The Thai Journal of Pharmaceutical Sciences

Volume 41
Issue 3 *2017*

Article 6

1-1-2017

High-performance thin-layer chromatography analysis of (E)-4-(3?,4?-dimethoxyphenyl)but-3-en-1-ol (compound D) in Zingiber cassumunar Roxb rhizome extract: An application on genetic differentiation

Prasan Tangyuenyongwatana

Piyarat Parinyapong Chareonsap

Wandee Gritsanapan

Follow this and additional works at: https://digital.car.chula.ac.th/tjps

Part of the Pharmacology Commons

Recommended Citation

Tangyuenyongwatana, Prasan; Chareonsap, Piyarat Parinyapong; and Gritsanapan, Wandee (2017) "High-performance thin-layer chromatography analysis of (E)-4-(3?,4?-dimethoxyphenyl)but-3-en-1-ol (compound D) in Zingiber cassumunar Roxb rhizome extract: An application on genetic differentiation," *The Thai Journal of Pharmaceutical Sciences*: Vol. 41: Iss. 3, Article 6.
Available at: https://digital.car.chula.ac.th/tjps/vol41/iss3/6

This Article is brought to you for free and open access by the Chulalongkorn Journal Online (CUJO) at Chula Digital Collections. It has been accepted for inclusion in The Thai Journal of Pharmaceutical Sciences by an authorized editor of Chula Digital Collections. For more information, please contact ChulaDC@car.chula.ac.th.

High-performance thin-layer chromatography analysis of (*E*)-4-(3′,4′-dimethoxyphenyl)but-3-en-1-ol in *Zingiber cassumunar* Roxb rhizome extract: Method validation and its application on studying compound-genetic relationship

Prasan Tangyuenyongwatana[1], Piyarat Parinyapong Chareonsap[2], Wandee Gritsanapan[3]

[1]Graduate Studies Office, College of Oriental Medicine, Rangsit University, Pathumthani 12000, Thailand, [2]Plant Genetic Conservation Project under the Royal Initiative of Her Royal Highness Maha Chakri Sirindhorn (RSPG), Chitralada Villa, Dusit Palace, Bangkok 10303, Thailand, [3]Phyto Product Research, 165 Soi Suwandee 3, Bangsue, Bangkok 10800, Thailand

Corresponding Author:
Prasan Tangyuenyongwatana, College of Oriental Medicine, Rangsit University, Pathumthani 12000, Thailand. Tel.: + (5140) 66 29972200. Fax: + (5160) 66 29972200. E-mail: prtang2000@yahoo.com

Received: May 17, 2017
Accepted: Sep 3, 2017
Published: Sep 30, 2017

Keywords:
(*E*)-4-(3′,4′-dimethoxyphenyl) but-3-en-1-ol, compound-genetic relationship, high-performance thin-layer chromatography, method validation, *Zingiber cassumunar*

ABSTRACT

Objectives: This study focused on developing high-performance thin-layer chromatography (HPTLC) quantitative analysis method for (*E*)-4-(3′,4′-dimethoxyphenyl)but-3-en-1-ol (Compound D), a major anti-inflammatory compound in *Zingiber cassumunar* extract. In addition, an amplified fragment length polymorphism (AFLP) technique was applied to explore the genetic differentiation relating to the yield of Compound D. **Materials and Methods:** HPTLC method validation included linearity, precisions, accuracy, limit of detection (LOD), limit of quantitation (LOQ), and robustness was performed according to International Conference of Harmonization guideline. For genetic differentiation in *Z. cassumunar*, AFLP method was applied to this study. **Results:** Linearity of the developed method showed a regression coefficient at 0.9958 and covered concentration range of 84-420 ng/band. The precisions were <2% (relative standard deviation [RSD]). The accuracy demonstrated the average recovery value of 98.31%. The LOD and LOQ were 10 and 40 ng. The results on robustness of the method were also <2% (RSD). When applied this method to determine the amounts of Compound D in *Z. cassumunar* rhizome extracts from 17 locations in Thailand, the results indicated some correlation between amount of Compound D and major genetic differentiation in *Z. cassumunar.* **Conclusion:** The validated HPTLC method has high potential in application of quality control of Compound D in raw material of different variety of *Z. cassumunar* rhizomes, and its herbal pharmaceutical products.

