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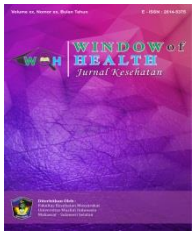

ARTICLE RESEARCH

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Angiotensin Converting Enzyme (ACE) Inhibitory Activity, Toxicity Test, and Phytochemical Analysis of Roselle Flower Extract

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ABSTRACT

Hypertension is still one of the biggest health problems in the world, including in Indonesia. The high prevalence rate has encouraged a lot of research to find antihypertensive drugs and other alternative healing methods, especially those using natural ingredients. One plant that has antihypertensive activity is the roselle flower (Hibiscus sabdariffa L.). This research aimed to observe the inhibitory activity of roselle flower extract on the angiotensin-converting enzyme (ACE), determine the potential toxicity of roselle flower extract on shrimp larvae (Artemia salina Leach), and determine the phytochemical content in it. Roselle flower simplicia was extracted using the maceration method using a 70% ethanol solvent. The extract obtained was then tested for its inhibitory activity against ACE photometrically using a microplate reader. The observed inhibitory activity was calculated in terms of IC_{50}. The toxicity of the extract was determined by the brine shrimp lethality test (BSLT) to see how toxic the roselle flower extract was to shrimp larvae. The secondary metabolite content in the extract was determined qualitatively and quantitatively. The research results showed that the extraction yield obtained was 32,63%. The IC_{50} value of roselle flower extract against ACE was 295,36 ppm. The toxicity test on shrimp larvae showed that the LC_{50} value obtained was 334,02 ppm. The results of qualitative phytochemical tests show that roselle flower extract contained flavonoids, quinones, and steroids. The flavonoids and phenolic content in roselle flower extract were 0,42% and 0,91%, respectively. Based on these results, the phytochemical content of roselle flower extract inhibited ACE activity, and its compounds can be used as ingredients for developing hypertension drugs.

Keywords: Antihypertensive; Angiotensin-converting enzyme; Brine shrimp lethality test

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INTRODUCTION

Hypertension is still one of the biggest health problems in the world, including in Indonesia. This disease is still a problem because many of the sufferers are not aware of it, so they do not undergo treatment properly, which makes the condition worse. [1] The high prevalence rate of hypertension has encouraged a lot of research and development to find antihypertensive drugs and other alternative methods of healing by prioritizing raw materials from nature to minimize the impact of drug dependence and undesired side effects. [2,3]

One of the enzymes that play an essential role in regulating blood pressure in the body is the *angiotensin-converting enzyme* (ACE), which is part of the renin-angiotensin-aldosterone hormonal system. ACE is an enzyme found in the inner endothelial layer of the pulmonary blood vessels that play an essential role in regulating blood pressure due to the conversion of angiotensin I into its active peptide form, namely angiotensin II. [3,4] Angiotensin II plays a role in narrowing blood vessels (vasoconstriction), which can trigger an increase in blood pressure, which can ultimately cause hypertension.[5,6] Thus, to avoid this increase in blood pressure, the process of converting angiotensin I to angiotensin II needs to be carried out, and this is one of the principles of hypertension management, namely through the ACE inhibition mechanism. Inhibition of ACE will cause the conversion of angiotensin I to angiotensin II to not occur. In this way, vasoconstriction or narrowing of blood vessels can be prevented, and blood pressure will remain stable. [7]

Basically, hypertension can be prevented or slowed down by taking antihypertensive medication regularly to keep blood pressure regular. The antihypertensive drugs currently used are mainly synthetic drugs that work as angiotensin-converting enzyme (ACE) inhibitors, beta-adrenergic antagonists, calcium channel blockers, beta-blockers, and diuretics. [8] However, the use of these drugs can cause side effects such as symptoms of hypersensitivity reactions in the form of itching and upper respiratory tract infections, especially if consumed for an extended period. [3] Therefore, the research and development of antihypertensive drugs need to consider aspects of safety, innovation, and economic value to find alternative prevention and treatment of hypertension, especially those sourced from natural ingredients. [2]

