava* L.) is known to possess SOD activity.

Objective: This research was performed to determine the activity of the superoxide dismutase enzyme in guava leaves extract (*Psidium guajava L.*) with ammonium sulfate concentrations of 25, 50, and 75%.

Methods: Guava leaves were extracted using phosphate buffer and centrifugation. The superoxide dismutase (SOD) enzyme was then purified using ammonium sulfate at concentrations of 25, 50, and 75%. The amount of protein was measured with the Lowry method, and the activity of the SOD enzyme was tested using the WST-1 assay to find out how much it was inhibited. The resulting data were statistically analyzed using one-way ANOVA.

Results: Guava leaves extract exhibits SOD enzyme activity. The total protein content of the crude guava leaf extract and the extracts purified with 25, 50, and 75% ammonium sulfate were 9.683, 6.958, 8.842, and 11.269 mg/mL, respectively. The corresponding percent inhibition values were 69.652, 35.323, 61.69, and 79.104%. The 75% ammonium sulfate concentration yielded the highest percent inhibition.

Conclusion: Guava leaves extract exhibits SOD enzyme activity, with the highest percent inhibition observed at an ammonium sulfate concentration of 75%.

Keywords: *Psidium guajava* L., free radicals, antioxidants, SOD, WST-1

1. Introduction

Contemporary lifestyles including unhealthy dietary patterns have adverse health implications. Furthermore, environmental pollution can reduce the quality of life due to lower production of compounds essential for physiological function. Natural antioxidants help maintain the health by mitigating the detrimental effects of free radicals generated by various environmental stressors, such as air pollution, radiation, hazardous chemicals, cigarette smoke, and solar radiation (Arnanda & Nuwarda, 2019).

Free radicals with unpaired electrons have high reactivity as they grab electrons from the surrounding molecules. This process induces oxidative stress, causing an imbalance between free radical production and antioxidant defenses and cellular damage. Oxidative stress occurs when the generation of reactive oxygen species (ROS) exceeds the available antioxidant capacity and overwhelm the cellular detoxification systems (Arief & Widodo, 2018).

Antioxidant counteracts the effects from free radicals by suppressing molecular oxidation. It neutralizes and inhibits the oxidation reactions that involve free radicals (Bintarti, 2014). Endogenous antioxidants, such as superoxide dismutase (SOD), offer a vital defense against oxidative

stress (Werdhawati, 2014) and protects cells from free radical-mediated damage, mitigating the risk of various diseases (Yanti *et al*., 2016). Furthermore, SOD may enhance the therapeutic efficacy of certain pharmaceuticals and holds potential as a nutritional supplement (Younus, 2018).

SOD can be obtained from plants that contain antioxidant compounds. Guava (*Psidium guajava L)* inhibits free radicals (Maulana *et al*., 2016) from its antioxidant compounds and it exhibits high SOD that reaches 100% in its leaves (Widowati *et al*., 2005). This research was performed to investigate the SOD activity, total protein content, and percentage inhibition of crude guava leaves extract purified with 25, 50, and 75% ammonium sulfate concentrations to determine the optimal purification concentration for SOD inhibition.

2. Method

2.1. Tools and materials

This research utilized the following tools: blender, analytical balance, knife, pH meter, measuring cup, beakers, Erlenmeyer flasks, refrigerator, centrifuge, flannel, jars, micropipettes, volumetric pipettes, volumetric flasks, magnetic stirrer, UV-Vis spectrophotometer, 96-well plate, incubator, and microplate reader. The materials included guava leaves, phosphate buffer, ammonium sulfate, distilled water (aquades and aquabides), bovine serum albumin (BSA), WST solution, vitamin C, Na_2CO_3, NaOH, sodium potassium tartrate, $CuSO_4 \cdot 5H_2O$, Folin-Ciocalteu phenol reagent, assay buffer, enzyme solution, and vitamin C.

2.2. Sampling and identification

Plant identification was conducted at the Center for Research and Development of Medicinal Plants and Traditional Medicines (B2P2TOOT) in Tawangmangu, Karanganyar Regency, Central Java, to ensure accurate species identification and prevent sample contamination (Klau & Hesturini, 2021). Guava (*Psidium guajava L.*) leaves were collected from a single, healthy tree in Plagan village, Klaten Regency, Central Java. Only undamaged, fresh leaves were selected.

2.3. SOD enzyme extraction

One hundred grams of guava leaves were cut into small pieces and homogenized in a 1:2 ratio with 0.02M phosphate buffer (pH 7.2) using a blender. The homogenate was filtered through flannel, cooled overnight at <4°C, and then centrifuged at 5.000 rpm for 30 minutes at 4°C. The resulting supernatant was collected as the crude extract (Male *et al*., 2014).

