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Original Research Article

A New Method of Xanthorrhizol Isolation from the Rhizome Extract of *Curcuma xanthorrhiza*

Emanuel D. Ramdani, Ujiatmi D. Marlupi, James Sinambela, Raymond R. Tjandrawinata*
Dexa Laboratories of Biomolecular Sciences (DLBS), Dexa Medica, Industri Selatan V Block PP No. 7, Jababeka Industrial Estate II, Cikarang 17550, West Java, Indonesia

***Corresponding author**
Raymond R. Tjandrawinata
Email: raymond@dexa-medica.com

Abstract: *Curcuma xanthorrhiza*, a plant originating from Indonesia, has been known for its medicinal uses for a long time, including its rhizomes. The major compound of *C. xanthorrhiza* essential oil is xanthorrhizol, which is responsible for many beneficial effects of *C. xanthorrhiza*. The present study aimed to develop simplified method for xanthorrhizol isolation from *C. xanthorrhiza* rhizome due to its limited availability and difficulties in the isolation method. Dried rhizome of *C. xanthorrhiza* was extracted using 80% ethanol and partitioned between *n*-hexane/H_2O. The organic layer was treated with acid-base reaction and acetylation followed by fractionation in a flash chromatography. The acetyl xanthorrhizol fraction was hydrolysed and purified using flash chromatography. The fractionation process produced xanthorrhizol with 0.8% yield and 99.57% purity that was analyzed using gas chromatography with flame ionization detector (GC-FID). The results of ^{1}H-NMR and ^{13}C-NMR spectra of nuclear magnetic resonance (NMR) and mass spectrometry (MS) analysis confirmed that the isolated compound was xanthorrhizol. It showed that xanthorrhizol has been successfully isolated using the present method. This research provides an alternative method for xanthorrhizol isolation and it gives a simpler fractionation method.
Keywords: Curcuma xanthorrhiza, acid-base reaction, acetylation, xanthorrhizol.

INTRODUCTION

Curcuma xanthorrhiza Roxb. (Zingiberaceae) is a plant that originally grows in Indonesia, especially in Java Island. Its rhizome part has been known as a medicinal herb to cure various illnesses for a long time, including stomach diseases, liver disorders, constipation, bloody diarrhea, dysentery, children's fever, hemorrhoid and skin eruption [1]. The uses of *C. xanthorrhiza* have also been scientifically proved as antibacterial, antinociceptive, gastroprotector, antioxidant and hepatoprotector [2-5].

Essential oil of *C. xanthorrhiza* contains various compounds, including xanthorrhizol (64.38%), camphene (8.27%), curcumin (5.85%), α-pinene (1.93%), α-thujene (0.16%), β-pinene (0.14%), myrcene (0.37%), linalool (0.27%) and zingiberene (0.10%) [6]. As the major compound of *C. xanthorrhiza* essential oil, xanthorrhizol is responsible for many effects of *C. xanthorrhiza*. Rukayadi [7] has studied the potential activity of xanthorrhizol as anti-candidal. Moreover, it has been found that xanthorrhizol has an activity in reducing *Candida albicans* biofilms. It showed that xanthorrhizol exhibited a potent activity against *C. albicans* biofilms *in vitro* and therefore might have potential therapeutic effects for treating *C. albicans*-related infections [8]. Other researchers have demonstrated that xanthorrhizol has an estrogenic [9] and antibacterial activities [1,10]. Combination of xanthorrhizol and curcumin has also been studied and the result exhibited an inhibition of human breast cancer cells (MDA-MB-231) growth via apoptosis induction [11].

Due to many beneficial effects of xanthorrhizol, development of optimum *C. xanthorrhiza* extraction method is necessary to be done to obtain an extract with high xanthorrhizol content. In the previous study, we have developed a high-technology method for *C. xanthorrhiza* extraction by supercritical fluid extraction using carbon dioxide (SCFE-CO_2). The xanthorrhizol content obtained in previous SCFE-CO_2 was higher than Soxhlet extraction and percolation process [12].

Recently, the presence of xanthorrhizol has become a unique marker for *C. xanthorrhiza* because it is able to differentiate the plant from other Curcuma species [13]. However, the availability of xanthorrhizol is limited due to the difficulties in its isolation method.

