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Original Article

METHOD VALIDATION OF (*E*)-4-(3', 4'-DIMETHOXYPHENYL)-BUT-3-EN-1-OL IN *ZINGIBER CASSUMUNAR* ROXB. WITH DIFFERENT EXTRACTION TECHNIQUES

JIRAPORNCHAI SUKSAEREE*, FAMEERA MADAKA, CHAOWALIT MONTON, APIRAK SAKUNPAK, TUN CHUSUT, AND TOSSATON CHAROONRATANA

Faculty of Pharmacy and Sino-Thai Traditional Medicine Research Center (Cooperation between Rangsit University, Harbin Institute of Technology, and Heilongjiang University of Chinese Medicine), Rangsit University, Pathum Thani 12000, Thailand.
Email: jirapornchai.s@rsu.ac.th

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ABSTRACT

Objective: To comparison of the RP-HPLC validation method of (*E*)-4-(3', 4'-dimethoxyphenyl)-but-3-en-1-ol (compound D) from different extraction methods. The compound D is the major compound of *Zingiber cassumunar* Roxb. The compound D was separated by HPLC instrument and separation with column chromatography on silica gel techniques.

Methods: First method, the *Z. cassumunar* powder was dissolved in absolute ethanol and then was injected into HPLC instrument for compound D collection at appropriated time elution. Second method, the *Z. cassumunar* powder was separated by column chromatography on silica gel technique with different ratio between hexane and ethyl acetate as eluted solvent to compound D collection. Then, the structure of compound D was identified by FTIR, ^{1}H-NMR, ^{13}C-NMR, and LC-MS. The compound D was dissolved in isotonic phosphate buffer pH 7.4, and incubated in isotonic phosphate buffer pH 7.4 and new born pig skin by sonication method. It was validated with HPLC in term of limit of detection, limit of quantitation, linearity, accuracy, and precision in ranges of 1-10 µg/mL.

Results: The FTIR, ^{1}H-NMR, ^{13}C-NMR, and LC-MS were identified structure of compound D which related to previous publication. The validation methods of different techniques were good linearity with good correlation coefficient (r^2) > 0.999 in ranges of 1-10 µg/mL. The limit of detection and limit of quantitation were 0.3, 0.8 µg/mL, respectively. The accuracy and precision were 95.38-104.76% and <2%, respectively for intraday preparation. The accuracy and precision were 88.94-102.43% and <5%, respectively for interday preparation.

Conclusion: This research successfully compared the RP-HPLC validation method using a gradient elution of 2% acetic acid in ultrapure water and methanol.

Keyword: *Zingiber cassumunar* Roxb., (*E*)-4-(3',4'-dimethoxyphenyl)-but-3-en-1-ol, Compound D, HPLC, Method validation.

INTRODUCTION

The *Zingiber cassumunar* Roxb., as known as Plai in Thai name, used for the treatment of asthma, as well as for muscle and joint pain that is the main herb in Thai herbal compress ball, as known LUK-PRA-KOP. In addition, the compound D was extracted from the rhizomes of *Zingiber cassumunar* Roxb. which the main active compound in *Z. cassumunar*. (*E*)- 4- (3', 4'-dimethoxy phenyl)-but-3-en-1-ol (compound D, Fig. 1) exhibits anti-inflammation activity by using various experimental models of inflammation [1-3]. Analgesic and antipyretic properties of compound D were also reported [2-4]. It is also used as topical treatment for sprains, contusions, joint inflammations, muscular pain, abscesses, and similar inflammation-related disorders [5-7].