2.4. Purification of SOD enzyme extract

Ammonium sulfate purification was performed at 25, 50, and 75% saturation. For each concentration, the appropriate amount of ammonium sulfate (4.02 g for 25%, 8.73 g for 50%, and 14.28 g for 75%) was gradually added to 30 mL of crude SOD extract while stirring with a magnetic stirrer at 4°C. Each mixture was then centrifuged at 5.000 rpm for 30 minutes at 4°C. Following centrifugation, the supernatant was discarded, and the resulting pellet was resuspended in phosphate buffer at a 1:2 ratio (Moon-Ai *et al*., 2012; Rahman *et al*., 2012).

2.5. The determination of total protein content using the Lowry method

Protein content was determined using the Lowry method (Lowry *et al*., 1951). Reagents were prepared as follows: reagent A (2% Na_2CO_3 in 0.10 N NaOH), reagent B (0.5% $CuSO_4 \cdot 5H_2O$ in 1% sodium potassium tartrate), reagent C (prepared fresh by mixing 50 mL of reagent A with 1 mL of reagent B), and reagent D (Folin-Ciocalteu phenol reagent diluted 1:1 with distilled water).

A BSA standard curve was generated using a 1000 ppm stock solution (50 mg BSA in 50 mL distilled water). Aliquots of the stock solution (0.5; 0.75; 1; 1.25; and 1.5 mL) were diluted to 10 mL with distilled water in volumetric flasks. One mL of each standard and a 100-fold diluted sample were treated as follows: 5 mL of reagent C was added, mixed, and incubated for 10 minutes. Then, 0.5 mL of reagent D was added, and the mixture was incubated for 25 minutes. Absorbance was measured at 750 nm using a UV-Vis spectrophotometer. Protein concentrations in the samples were determined using the linear regression equation derived from the BSA standard curve (Lowry *et al*., 1951).

2.6. SOD enzyme activity test using the Water Soluble Tetrazolium Salt-1 (WST-1) method

Crude extracts of guava leaves SOD, ammonium sulfate concentration, positive control, and negative control were added with 200 µL of WST Working Solution and 20 µL of enzyme working solution. **Table 1** shows the composition of sample solutions, blank 1, blank 2, and blank 3.

Table 1. Composition of sample solutions, positive and negative controls, and blanks (Qwele *et al*., 2013)

	Control (+) (µL)	**Control (-) (µL)**	**Sample (µL)**	**Blank 1 (µL)**	**Blank 2 (µL)**	**Blank 3 (µL)**
Sample solution			20		20	
ddH2O				20		20
WST working solution	200	200	200	200	200	200
Enzyme working solution	20	20	20	20		
Solution		20			20	20
Vitamin C	20					

The standard solutions, positive control, negative control, samples, blank 1, blank 2, and blank 3 were each added to a 96-well plate and incubated at 37°C for 20 minutes. Subsequently, the absorbance of each well was measured using a microplate reader at a wavelength of 450 nm (Qwele *et al.*, 2013). SOD activity was then calculated using the percentage inhibition in the following formula:

$$\%\ inhibition = \frac{(A\ blank\ 1 - A\ blank\ 3) - (A\ sample - A\ blank\ 2)}{(A\ blank\ 1 - A\ blank\ 2)}\ x100\%$$

Description :
A_{sample} =Sample absorbance
$A_{blank\ 1}$ = Blank 1 absorbance
A_{blank2} = Blank 2 absorbance
A_{blank3} = Blank 3 absorbance

2.7. Results analysis

Data was analyzed using a one-way ANOVA parametric statistical test. Prior to ANOVA, data normality was assessed using the Shapiro-Wilk test (significance level > 0.05), and homogeneity of variance was confirmed using Levene's test (significance level > 0.05). A Tukey post-hoc test was then performed to determine significant differences between groups.

3. Results and discussion

3.1. SOD enzyme extraction

Enzyme extraction aimed to separate the enzyme from guava leaf tissue cells, yielding a supernatant after centrifugation. Extracting SOD enzyme from 100 g of guava leaves with 200 mL of phosphate buffer resulted in 150 mL of supernatant and 20.541 g of sediment. The SOD enzyme was extracted by homogenizing the leaves with 0.02 M phosphate buffer (pH 7.2), using a buffer volume twice that of the leaf sample. This buffer addition maintains cell component integrity, preserving optimal cellular conditions (Arjita, 2009).

