International Journal of Applied Pharmaceutics

ISSN- 0975-7058 Vol 11, Issue 4, 2019

Original Article

APPLICATION OF ^{1}H-NMR SPECTA AND MULTIVARIATE ANALYSIS FOR THE AUTHENTICATION OF *CURCUMA XANTHORRHIZA* FROM *ZINGIBER CASSUMUNAR*

THERESIA WIJAYANTI[1], SUGENG RIYANTO[1], ENDANG LUKITANINGSIH[1], ABDUL ROHMAN[1,2*]

[1]Departement of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, 55281, Indonesia, [2]Institute of Halal Industry and Systems, Universitas Gadjah Mada, Yogyakarta, 55281, Indonesia
Email: abdulkimfar@gmail.com

Received: 05 Feb 2019, Revised and Accepted: 10 May 2019

ABSTRACT

Objective: This study was aimed to apply metabolite fingerprinting for the authentication of *Curcuma xanthorrhiza* adulterated with *Zingiber cassumunar* using ^{1}H-NMR spectroscopy and multivariate analysis (chemometrics) methods, namely principal component analysis (PCA) and partial least square–discriminant analysis (PLS-DA).

Methods: The pure dried powder samples of *C. xanthorrhiza* from different regions, *Z. cassumunar*, and its binary mixtures of *C. xanthorrhiza* with various concentrations of *Z. cassumunar* as adulterants were prepared for ^{1}H-NMR measurements. The binary mixtures were prepared by mixing *C. xanthorrhiza* with various concentrations (10%, 25%, 40%, 50%, and 75%) of *Z. cassumunar*. ^{1}H-NMR spectra were subjected to multivariate analysis for classification using PCA and PLS-DA.

Results: A diverse group of metabolites could be detected by ^{1}H-NMR spectroscopy. PCA using the chemical shift in ^{1}H-NMR spectra of the plant extracts as variables clearly discriminated pure *C. xanthorrhiza* extracts from different origins and *C. xanthorrhiza* extract adulterated with *Z. cassumunar*. PLS-DA employed to enhance the separation obtained from the PCA model resulted in well separation and good classification of pure *C. xanthorrhiza* from the adulterated ones.

Conclusion: The developed method could be a useful and powerfull tools to assess adulteration practice and to evaluate the authentication of *C. xanthorrhiza* extracts.

Keywords: Metabolite fingerprinting, ^{1}H-NMR spectroscopy, *C. xanthorrhiza*, Authentication, Principal component analysis, Partial least square-discriminant analysis

© 2019 The Authors. Published by Innovare Academic Sciences Pvt Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/)
DOI: http://dx.doi.org/10.22159/ijap.2019v11i4.32417

INTRODUCTION

In the last decade, the metabolomic approach has been established as a well-known technique in the field of plant science and natural product chemistry with the main objective to identify and quantify all the metabolites contained in an organism under certain condition [1, 2]. One of the metabolomic approaches is metabolite fingerprinting, a method to classify and discriminate samples based on its metabolite pattern or 'fingerprint'. These metabolite patterns can be provided by various high-throughput analytical methods such as mass spectrometry (MS), nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS), or liquid chromatography-nuclear magnetic resonance (LC-NMR) [2, 3]. One of the most popular analytical methods used in metabolite fingerprinting in the last recent years is nuclear magnetic resonance (NMR) spectroscopy. Proton (1H)-NMR spectroscopy gained its popularity because of its capability for simultaneous detection of diverse groups of secondary metabolites, fast, convenient, and effective for discriminating between groups of samples with relatively simple sample preparation [1, 2].

NMR-based metabolite fingerprinting has been applied in many different fields. Some of them were discrimination of wild or transgenic plants and also monitoring the response of plants to stress or wounds. Another important application of NMR-based metabolite fingerprinting is for classification and characterization of different species of medicinal plants, such as *Curcuma xanthorrhiza*. This approach can be a very powerful tool for quality control and authentication of *C. xanthorrhiza* used as a raw material in herbal medicine industries [1].

