SYNTHESIS OF ACETYL AND BENZOYL ESTERS OF XANTHORRHIZOL AND ITS OXIDATION PRODUCTS AND EVALUATION OF THEIR INHIBITORY ACTIVITY AGAINST NITRIC OXIDE PRODUCTION

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ABSTRACT

Objective: Xanthorrhizol is known to have anti-inflammatory activity. However, new xanthorrhizol derivatives with improved anti-inflammatory activity and reduced toxicity are needed.

Methods: In this study, the derivatives of xanthorrhizol were synthesized and spectroscopically characterized, and their inhibitory activities against nitric oxide (NO) production were evaluated in RAW 264.7 macrophage cells.

Results: The first stage of synthesis produced compounds 2a and 2b in 58.49% and 63.26% yields, respectively. Compounds 2a and 2b were oxidized using potassium permanganate, giving compounds 3a and 3b in yields of 51.92% and 43.78%, respectively. Compounds 1, 2a, 3a, and 3b along with diclofenac sodium (the positive control) exhibited IC50 values for NO production of 31.82, 73.85, 354.05, 97.19, and 78.43 µM, respectively. In contrast, compound 2b did not show any inhibitory activity. Based on cytotoxicity assay, compounds 1, 2a, 2b, 3a, 3b, and diclofenac sodium had LD50 values of 30.97, 65.15, 31.15, 117.86, 53.40, and 5.167 µM, respectively. The NO inhibitory activities of compounds 2a, 3a, and 3b were lower than that of xanthorrhizol (compound 1). However, cytotoxicity tests showed that compounds 2a, 3a, and 3b had reduced toxicities compared to xanthorrhizol.

Conclusion: The modification of xanthorrhizol through esterification and oxidation produced derivative compounds with weaker anti-inflammatory activity but reduced cytotoxicity.

Keywords: Xanthorrhizol, Oxidation, Potassium permanganate, Nitric oxide, RAW 264.7 cells.

INTRODUCTION

Inflammation is a protective immune response designed to protect against pathogenic infections and tissue injuries [1]. To reduce inflammation, nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed. NSAIDs work by inhibiting cyclooxygenase (COX) enzymes, which catalyze the conversion of arachidonic acid into the inflammatory mediators prostaglandin and thromboxane [2,3]. However, NSAIDs, which inhibit the COX-1 enzyme activity, have toxic effects on the gastrointestinal tract, especially the stomach, and the inhibition of COX-2 enzyme increases cardiovascular risk [4]. Therefore, novel anti-inflammatory drugs with different targets are desired.

When developing anti-inflammatory drugs, an alternative to COX enzyme inhibition is the hindrance of nitric oxide (NO) production. In addition to COX inhibition, some NSAIDs also inhibit NO production by restraining inducible NO synthase (iNOS) expression. For example, diclofenac sodium, an NSAID, inhibits iNOS gene expression at the transcription level by suppressing nuclear factor kappa B activity. The inhibition of iNOS gene expression ultimately results in a reduction in NO production [5] and NOS inhibitors found to be effective in treating experimentally induced arthritis [6].

Xanthorrhizol, a major component of Curcuma xanthorrhiza, is known anti-inflammatory agent [7-10]. The inhibition of NO production by xanthorrhizol was previously investigated [11]. However, xanthorrhizol has been found to restrict iNOS and COX expression in the skin of mice with acute inflammation under stimulation by 12-O-tetradecanoylphorbol-13-acetate [7]. Xanthorrhizol derivatization has been carried out through modifications to the xanthorrhizol structure, including the conversion of hydroxyl groups into ester groups [12,13] and the addition of epoxides and diols into the unsaturated chain using a weak oxidizing agent [12]. However, a new process to obtain novel derivatives of xanthorrhizol is needed to obtain compounds with enhanced anti-inflammatory ability and lower toxicity. Potassium permanganate (KMnO4) is an important strong oxidizing agent which is very useful for oxidizing carbon-carbon double bonds in organic compounds [14]. We have recently reported the oxidation of 1-o-acetyl-xanthorrhizol using permanganate to obtain an α-hydroxyl ketone of the compound, but the synthesis of other derivatives and evaluation of their biological activities have not been reported [15].

