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Research Article

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 51.67 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.

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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 a known anti-inflammatory agent [7-10]. The inhibition of NO production by xanthorrhizol in RAW 264.7 cells stimulated by lipopolysaccharide (LPS) was previously investigated [11]. 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 ($KMnO_4$) 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 ($CDCl_3$) 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, 10.209 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_2SO_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. Benzoyl chloride (0.581 mL, 5 mmol) was added to the mixture followed by stirring until no benzoyl 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 (11 mL), and distilled water (3.81 mL) were added to a three-neck boiling flask. After conditioning the mixture at 0°C–5°C, $KMnO_4$ 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_2O_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 [11,19]. 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 1×10^5 cells/well and incubated at 37°C in an incubator containing 5% CO_2 for 24 h. After incubation, the medium was discarded and replaced with various concentrations of each sample in serum (2% FBS) for 2 h. LPS was added to each well to achieve a final LPS concentration of 1 ppm. The cells were then reincubated for 24 h. 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:

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

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 left to attach for 24 h in an incubator at 37°C with 5% CO_2. After cell growth, the medium was removed and replaced with various concentrations of each sample in serum (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 agitation for 10 min in the dark. The absorption at 570 nm (*T*) was measured using a spectrophotometer, and cell viability was calculated as follows:

$$\text{Cell viability}(\%) = 100 - \left(\frac{C - T}{C} \times 100\%\right)$$

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 benzoyl 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) cm^{-1}, 2964.21 and 2926, 13 (C-H aliphatic), 1768.06 (C=O, ester), 1214.16 (C–O, ester). ^{1}H-NMR (500 MHz, $CDCl_3$), δ (ppm): 7.14 (1H, d, J = 7.8, Ar-H), 6.97 (1H, d, J = 6, Ar-H), 6.81 (1H, s, Ar-H), 5.07 (1H, m, CH_2-CH=C), 2.67, (1H, m, CH–Ar), 2.31 (3H, s, CH_3 acetate), 2.14 (3H, s, CH_3–Ar), 1.89 (2H, m, CH_2–CH_2–CH), 1, 66 (3H, s, CH_3–C, 1.58 (2H, m, CH–CH_2–CH_2), 1.52 (3H, s, CH_3–C), 1, 22 (3H, d, J = 1.39, CH_3–CH), ^{13}C-NMR (500 MHz, CDCl3) δ/ppm: 169.35 (C=O acetate), 149.42 (C-O acetate), 147.07 (CAr), 131.65 (CH=C–$(CH_3)_2$ aliphatic), 130.9 (CAr), 127.31 (CAr), 124.85 (CAr), 124.51 (CH_2–CH=C), 120.47 (CAr), 38.99 (CH–Ar), 38.48 (CH–CH_2–CH_2), 26.21 (CH_2–CH_2–CH), 25.85 (CH_3–C), 22.30 (CH_3–CH), 20.97 (CH_3–acetate), 17.81 (CH_3–C), 15.93 (CH_3–Ar). HR ESI-MS m/z: 283.1680 $[M+Na]^+$ calculated for $C_{17}H_{24}O_2Na$: 283.1674.

*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) cm^{-1}, 2962.75 and 2924.22 (C–H aliphatic), 1737.90 (C=O ester), 1263.59 (C–O ester). ^{1}H-NMR (500 MHz, $CDCl_3$), δ (ppm): 8.23 (1H, d, J = 8.55, Ar-H), 7.52 (1H, m, Ar-H), 7.64 (1H, m, Ar-H), 7.18 (1H, d, J = 7.8, Ar-H), 7.02 (1H, d, J = 7.75, Ar-H), 6.96 (1H, s, Ar-H), 5.09 (1H, m, CH_2–CH=C), 2.7 (1H, m, CH–Ar), 2.19 (3H, s, CH_3–Ar), 1.92 (2H, m, CH_2–CH_2–CH), 1.67 (3H, s, CH_3–C), 1.6 (2H, m, CH–CH_2–CH_2), 1.54 (3H, s, CH_3–C),

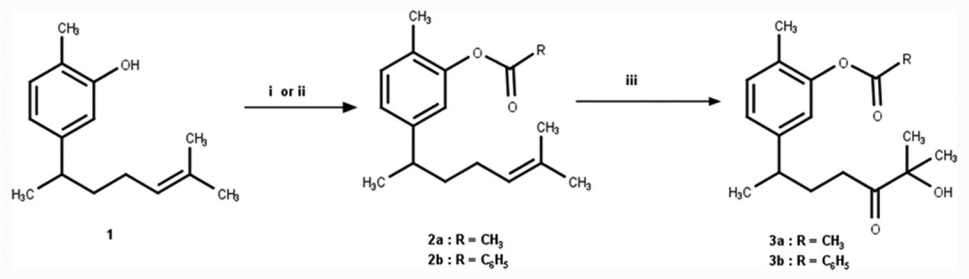

Fig. 1: Structures and synthetic routes of xanthorrhizol derivatives. Reaction conditions: (i) Acetic anhydride $NaHCO_3$ ethyl acetate, rt; (ii) benzoyl chloride, NaOH, cold (0°C–5°C); and (iii) $KMnO_4$, acetic acid, acetone, water, 0°C–5°C

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

Compound	IC_{50} (μM)[1]	LD_{50} (μM)[2]
1	31.82	30.97
2a	73.85	65.15
2b	-	31.15
3a	354.05	117.86
3b	97.19	53.40
Diclofenac sodium	78.43	51.67