C. xanthorrhiza, also known as *temulawak* belongs to family Zingiberaceae and is widely cultivated around the world, especially in Southeast Asia due to its variety of pharmacological activities such as anticancer, antimicrobial, anti-inflammatory, antioxidant, anticandidal, antihyperglycemic, and antihypertensive effects [4-6]. This herbal plant was commonly used as food supplements, herbal drink, syrup, and has recently gained popularity as raw material in herbal industries [7, 8]. Most of the potential pharmacological activities of *C. xanthorrhiza* are believed to be related to various bioactive and phytochemical compounds, which include xanthorrhizol (1.48-1.63%), curcuminoids such as curcumin and demethoxycurcumin (1-2%), phelandren, camphor, tumerol, sineol, borneol, flavonoids, and sesquiterpenes [9].

Because the adulteration practice of herbal plant used as raw material has became a serious problem, the authentication of *C. xanthorrhiza* needs to be performed in order to ensure its quality, safety, and efficacy [10, 11]. The adulteration practice, either intentionally or unintentionally, usually includes partial or full substitution of original crude drugs with other substances which is cheaper, either free from or inferior in therapeutic properties. The most common rhizome used as an adulterant in *C. xanthorrhiza* is *Zingiber cassumunar* which also belongs to family Zingiberaceae, due to the similar color and similar metabolites content of both rhizomes [12]. Proton NMR-based metabolite fingerprinting combined with chemometric methods of principal component analysis (PCA) and partial least square–discriminant analysis (PLS-DA), could be an appropriate method to assess the authentication of *C. xanthorrhiza* extract.

Proton (1H)-NMR metabolite fingerprinting and chemometrics has been used for the authentication of *Curcuma longa* adulterated with *Curcuma manga* [13]. In our best knowledge, there is no publication regarding authentication of *C. xanthorrhiza* extract which is adulterated with *Z. cassumunar*. This study proposed the application of metabolite fingerprinting for the authentication of *C. xanthorrhiza* adulterated with *Z. cassumunar* using ^{1}H-NMR spectroscopy and chemometric methods of PCA and PLS-DA. With PCA, separation of samples based on the pattern of the signals in the spectra could be accomplished. Meanwhile, by conducting PLS-DA, classification of

pure and adulterated samples could be developed. Therefore, this study was aimed to develop sufficient and suitable techniques (combination of 1H-NMR spectra and chemometrics) as very powerful tools for the authentication of *C. xanthorrhiza* extracts.

MATERIALS AND METHODS

Plant materials

Curcuma xanthorrhiza rhizomes were collected from Sleman (Yogyakarta), Karanganyar, Pati, Wonogiri, and Sukolilo (Central Java), meanwhile, *Z. cassumunar* rhizomes were obtained from Yogyakarta. The identification of rhizomes used in this study was performed in the Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia. These rhizomes were washed and then chopped into small and thin pieces, followed by air drying. The dried thin pieces of rhizomes were then ground into fine powder form and were used as samples for ^{1}H-NMR measurements.

Sample preparation for ^{1}H-NMR measurements

Pure dried powder samples of *C. xanthorrhiza* from each region, *Z. cassumunar*, and binary mixtures of *C. xanthorrhiza* with various concentrations of *Z. cassumunar* as adulterants were prepared for ^{1}H-NMR measurements. The binary mixtures were prepared by mixing *C. xanthorrhiza* with various concentrations (10%, 25%, 40%, 50%, and 75%) of *Z. cassumunar* as adulterants in a total weight of 5 g.

^{1}H-NMR spectra measurements

Twenty-five mg of each dried powder samples (pure and binary mixtures) were weighed and transferred in 2 ml micro-tubes and then added with 500 µl of CD_3OD and 500 µl of KH_2PO_4 buffer (pH 6.0) in D_2O containing trimethylsilyl propionic acid (TSP) 0.01%. The mixture was vortexed (Vortex Mixer Maxi Mix™ II, Thermo Fisher Scientific, Waltham, MA USA) for 1 min followed by ultrasonication for 20 min and centrifugation (Centrifuge MPW-260, MPW Med. Instruments, Warsaw, Poland) at 13000 rpm for 10 min to obtain a clear supernatant. Approximately of 600 µl of the supernatant was transferred to an NMR tube and was immediately subjected to ^{1}H-NMR measurements using a preset setting for all the samples. The ^{1}H-NMR measurements were implemented using a 500 MHz NMR Spectrometer (JEOL ECZR, JEOL Ltd., Tokyo, Japan).