Herein, we report the synthesis of other derivatives of xanthorrhizol and the evaluation of anti-inflammatory activities of all obtained derivatives by testing their inhibition of NO production in vitro using RAW 264.7 macrophage cells stimulated by LPS.

EXPERIMENTAL METHODS

Materials and instruments

All solvents, chemicals, and reagents were commercially purchased. Xanthorrhizol was supplied by Java Plant (Karanganyar, Indonesia). The purity of xanthorrhizol was evaluated by thin-layer chromatography (TLC) on silica gel 60 F254 plates (Merck). Fourier transform infrared (FTIR) spectra were recorded using an FTIR spectrophotometer (Nicolet iS10, Thermo Fisher Scientific, Waltham, MA, USA). Nuclear magnetic resonance (NMR) spectra were collected using an NMR spectrometer (Agilent, Santa Clara, CA, USA) at 500 MHz for protons and 125 MHz for carbon atoms with deuterated chloroform (CDCl3) as
solvent and tetramethylsilane as an internal standard. High-resolution mass spectrometry (HR-MS) was carried out on an LCT Premier XE-TOF mass spectrometer (Waters Corp., Milford, MA, USA) in positive electrospray ionization (ESI) mode.

**Chemistry**

**Synthesis of 2-methyl-5-(6-methylhept-5-en-2-yl)phenyl acetate (2a)**

Compound 2a was prepared using the previously reported method with slight modification [16,17]. The salt used in this study was dried at 90°C before the synthesis. In a boiling flask, xanthorrhizol (5 mmol, 1091.65 mg) was dissolved in 30 mL ethyl acetate. NaHCO$_3$ (50 mmol, 4200.35 mg) and acetate anhydride (100 mmol, 10209 mg) were then added and stirred at room temperature for 24 h. The reaction was monitored by TLC until the reaction was complete. The formed precipitate was filtered, and the filtrate was concentrated. The obtained residue was then extracted using dichloromethane (15 mL) and water (5 mL). The organic phase was collected and dried by adding Na$_2$SO$_4$. Subsequently, the product was concentrated by evaporating the solvent using a rotary evaporator. Finally, the product was purified by column chromatography using a mixture of hexane and ethyl acetate (9:1 v/v) to obtain pure 2a.

**Synthesis of 2-methyl-5-(6-methylhept-5-en-2-yl)phenyl benzoate (2b)**

Compound 2b was synthesized using a reported method with slight modification [16,17]. Xanthorrhizol (1.084 g, 5 mmol) was dissolved in 10 mL of 5% NaOH at 0°C-5°C. Benzoic chloride (0.581 mL, 5 mmol) was added to the mixture followed by stirring until no benzoic chloride fumes were present. The product was separated using dichloromethane and concentrated. The product was then purified by column chromatography using a mixture of hexane and ethyl acetate (9:1 v/v) to obtain pure 2b.

**Synthesis of 5-(6-hydroxy-6-methyl-5-oxoheptan-2-yl)-2-methylphenyl acetate (3a)**

Compound 3a was prepared according to the procedure reported in our previous publication [15].

**Synthesis of 5-(6-hydroxy-6-methyl-5-oxoheptan-2-yl)-2-methylphenyl benzoate (3b)**

Compound 3b was synthesized using a previously reported method[18]. Compound 2b (2.206 mmol), glacial acetic acid (4.941 mL), acetone (5.9 mL), and distilled water (3.81 mL) were added to a three-neck boiling flask. After conditioning the mixture at 0°C-5°C, K$_2$MnO$_4$ crystals (2.42 mmol, 386.62 mg) were added slowly for 6 h followed by stirring for an additional 1 h. The reaction was monitored until completion using TLC. Subsequently, the solution was transferred into 10.83 mL of distilled water. H$_2$O$_2$ was then added until a clear solution formed. The obtained product was extracted using dichloromethane and then purified by column chromatography using a mixture of hexane, ethyl acetate, and methanol (8:2:1 v/v/v).

