Synthesis and Antiplatelet Activity of 4-Hydroxy-3-Methoxycinnamic Acid

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Abstract

Background: Cinnamic acid and its derivatives have been widely studied for their efficacy because of the pharmacological effect on good health and well being. Microwave irradiation is more time effective to synthesize than conventional heating method because it conducts heat faster and shortens the reaction time. Objective: This study aimed to synthesize 4-hydroxy-3-methoxycinnamic acid using microwave irradiation and its antiplatelet activity by blood clotting time method. Methods: Synthesis of 4-hydroxy-3-methoxycinnamic acid with malonic acid and 4-hydroxy-3-methoxybenzaldehyde as a starting material using ammonium acetate catalyst with microwave irradiation (960 Watt, 4 minutes). The synthesis results were tested for purity by thin-layer chromatography, a melting point determination and structure identification (UV-Vis, infrared, and proton NMR spectroscopy). The antiplatelet activity test consisted of a negative control group CMC-Na, a positive control acetosal, cinnamic acid, and 4-hydroxy-3-methoxycinnamic acid, each group consisted of 3 different doses, namely 0.0037 mmol/Kg (I), 0.0069 mmol/Kg (II) and 0.0139 mmol/Kg (III). Results: Synthesis of 4-hydroxy-3-methoxycinnamic acid had a yield percentage of 30.55%. The test results showed that the 4-hydroxy-3-methoxycinnamic acid compound has antiplatelet activity with an ED50 value of 1.3080 mg/Kg BW and antiplatelet activity comparable to acetosal. Conclusion: 4-hydroxy-3-methoxycinnamic acid can be synthesized by microwave irradiation and had antiplatelet activity 1.7 fold greater than cinnamic acid.

Keywords: 4-hydroxy-3-methoxycinnamic acid, antiplatelet, good health and well being, microwave irradiation
INTRODUCTION

Thromboembolism is a pathological process that leads to the formation of intravascular clots that can reduce blood flow to vital organs. Therapy for diseases associated with thromboembolism is antithrombotic, including antiplatelet (Acosta et al., 2016). Antiplatelets work by reducing platelet aggregation to prevent thrombus formation. Platelet inhibitory drugs have been identified, such as cyclooxygenase-1 inhibitors, adenosine diphosphate (ADP) antagonists and blockade of glycoprotein IIb/IIIa receptors on platelets (Hoffman et al., 2018). However, it is necessary to synthesize compounds with better therapeutic effects and minimal side effects.

Cinnamic acid derivatives have various pharmacological effects such as anti-inflammatory, antiplatelet, antimicrobial and antidiabetic (Sharma, 2011). The compound 4-hydroxy-3-methoxy cinnamic acid or ferulic acid (Figure 1) is one of the cinnamic acid derivatives that can be synthesized by Knoevenagel condensation. Knoevenagel condensation is a reaction involving an active methylene compound (a CH₂ flanked by two electron-withdrawing groups) and an aldehyde or ketone to yield an α,β-unsaturated product (McMurry, 2016). Ferulic acid is a cinnamic acid derivative, estimated to have the exact mechanism in inhibiting platelet aggregation by inhibiting the Ca²⁺ channels on the cell membrane surface and interfering with phospholipase C in releasing Ca²⁺ to reduce the interaction between ADP and P2Y₁₂ receptors and inhibiting the synthesis of thromboxane A₂ (TXA₂), which can activate platelet aggregation (Yang et al., 2013).

This study aims to synthesize 4-hydroxy-3-methoxycinnamic acid compound by reacting malonic acid with 4-hydroxy-3-methoxybenzaldehyde and ammonium acetate as catalyst using microwave irradiation. The synthesized product was then compared to cinnamic acid and acetosal for antiplatelet activity utilizing a blood clotting time method with mice blood. Acetosal was chosen for this study because it is commonly used as an antiplatelet medication.

MATERIALS AND METHODS

Materials
Malonic acid (Sigma, Aldrich), 4-hydroxy-3-methoxybenzaldehyde (Merck, Germany), ammonium acetate, chloroform p.a. (Mallinckrodt, USA), ethanol p.a. (Mallinckrodt, USA), methanol p.a. (Mallinckrodt, USA), and silica gel 60 F₂₅₄ (MACHELEY-NAGEL GmbH & Co. KG, Jerman).

Tools
Analytical balance (Sartorius, Germany), Sakura MW 9600 microwave oven with an output power of 1600 W with a frequency of 2,450 MHz, hot plate-magnetic stirrer, melting point apparatus (Stuart Scientific SMP1, UK), UV spectrophotometer (Hitachi UV-Vis U2910, Japan), infrared spectrophotometer (UATR Two Perkin Elmer, USA), nuclear magnetic resonance spectrometer (FT-NMR JEOL ECS-400, USA).