INTRODUCTION

Zingiber cassumunar Roxb. (Zingiberaceae), known as "Plai" in Thai, has been widely used as traditional medicinal plant in Thailand. The rhizomes possess many compounds which showed diverse biological activities such as anti-asthmatic [1], smooth muscle relaxant [2] and anti-inflammatory activities [3,4]. Compound D (1) or (*E*)-(3′,4′-dimethoxyphenyl)but-3-en-1-ol is one essential anti-inflammatory compound (Figure 1) of *Z. cassumunar*. When assessed with various inflammatory models in comparison with indomethacin, aspirin, and prednisolone, Compound D was able to treat acute inflammation. It has also been shown to inhibit carrageenan -induced rat paw edema and exudate formation [5].

A study by Chairul and Chairul has shown that Compound D exerted significant immunostimulant activity

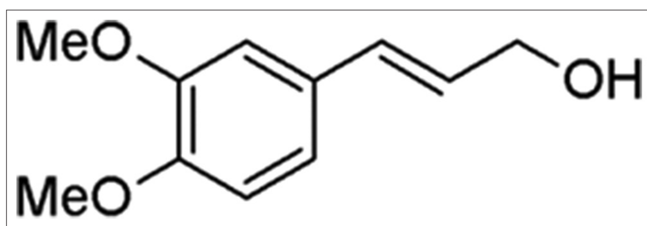

Figure 1: Chemical structure of (*E*)-(3′,4′-dimethoxyphenyl)but-3-en-1-ol or Compound D

on phagocytosis potential [6]. Although it is not an active substrate of cyclooxygenase-2 enzyme [4], Compound D has been found to act as an upstream inhibitor of the catabolic cascade in the case of chronic joint erosion [7]. Khemawoot *et al*. have recently examined the pharmacokinetic profiles of Compound D in male Wistar rats [8]. An extract of *Z. cassumunar* rhizome containing 4% (w/w) of Compound D was administered intravenously at 25 mg/kg. They found that the concentration of Compound D ranging from 10 to 100 μg/L in blood tissues, urine, and feces samples and reached to maximum level approximately 0.15 h after oral dosing [8].

High-performance thin-layer chromatography (HPTLC) is a method with an advantage of rapid determination of the amount of interesting compounds, comparing to other methods such as high-performance liquid chromatography (HPLC) or gas chromatography. For the active compounds in *Z. cassumunar* rhizome, there are few reports on the quantitative analysis of compound D using HPLC [9,10]. However, there have no reports on Compound D analysis by HPTLC method. In this study, we focused on the method validation of Compound D in *Z. cassumunar* extract with HPTLC. In addition, we analyzed the amount of Compound D in *Z. cassumunar* rhizome extracts from 17 known locations in Thailand by proposed technique. Finally, we used this method to evaluate the correlation between the amount Compound D and the genetic variation of the plants from various locations in Thailand that would be of value for primary information for future usage of the Compound D and its commercial and medical preparation.

MATERIALS AND METHODS

Preparation of Compound D

All reagents and solvents were reagent grade and used without further purification. Compound D was synthesized in four steps at our laboratory according to the previous report [11] with the purity more than 95%. Briefly, 3,4-dimethoxybenzaldehyde (2) was mixed with acetone and the sodium hydroxide was added to give (*E*)-4-(3´,4´-dimethoxyphenyl)butane-3-one (3). Next, ketone functional group of compound 3 was converted to secondary alcohol using sodium borohydride to obtain (*E*)-4-(3´,4´-dimethoxyphenyl)but-3-en-2-ol (4). Then, the alcohol was eliminated using catalytic amount of *p*-toluenesulfonic acid in benzene to give (*E*)-4-(3´,4´-dimethoxyphenyl) butadiene (5). The last step was the terminal hydroxylation of the compound (5) with 9-BBN (9-borabicyclo[3.3.1] nonane) and hydrogen peroxide (H_2O_2) to give (*E*)-4-(3´,4´-dimethoxyphenyl)but-3-en-1-ol (1, Compound D) as colorless oil; EI-MS: m/z = 208 [M^+]; ^{1}H-nuclear magnetic resonance (NMR) ($CDCl_3$): δ = 2.48 (2H, td, *J* = 13.4, 6.2 Hz, H-2), 3.76 (2H, t, *J* = 6.3 Hz, H-1), 3.88 (3H, s, 3-OMe), 3.90 (3H, s, 4-OMe), 6.08 (1H, dt, *J* = 15.8, 7.2 Hz, H-3), 6.44 (1H, d, *J* = 15.8, Hz, H-4), 6.81 (1H, d, *J* = 8.2 Hz, H-6´), 6.88-6.93 (1H, m, H-5´), 6.92 (1H, s, H-2´): ^{13}C-NMR ($CDCl_3$): δ = 36.3 (C-2), 55.7 (OMe), 55.8 (OMe), 62.0 (C-1), 108.4 (C-2´), 111.0 (C-5´), 119.1 (C-6´), 124.2 (C-3), 130.3 (C-1´), 132.4 (C-4), 148.5 (C-4´), 148.9 (C-3´).