In the present study, simplified isolation method of xanthorrhizol was developed in order to obtain a more effective method for xanthorrhizol isolation.

MATERIALS AND METHODS

General experiment

Flash chromatography was performed in a Sepacore Flash Chromatography (Buchi, Switzerland) using Silica Gel 60 with 0.063-0.2 mm mesh (Merck, Germany). Thin-layer chromatography (TLC) was done using an Automatic TLC Sampler (ATS) 4 (CAMAG, Switzerland) in silica gel 60 F254 TLC plates (Merck, Germany) and the results were documented by Reprostar3 (CAMAG, Switzerland). High resolution mass spectra (HRMS) were obtained from an LCT Premier XE (TOF) using electrospray ionization (ESI) instrument (Waters, United Kingdom) by direct injection. Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL JNM-ECA 500 (JEOL, Japan) NMR spectrometer (500 and 125 MHz for ^{1}H and ^{13}C NMR, respectively). Results were recorded as follows: chemical shift values were expressed as δ units acquired in CD_3OD, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet), coupling constants (J) in Hertz (Hz) and integration. The purity was analyzed using a gas chromatography Clarus 680 apparatus analysis with a flame ionization detector (FID) (Perkin Elmer, USA).

Chemical reagents

Ethanol (PT Molindo Raya Industrial, Indonesia), *n*-hexane (Exxon mobil, USA), ethyl acetate (PT Showa Esterindo, Indonesia), methylene chloride, *n*-hexane, ethyl acetate, acetic anhydride, methanol, acetonitrile for LC-MS, sulphuric acid, hydrochloric acid, potassium hydroxide, TLC plates silica gel 60 F254 (Merck, Germany), packed silica gel (BUCHI, Switzerland).

Extraction method

Approximately 500 g of sliced and dried *C. xanthorrhiza* rhizomes were extracted with 80% ethanol. It was then evaporated under reduced pressure. The ethanolic extract was dissolved in aqueous methanol and liquid-liquid partitioned with *n*-hexane. Thus, the *n*-hexane phase was separated and evaporated under reduced pressure to obtain 45.6 g of *n*-hexane fraction.

Fractionation and purification method

45.6 g *n*-hexane fraction was dissolved in methylene chloride and partitioned with 5% potassium hydroxide in water. The water fraction was acidified with concentrated hydrochloric acid until pH 1. It was then partitioned by *n*-hexane followed by evaporation. Pyridine and acetic anhydride were added into the evaporated organic phase and the mixture was stirred at room temperature for 24 h. After that, the mixture was, once again, liquid-liquid partitioned by n-hexane and H_2O. The obtained *n*-hexane layer was fractionated using flash chromatography with silica gel as stationary phase and eluted with *n*-hexane:ethyl acetate mixture. Targeted fractions were combined and evaporated under reduced pressure. The residue was reacted with 10% potassium hydroxide in methanol for 2 h and partitioned with *n*-hexane. The organic fraction was further fractionated using flash chromatography with silica gel as stationary phase and eluted with *n*-hexane. This process resulted in pure xanthorrhizol in colorless oil form with yield of 0.8%.

Analysis method

MS analysis

Isolated compound was checked for its molecular mass by mass spectrometry (MS). The isolated compound was directly injected into a Waters LCT Premier Xe (Waters, UK). Mass spectrometry condition was performed on ESI negative with *m/z* 100-500.

GC-FID analysis

Gas chromatography (GC) analysis was performed using a Perkin Elmer Clarus 680 apparatus with a flame ionization detector (FID). Operating conditions including an Elite-5 capillary column (dimethyl polysiloxane (5% diphenyl)), 30 m x 0.25 mm i.d. with film thickness of 0.25 µm, temperature program of 160-230°C (14 min) at 5°C/min, at injector and detector temperatures of 220°C and 240°C, respectively. Nitrogen was used as carrier gas for GC analysis at 1 ml/min flow rate.

