Identification of Phenolic acid from ethanol extract leaves binahong (*Anredera cordifolia* (ten) stennis) and antioxidant activity test

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Abstract. Binahong (*Anredera cordifolia* (Ten.) Stennis) is a plant which comes from Basellaceae family. Also, this plant is also known to have biological activity due to the presence of the bioactive compound. Phenolic acids are bioactive compound and widely used as antioxidants. Identification and testing of antioxidant activity using ethanol extract have been done in this research. Phenolic acids were isolated without hydrolysis (TH fraction), and by acid hydrolysis (fraction HA), and alkaline hydrolysis (HB fraction). Based on the identification using TLC with co-chromatography, UV-Vis and FTIR spectrophotometer, the ethanolic extract of binahong leaves was allegedly suspected contain γ-coumaric acid. Based on the results of quantitative analysis using TLC Scanner, it can be seen that the γ-coumaric acid content in fractions TH, HA, and HB, 11.4220%; 2.8642%; and 23.3563%, respectively. The result of the antioxidant activity test on ethanol extract was 866.89831 mg / L, while (IC$_{50}$) value on isolate B was 1263.3333 mg / L.

1. Introduction
Binahong is a plant belonging to the Basellaceae family. Binahong leaves have been reported to have activities such as antidiabetic [1], antifungal [2], antibacterial [3,4], antihematomata [5], antihyperlipidemia [6], and antioxidants [7,8]. Sukandar et al. [9] reported that ethanol extract of *Anredera cordifolia* leaves could improve kidney function in rats. Ethanol extract of *Anredera cordifolia* leaves was also reported Widyarini et al. [10] could be developed as an anti-hyperuricemia agent. The same extract reported by Garmana et al. [11] can reduce blood pressure in mice. Methanol extract reported by Sukrama et al. [12] can accelerate the healing of burns. The existence of these activities is due to the presence of secondary metabolites found in binahong leaves. Binahong leaves are reported to contain secondary metabolites namely flavonoids, phenolics, alkaloids, tannins, steroids, triterpenoids, saponins [5,13,14], and essential oils [15]. One of the phenolic compounds is phenolic acid. Phenolic acid is found in many types of plants and is beneficial for health [16]. Phenolic acid is a type of secondary metabolite which is found in many types of plants. Hydroxybenzoic acid derivatives and hydroxycinnamic acid are types of phenolic acids that are widely found in plants [17]. Research on phenolic acids in ethanol extract is very slightly, so it is very interesting to isolate phenolic acids and test their antioxidant activity.
2. Materials and method

2.1. Tools
Research tools used are glass that commonly used in the laboratory, Analytical balance (Kern-870), hot plate with magnetic stirrer, rotary vacuum evaporator (Buchii B480), reflux set, a set of thin layer chromatography tools, Spectroline ENF-24/F, UV spectrophotometer (Shimadzu UV – 1601), spectrophotometer FTIR (Shimadzu Prestige-2), TLC Scanner (Camag 3).

2.2. Material
Distilled water, hexane, ethanol, sulfuric acid p.a (Merck), sodium hydroxide p.a (Merck), sodium bicarbonate p.a (Merck), diethylether, chloride acid p.a (Merck), ammonia, magnesium powder, acetic acid anhydride p.a (Merck), Meyer reagent, Dragendorf reagent, ion (III) chloride 1%, chloroform p.a (Merck), sodium acetate p.a (Merck), anhydrous sodium sulfate p.a (Merck), silica gel GF254 plates (Merck), acetic acid p.a (Merck), benzene p.a (Merck), methanol p.a (Merck), p- aminoaniline p.a (Merck), sodium carbonate p.a (Merck), gallic acid p.a (Sigma), caffeic acid p.a (Sigma), ferulic acid p.a (Merck), p-coumaric acid p.a (Merck), 1,1-diphenyl-2-picrylhydrazyl p.a (Sigma).

2.3. Method
2.3.1. Sample preparation
Binahong leaves were obtained from the Research Institute for Medicinal Plants, Tawangmangu, central Java. Binahong leaves are washed, dried by aerated, thinly sliced, and mashed so that binahong leaves powder is obtained.