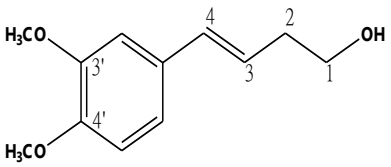

Fig. 1: Chemical structure of (*E*)-4-(3', 4'-dimethoxyphenyl)-but-3-en-1-ol (compound D)

More than 400 years, the Thai herbal compress ball, also known as herbal ball or LUK-PRA-KOP, has been used in Thailand and in Thai traditional fields. It is a ball of Thai herbs used as a hot compress to relieve aches, inflammation, and pains of the body by opening the pores and bringing a medicinal heat to induce muscle relaxation. It is composed of various dried herbs such as *Zingiber cassumunar* Roxb., *Curcuma longa* Linn., *Citrus hystrix* DC., *Cymbopogon citratus* Stapf., *Acacia rugata* Merr., and *Tamarindus indica* Linn. These are wrapped in cotton clothes traditionally used in Thai medicine [8, 9]. This research prepared and separated the compound D by HPLC instrument and separation with column chromatography on silica gel techniques from *Z. cassumunar* powder that was dissolved in ethanol. Then, its structure was confirmed by fourier transform infrared spectroscopy (FTIR), proton nuclear magnetic resonance (^{1}H-NMR), carbon nuclear magnetic resonance (^{13}C-NMR), and liquid chromatography–mass spectrometry (LC-MS). Finally, the compound D was dissolved in isotonic phosphate buffer pH 7.4 and validated by the RP-HPLC method. The compounds D was incubated in composition of isotonic phosphate buffer pH 7.4 and new born pig skin by sonication method, and were then validated by the RP-HPLC method.

MATERIALS AND METHODS

Materials

Z. cassumunar (Plai) powder was purchased from Charoensuk Osod, Thailand. All organic solvents were of analytical grade obtained from Merck KGaA (Germany). The ultrapure water was produced by Puris, Expe-UP water system (model: Expe-UP series, Korea) that had been purified to stringent specifications with 18.2 MΩ·cm at 25ºC of resistivity, 5 – 10 ppb of total organic carbon (TOC), <0.05 ppb of inorganics, and <1 cfu/mL of bacteria.

Separation of compound D by RP-HPLC instrument technique

The *Z. cassumunar* (Plai) powder was extracted in 95% ethanol and filtered through a 0.45 µm of polyamide membrane to obtained crude *Z. cassumunar* (Plai). The crude *Z. cassumunar* (Plai) was dissolved in absolute ethanol and was analyzed by the HPLC system using an Agilent 1260 Infinity system that described in HPLC method I as below section.

Separation of compound D by column chromatography on silica gel technique

The *Z. cassumunar* (Plai) powder was extracted in 95% ethanol and filtered through a 0.45 µm of polyamide membrane to obtained crude *Z. cassumunar* (Plai). The crude *Z. cassumunar* (Plai) was dissolved in ethyl acetate.

The 20 g ethyl acetate extract was then adsorbed on 200 g of silica gel by vacuum liquid chromatography. The column was eluted in ascending polarity manner with hexane: ethyl acetate in ratio of 9:1, 7:3, 6:4 and 5:5 to afford seven fractions (E1-E8). E6 (3.31g) was subjected to column chromatography on 100 g of silica gel eluted with hexane/ethyl acetate (in ratio of 7:3 up to 5:5) to give six sub-fractions (E1a - E6a).

Further column chromatography of the sub-fraction E3a (75 mg) was performed on 50 g of silica gel to give many sub-fractions which were combined to give sub-fractions 1 to 10. Sub-fraction 5 was purified by preparative TLC with hexane/ethyl acetate in ratio of 5:5 (eluted 5 times) to afford compound D as pale yellow oil (20 mg).

FTIR study

The compound D was homogeneously mixed with potassium bromide (KBr) and was pressed into disc. The compound D disc was examined by the FTIR technique. It was scanned at a resolution of 4 cm^{-1} with 16 scans over a wavenumber region of 400 - 4000 cm^{-1} using the FTIR spectrophotometer (model: Nicolet 6700, DLaTGS detector, Thermo Scientific, USA.). The characteristic peaks of IR transmission spectra were recorded.