Natural ingredients, whether in the form of leaves, fruit, bark, or plant roots, contain secondary metabolites that generally have certain bioactivity. Secondary metabolite compounds that can inhibit ACE are the flavonoids group. One example of a natural ingredient that contains flavonoids is the roselle flower (*Hibiscus sabdariffa* L.). This plant is known to have antibacterial, [9] antioxidants, [10,11] anti-diabetic,[12] and anti-inflammatory activity.[13] Previous research reported that roselle flower syrup was effective in lowering blood pressure in hypertensive patients with cholesterolemia. However, the effective dose and toxicity tests have yet to be carried out. [14] In other studies, infusion from roselle flowers has been proven to be able to inhibit ACE, and when combined with cucumber, both infusions show an even more significant inhibitory effect. However, bioactivity tests of extracts from organic solvents and toxicity tests have yet to be carried out. [15] Therefore, in this research, roselle flower extract

was made using an organic solvent, namely 70% ethanol, to see the differences in inhibitory activity that occurred. Apart from that, this research also carried out a toxicity test to observe the toxic effects caused by the extract. Qualitative phytochemical tests were also carried out to identify secondary metabolite compounds in the extract. The quantitative determination of the total flavonoids and phenolic content was also carried out.

This research aimed to observe the activity of roselle flower extract in inhibiting ACE, determine the potential toxicity of roselle flower extract, determine the content of secondary metabolites in the extract that might play a role in inhibiting ACE, and measure the total flavonoids and total phenolic content in the extract. ACE inhibitory activity was observed using a photometric method based on absorbance measurements using a microplate reader. The toxicity test was conducted using the toxicity analysis method on *Artemia salina* Leach shrimp larvae (brine shrimp lethality test, BSLT). Meanwhile, phytochemical screening was conducted qualitatively on secondary metabolite components of flavonoids, alkaloids, saponins, tannins, steroids, and triterpenoids. The calculation of total flavonoids and total phenolic content from the extract was carried out quantitatively by spectrophotometry.

METHODS

Roselle flowers were obtained from roselle plantations in the Tangerang area, Banten. Plant identification and determination were carried out at UPT Laboratorium Herbal Materia Medica Batu, Malang, to ensure the accuracy of the plant species used. After ensuring that the species name was correct, the fresh roselle flowers that had been harvested were cleaned and washed thoroughly with running water. Next, the flowers were dried in the sun. The dried roselle flowers were then ground using a blender until a coarse powder of dried roselle flower simplicia was obtained. The simplicia powder was then stored in an airtight container and ready to be extracted. Extraction of roselle flower simplicia was carried out using the maceration method. A total of 500 g of simplicia was given 70% ethanol solvent in a ratio of 1:10 until the simplicia powder was completely submerged. The extraction was carried out for 1x24 hours with occasional stirring to maximize contact of the simplicia with the solvent. After 1x24 hours, filtering was carried out to separate the filtrate (macerate) from the simplicia. The filtrate obtained was separated from the residue, then the residue was added to 70% ethanol solvent again as previously, and the second maceration was carried out again. This process was carried out up to 3 repetitions. The macerate from each repetition was collected and concentrated using a rotary evaporator until a solvent-free extract was obtained, then the extraction yield was calculated. The extract was then put into a clean, closed bottle and stored in the freezer until the following analysis was carried out.

Antihypertensive activity testing was carried out on roselle flower extract using the WST Dojindo ACE kit with slight modifications.[15,16] Extract solutions were prepared in concentrations of 5, 10, 25, 50, 100, 250, 500, and 1,000 ppm by dissolving the extract in distilled water. The procedure for preparing the substrate, enzyme (ACE), and indicator solution referred to the technical procedures provided by the