2.3.1.2. Phytochemical screening
Binahong leaves powder, n-hexane extract, and ethanol extract was carried out by phytochemical screening tests to determine the content of the chemical compounds. Phytochemical screening tests include tests of phenolic, alkaloids, flavonoids, tannins, saponins, steroids and triterpenoids [18].

2.3.1.3. Extraction
Binahong leaves powder as much as 950 g was macerated with n-hexane, every 24 hours the solvent was replaced with a new one until the filtrate was colourless. The n-hexane extract was then concentrated using a rotary vacuum evaporator to obtain concentrated n-hexane extract. Binahong leaves pulp is dried by air and macerated with ethanol, every 24 hours the solvent is replaced until the colourless filtrate. Ethanol extract was then concentrated using a rotary vacuum evaporator to obtain concentrated ethanol extract. Concentrated ethanol extract was dissolved in ethanol, then added with distilled water in a ratio of 1: 1 and allowed to stand for 24 hours to remove chlorophyll. The mixture is then filtered to separate between chlorophyll and ethanol-water extract.

2.3.1.4. Isolation of phenolic acid
Phenolic acid is isolated in three methods, namely acid hydrolysis (HA), base hydrolysis (HB) and without hydrolysis (TH). Acid hydrolysis is carried out using 2N H2SO4 by heating for 2 hours at 60°C. Base hydrolysis is carried out using 1N NaOH in a dark room for 24 hours. After the hydrolysis process is completed, the three fractions are then extracted using ether and were dried with anhydrous Na2SO4. The extraction results are then dried and dissolved in methanol [19].

2.3.1.5. Separation of phenolic acid
The separation of phenolic acids was carried out on TH, HA, and HB fractions using thin layer chromatography (TLC) with silica gel GF254 plate and eluent mixture of benzene, acetic acid, and methanol (50:50:1). The stains obtained are then compared with standard phenolic acids. The standard phenolic acids used are gallic acid, caffeic acid, ferulic acid, and p-coumaric acid. Stains that have an RF value are parallel to the comparative identified by TLC and quantitative analysis with the TLC scanner Camag 3. Analysis with the TLC Scanner was carried out by varying the concentration of standard phenolic acid solutions that were parallel to the stain. The concentrations used were 50 ppm,
100 ppm, 250 ppm, 500 ppm, and 1000 ppm. Then TLC was carried out with an eluent mixture of benzene: acetic acid: methanol (50: 50: 1). The results of the TLC are then scanned by using the TLC Scanner Camag 3.

Identification of phenolic acids isolates was carried out using TLC, UV-Vis spectrophotometer and FTIR. Quantitative analysis of phenolic acids was carried out on the fractions of TH, HA and HB using the TLC Scanner. p-coumaric the acid content in ethanol extract was determined using the standard p-coumaric acid curve regression equation.

2.3.6. Antioxidant Activity Test
Ethanol extracts were made at concentrations of 200, 400, 600, 800, and 1000 mg/L. Each concentration of ethanol extract as much as 0.2 mL was pipetted and put into a vial bottle, then added 4 mL of 0.1 mM DPPH solution. The mixture is homogenized and left for 30 minutes in a dark place. This solution is then measured absorbance at 516.8 nm wavelength.

The same treatment was carried out isolates B (phenolic acid isolates) (250, 500, 750, 1000, and 1250 mg/L) and standard gallic acid (50, 100, 150, 200, and 250 mg/L).

The ability to reduce DPPH radical (inhibition) can be calculated using the following equation:

$$\% \text{ Inhibisi} = \frac{A_{CFFH} - A_{\text{sample}}}{A_{CFFH}} \times 100\%$$

The amount of concentration of the test solution to reduce 50% DPPH free radical activity was determined by the IC$_{50}$ value calculated from the percentage inhibition of various concentrations using the equation obtained from the linear regression curve.