NMR analysis

NMR spectra of compound D was recorded in $CDCl_3$, on a fourier transform NMR spectrometer 500 MHz (Unity Inova, Varian, Germany), operating a 500 MHz for protons, and 75 MHz for carbons.

LC-MS analysis

The LC-MS studies were performed by coupling the LC system with a Dionex Ultimate TM3000. A 2.1 mm × 150 mm diameter, 2.7 µm particle size C18 column (Poroshell 120), and injection volume of 10 µL were used for this experiment. The mobile phase was a gradient elution of 0.2% formic acid in ultrapure water (A) and methanol (B) of 60 to 50% of A, 50 to 30% of A, 30 to 20% of A, 20 to 50% of A, 50 to 60% of A, and 60% of A for 0 – 5 min, 5 – 15 min, 15 – 25 min, 25 – 30 min, 30 – 32 min, and 32 – 40 min, respectively. The mass determinations were made in positive ESI mode in capillary ± 4,500 volt nebulizer 2.00 bar, dry gas 7.0 L/min, dry temp. 200 oC, range of MW 70-2,000 in full scan (Bruker Amazon SL)

Preparation of isotonic phosphate buffer pH 7.4

Isotonic phosphate buffer pH 7.4 was prepared by mixing two stock solutions, 200 mL of a stock solution containing 8 g of monobasic sodium phosphate (NaH2PO4) per liter and 800 mL of a stock solution containing 9.47 g of dibasic sodium phosphate (Na2HPO4) per liter, the weights being on an anhydrous basic.

Then, the obtained solution was adjusted with respect to tonicity by adding 4.4 g of sodium chloride (NaCl). The obtained isotonic phosphate buffer pH 7.4 was filtered through a 0.45 µm of polyamide membrane and degassed by sonication before use [10].

Preparation of compound D in isotonic phosphate buffer pH 7.4

The compound D was dissolved in absolute ethanol for 200 µg/mL in concentration as stock solution. Then, the initial compound D stock solution was dilute with isotonic phosphate buffer pH 7.4 in different concentration of 2 – 40 µg/mL for HPLC method validation by HPLC condition method I and II.

Preparation of compound D in isotonic phosphate buffer pH 7.4 and new born pig skin

The compound D was dissolved in absolute ethanol for 200 µg/mL in concentration as stock solution. The new born pig skin was soaked in isotonic phosphate buffer pH 7.4 for overnight and then the initial compound D stock solution was dilute with mixture of isotonic phosphate buffer pH 7.4 and new born pig skin in different concentration of 2 – 40 µg/mL. After that, they were mixed by sonication method for 30 min. The compound D solution in various concentrations was analyzed for HPLC method validation by HPLC condition method II.

HPLC condition method I

Z. cassumunar (Plai) was analyzed by the RP-HPLC system using an Agilent 1260 Infinity system (Agilent Technologies, USA.) with detection at 260 nm. A 4.6 mm × 100 mm diameter, 3.5 µm particle size C18 column (Agilent Technologies, USA.), a flow rate of 1 mL/min, and injection volume of 10 µL were used for this experiment. The mobile phase was a gradient elution of 2% acetic acid in ultrapure water (A) and methanol (B) of 60 to 50% of A, 50 to 30% of A, 30 to 20% of A, 20 to 50% of A, 50 to 60% of A, and 60% of A for 0 – 5 min, 5 – 15 min, 15 – 25 min, 25 – 30 min, 30 – 32 min, and 32 – 40 min, respectively.