Plant Materials

The rhizomes of *Z. cassumunar* were collected from 17 locations in four different parts (North, Central, North-East, and South) of Thailand. They were identified by comparing with specimens at the Forest Herbarium, Department of National Park, Wildlife and Plant Conservation, Ministry of Natural Resources and Environments, Bangkok. The voucher specimens (*Z. cassumunar*: OMD101-117) were deposited at the College of Oriental Medicine, Rangsit University, Pathum Thani, Thailand.

HPTLC and Amplified Fragment Length Polymorphism (AFLP) Instruments

HPTLC was performed on CAMAG (Muttenz, Switzerland) equipped with a spotting device Linomat 5 automatic sample spotter, syringe 100 μL (Hamilton, Bonaduz, Switzerland), TLC chamber glass twin-trough chamber (20 cm × 10 cm), densitometer-TLC scanner 3 with winCATS software. The HPTLC plate was obtained from Merck (Damstadt, Germany); size 20 cm × 10 cm, 0.2 mm. layer thickness precoated with silica gel 60 F_{254}, Cat. No. 1.05548.0001. DNA extraction and AFLP procedure were performed on Hoefer DQ 200 fluorometer (Hoefer, USA), Px2 Thermal Cycler (Thermo Electron Corporation, USA) and Sequi-Gen GT Sequencing Cell (Bio-Rad, USA).

Preparation of Standard Solution of Compound D

Stock standard solution was prepared by dissolving Compound D (21.0 mg with more than 95% purity) with methanol (15 mL) in a 25 mL volumetric flask. The stock standard solution (1.0 mL) was pipetted into a 10 mL volumetric flask and adjusted to the volume with methanol. Various amounts of the standard solution were spotted on the TLC plate to obtain final concentrations at 84, 168, 252, 336, and 420 ng/spot.

Sample Preparation

Dried powder of rhizomes of *Z. cassumunar* (1.50 g) was accurately weighed and transferred to a 100 mL volumetric flask. Methanol (50 mL) was added and the flask was placed onto ultrasonic bath and sonicated for 15 min. The methanol extract was collected and another portion of methanol (50 mL) was added and sonicated as previously mention. The extraction was exhaustively done and monitored by TLC. All methanol extracts were combined and concentrated using a rotary evaporator. The dried extract was dissolved again with methanol, transferred to a 25 mL volumetric flask and then adjusted to volume with methanol. This sample was ready for analysis with HPTLC.

HPTLC Condition for Quantitative Analysis of Compound D in *Z. cassumunar* Extracts

Samples were applied by Linomat 5 to the plate 15 mm from the side edges and 10 mm from the bottom. Sample solution (2 μL) was applied for each spot in triplicate as thin band of 6 mm length. The rate of application was 150 nL/s. The mobile phase consisted of dichloromethane:methanol (98:2, v/v) and each TLC plate was developed using 50 mL of the mobile phase. The chamber was saturated with the mobile phase for 25 min at room temperature (25 ± 2°C). The distance for each development was 8 cm and took about 10 min for each run. Next, the spots were scanned using the TLC scanner 3 in the reflectance-absorbance mode at 254 nm which operated by winCATS software. The amount of compound D in *Z. cassumunar* extract was calculated using calibration graph.