**Anti-inflammatory activity**

**Cell culture**

Murine macrophage cells (RAW 264.7, BPPT collection) were cultured at 37°C in Roswell Park Memorial Institute medium containing 10% fetal bovine serum (FBS) in a humidified incubator with 5% CO$_2$.

**Measurement of NO**

NO was determined by calculating the quantity of nitrite from sodium nitrite in the cell culture medium using Griess reagent as reported previously [1,11,9]. NO inhibition was analyzed in RAW 264.7 cells that had been stimulated using LPS. Seeded cells were placed in 96-well culture plates at a density of 1×10$^4$ cells/well and incubated at 37°C before the synthesis. In a boiling flask, xanthorrhizol (5 mmol, 1091.65 mg) was dissolved in 30 mL ethyl acetate. NaHCO$_3$ (50 mmol, 4200.35 mg) and acetate anhydride (100 mmol, 10209 mg) were then added and stirred at room temperature for 24 h. The reaction was monitored by TLC until the reaction was complete. The formed precipitate was filtered, and the filtrate was concentrated. The obtained residue was then extracted using dichloromethane (15 mL) and water (5 mL). The organic phase was collected and dried by adding Na$_2$SO$_4$. Subsequently, the product was concentrated by evaporating the solvent using a rotary evaporator. Finally, the product was purified by column chromatography using a mixture of hexane and ethyl acetate (9:1 v/v) to obtain pure 2a.

**Synthesis of 2-methyl-5-(6-methylhept-5-en-2-yl)phenyl benzoate (2b)**

Compound 2b was synthesized using a reported method with slight modification [16,17]. Xanthorrhizol (1.084 g, 5 mmol) was dissolved in 10 mL of 5% NaOH at 0°C-5°C. Benzoic chloride (0.581 mL, 5 mmol) was added to the mixture followed by stirring until no benzoic chloride fumes were present. The product was separated using dichloromethane and concentrated. The product was then purified by column chromatography using a mixture of hexane, ethyl acetate, and methanol (8:2:1 v/v/v).

**Cell culture supernatant (75 µL) was mixed with Griess reagent (75 µL) in a new 96-well plate in the dark for 20 min. Finally, the absorption was measured spectrophotometrically at 540 nm. The NO concentration (A) was calculated using a sodium nitrite standard curve, and the inhibition of NO was then calculated as follows:**

\[
\% \text{NO inhibition} = \frac{A - B}{A} \times 100
\]

Where, B is the NO concentration after treatment.

**Cytotoxicity assay against RAW 264.7 macrophage cells**

Cytotoxicity of the synthesized compounds on RAW 264.7 macrophages was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method [11]. RAW 264.7 cells were added to 96-well culture plates at a density of 1×10$^4$ cells/well and incubated at 37°C for 24 h in an incubator containing 5% CO$_2$. After cell growth, the medium was removed and replaced with various concentrations of each sample in 2% FBS for 24 h. After incubation, the culture medium was discarded, and (MTT: 0.5 mg/mL, 100 µL) solution was added to each cell. The cells were then reincubated at 37°C in an incubator containing 5% CO$_2$ for 4 h. After removing the culture supernatants, 200 µL dimethyl sulfoxide was added followed by agitating for 10 min in the dark. The absorption at 570 nm (T) was measured using a spectrophotometer, and cell viability was calculated as follows:

\[
\text{Cell viability} = \frac{100 - (C - T)}{C} \times 100\%
\]

Where, C is the absorption of the control. The IC$_{50}$ value was calculated by plotting cell viability against log concentration (logC) of the tested compounds using SPSS software.