manufacturer.[17] The positive control used was *Captopril* 25 ppm. After all the reagents were prepared, each reagent was then put into a 96-well plate with the composition and placement referring to the manufacturer's technical procedures. After the plate was filled with the appropriate reaction system, the 96-well plate was then incubated at 37°C for 1 hour, then the indicator solution was added and incubated again at room temperature for 10 minutes. After that, the absorbance was read using a microplate reader at a wavelength of 450 nm. The percentage of inhibition observed from each well was then calculated, and the toxicity of the extract to shrimp larvae was determined through probit analysis.[18,19] *Artemia salina* Leach shrimp eggs were weighed as much as 1 gram and then placed in an Erlenmeyer flask containing 500 ml of filtered seawater and given an aerator. The flask was left for 48 hours under a lamp until the eggs hatched into shrimp larvae (*nauplii*) and were ready to be used for toxicity testing. Before the test was carried out, the extract stock solution was prepared in a concentration of 2,000 ppm by dissolving the extract in seawater. If the extract was not dissolved in seawater, then five drops of 1% dimethyl sulfoxide (DMSO) were added. The extract solution was then diluted until concentrations of 5, 10, 25, 50, 100, 250, 500 and 1,000 ppm were obtained. After that, nine containers were prepared containing 20 48-hour-old shrimp larvae each for testing the toxicity of each extract concentration, and one control container was used without the addition of the extract solution. Observations were carried out for 24 hours. During the observation, aeration was carried out, and the container was placed under the light. After 24 hours, the percentage of dead shrimp larvae was calculated. Mortality percentage data were converted into probit values, and logarithm concentration values were calculated. The curve of the relationship between these two values was then used to calculate the LC_{50} of the extract based on the linear regression equation obtained.

Phytochemical analysis was carried out to determine the presence of flavonoids, alkaloids, saponins, tannins, quinones, steroids, and triterpenoids.[20,21] The presence of flavonoids was tested by dissolving the extract in methanol, then putting it into a test tube and adding magnesium powder. After that, 1 ml of concentrated HCl was added. The presence of flavonoids was indicated by the color changes of the solution to yellow, orange, red, or green. The presence of alkaloids was tested by putting a thick extract into a test tube, then giving chloroform and a few drops of ammonia solution, and then shaking and filtering. The solution was then treated with 2N sulfuric acid and shaken until two layers formed. The top layer was separated into three different test tubes. The first tube was given three drops of Dragendorff's reagent, the second tube was given three drops of Meyer's reagent, and the third tube was given three drops of Wagner's reagent. The presence of alkaloids was indicated by the formation of a reddish-brown precipitate in the first and third tubes and a white precipitate in the second tube. Saponins examination was carried out by dissolving the extract with a little warm distilled water in a test tube, then adding 5 ml of distilled water. After that, the solution was vigorously shaken until foam formed. The presence of saponins was indicated by a stable foam formed in the solution. The presence of tannins was tested by dissolving a small amount of the extract with methanol in a test tube and then adding three drops of 5% $FeCl_3$. The presence of tannins was indicated by a blackish-green or dark blue color formed.

The presence of quinones was tested by putting a thick extract and dissolving it with benzene in a test tube, then giving a few drops of NaOH solution. The extract contains quinones if the color changes from yellow to red. The presence of triterpenoids and steroids was tested by dissolving the extract with n-hexane in a test tube, then adding 1 ml of glacial acetic acid and 1 ml of concentrated sulfuric acid. The presence of a reddish-brown ring at the boundary of the two layers indicates the presence of triterpenoids, while the presence of a blue or green ring indicates the presence of steroids.