Method
Synthesis of 4-hydroxy-3-methoxycinnamic acid (ferulic acid)
Malonic acid was being crushed as much as 1.56 g (15 mmol) with ammonium acetate 770.8 mg (10 mmol) in an Erlenmeyer flask. 4-hydroxy-3-methoxybenzaldehyde 760.75 mg (5 mmol) was added to the mixture and stirred for several seconds. Erlenmeyer was removed from the microwave oven (960 Watt, 4 minutes) after the reaction, cooled, and added 2 N HCl little by little while stirring until a precipitate formed, filtered and washed with water to remove residual acid. The precipitated of ferulic acid was recrystallized from 70% ethanol.

Figure 1. Scheme of 4-hydroxy-3-methoxycinnamic acid reaction
Purity test and identification of synthesized compounds

The purity test was conducted by determining the melting point and thin-layer chromatography (TLC) test using 3 mobile phases with different polarity indexes and using 4-hydroxy-3-methoxybenzaldehyde as a standard.

Structure identification of the synthesized compounds was carried out by ultraviolet (uv) spectrophotometric test with ethanol solvent, infrared (IR) spectrophotometric test at a wavelength of 4000 - 600 cm\(^{-1}\) and proton NMR (\(^{1}\)H-NMR) spectrometry test with acetone-d6 solvent.

Antiplatelet activity test

Healthy mice aged 8-12 weeks and weighing 20-22 grams were used to test the antiplatelet activity of 4-hydroxy-3-methoxy cinnamic acid compounds. Mice were divided into ten treatment groups, namely the negative control group (CMC-Na 0.5%), positive control (acetosal) doses of 40 mg, 75 mg and 150 mg, groups of cinnamic acid and ferulic acid compounds equivalent to doses of acetosal 40 mg, 75 mg and 150 mg, specifically 0.0037 mmol/Kg (I), 0.0069 mmol/Kg (II) and 0.0139 mmol/Kg (III).

Test compounds such as acetosal, cinnamic acid and ferulic acid were prepared in suspension form using 0.5% CMC-Na solution because the test compounds are not soluble in water. Acetosal is less stable and easily hydrolyzed in water, so the test solution is made every day. The maximum administration volume was 0.3 mL because the test compound was administered after eating, so it did not irritate the stomach because it was acidic.

Each mouse was conditioned for one week, and the length of time for fibrin formation was measured in the mice's blood samples on day 0. The mice's tale was cleaned with 70% alcohol, then pierced with a scalp as far as 2 cm from the edge of the tail with a wound depth of 2 mm. The blood sample was then dripped into an object-glass and observed every 15 seconds to determine the onset of fibrin formation. Afterwards, the wounds in mice were given an antiseptic solution. Each test solution was administered orally for seven days. Observation of the onset of fibrin formation was analyzed on day 8 to determine the effect of the test solution. The results were then statistically tested by the one-way ANOVA method. The Ethical Commission approved the antiplatelet activity test in this experiment of Universitas Airlangga No.: 2.KE.103.11.2020.

RESULTS AND DISCUSSION

4-Hydroxy-3-methoxycinnamic acid (ferulic acid)

The 4-hydroxy-3-methoxycinnamic acid compound was light yellow crystals as shown in Figure 2. The synthesis of 4-hydroxy-3-methoxycinnamic acid was replicated three times. The percentage yield of the synthesis results were 31.92%, 28.83% and 30.89%, respectively, with an average of 30.55% ± 1.57. The results of determining the melting point obtained an average result of 170 - 171.5°C. In a study conducted by Ekowati (2016), the melting point test of 4-hydroxy-3-methoxycinnamic acid compounds obtained an average of 170 - 171°C. That melting point range is the same as the one produced in this study. Each replication of ferulic acid was tested by TLC with some mobile phases such as ethyl acetate: n-hexane (1:1, v/v; Rf 0.27), acetone: chloroform (1:4, v/v); Rf 0.38), and methanol: ethyl acetate (7:3, v/v; Rf 0.61).

![Figure 2. 4-Hydroxy-3-methoxycinnamic acid crystal](image)

Structure identification of ferulic acid was carried out by analyzing the test results of the ultraviolet, infrared and \(^{1}\)H-NMR spectra. The UV spectra of ferulic acid gave a maximum absorption peak at 313 nm and the starting material, 4-hydroxy-3-methoxybenzaldehyde, gave an absorption peak at 309 nm (Figure 3). The presence of conjugated double bonds cause the wavelength of the light absorbed becomes longer (Pavia, 2015). The IR spectrum (Figure 4) of ferulic acid shows the absorption at wave numbers 3433 cm\(^{-1}\) (phenolic OH), 1688 cm\(^{-1}\) (conjugged C=O), 1590 and 1431 cm\(^{-1}\) (aromatic C=C), 1265 and 1033 cm\(^{-1}\) (C=O ether). \(^{1}\)H NMR (400 MHz; acetone-\(D_{6}\), δ ppm) showed signals at 8.08 (s, 1H), 7.57 (d, J = 15.9 Hz, 1H), 7.30 (d, J = 1.9 Hz, 1H), 7.11 (dd, J = 8.2, 2.0 Hz, 1H), 6.84 (d, J = 8.2 Hz, 1H), 6.34 (d, J = 15.9 Hz, 1H), 3.89 (s, 3H).
Figure 3. UV spectra overlays of 4-hydroxy-3-methoxybenzaldehyde and 4-hydroxy-3-methoxycinnamic acid