NMR analysis

Nuclear magnetic resonance (NMR) analysis was done to perform ^{1}H NMR and ^{13}C NMR spectroscopy. ^{1}H NMR and ^{13}C NMR spectra were recorded on a JEOL spectrometer at 500 MHz and 125 MHz (JEOL, Japan), respectively. Chemical shifts are determined as δ values.

RESULTS AND DISCUSSION

Xanthorrhizol, colorless oil; ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.22 (d, 3H, J=7.15 Hz, H-14), 1.55 (s, 3H, H-15), 1.62 (m, 2H, H-8), 1.69 (s, 3H, H-12), 1.89 (m, 2H, H-9), 2.23 (s, 3H, H-13), 2.63 (sextet, 1H, H-7), 5.10 (m, 1H, H-10), 6.61 (d, 1H, J=1.95 Hz, H-5), 6.68 (dd, 1H, J=1.95 Hz, 7.8Hz, H-4), 7.02 (d, 1H, J=7.75 Hz, H-2); ^{13}C NMR (125 MHz, $CDCl_3$): δ 15.53 (C-13), δ 17.88 (C-15), δ 22.58 (C-14), δ 25.91 (C-12), δ 26.32 (C-9), δ 38.55 (C-8), δ 39.21 (C-7), δ 113.69 (C-2), δ 119.63 (C-4), δ 120.97 (C-6), δ 124.68 (C-10), δ 130.95 (C-5), δ 131.62 (C-11), δ 147.41 (C-3), δ 153.76 (C-1).

Compared to NMR results of previous xanthorrhizol isolate [14], ^{1}H-NMR ($CDCl_3$, 500 MHz) showed similar chemical shifts to the present study. The presence of aromatic ring was shown by the occurrence of three proton signals as ABX system at δ 6.61 (d, 1H,

J=1.95 Hz), 6.68 (dd, 1H, J=1.95 Hz, 7.8 Hz) and 7.02 (d, 1H, J=7.8). Other protons gave signal at δ 1.22 (d, 3H, J=7.15 Hz, H-14), δ 1.55 (s, 3H, H-15), δ 1.62 (m, 2H, H-8), δ 1.69 (s, 3H, H-12), δ 1.89 (m, 2H, H-9), δ 2.23 (s, 3H, H-13), δ 2.63 (sextet, 1H, H-7) and δ 5.10 (m, 1H, H-10).

It was showed in ^{13}C NMR spectrum that the isolated compound possessed 15 carbon atoms. Six carbons were in aromatic ring at δ 113.69, 119.63, 120.97, 130.95, 147.41 and 153.76, and three of the carbons were substituted carbons. One carbon at δ 153.76 indicated the presence of hydroxy group and one carbon at δ 120.97 that was bound to methyl (δ 15.53). Another carbon from three substituted aromatic carbons (δ 147.41) was bound to an alkyl which contains two carbons as tri-substituted alkene (δ 124.68 and 131.62), a methine carbon (δ 39.21), two methylene carbons (δ 26.32 and 38.55) and three methyl carbons (δ 17.88, 22.58, and 25.91). In conclusion, the isolated compound has similar characteristics to xanthorrhizol based on its ^{1}H-NMR spectrum and ^{13}C-NMR spectrum.

Table 1. Chemical shifts data from ^{1}H NMR and ^{13}C NMR spectrum of xanthorrhizol

C Position	Isolated Compound (δ)		Xanthorrhizol (δ) [14]	
	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR
C-1	-	153.76	-	153.51
C-2	7.02 (d, 1H, J=7.8 Hz)	113.69	7.01 (d, 1H, J=7.6 Hz)	113.50
C-3	-	147.41	-	147.16
C-4	6.61 (d, 1H, J=1.95 Hz)	119.63	6.59 (br s, 1H)	119.42
C-5	6.68 (dd, 1H, J=1.95 Hz, 7.8Hz)	130.95	6.66 (br d, 1H)	130.74
C-6	-	120.97	-	119.42
C-7	2.63 (sextet, 1H)	39.21	2.59 (q, 1H)	38.98
C-8	1.62 (m, 2H)	38.55	1.57 (dt, 2H, J=7.1, 7.2 Hz)	38.32
C-9	1.89 (m, 2H)	26.32	1.85 (dt, 2H, J=7.0, 7.2 Hz)	26.10
C-10	5.10 (m, 1H, J=7.15 Hz)	124.68	5.08 (t, 3H, J=7.0, 7.2 Hz)	124.48
C-11	-	131.62		131.39
C-12	1.69 (s, 3H)	25.91	1.67 (s, 3H)	25.67
C-13	2.23 (s, 3H)	15.53	2.20 (s, 3H)	15.31
C-14	1.22 (d, 3H, J=7.15 Hz)	22.58	1.18 (d, 3H, J=7.1 Hz)	22.3
C-15	1.55 (s, 3H)	17.88	1.52 (s, 3H)	17.64