Bucketing of ^{1}H-NMR spectra and principal component analysis

The ^{1}H-NMR spectra were automatically reduced to ASCII files using MestreNova software (v. 12.0, Mestrelab Research, Santiago de Compostela, Spain). Spectra intensities were reduced to buckets with the spectral width δ 0.04 forming a region of 0.08-10.04 ppm and the total variables of 250 chemical shifts bin were generated for each ^{1}H-NMR spectrum. The generated ASCII files were subjected to PCA and PLS-DA using XLSTAT add-ins (v. 2018.2.50198) integrated with Microsoft Excel.

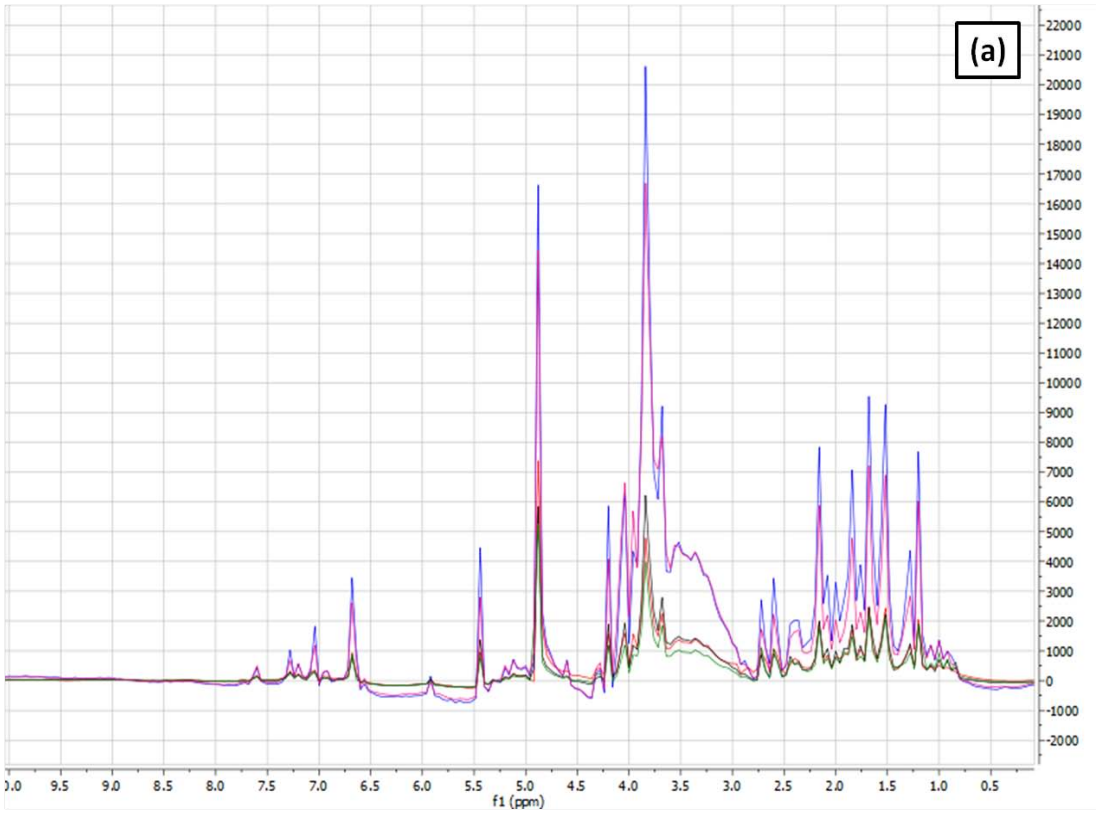
Statistical analysis

Multivariate analysis of principal component analysis (PCA) and partial least square-discriminant was carried with the software of XL-STAT and Microsoft Excel (Microsoft Inc.), respectively.