[1]NO production inhibition, [2]Cytotoxicity. NO: Nitric oxide, LPS: Lipopolysaccharide

1.25 (3H, d, J = 6.9 CH_3–CH), ^{13}C-NMR (500 MHz, $CDCl_3$) δ (ppm): 164.97 (C=O), 149.61 (C–O), 147.13 (CAr), 133.61 (CAr), 131.65 (CH=C–$(CH_3)_2$, 131.02 (CAr), 130.28 (CAr), 129.8 (CAr), 128.71 (CAr), 127.53 (CAr), 124.88 (CAr), 124.54 (CH_2–CH=C), 120.61 (CAr), 39.05 (CH–Ar), 38.51 (CH–CH_2–CH_2), 26.24 (CH_2–CH–CH), 25.86 (CH_3–C), 22.35 (CH_3–CH), 17.83 (CH_3–C), 16.04 (CH_3–Ar), HR ESI-MS m/z: 345.1827 $[M+Na]^+$ calculated for $C_{22}H_{26}O_2Na$: 345.1831.

*5-(6-Hydroxy-6-methyl-5-oxoheptan-2-yl)-2-methylphenyl acetate (**3a**)*

The spectroscopic data of compound **3a** were reported previously [15].

*5-(6-Hydroxy-6-methyl-5-oxoheptan-2-yl)-2-methylphenyl benzoate (**3b**)*

Compound **3b** was obtained as a yellow oil in 43.78% yield: FTIR (KBr) cm^{-1}, 3492.04 (O–H), 2969.65 and 2929.93 (C–H aliphatic), 1736.23 (C=O ester), 1710.52 (C=O ketone), 1266.26 (C–O ester), ^{1}H-NMR (500 MHz, $CDCl_3$), δ (ppm): 8.21 (2H, d, J = 6.55, Ar-H), 7.52 (2H, m, Ar-H), 7.65 (1H, m, Ar-H), 7.2 (1H, d, J = 7.8, Ar-H), 7 (1H, d, J = 6,1, Ar-H), 6.93 (1H, s, Ar-H), 3.74 (1H, s, –OH), 2.71 (1H, m, CH–Ar), 2.2 (3H, s, CH_3–Ar), 2.4 (2H, m, CH_2–CH_2–C=O), 1.29 (3H, s, CH_3–C), 1.96 and 1.87 (2H, m, CH–CH_2–CH_2), 1.29 (3H, s, CH_3–C), 1.28 (3H, d, J = 7, 1, CH_3–CH), ^{13}C-NMR (500 MHz, $CDCl_3$) δ (ppm): 214.69 (C=O ketone), 165.09 (C=O benzoyl), 149.72 (C–O benzoyl), 145.46 (CAr), 133.73 (CAr), 131.36 (CAr), 130.27 (CAr), 129.66 (CAr), 128.75 (CAr), 128.17 (CAr), 124.77 (CAr), 120.78 (CAr), 38.93 (CH–Ar), 31.83 (CH–CH_2–CH_2), 33.62 (CH_2–CH_2–C=O), 26.57 (CH_3–C), 26.7 (CH_3–C) 22.73 (CH_3–CH), 16.03 (CH_3–Ar), HR ESI-MS m/z: 377.1718 $[M+Na]^+$ calculated for $C_{22}H_{26}O_4Na$: 345.1729.

The FTIR spectra of **2a** and **2b** show bands corresponding to C=O in ester groups at 1768.06 and 1737.9 cm^{-1}, respectively. No bands of–OH groups (expected at 3400 cm^{-1}) are observed, indicating that the hydroxyl groups were all replaced by ester groups. This conclusion is supported by the ^{12}C-NMR spectra, in which the signals of the carbon atoms in the ester groups are observed at 169.35 and 164.97 ppm for **2a** and **2b**, respectively.

The FTIR spectra of **3a** and **3b** show peaks corresponding to–OH groups at 3482.04 and 3492.04 cm^{-1}, respectively. Ketone groups are also indicated by the peaks at 1709.98 and 1710.52 cm^{-1} in the spectra of **3a** and **3b**, respectively. The previously formed ester groups (in **2a** and **2b**) remained in **3a** and **3b**. The FTIR results were supported by the ^{13}C-NMR spectra of **3a** and **3b**, in which the peaks corresponding to the carbon atoms of ketone groups are found at 214.5 and 214.69 ppm, respectively, and the peaks of the carbon atoms of–OH groups are located at 76.33 and 76.36 ppm, respectively. The ^{1}H-NMR and HR-MS data also supported the reported structures of all compounds.

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 Abelson 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 IC_{50} 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 IC_{50} values obtained for xanthorrhizol (1) and diclofenac sodium were 31.82 and 78.43 μM, respectively. Thus, **2a, 3a**, and **3b** had lower inhibitory activities for NO production compared to 1.

Based on cytotoxicity assay, the LD_{50} values of compounds **2a**, **2b**, **3a**, and **3b** were 65.15, 31.15, 117.86, and 53.40 μM, respectively (Table 1). The LD_{50} values of compounds 1 and diclofenac sodium were 30.97 and 51.67 μM, respectively (Table 1). Thus, the cytotoxicities of the xanthorrhizol derivatives **2a, 2b, 3a**, and **3b** were lower than that of 1. These results indicate that all structural modifications of xanthorrhizol reduced the cytotoxicity. Xanthorrhizol has potential as an anti-inflammatory agent, but it also was reported to have a high cytotoxic potential [7-10]. Further studies should be done to explore the relation of its pharmacological activity and cytotoxicity.

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** and **2b** with $KMnO_4$ 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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