**RESULTS AND DISCUSSION**

**Chemistry**

The synthetic method used to obtain xanthorrhizol derivatives is shown in Fig. 1. The hydroxyl groups of xanthorrhizol were first esterified either using acetic anhydride with NaHCO$_3$ as a catalyst to give 2a or using benzoic chloride with NaOH as a catalyst to give 2b. Second, the oxidation of 2a and 2b using a strong oxidizing agent (KMnO$_4$) in the presence of weak acid (acetic acid) at 0°C-5°C and water/acetone as solvent gave compounds 3a and 3b.

**Spectroscopy data of the synthesized compounds**

**2-Methyl-5-(6-methylhept-5-en-2-yl)phenyl acetate (2a)**

Compound 2a was obtained as a yellow oil in 58.49% yield: FTIR (KBr) 3087 (vC=C, aliphatic), 1309 (vC=C, benzene), 2935 (vCH$_3$), 2876 (vCH$_2$), 1732 (vC=O). HR ESI-MS m/z: 283.1680 [M+Na].

**2-Methyl-5-(6-methylhept-5-en-2-yl)phenyl benzoate (2b)**

Compound 2b was obtained as a yellow oil in 63.2% yield: FTIR (KBr) 3062 (vC=C, aliphatic), 1737 (vC=O), 1606 (vC=C, benzene), 3028 (vCH$_3$), 2928 (vCH$_2$), 1594 (vC=C). HR ESI-MS m/z: 283.1680 [M+Na].

2-Methyl-5-(6-methylhept-5-en-2-yl)phenyl benzoate (2b)

Cell culture supernatant (75 µL) was mixed with Griess reagent (75 µL) in a new 96-well plate in the dark for 20 min. Finally, the absorption was measured spectrophotometrically at 540 nm. The NO concentration (A) was calculated using a sodium nitrite standard curve, and the inhibition of NO was then calculated as follows:
Inhibition of NO production

The inhibition of NO production by the synthesized products was evaluated in RAW 264.7 monocyte macrophage-like cells from BALB/c mice induced by Aebelson leukemia virus. These cells have the ability to undergo pinocytosis and phagocytosis [20]. LPS, the main component of the bacterial cell wall, was used to stimulate NO production in the cells [21]. The NO inhibition assay indicated IC₅₀ values for NO production of 73.85, 354.05, and 97.19 µM for compounds 2a, 3a, and 3b, respectively. In contrast, 2b did not inhibit NO production (Table 1). The IC₅₀ values obtained for xanthorrhizol (1) and diclofenac sodium were 3.18 and 78.43 µM, respectively. Thus, 2a, 3a, and 3b had lower inhibitory activities for NO production compared to 1.

Table 1: Cytotoxicities and inhibitory activities for NO production of different compounds in RAW 264.7 cells induced by LPS

| Compound                        | IC₅₀ (µM)¹ | LD₅₀ (µM)² |
|---------------------------------|-----------|-----------|
| 1                               | 31.82     | 30.97     |
| 2a                              | 73.05     | 65.15     |
| 2b                              | 31.15     |           |
| 3a                              | 354.05    | 117.86    |
| 3b                              | 97.19     | 53.40     |
| Diclofenac sodium               |           | 51.67     |

¹NO production inhibition, ²cytotoxicity. NO: Nitric oxide, LPS: Lipopolysaccharide.

CONCLUSION

Ester derivatives of xanthorrhizol and its oxidation products were successfully synthesized. Compounds 2a and 2b were generated in yields of 58.49% and 63.26%, respectively, through the esterification of the hydroxyl groups of xanthorrhizol. Compounds 3a and 3b were produced in yields of 51.92% and 43.78%, respectively, by oxidizing 2a, 2b, and 3b with KMnO₄ to break the double bonds in the alkyl groups. The anti-inflammatory activities of the products were tested by NO inhibition assay using RAW 264.7 macrophage cells. The results indicated that the xanthorrhizol derivatives were less active against NO production compared to xanthorrhizol. However, cytotoxicity assay revealed that the modification of xanthorrhizol successfully reduced its toxicity.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.
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