RESULTS AND DISCUSSION

^{1}H-NMR measurements and principal component analysis (PCA)

Representative one-dimensional ^{1}H-NMR spectra of *C. xanthorrhiza* extracts from different origins, *Z. cassumunar* extract, and *C. xanthorrhiza* adulterated with *Z. cassumunar* were shown in fig. 1. where a diverse range of metabolites of *C. xanthorrhiza* and *Z. cassumunar* could be observed. Proton signals in the regions of 3.90, 7.22, 7.28, and 6.80 ppm corresponds to curcumin, whereas proton signals in the regions of 3.94, 5.89, and 6.92 ppm is correlated to demethoxycurcumin. Besides of that, signals in the regions of 1.52, 1.66, 7.02, and 6.64 ppm is characterized to be the signals of xanthorrhizol. Proton signals correspond to glucose and sucrose are observed in the regions of 4.58–5.38 ppm [14].



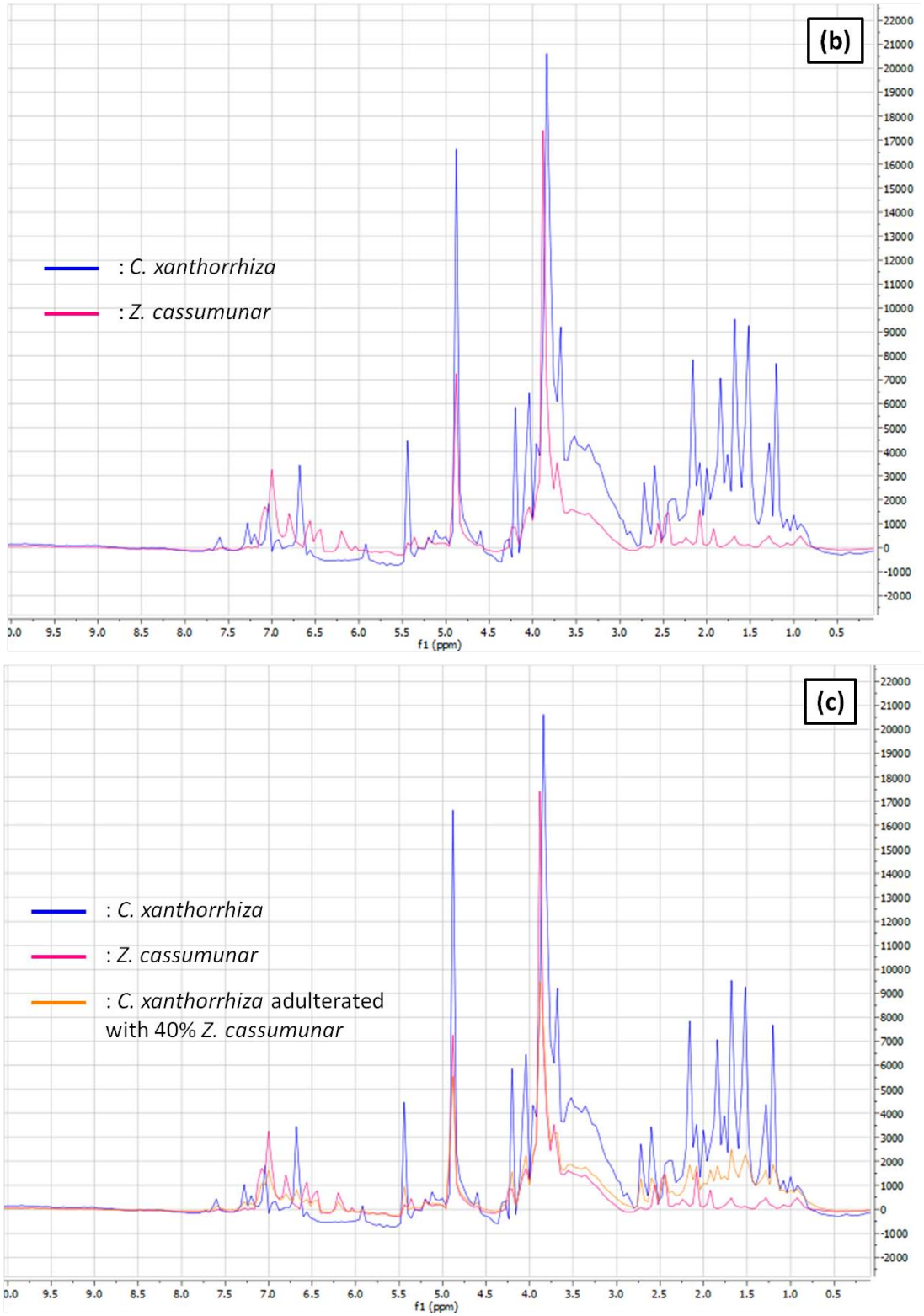


Fig. 1: ^{1}H-NMR spectra of (a): *Curcuma xanthorrhiza* from different regions; (b): *Curcuma xanthorrhiza* and *Zingiber cassumunar*; (C): *Curcuma xanthorrhiza*, *Zingiber cassumunar*, and *Curcuma xanthorrhiza* adulterated with 40% *Zingiber cassumunar*