Validation of HPTLC Method

The method was validated for linearity, precision, accuracy, limit of detection (LOD), and limit of quantitation (LOQ) according to the International Conference of Harmonization guidelines. First, linearity relationship between peak area and concentration of the standard was evaluated over five concentration levels in the range of 84-420 ng/spot by making triplicate for each concentration. Next, the precision which included intra-day and inter-day precisions of the developed method were conducted. They were expressed in term of relative standard deviation (RSD) of peak area. Intra-day precision was performed by analyzing the standard in triplicate within the same day at three concentrations (168, 252, and 336 ng/spot) of Compound D. Inter-day precision was done by analyzing the same three concentrations on three different days. The accuracy of the method was assessed by carrying out recovery studies by adding known amount of the reference compound at three levels (84, 168, and 210 ng/spot) to the methanol extract sample. Then, the solutions were performed on HPTLC and analyzed amount for Compound D and the recoveries were calculated. The LOD and LOQ were determined by firstly scanning the blank (methanol) spot and detected the noise. Then, a series of concentration of Compound D standard solution (10-100 ng/spot) were spotted on the TLC plate. Signal-to-noise ratios of 3:1 and 10:1 were considered as LOD and LOQ, respectively. Robustness was determined by introducing small changes in the chromatographic condition of analysis. The changes included ±5% changes in temperature, amount of mobile phase, mobile phase composition, and chamber saturation time. The results were calculated as %RSD variation.

Genomic DNA Extraction and AFLP Procedure

Z. cassumunar fresh leaf (5.0 g) was ground in liquid nitrogen with mortar and pestle. Total DNA was isolated according to the procedure described by Doyle and Doyle [12], which is a CTAB-based extraction. DNA concentration was evaluated using spectrofluorometric measurement with H 33258 fluorescent dye. The AFLP procedure was performed according to Vos *et al*. [13]. Briefly, DNA (100 ng/μL) was digested by restriction enzymes, *Eco*RI and *Mse*I and incubated for 2 h at 37°C for *Eco*RI and *Mse*I followed by 1 h at 65°C for restriction enzyme deactivation. The restricted DNA fragment was ligated to *Eco*RI-adapter and *Mse*I-adapter overnight at 37°C to generate template DNA for amplification. The 1:10 diluted DNA template (5 μL) was first preamplified in Px2 Thermal Cycler using *Eco*RI + A and *Mse*I + C primers [14]. Then, the pre-amplified DNA was diluted to 1:9 with sdH_2O and the product (3 μL) was used for selective amplification in a reaction tube containing selective amplification mixtures (20 μL). AFLP adapters and nine primer pairs (ER-AGC/MS-CTT, ER-AGG/MS-CTA, ER-AGG/MS-CTC, ER-AGG/MS-CGA, ER-AGG/MS-CGC, ER-AGG/MS-CTT, ER-AGC/MS-CGG, ER-AGG/MS-CGT and ER-AGG/MS-CCA) were used for the selective amplification as described in Vos *et al*. The final polymerase chain reaction products were separated on a 4.5% denaturing polyacrylamide gel electrophoresis in ×1 tris/borate/EDTA buffer in a Sequi-Gen GT sequencing cell. DNA fragments on gels were visualized using silver nitrate staining. The gel was washed with distilled water and air-dried on mirror plates.

Data Analysis

For genetic diversity analysis, the distinctive bands from each sample were documented manually. A band was considered polymorphic if it was present in at least one genotype and absent in others. Each present band was scored "1" and as "0" if absent. A genetic similarity matrix was evaluated according to Nei and Li similarity index. Genetic diversity estimates were considered as 1 minus Nei and Li Similarity Coefficient and multiplying the result by 100 $[(1-S_{ij}) \times 100]$. A dendrogram was assembled from the matrix of similarity coefficients using the unweighted pair-group method of the arithmetic average (UPGMA) technique by the NTSYS-pc software package version 2.11T [15].

RESULTS AND DISCUSSION

Validation of HPTLC Method

The rhizome extract of *Z. cassumunar* possesses many phenylpropanoids which include Compound D. We developed a HPTLC method for evaluating the amount of Compound D. The TLC solvent system that efficiently separated Compound D from other compounds in the *Z. cassumunar* extract appeared to be a mixture of dichloromethane:methanol (98:2, v/v). The chromatograms of HPTLC of 17 samples were not much different from each other. There is one possible reason why chromatogram of the extract in Figure 2 looks so simple. The reason is the HPTLC solvent system which is an isocratic system. This solvent system can separate Compound D from other compounds but the resolving power of the solvent system cannot separate other compounds in the extract. The mobile phase moves all other compounds combining to the peaks at higher R_f values.