Determination of total flavonoids was carried out by weighing 200 mg of the extract and then placing it in a round bottom flask. The hydrolysis system was carried out by adding 1 ml of 0.5% (w/v) hexamethylenetetramine, 20 ml of acetone, and 2 ml of 25% HCl solution, then heated until boiling for 30 minutes and filtered. All filtrate was collected into a volumetric flask. 20 ml of the filtrate from hydrolysis was taken and then put into a separating funnel. After that, 15 ml of ethyl acetate was added and then shaken. The ethyl acetate fraction was collected into a volumetric flask. 10 ml of filtrate was added to 1 ml of 2% $AlCl_3$ solution in 5% glacial acetic acid (v/v) and adjusted. The absorbance of the solution was measured using a UV-Vis spectrophotometer at a wavelength of 425 nm. The total flavonoids were determined compared with standard quercetin, and the result was expressed as quercetin equivalent (%).[22] Meanwhile, the total phenolic content in the extract was determined based on the method in the Indonesian Herbal Pharmacopoeia.[23] Determining total phenolic content began with making a standard curve for gallic acid. A stock solution of gallic acid with a concentration of 500 ppm was prepared by weighing 12.5 mg of gallic acid and dissolving it with pro-analysis methanol in a 25 ml volumetric flask. A standard concentration range of 0, 10, 30, 50, 70, and 100 ppm was made in a 25 ml measuring flask. 1 ml of each solution was pipetted into a test tube, then 5 ml of 7.5% Folin-Ciocalteu reagent was added, vortexed, and incubated in the dark for about 8 minutes. After that, 4 ml of 1% NaOH was added, vortexed, and incubated again in the dark for 1 hour. The entire solution was measured using a UV-Vis spectrophotometer at a wavelength of 730 nm, and then a standard curve for gallic acid was created. Then, 10 mg of the extract was weighed and dissolved in methanol, then 1 ml solution was pipetted into a test tube. After that, 5 ml of 7.5% Folin-Ciocalteu reagent was added, vortexed, and incubated in the dark for approximately 8 minutes. After that, 4 ml of 1% NaOH was added, vortexed, and incubated again in the dark for 1 hour. Finally, the absorbance of the solution was measured using a UV-Vis spectrophotometer at a wavelength of 730 nm. The total phenolic content was compared with standard gallic acid, and the result was calculated as gallic acid equivalent (%).

RESULTS

The roselle plants used in the research were first determined at UPT Laboratorium Herbal Materia Medica Batu, Malang. The determination result, document number 074/713/102.20-A/2022, confirmed that the plant used in this research was *Hibiscus sabdariffa* L. or the roselle plant. After extracting 500 g of dried simplisia of roselle flowers, the extract weight obtained was 163.17 g. Thus, the extraction yield obtained was 32.63%.

The inhibitory activity of roselle flower extract against ACE can be seen in Table 1 below.

Table 1. Inhibitory activity of roselle flower extract against ACE

Extract concentration (ppm)	log concentration	Inhibitory activity against ACE (%)
5	0.699	-*
10	1,000	1,82
25	1,398	3.26
50	1,699	12,88
100	2,000	32,17
250	2,398	52,06
500	2,699	58,62
1.000	3,000	72,49
Captopril 25 ppm	1,398	92,37

*not observed

Concentration analysis was carried out using linear regression calculations. The absorbance value obtained from measurements with the microplate reader was used to calculate the IC_{50} of the extract. The IC_{50} value was calculated using Microsoft Excel by plotting the logarithm value of roselle flower extract concentration and its inhibitory activity against ACE. The linear regression equation was then used to calculate the IC_{50} value of the extract. Based on the data in Table 1, the linear regression equation obtained was $y = 34.23x - 34.56$ with an R^2 value of 0.9352 (Figure 1). From the equation, the IC_{50} of the extract was 295.36 ppm.

The toxicity test results of roselle flower extract on shrimp larvae are presented in Table 2 below.

Table 2. Toxicity test results of roselle flower extract

Extract concentration (ppm)	log concentration	Total dead	Mortality (%)	Probit value
5	0.699	1	5	3,36
10	1,000	1	5	3,36
25	1,398	2	10	3,72
50	1,699	4	20	4,16
100	2,000	4	20	4,16
250	2,398	8	40	4,87
500	2,699	13	65	5,39
1.000	3,000	14	70	5,52

The LC_{50} value for roselle flower extract was calculated using the probit quantile regression analysis method using Microsoft Excel. The number of shrimp larvae that died for each observed extract concentration was calculated as a percentage of larval death (mortality). This mortality number was converted into a probit number with the help of a percentage transformation table to probit values. Then, a curve was created between the logarithm of the extract concentration and the probit value, and a linear regression equation was determined from this curve. The LC_{50} was calculated by finding the logarithm value of the extract concentration, which resulted in a shrimp larval mortality value of 50%.