Whitaker (1994) mentioned that phosphate buffer maintains a neutral pH during intracellular enzyme extraction from plant tissue. Its properties do not interfere with protein binding or enzyme activity, making it suitable for this purpose. The homogenate was cooled overnight at 4°C before filtration to prevent enzyme degradation and allow residual leaf fibers to settle. Filtration using flannel separated the filtrate (containing cell components) from the residue (containing cell walls).

Subsequent centrifugation at 5000 rpm for 30 minutes separated the supernatant (crude enzyme extract) from remaining leaves fiber. Centrifugation separates components based on size and

molecular weight, and the low temperature minimizes enzyme activity loss (Masruroh *et al*., 2018). This process yielded two distinct fractions: the supernatant (containing the crude enzyme extract) and the sediment (impurities from the extraction process).

3.2. Purification using amonium sulfat

In this research, precipitation with ammonium sulfate was carried out at three concentrations: 25, 50, and 75%. The weights of the pellets resulting from partial purification were 0.1622 g at 25% ammonium sulfate, 0.7752 g at 50%, and 1.628 g at 75%.

These pellet weights were obtained from the precipitated protein during partial purification. The 75% ammonium sulfate concentration yielded the highest pellet weight, while the 25% concentration yielded the lowest. These partial purification pellet results showed that increasing the ammonium sulfate concentration resulted in more precipitated protein. Protein precipitation does not denature the protein; it only reduces its solubility. The protein can be concentrated by removing the remaining ammonium sulfate solution and redissolving the protein in an appropriate buffer solution (Wingfield, 2001).

By adding high concentrations of ammonium sulfate, water molecules attached to the protein's hydrophobic surface bind to the salt. As more water molecules bind to salt ions, the proteins interact, combine, and precipitate known as salting out (Bintang, 2010).

3.3. Protein measurement using Lowry method

Protein levels in this research were measured using the Lowry method. The maximum wavelength obtained was 750 nm, with an absorbance value of 0.5402, and the optimal operating time (OT) was determined to be 35–45 minutes. Bovine serum albumin (BSA) was used as a standard in the Lowry method, with a concentration series of 50, 75, 100, 125, and 150 ppm. BSA was chosen as a standard because it exhibits good linearity, produces an accurate standard curve, and is a widely used protein standard (Walker, 2002). **Table 3** presents the UV-Vis spectrophotometry absorbance values for the BSA concentration series.

Table 3. Absorbance values of BSA solution concentration series (*Bovine Serum Albumin*)

Concentration (ppm)	Absorbance
50	0.262
75	0.390
100	0.489
125	0.597
150	0.738

Standard solutions with several concentrations were prepared to determine the protein content in the samples. A linear regression equation was generated using the straight line obtained from the standard curve. This equation was based on the absorbance values (y) and the corresponding BSA concentrations (x) of the five BSA standard solutions.

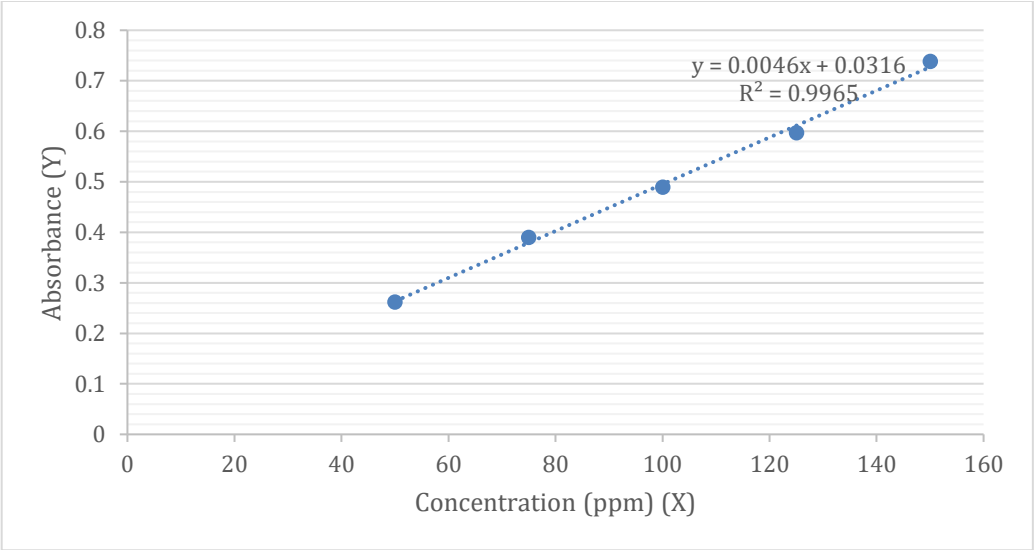

Figure 1. Linear regression equation of BSA absorbance concentration

The slope curve and the slope of the straight line are close to 1 (R^2 = 0.9965). The linear regression line equation y = a + bx and the equation y = 0.0316 + 0.0046x were obtained (**Figure 1**).