After the ^{1}H-NMR spectra were reduced to buckets and converted to ASCII files, principal component analysis (PCA) was carried out to observe the differences between the spectra of each sample. PCA is an unsupervised pattern recognition method used to decrease the dimensionality of the multivariate data set while conserving most of the variance within it [15, 16]. Fig. 2 showed PCA score plot of the ^{1}H-NMR spectra of *C. xanthorrhiza* extract from different origins. *C. xanthorrhiza* from various regions were mapped in three well-separated clusters that took place in the first two factors (Fs) which cumulatively account for 95.43% of the variation. The first factor (F1) unfold the differences of *C. xanthorrhiza* from various regions for 68.50% variance. *C. xanthorrhiza* from Sukolilo, Sleman, and Pati were located in negative side of F1 and F2, and were clearly separated from either *C. xanthorrhiza* from Karanganyar or Wonogiri.

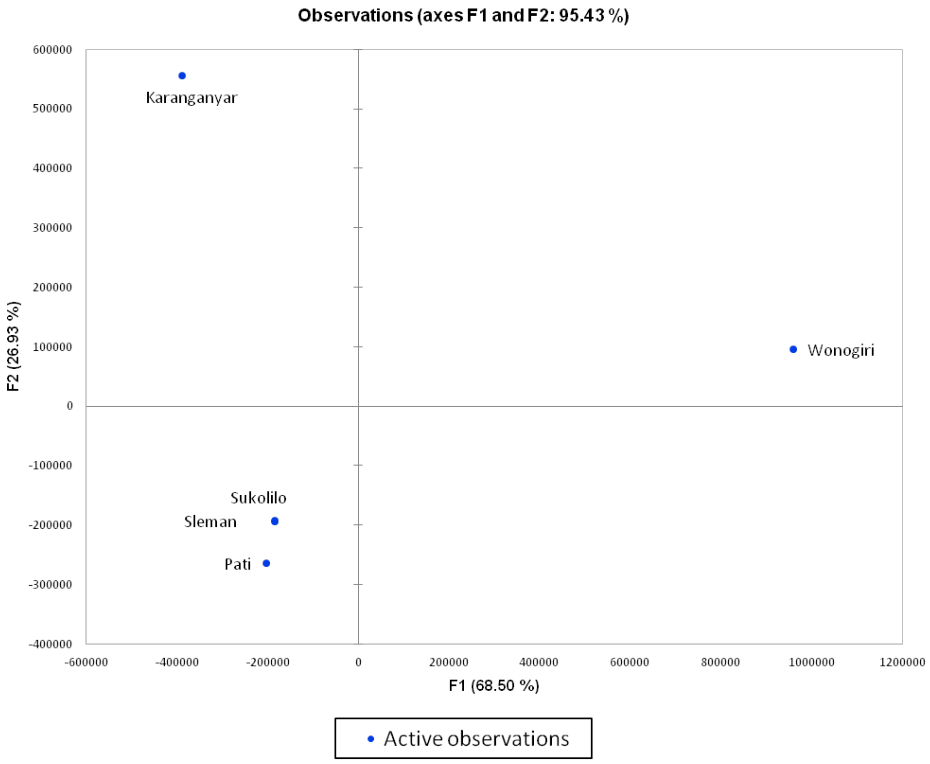


Fig. 2: Principal component analysis (PCA) score plot of *Curcuma xanthorrhiza* from different regions