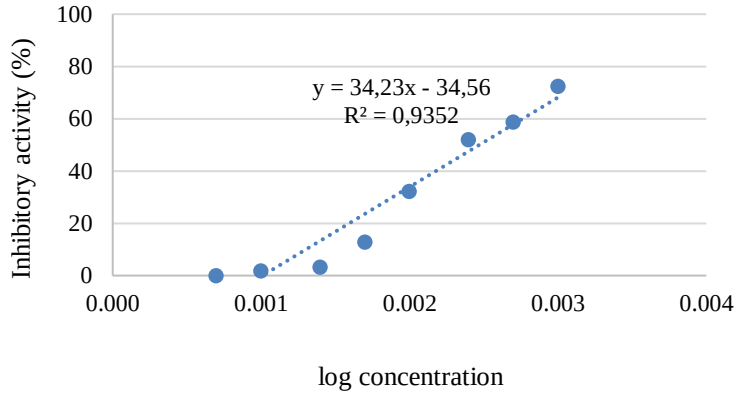

Figure 1. Inhibitory activity of roselle flower extract against ACE

The logarithm value was then converted to its antilogarithm value, representing the LC_{50} of the extract. The extract concentration resulting from the calculation was set as the LC_{50} value. Based on the data in Table 2, the linear regression curve between the logarithm of concentration and the probit value can be plotted as follows.

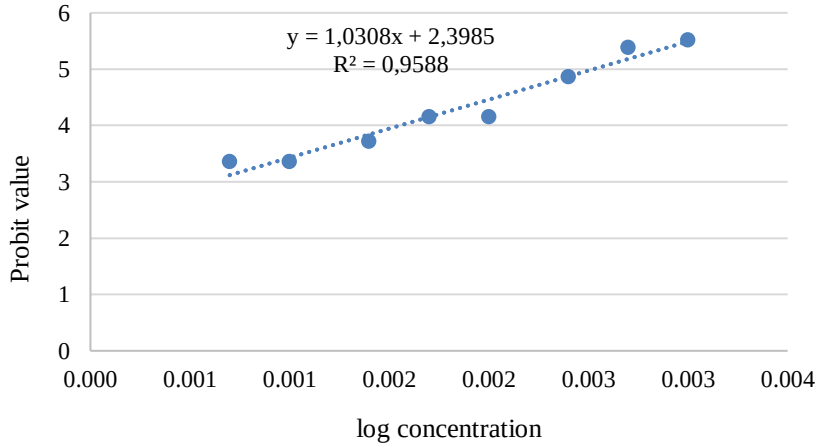

Figure 2. The relationship between log concentration and probit value

The results of qualitative phytochemical screening of roselle flower extract are presented in the following table.

Table 3. Phytochemical profile of roselle flower extract

Phytochemical compounds	Remark*
Flavonoid	+
Alkaloid (Dragendorff)	-
Alkaloid (Meyer)	-
Alkaloid (Wagner)	-
Saponin	-
Tanin	-
Kuinon	+
Triterpenoid	-
Steroid	+

*(+): presence, (-): absence

Meanwhile, the quantitative analysis results of total flavonoids and total phenolic content in roselle flower extract showed that the total flavonoids contained in the extract was 0.42% (quercetin equivalent) and the total phenolic content was 0.91% (gallic acid equivalent).

DISCUSSION

Extraction of roselle calyces simplicia with 70% ethanol solvent resulted in a yield of 32.63%. This yield percentage is relatively high because the extract obtained is almost a third of the total simplicia used in the extraction. Previous research showed that extraction of roselle flowers with 96% ethanol solvent produced a yield of 25.68%,[24] while extraction with 70% ethanol solvent produced 25.8% [25] and 31% yields.[26] This yield was in line with the requirements of the Indonesian Herbal Pharmacopoeia, which states that extraction of roselle flowers with ethanol solvent produces an extract yield of no less than 16.3%.[23] The difference in the yield percentage might be influenced by the length of extraction time, the type and quality of solvent used, the solvent evaporation process, and the amount of simplicia used.[27]