The R_f value of compound D was 0.38 ± 0.02 (Figure 3). The specificity of Compound D in standard and *Z. cassumunar* extract were evaluated by comparing ultraviolet (UV) absorption spectra range from 200 to 500 nm (Figure 4). The UV absorption of Compound D in standard and sample were

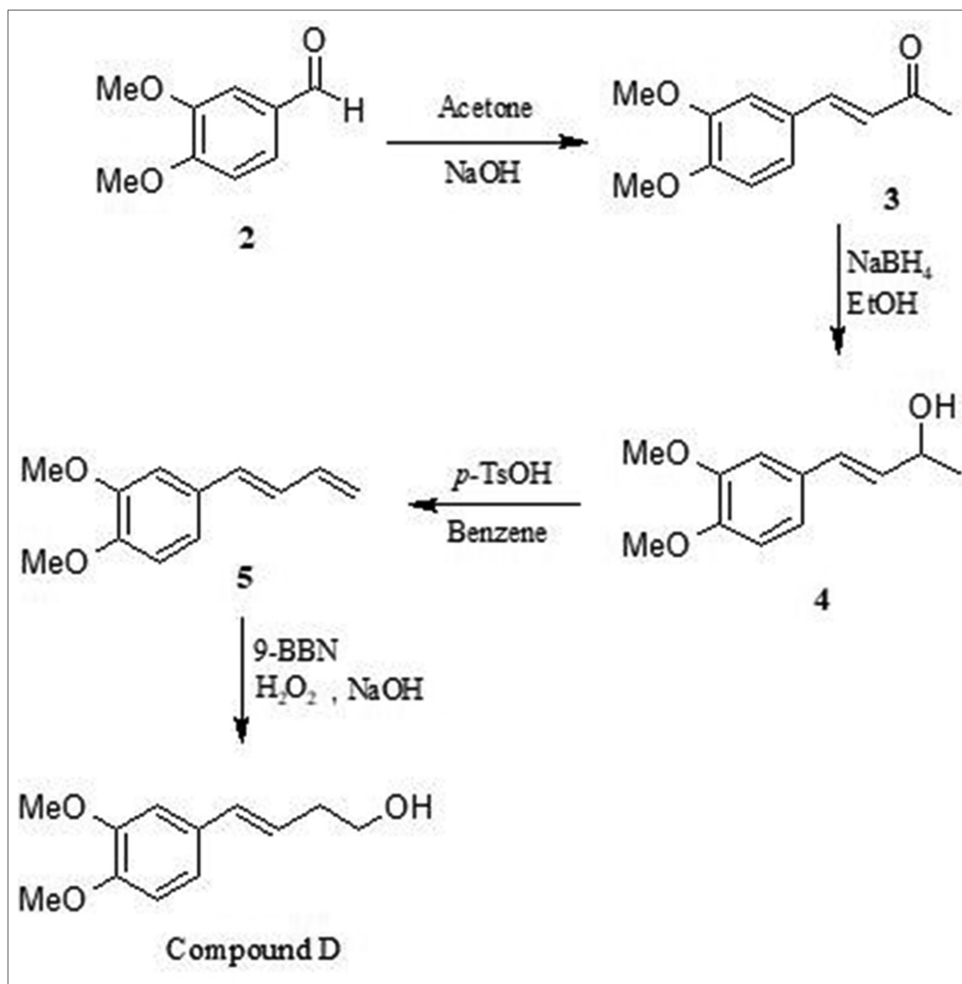

Figure 2: Synthetic pathway of Compound D

overlaid to compare the specificity or purity of compound which obtained high correlation coefficients which were 0.9998 $r(s, m)$ and 0.9997 $r(m, e)$ where s, m and e stand for start, apex and end, respectively.

The parameters of method validation included linearity, accuracy, precision, LOD, and LOQ. The method yielded an acceptable linear relationship between peak area and compound D concentrations with the correlation coefficient (r^2) of 0.9958 and within the concentration range of 84-420 ng/spot (Table 1). The inter-day and intra-day precisions of Compound D demonstrated the satisfactory precision at three different concentration levels of 168-336 ng/spot with RSD <2% (Table 2). The accuracy of the method was focused on the percent recovery at three levels of compound D which was 98.85%, 97.89%, and 98.19% with an average value of 98.31% (Table 3). The robustness test was also evaluated and deviation of peak area of Compound D was <2% (RSD) under all parameters (Table 4). Variation the amount of mobile phase caused the main deviation of the result than other parameters. The contents of Compound D in 17 varieties of *Z. cassumunar* from different parts of Thailand were determined by the proposed HPTLC method as shown in Figure 5.