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Ultra violet spectrum pattern of 4-hydroxy-3-methoxybenzaldehyde

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Ultra violet spectrum pattern of 4-hydroxy-3-methoxycinnamic acid

Figure 4. IR spectrum of 4-hydroxy-3-methoxycinnamic acid

The benzene ring in the synthesized compound can be proven based on IR spectrum data at wave numbers 1590 dan 1431 cm\(^{-1}\) which shows the presence of aromatic C=C bonds and aromatic C-H sp\(^2\) which is indicated at an absorption of 3012 cm\(^{-1}\). This is also evidenced by data on the \(^1\)H-NMR spectrum (Figure 5) with a chemical shift of 7.30 ppm and a multiplicity of doublets, 7.11 ppm and multiplicity of double doublets, as well as 6.84 ppm and multiplicity of doublets. These three kinds of chemical shifts indicate the presence of 3 types of protons attached to the benzene ring because of the presence of absorption at a chemical shift of 6.5 - 8 ppm (Smith, 2011). The alkene group conjugated with carboxylate in the \(^1\)H-NMR spectrum data is indicated by doublet absorption with each absorption showing 1 proton at a chemical shift of 7.57 ppm with \(J = 15.9\) Hz and at a chemical shift of 6.34 ppm \(J = 15.9\) Hz. Based on these data, it was concluded that the protons in C\(\alpha\) dan C\(\beta\) are in the trans conformation indicated by a coupling constant in the 11 - 18 Hz range representing protons in an alkene with the trans conformation (Smith, 2011). The company of a hydroxyl group (-OH) bound to the benzene ring is indicated by IR absorption at a wavenumber of 3433 cm\(^{-1}\) and \(^1\)H-NMR spectrum data with a chemical shift at 8.08 ppm with singlet multiplicity and 1 proton. The H atom bound to the phenolic O atom has a chemical change in 4.0 - 7.0 ppm, but this chemical shift can vary depending on the concentration, temperature and solvent (Pavia et al., 2015). The presence of a methoxy group (-OCH\(_3\)) bound to the benzene ring is indicated by IR absorptions at wavenumbers 1265 dan 1033 cm\(^{-1}\) by C-O eter dan C-H sp\(^3\) bonds at wavenumbers 2969 cm\(^{-1}\) and \(^1\)H-NMR spectrum data with a chemical shift of 3.89 ppm (\(s, 3H\)) which according to the literature, namely C-H sp\(^3\) bound to the O atom undergoes a chemical shift in the range of 2.5 - 4 ppm (Smith, 2011).

**Antiplatelet activity test**

The test compounds consisted of positive control (acetosal) at doses of 40 mg, 75 mg and 150 mg, groups of cinnamic acid and ferulic acid compounds which were equivalent to the molar doses of acetosal 40 mg, 75 mg and 150 mg, namely \(3.7 \times 10^{-3}\) mmol/Kg (I), \(6.9 \times 10^{-3}\) mmol/Kg (II) and \(13.9 \times 10^{-3}\) mmol/Kg (III) which can be seen in Table 1. The selection of these three doses refers to the dose of acetosal as an antiplatelet, which is 75 - 325 mg (Katzung, 2018), a dose of 40 mg to see whether the potential test compound can provide antiplatelet activity in low doses. The dose of 75 mg was chosen because it is the minimum dose as an antiplatelet, and the dose of 150 mg was chosen to see the potential of the test compound if it was increased twice from the minimum dose.
Table 1. % Antiplatelet activity and ED$_{30}$ of the test compounds

| Compounds                          | Dosage (mg/Kg) | % Activity | ED$_{30}$ mg/kg BW | mmol/kg  |
|------------------------------------|----------------|------------|-------------------|----------|
| Acetosal (MW : 180.16 mg/mmol)     | 8.2            | 24.69      | 1.004             | 5.81 x 10$^{-3}$ |
| Cinnamic Acid (MW : 148.16 mg/mmol) | 8.2            | 8.68       | 1.695             | 11.45 x 10$^{-3}$ |
| 4-Hydroxy-3-methoxycinnamic acid (MW : 194.18 mg/mmol) | 8.2            | 22.27      | 1.308             | 6.74 x 10$^{-3}$ |

Figure 5. a) $^1$H-NMR spectrum of 4-hydroxy-3-methoxycinnamic acid using acetone-d6 solvent; b) magnification of chemical shift : 6.324 - 8.075 ppm.
The difference between the time of fibrin formation after and before being administered the test solution, as shown in Figure 6, was used to calculate the blood clotting time in each test compound dose. This indicates that ferulic acid has an antiplatelet effect with a longer onset of fibrin formed and has activity comparable to acetosal at a dose of $3.7 \times 10^{-3}$ mmol/Kg (I).