Molecular mass of xanthorrhizol was identified by Waters LCT Premier Xe (Waters, UK). As shown in Figure 1, the isolated compound has *m/z* $[M-H]^-$ 217.1093 in the ESI negative ion mode. Based on the result, it showed that molecular mass of the isolated compound is 218.1093, which was similar to xanthorrhizol mass.

The purity of isolated compound was analyzed using GC-FID. Purity of isolated xanthorrizol was 99.57%.

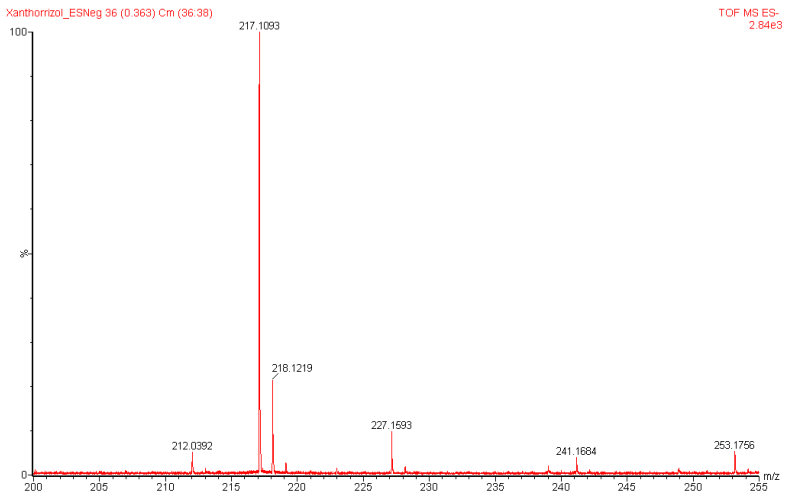

Fig-1. Mass profile of isolated compound

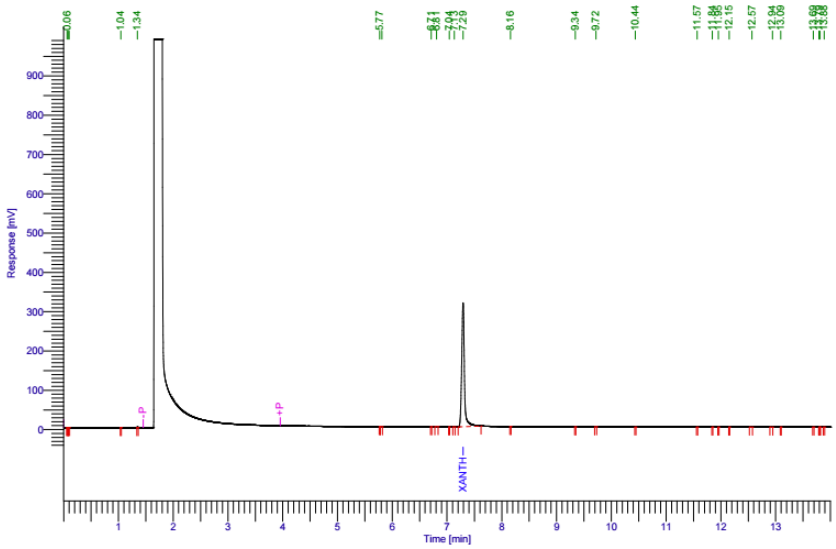

Fig-2. GC-FID result of isolated compound

Isolation of xanthorrhizol has become important due to its use as a specific marker for *C. xanthorrhiza* and its limited availability. However, xanthorrhizol cannot be separated by normal column chromatography using mixture of solvents with different polarity because other compounds were always eluted together as co-eluents [11]. In order to solve this problem, Hwang et al. [14] have proposed a new method for xanthorrhizol isolation. The main feature in this method was the acetylation which converted xanthorrhizol into acetyl xanthorrhizol, thus it was able to be separated from other compounds.