3. Results and discussion
3.1. Binahong extraction and Phytochemical screening
Ethanol extract of binahong leaves obtained yield 6.176%. The Results of phytochemical screening of binahong leaves ethanol extract were an alkaloid, flavonoid, tannin, phenolic, saponin, steroid, and triterpene. The phytochemical screening results above are following Kumalasari dan Sulistyani’s research[2] and Widyarini et al. [10]

3.2. Isolation of phenolic acid
Isolation of phenolic acids in ethanol extract was carried out by hydrolysis (acid or base) and without hydrolysis (TH) to extract free phenolic acid. Hydrolysis with acid (HA) to take phenolic acid in the form of glycosides, and alkaline hydrolysis (HB) for phenolic acids in the form of esters [19]. TH, HA, and TLC then identified HB fractions.

3.3. Separation of phenolic acid
Separation of phenolic acids was carried out by TLC with silica gel GF$_{254}$ stationary phase and an eluent mixture of benzene: acetic acid: methanol (50: 50: 1) as the mobile phase. The results of TLC fraction of HB, TH, and HA, and standard phenolic acid as a comparison with silica gel GF$_{254}$ plate and mixture eluent benzene: acetic acid: methanol (50: 50: 1) shown in figure 1. Standard phenolic acids used are ferulic acid, p-coumaric acid, caffeic acid and gallic acid.
Figure 1. The results of TLC fraction of HB, TH, and HA, and standard phenolic acid as a comparison with silica gel GF254 plate and mixture eluent benzene: acetic acid: methanol (50: 50: 1)

In figure 1, there is one spot that can be separated, namely spot B, T, and A. The resulting spot has an Rf that is parallel to the P (standard p-coumaric acid) spot that is 0.89 so that the spot B, T, A that is produced is suspected p-coumaric acid. The fractions HB, TH, and HA, were then separated using preparative TLC to obtain isolates B, T, A. Isolates were tested for purity using TLC and 2-dimensional TLC. The results of the purity test shown that the isolate was pure so that structural identification was carried out. Identification of isolates B, T, and A was carried out using the co-chromatographic TLC method. The result of identification with co-chromatographic TLC of fraction HB, HA, TH, p-coumaric acid standard are presented in figure 2.

Figure 2. The results of TLC by co-chromatography of HB, TH, HA, and compared with p-coumaric acid standard, with silica gel GF254 plate, mixture eluent benzene: acetic acid: methanol (50: 50: 1)

The result of identification with co-chromatographic TLC shown that the price of Rf spot B, T and A is parallel to RF spot P. Based on the results of identification using TLC, the spots B, T, and A on each fraction are estimated to be p-coumaric acid compounds.
Identification structure was carried out using UV-Vis spectrophotometer and FTIR. The identification results with UV-Vis spectrophotometer are presented in Table 1.

### Table 1. Measurement results of the maximum wavelength (λmax) of isolates B, T and A and a \( p \)-coumaric acid standard by UV spectroscopy

| Sample                        | Wavelength (nm) |
|-------------------------------|-----------------|
| \( p \)-coumaric acid standard | 290,5           |
| Fraction                      | Isolate         |
| Base hydrolysis (HB)          | B               |
| Without Hydrolysis (TH)       | T               |
| Acid hydrolysis (HA)          | A               |

Table 1. shows that isolate B has a maximum wavelength (λmax) which is closer to the standard \( p \)-coumaric acid λmax than isolates T and A. Therefore, identification using FTIR spectrophotometer was carried out on isolates B. Analysis with FTIR spectrophotometer aims to determine the functional groups contained in isolate B and standard \( p \)-coumaric acid as a compare.

Analysis of isolate B and standard \( p \)-coumaric acid with FTIR spectrophotometer are presented in Table 2.