The ACE inhibitory activity testing is used to evaluate the antihypertensive potential of natural products. Naturally, the ACE converts angiotensin-I to angiotensin-II, a potent vasopressor that causes an increase in blood pressure. This change must be prevented to maintain normal blood pressure.[28] In this study, the inhibitory activity of roselle flower extract against ACE was carried out using the ACE kit-WST (Dojindo Laboratory, Japan). This test is based on the detection of 3-hydroxybutyric acid (3HB), which is produced from the breakdown of the substrate 3-hydroxybutyrylglycylglycylglycine (3HBGGG) in the presence of ACE and aminoacylase.[16,17] Samples containing secondary metabolites with inhibitory activity against ACE will be able to prevent the formation of 3HB. Therefore, the lower the absorbance read by the microplate reader, the higher the inhibitory activity measured.

The ACE inhibitory activity of roselle flower extract shows that the inhibition rate increases with increasing concentration. The highest ACE inhibition value was obtained at a concentration of 1,000 ppm, 72.49%. This inhibitory activity is relatively high, but the activity shown by the extract is still below the inhibitory ability shown by *Captopril*, namely 92.37%. The IC_{50} value of the extract observed in this study was 295.36 ppm. Meanwhile, the toxicity test results showed that the LC_{50} value for roselle flower extract was 334.02 ppm. Phytochemical screening of natural products with medicinal potential cannot be separated from testing their efficacy on certain living organisms, known as bioassays. Bioassay is an analysis that determines the presence of a substance and its potency by observing the effects that appear on certain living organisms, both animals and plants. Commonly performed bioassays usually involve living cells (for example, cancer cells used in testing anticancer activity), are expensive, and employ complex analytical techniques. Meanwhile, the phytochemical properties of plants that show certain activities may be able to influence other living things in simple zoological systems. Thus, bioassays to observe any physiological activity of certain phytochemicals can also be observed in simple organisms, one of which is the small crustacean group, namely brine shrimp larvae. These larvae can be

used in various bioassays because they are sensitive to phytochemicals. Toxicity testing on shrimp larvae has been proven effective in multiple tests, such as observing antifungal activity, mycotoxins, waste contamination, pesticide residues, and other pharmacological activities, so this method is quite effective and efficient in observing the pharmacological effects of a plant extract.[18,19]

The extract's phytochemical content plays a distinguished role in its potential biological activity. The potent inhibitory activity of roselle flower extract against ACE is possibly due to flavonoids, quinones, and steroids contained in the extract (Table 3). The active compound that plays the most role in this activity is thought to be the flavonoid group, as evidenced by the presence of total flavonoids and total phenolic content in the extract. Previous research reported that flavonoids showed inhibitory activity against ACE and could reduce blood pressure. [4]

The inhibition mechanism between secondary metabolites and the ACE enzyme suggested that the inhibition mechanism was competitive. Previous research showed that flavonoids could inhibit ACE activity due to the formation of a chelate complex with a zinc (Zn) atom in the active center of the ACE.[29] With this interaction, the enzyme loses its affinity to bind to the substrate, resulting in no conversion of angiotensin-I to angiotensin-II.[30] In addition, the total phenolic and anthocyanins content contained in roselle flowers is known to have a vasodilation effect, which can help dilate blood vessels, thereby helping to lower blood pressure.[31]

CONCLUSION AND RECOMMENDATIONS

The yield of roselle flower extract obtained was relatively high, namely 32.63%. The IC_{50} value of roselle flower extract against ACE is 295.36 ppm, which shows that roselle flower extract has potential as an antihypertensive, although its activity is still below that of *Captopril*. The toxicity test on shrimp larvae showed that the LC_{50} value obtained was 334.02 ppm. Phytochemical test results showed that roselle flower extract contains flavonoids, quinones, and steroids, while the total flavonoids and phenolic content in roselle flower extract are 0.42% and 0.91%. Based on these results, the phytochemicals in roselle flower extracts have the potential to become natural ingredients for developing hypertension drugs.

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