Table 1: Summary of parameters involved in the validation method of the HPTLC used for determination of Compound D

Parameter	Results
Linear range	84-420 ng/spot
Linear regression equation	$y=7.798\mathbf{x}+2530.968$[a]
Correlation coefficient (r^2)	0.9958
LOQ, ng	40
LOD, ng	10

[a]**x** is the amount of Compound D in ng, y is the peak area at 254 nm. LOD: Limit of detection, LOQ: Limit of quantitation, HPTLC: High-performance thin-layer chromatography

Table 2: Intra-day and inter-day precision for Compound D[a]

Concentration ng/spot	Intra-day precision, %	Inter-day precision, %
168	0.44	1.45
252	0.21	2.07
366	0.65	0.79

[a]RSD, % (n=3). RSD: Relative standard deviation

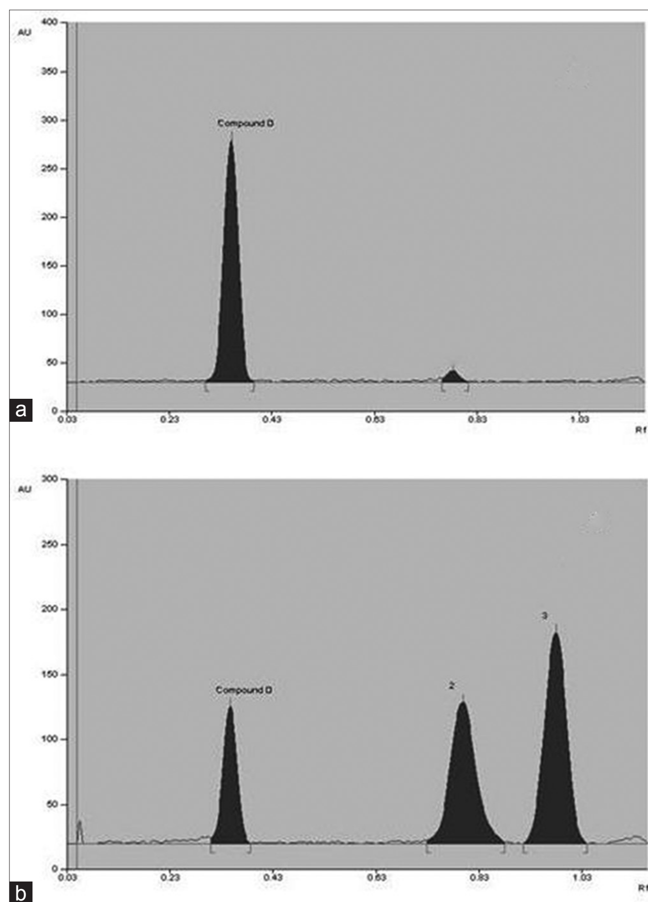

Figure 3: High-performance thin-layer chromatography chromatogram of (a) standard Compound D, (b) *Zingiber cassumunar* extract

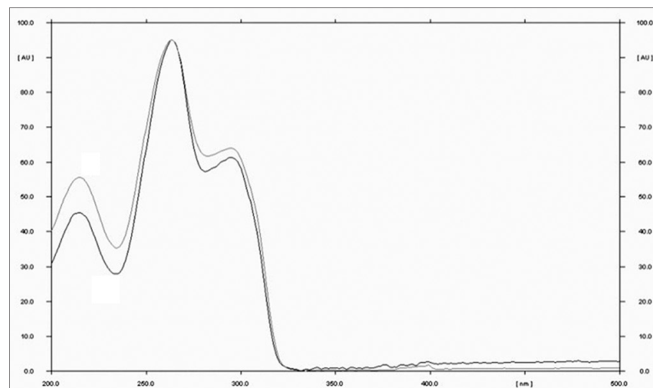

Figure 4: Peak purity of (a) standard compound D, (b) Compound D in *Zingiber cassumunar* extract

Genetic Differentiation Using AFLP Method

For AFLP method, Kladmook *et al*. [16] assessed the genetic relationship among 132 samples of *Z. cassumunar* in Thailand using AFLP method. However, they are only giving the information on the genetic differentiation in *Z. cassumunar* in Thailand, without doing the correlation with the amounts of major metabolites. We did this using nine primers to generate 245 fragments and 136 bands were polymorphic. A dendrogram

Table 3: Recovery study of Compound D

No.	Amount present in the extract, ng	Amount added, ng	Amount found[a], ng	Recovery[a,b], %
1	142.3	84.0	83.05±0.86	98.85±1.04
2	142.3	168.0	164.31±0.96	97.89±1.64
3	137.0	210.0	206.22±0.41	98.19±0.20