HPLC condition method II

Z. cassumunar (Plai) was analyzed by the RP-HPLC system using an Agilent 1260 Infinity system (Agilent Technologies, USA.) with detection at 260 nm. A 4.6 mm × 250 mm diameter, 5 µm particle size C18 column (ACE 5, DV12-7219, USA.), a flow rate of 1 mL/min, and injection volume of 10 µL were used for this experiment. The mobile phase was a gradient elution of 2% acetic acid in ultrapure water (A) and methanol (B) of 60 to 50% of A, 50 to 30% of A, 30 to 20% of A, 20 to 50% of A, 50 to 60% of A, and 60% of A for 0 – 5 min, 5 – 15 min, 15 – 25 min, 25 – 30 min, 30 – 32 min, and 32 – 40 min, respectively.

HPLC validation method

The HPLC method in term of limit of detection, limit of quantitation, linearity, accuracy, and precision was validated in ranges of 2 – 40 µg/mL following by the practice of ICH Guidance [11].

RESULTS AND DISCUSSION

Compound D isolated as a pale yellow oil, IR ν_{max}: 3478-3417 (broad, OH), 1514 (aromatic), 2931, 2361 cm^{-1} (weak, short chain aliphatic) (Fig. 2). ^{1}H-NMR; δ 2.45 (*q*, 2H), 3.73 (*t*, 2H), 3.86 (*s*, 3H), 3.88 (*s*, 3H), 6.05 (*m*, 1H), 6.42 (*d*, 1H), 6.79 (*d*, 1H), 6.87 (*dd*, 1H), and 6.91 (*d*, 1H) (Fig. 3). ^{13}C-NMR; δ 36.48 (C-2), 55.79 (OMe), 55.90 (OMe), 62.07(C-1), 108.44 (C-2′), 111.04 (C-5′), 119.12 (C-6′), 124.27 (C-3), 130.32 (C-1′), 132.53 (C-4), 148.53 (C-4′) and 148.96 (C-3′) (Fig. 4). MS showed the MS m/z 208 $[M]^+$. These results were related to previous report [12].

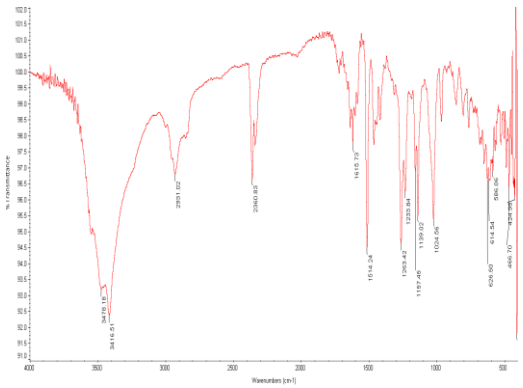

Fig. 2: FTIR spectra of (*E*)-4-(3', 4'-dimethoxyphenyl)-but-3-en-1-ol (compound D)

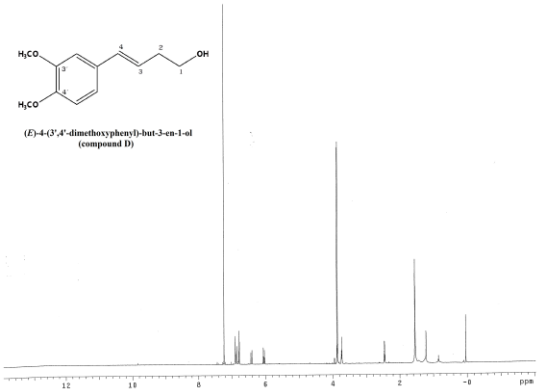

Fig. 3: ^{1}H NMR spectra of (*E*)-4-(3', 4'-dimethoxyphenyl)-but-3-en-1-ol (compound D)

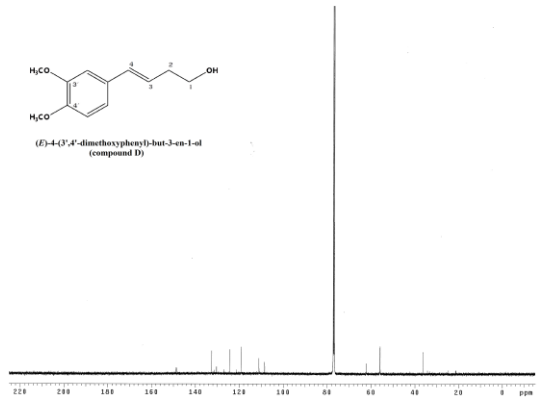

Fig. 4: ^{13}C NMR spectra of (*E*)-4-(3', 4'-dimethoxyphenyl)-but-3-en-1-ol (compound D)