Table 4. Protein content results using the Lowry method

Sample	Content (mg/mL; mean ± SD)
Crude extract	9.683 ± 0.043
Ammonium sulfate 25%	6.958 ± 0.054
Ammonium sulfate 50%	8.842 ± 0.067
Ammonium sulfate 75%	11.269 ± 0.121

Based on the total protein content analysis, the pellets purified with 75% ammonium sulfate contained more protein than the crude extracts, as did the pellets purified with 25 and 50% ammonium sulfate. Higher concentrations of ammonium sulfate promote increased protein precipitation, which explains this result. SPSS analysis revealed a statistically significant difference in protein content among the crude extract and the samples purified with 25, 50, and 75% ammonium sulfate. This indicates that increasing the concentration of ammonium sulfate leads to a higher protein yield (Boschetti & Righetti, 2013).

The Lowry method is advantageous due to its 100-fold greater sensitivity compared to the biuret method, allowing for the use of smaller sample volumes. However, this increased sensitivity also makes the Lowry method more susceptible to interference, even though its detection limit is as low as 0.01 mg/mL (Purwanto, 2014).

3.4. SOD enzyme activity test

A quantitative activity assay was performed on the crude superoxide dismutase (SOD) extract from guava leaves to determine its SOD activity. A microplate reader was used for sample detection. This method was chosen due to its ability to accurately analyze small sample volumes (Qwele *et al*., 2013). The absorbance values for blanks 1, 2, and 3 were 0.168, 0.034, and 0.101, respectively.

The blank solutions served to calibrate the absorbance measurements. The variations in absorbance among the blanks likely reflect differences in their compositions. These blank absorbance values, along with the sample absorbance values, were then used to calculate the percentage inhibition using a specific formula. The resulting percentage inhibition values are presented in **Table 6**.

Table 6. Inhibition percentage of SOD enzyme

Sample	% of inhibition (Mean ± SD)
Positive Control	82.089 ± 1.492
Negative Control	2.985 ± 1.493
Crude extract	69.652 ± 3.756
Ammonium sulfate 25%	35.323 ± 2.279
Ammonium sulfate 50%	61.691 ± 2.280
Ammonium sulfate 75%	79.104 ± 1.492

Description:
Positive control : Vitamin C
Negative control : Buffer phosphate

The highest SOD activity was observed in the 75% ammonium sulfate purification, while the lowest activity was found in the 25% purification. The SOD activity at 75% ammonium sulfate was comparable to that of the positive control, indicating that the crude guava leaf extract retained substantial SOD activity at this concentration. This increase in activity is attributed to the higher purity of the enzyme obtained by increasing ammonium sulfate concentration.

The variation in SOD activity among samples reflects differences in superoxide anion reduction, which is linearly related to xanthine oxidase (XO) activity and inhibited by SOD. The SOD assay measures the reaction between WST-1 and superoxide radicals, producing yellow WST-1 formazan. SOD converts superoxide, thereby inhibiting the reduction of WST-1 to WST-1 formazan. SOD competes with WST-1 for superoxide, inhibiting dye formation. SOD activity is quantified by measuring the degree of inhibition of dye formation at 450 nm (Widowati *et al*., 2005). Vitamin C was used as a positive control due to its SOD-mimicking properties, while phosphate buffer served as a negative control, as it lacks SOD activity.

SPSS analysis confirmed SOD activity in the positive control, negative control, and all ammonium sulfate purifications (25, 50, and 75%). The negative control exhibited the lowest activity, while the 75% ammonium sulfate purification showed the highest, with no significant difference

observed between this purification and the positive control. Significant differences were found between the crude extract and each of the ammonium sulfate purifications (25, 50, and 75%).

4. Conclusion

Guava leaves extract (*Psidium guajava L.*) exhibits SOD activity. The total protein content in the crude SOD extract was 9.683 mg/mL. Following ammonium sulfate purification at 25, 50, and 75% concentrations, the protein content was 6.958, 8.842, and 11.269 mg/mL, respectively. The percentage inhibition values were 69.652% for the crude extract, and 35.323, 61.691, and 79.104% for the 25, 50, and 75% ammonium sulfate purifications, respectively. Therefore, the 75% ammonium sulfate concentration yielded the most optimal percentage inhibition.

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