[a]Expressed as mean standard deviation (n=3). [b]Average recovery=98.31%

Table 4: Results from robustness studied

Parameter	RSD % in peak area Compound D
Mobile phase composition	1.46
Amount of mobile phase	1.69
Temperature	1.34
Chamber saturation time	0.56

RSD: Relative standard deviation

of 17 varieties of *Z. cassumunar* by AFLP technique was constructed from the matrix of similarity coefficients using the UPGMA technique (Figure 5). The results showed that the similarity coefficient index (Jaccard's coefficient) was in the range of 0.10-0.78, which could separate *Z. cassumunar* into three groups at 0.15 level of similarity.

For genetic evaluation, the relationship between genetic differentiation UPGMA tree and the amount of Compound D revealed obvious correlation. Group 2 had no active Compound D in the extracts while Groups 1 and 3 contained Compound D in the range of 0.152-0.913% (w/w) of dried powder. Furthermore, the samples containing similar amount of Compound D were in the same cluster and grouped close to each other.

For example, *Z. cassumunar* from BURR and UBON were grouped at similarity level of 0.71, having Compound D at 0.845 and 0.913% (w/w), respectively while *Z. cassumunar* from RACH and SMPA were grouped at similarity level of 0.76 having Compound D at 0.680 and 0.584% (w/w), respectively. These two groups were combined into a group at similarity level of 0.625. In conclusion, these four varieties of *Zingiber cassumunar* have higher amount of Compound D than other *Z. cassumunar*.

CONCLUSIONS

This HPTLC method was effectiveness, precise and sensitive for the analysis of Compound D in *Z. cassumunar* rhizome extract. When combined with AFLP technique, the result of the relationship between genetic differentiation UPGMA tree and the amount of Compound D revealed some corresponding correlation. This information suggests a potential application for standardization of Compound D in raw material of *Z. cassumunar* rhizomes, and its herbal pharmaceutical products.

ACKNOWLEDGMENTS

The authors would like to thank College of Oriental Medicine, Rangsit University and Plant Genetic Conservation Project

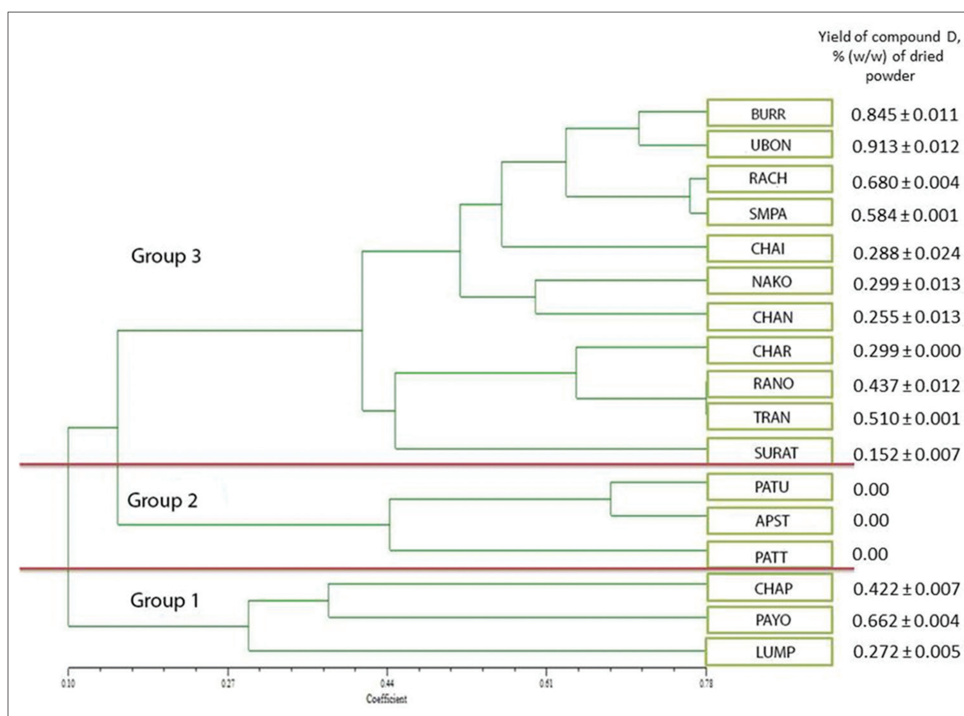

Figure 5: Dendogram of 17 varieties of *Zingiber cassumunar* in four parts of Thailand and the yields of Compound D. BURR (Buriram), UBON (Ubon ratchatani), RACH (Ratchaburi), SMPA (Samut prakan), CHAI (Chaing mai), NAKO (Nakon Ratchasima), CHAN (Chainat), CHAR (Chaing rai), RANO (Ranong), TRAN (Trang), SURA (Surat thani), PATU (Pathum thani), APST (Nakon Ratchasima), PATT (Pattani), CHAP (Chaiyaphum)