### Table 2. Analysis of isolate B and \( p \)-coumaric acid standard with FTIR spectrophotometer

| Type of vibration          | Wave number (cm\(^{-1}\)) |
|----------------------------|----------------------------|
|                            | Isolat B                   | \( p \)-Coumaric acid standard |
| O-H str                    | 3394,72                    | 3387,00                        |
| =C-H stralkena             | 3080,90                    | 3101,20                        |
| =C-H straromatik           | 3002,01                    | 3032,10                        |
| C=O str carboxylic acid    | 1674,21                    | 1674,21                        |
| C=C stralkena              | 1625,10                    | 1627,92                        |
| C=C aromatic               | 1604,77 ; 1512,19          | 1604,77 ; 1512,19              |
| =C-H bend alkena           | 1450,47 ; 1381,03          | 1450,47 ; 1381,03              |
| =C-H bend aromatic         | 1249,87 ; 1229,55          | 1249,87 ; 1219,01              |
| C-O carboxylic acid        | 1172,72                    | 1172,72                        |
| C-O alcohol                | 1111,00                    | 1111,00                        |
| substituted benzene        | 941,26 ; 839,25            | 941,26 ; 833,25                |

Analysis of isolate B by FTIR spectrophotometer was shown in Figure 3.
Based on UV-Vis and FTIR spectroscopic data, isolate B is suspected to be p-coumaric acid. The p-coumaric acid structure was shown in Figure 4.

![FTIR spectrum of isolate B](image)

**Figure 3.** The result analysis FTIR of isolate B

Quantitative analysis of phenolic acid was carried out using the TLC Scanner. Quantitative analysis was used to determine p-coumaric acid levels in binahong leaves ethanol extract based on the spot area on the TLC plate. TLC Scanner analysis is done by measuring absorbance at a wavelength of 290 nm, and the sample area eluted using a mixture of benzene eluent: acetic acid: methanol (50: 50: 1). The p-coumaric acid content in ethanol extract was determined using the standard p-coumaric acid regression equation. Standard p-coumaric acid curves can be seen in Figure 5.
Based on the $p$-coumaric acid standard curve, the regression equation $y = 17863x - 26595$ is obtained with a correlation coefficient ($r$) of 0.979. The value of $r$ that approaches one indicates the regression equation is linear and can be used because of the concentration that affects the area by 98% $p$-coumaric acid levels in the HB, TH, and HA fractions were determined based on calculations using the linear regression equation of the standard curve. Calculation results can be seen in table 3.

| Fraction         | Isolate | area (mm) | Concentration (mg/L) | Levels (%) |
|------------------|---------|-----------|----------------------|------------|
| Base hydrolysis (HB) | B       | 21160.1   | 467.126              | 23.3563    |
| Without hydrolysis (TH) | T       | 12885.1   | 228.440              | 11.422     |
| Acid hydrolysis (HA) | A       | 6951.3    | 57.284               | 2.8642     |

Table 3 shows that isolate B had higher levels than isolates T and A, so isolates B was used for testing antioxidant activity. This is following Chibbar's research, et al. (2009)[21] that base hydrolysis fractions produce high levels of $p$-coumaric acid

3.4. Antioxidant Activity Test

Antioxidant activity test was carried out on ethanol extract, isolate B, and gallic acid as a comparison using DPPH method there was $\lambda$ 516.8 nm. Quantitative determination of antioxidant activity is determined by IC$_{50}$ value. Graph of antioxidant activity is shown in Figure 6
The calculation results show IC\textsubscript{50} values of ethanol extract, isolate B, and comparable gallic acid was 866.8983 mg / L, 1263.333 mg / L, and 66.0894 mg / L, respectively. Standard gallic acid has a smaller IC\textsubscript{50} value than ethanol extract and B isolate, but IC\textsubscript{50} value of ethanol extract is smaller than that of isolating B. Large IC\textsubscript{50} values of isolate B show that the antioxidant activity of isolate B is low, this is because the isolate is suspected to be \textit{p}-coumaric acid which is known to have low antioxidant activity (Chibbar et al., 2009)[21].

4. Conclusion

Phenolic acid contained in the ethanol extract of binahong leaves is suspected to be \textit{p}-coumaric acid. The isolation results of phenolic acids (\textit{p}-coumaric) from the base (HB), acid (HA) and without hydrolysis (TH) were obtained at 23.3563; 2,8642 and 11,422\% respectively. Antioxidant activity of ethanol extract and isolate B (\textit{p}-coumaric acid) showed IC\textsubscript{50} values of 866.8983 mg / L and 1263.3333 mg / L respectively.

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