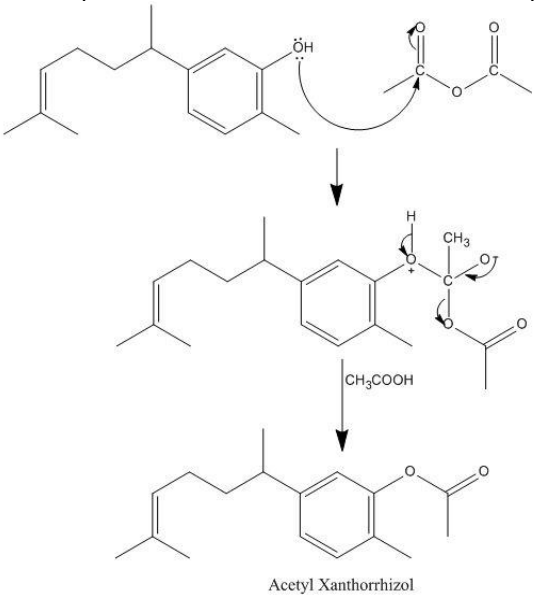

Fig- 3. Acetylation of xanthorrhizol

Three steps of column chromatography were performed by Hwang et al. [14] in order to isolate xanthorrhizol. The first step of column chromatography was carried out to separate xanthorrhizol fraction from other polar compounds because non-polar solvent was used for elution process. The second step of fractionation was performed after acetylation, so that acetyl xanthorrhizol formed during acetylation could easily be separated from impurities. Lastly, after deacetylation process, the third column chromatography was done using cation-exchange resin in order to purify xanthorrhizol.

In the present study, modification was performed to improve the efficiency of separation. The core reaction, which was acetylation, was not removed. Instead, the pre-acetylation was changed from column chromatography fractionation to acid-base reaction by utilizing the acidic properties of phenolic group of xanthorrhizol.

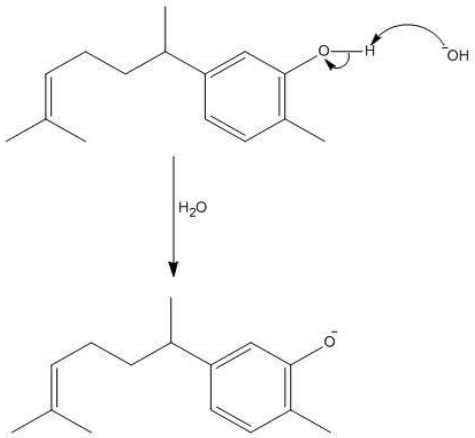

Fig-4. Reaction between xanthorrhizol and strong base

Reaction between xanthorrhizol and strong bases converted xanthorrhizol into its ionic form. It gave a particular polar xanthorrhizol form. Thus, it became easier to be separated from other non-polar compounds. Reversion of xanthorrhizol into its base compound also made it possible to be separated from other semi-polar and polar compounds. These processes, followed by acetylation, were made it possible to be separated from impurities when it is fractionated in normal phase column chromatography.

Obtained acetyl xanthorrhizol was then hydrolyzed and fractionated in column chromatography with silica gel to obtain xanthorrhizol with a purity of 99.57% and yield of 0.8% in the form of colorless oil. Results of this experiment will add more values to the growing knowledge of herbal materials which were previously studied by scientists in our laboratory [15-18].

CONCLUSION

Modification of xanthorrhizol isolation method has been completely done in this study. Acid-base reaction was able to reduce the number of column chromatography compared to previous method. Therefore, the effectiveness of xanthorrhizol isolation was greatly improved and the present method leads to a faster isolation method.

CONFLICT OF INTEREST

The authors declared no conflict of interest with respect to the authorship and/or publication.

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