under the Royal Initiative of Her Royal Highness Maha Chakri Sirindhorn (RSPG) for supporting in this research.

REFERENCES

1. Kiatyingungsulee N, Wangmud M, Swasdimongkol S, Mokkhasmit M. Some pharmacological studies of active constituents in Plai (*Zingiber cassumunar* Roxb.). Bull Dept Med Sci 1979;21:13-24.
2. Anantasan V. A pharmacological study of Plai (*Zingiber cassumunar* Roxb.) water extract on smooth muscle (Section II). J Natl Res Counc Thai 1982;14:1-24.
3. Jeenapongsa R, Yoovathaworn K, Sriwatanakul KM, Pongprayoon U, Sriwatanakul K. Anti-inflammatory activity of (E)-1-(3,4-dimethoxyphenyl) butadiene from *Zingiber cassumunar* Roxb. J Ethnopharmacol 2003;87:143-8.
4. Han AR, Kim MS, Jeong YH, Lee SK, Seo EK. Cyclooxygenase-2 inhibitory phenylbutanoids from the rhizomes of *Zingiber cassumunar*. Chem Pharm Bull 2005;53:1466-8.
5. Panthong A, Kanjanapothi D, Niwatananun V, Tuntiwachwuttikul P, Reutrakul V. Anti-inflammatory activity of compounds isolated from *Zingiber cassumunar*. Planta Med 1990;56:655.
6. Chairul P, Chairul SM. Phagocytosis effectivity test of phenylbutanoid compounds isolated from Bangle (*Zingiber cassumunar* Roxb.) rhizome. Biodiversitas 2009;10:40-3.
7. Chaiwongsa R, Ongchai S, Boonsing P, Kongtawelert P, Panthong A, Reutrakul V. Active compound of *Zingiber cassumunar* Roxb. Down-regulates the expression of genes involved in joint erosion in a human synovial fibroblast cell line. Afr J Tradit Complement Altern Med 2013;10:40-8.
8. Khemawoot P, Hunsakunachai N, Anukunwithaya T, Bangphumi K, Ongpipattanakul B, Jiratchariyakul W, *et al.* Pharmacokinetics of compound D, the major bioactive component of *Zingiber cassumunar*, in Rats. Planta Med 2016;82:1186-91.
9. Tangyuenyongwatana P, Gritsanapan W. A study on artifacts formation in the Thai traditional medicine Prasaplai. Planta Med 2008;74:1403-5.
10. Suksaeree J, Madaka F, Monton C, Sakunpak A, Chusut T, Charoonratana T. Method validation of (*E*)-4-(3´,4´-dimethoxyphenyl)but-3-en-1-ol in *Zingiber cassumunar* Roxb. With different extraction techniques. Int J Pharm Pharm Sci 2014;6:295-8.
11. Tangyuenyongwatana P, Gritsanapan W. Biological evaluations of fatty acid esters originated during storage of Prasaplai, a Thai traditional medicine. Nat Prod Res 2007;21:990-7.
12. Doyle JJ, Doyle JL. Isolation of plant DNA from fresh tissue. Focus 1990;12:13-5.
13. Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, *et al.* AFLP: A new technique for DNA fingerprinting. Nucleic Acids Res 1995;23:4407-14.
14. Loh JP, Kiew R, Set O, Gan LH, Gan YY. Amplified fragment length polymorphism fingerprinting of 16 banana cultivars (Musa cvs.). Mol Phylogenet Evol 2000;17:360-6.
15. Rohlf FJ. NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System Version 2.11T, User Guide, Exeter Software. Stony Brook, N.Y.: State University of New York; 2000.
16. Kladmook M, Chidchenchey S, Keeratinijakal V. Assessment of genetic diversity in cassumunar ginger (*Zingiber cassumunar* Roxb.) in Thailand using AFLP markers. Breeding Sci 2010;60:412-8.