The blood clotting time in each test compound in dose II it was stated that all test groups had an effect on mice and produced antiplatelet activity with a longer time for fibrin formation Figure 6. The results in the ferulic acid group gave significantly different results from the cinnamic acid group ($P < 0.05$), where blood clotting time in ferulic acid was longer, indicating that its activity is higher than in cinnamic acid. However, the antiplatelet activity of ferulic acid compared to acetosal did not give significantly different results, so that it can be said to have similar activity ($P > 0.05$).

The blood clotting time in each test compound at dose III, ferulic acid and cinnamic acid groups compared to acetosal did not give significantly different results, so it can be said that all test groups provided antiplatelet activity Figure 6. Comparison of blood clotting time at doses I, II and III of each test compound can be seen in Figure 6.

The $ED_{30}$ value is the amount of dose that can produce 30% activity. The $ED_{30}$ value in determining antiplatelet activity aims to compare the antiplatelet activity of ferulic acid with acetosal and cinnamic acid to assess the effect of adding hydroxy and methoxy groups. The correlation coefficient ($r$) of the dose linearity equation to the percent activity of the test group consisting of ferulic acid, cinnamic acid and acetosal is 0.98. The graph of the linearity equation of the molar dose of each test compound to the percent antiplatelet activity at each dose is shown in Figure 7.

The $ED_{30}$ value Table 1 from the results of the regression equation for each compound namely ferulic acid, cinnamic acid and acetosal was $67.4 \times 10^{-3}$ mmol/Kg or 1.080 mg/Kg; $11.45 \times 10^{-3}$ mmol/Kg;
or 1.6958 mg/Kg; and 58.1 x 10⁻³ mmol/Kg or 1.0466 mg/Kg. The test compound ferulic acid had better activity than cinnamic acid which could be due to the presence of hydroxy and methoxy groups substituted in the cinnamic acid compound. The ED30 value of ferulic acid proves that this compound has a better activity of 1.7 times compared to cinnamic acid because it has a smaller ED30 value by comparing the ED30 value in mmol/Kg.

Based on research conducted by Ekowati et al. (2019), the addition of hydroxy and methoxy groups can increase antiplatelet activity. Referring to the study by Ekowati et al. (2019) regarding testing the antiplatelet activity of p-coumaric acid derivative compounds, including p-methoxycinnamic acid and p-hydroxycinnamic acid in a dose of 80 mg with the same method as this study, which produced the same antiplatelet activity compared to acetosal did not give significantly different results, so it can be said to have comparable activity (P > 0.05).

However, the antiplatelet activity of ferulic acid compared to acetosal did not give significantly different results from the cinnamic acid group (P < 0.05), where the length of time fibrin formed in ferulic acid was longer, indicating that its activity is higher than that of cinnamic acid. However, the antiplatelet activity of dose II of ferulic acid compared to acetosal did not give significantly different results, so it can be said to have comparable activity (P > 0.05).

CONCLUSION

4-Hydroxy-3-methoxy cinnamic acid can be synthesized with the help of microwave irradiation using malonic acid and 4-hydroxy-3-methoxy benzaldehyde as the starting material and ammonium acetate as a catalyst at 960 Watt (P60) for 4 minutes which produces a yield percentage of 30.55%. Ferulic acid has antiplatelet activity which was tested by the method of measuring the length of time fibrin formed in the blood of mice with an ED₃₀ value of 1.308 mg/KgBW.

The test for blood clotting time in the ferulic acid group gave significantly different results from the cinnamic acid group (P < 0.05), where the length of time fibrin formed in ferulic acid was longer, indicating that its activity is higher than that of cinnamic acid. However, the antiplatelet activity of dose II of ferulic acid compared to acetosal did not give significantly different results, so it can be said to have comparable activity (P > 0.05).

AUTHOR CONTRIBUTIONS

Conceptualization, J.E.; Methodology, J.E.; Software, E.C.M.; Validation, T.B.; Formal Analysis, T.B.; Investigation, E.C.M.; Resources, E.C.M.; Data Curation, J.E.; Writing - Original Draft, T.B.; Writing - Review & Editing, J.E.; Visualization, E.C.M.; Supervision, T.B.; Project Administration, T.B.; Funding Acquisition, J.E.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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