For both HPLC method I and II, a limit of detection was 0.2 µg/mL, limit of quantitation was and 0.8 µg/mL, the intraday and interday precision of injection results demonstrated relative standard deviation of less than 2% and 5%, respectively, the mean recovery values were 95.38-104.76% and 88.94-102.43% for intraday and interday accuracy, respectively.

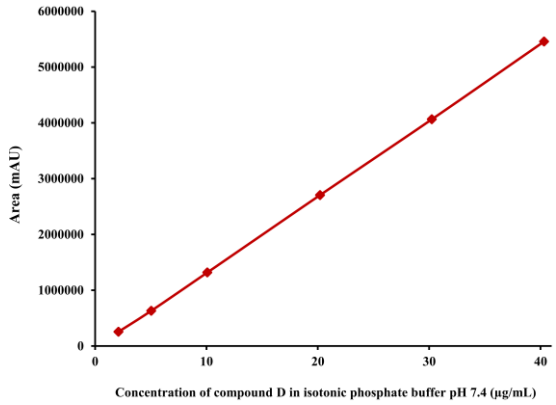

Fig. 5: The linearity curve of (*E*)-4-(3', 4'-dimethoxyphenyl)-but-3-en-1-ol (compound D) in isotonic phosphate buffer pH 7.4 from HPLC method II

The linearity of compound D in isotonic phosphate buffer pH 7.4 was analyzed by HPLC method I was y = 1722000.15x - 549390.70. Linear regression equations had a correlation coefficient (r^2) > 0.9992 (the data showed in our previous publication [9]). The linearity of compound D in isotonic phosphate buffer pH 7.4 was analyzed by HPLC method II was y = 136283.38x - 47907.47 which had good correlation coefficient (r^2) > 0.9999 (Fig. 5). In addition, the linearity of compound D in isotonic phosphate buffer pH 7.4 and new born pig skin was analyzed by HPLC method II was y = 128765.18x + 53635.01 which had good correlation coefficient (r^2) > 0.9997 (Fig. 6).

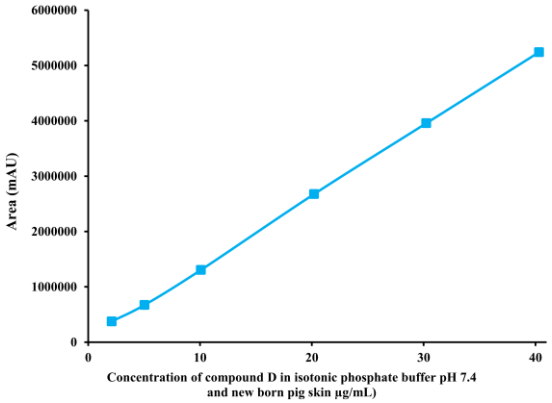

Fig. 6: The linearity curve of (*E*)-4-(3', 4'-dimethoxyphenyl)-but-3-en-1-ol (compound D) in isotonic phosphate buffer pH 7.4 and new born pig skin from HPLC method II

CONCLUSION

This research successfully compared the RP-HPLC validation method using a gradient elution of 2% acetic acid in ultrapure water and methanol. The separation of compound D was successfully purified from *Z. cassumunar* (Plai) powder by HPLC instrument and separation with column chromatography on silica gel techniques. In the further, these methods will be used for analyze the compound D from *Z. cassumunar* (Plai) formulations for medical and pharmaceutical applications.

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