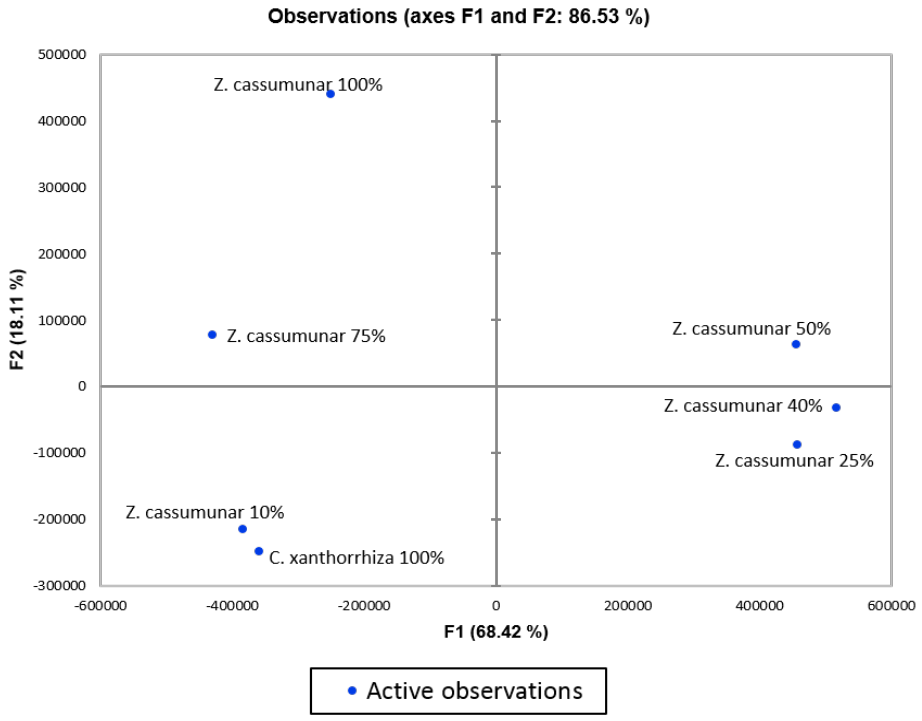


Fig. 3: PCA score plot of *Curcuma xanthorrhiza* and *Curcuma xanthorrhiza* adulterated with various concentrations of *Zingiber cassumunar*

PCA was also implemented to ^{1}H-NMR spectra obtained from *C. xanthorrhiza* and *C. xanthorrhiza* adulterated with various concentration of *Z. cassumunar* to discriminate the pure *C. xanthorrhiza* extracts from adulterated ones. Fig. 3 displayed the score plot and loadings plot of *C. xanthorrhiza* and *C. xanthorrhiza* adulterated with various concentration of adulterants. The PCA score plot exhibits three clearly separated clusters (fig. 3a.), with the most variance, was displayed by F1 at 68.2%. Pure *C. xanthorrhiza* and *C. xanthorrhiza* adulterated with 10% of *Z. cassumunar* were located in the negative side of F1 and F2, which were well separated from pure *Z. cassumunar* and *C. xanthorrhiza* adulterated with 75% of *Z. cassumunar*. Meanwhile, *C. xanthorrhiza* adulterated with 25, 40, and 50% of *Z. cassumunar* was clustered separately from other samples in the positive side of F1. The adulterated *C. xanthorrhiza*

extracts were clustered between pure *C. xanthorrhiza* and pure *Z. cassumunar* according to F2 score plot.

Classification using partial least square–discriminant analysis (PLS-DA)

In order to improve the separation obtained from PCA model of *C. xanthorrhiza* and *C. xanthorrhiza* adulterated with various concentrations of *Z. cassumunar* as adulterants, the supervised pattern recognition method PLS-DA was carried out. PLS-DA belongs to supervised pattern recognition method because of its ability to classifying objects to certain class or group if the required passing grade of the group is achieved [17]. In this study, PLS-DA was engaged to discriminate pure *C. xanthorrhiza* and adulterated *C.* xanthorrhiza. Table 1 compiled the classification process of each observation into the class or group for which the function value is the highest. The results of this classfication process could be seen in fig. 4 that displayed the observations on the axes.

Table 1: Pre-and post-classification and scores of *Curcuma xanthorrhiza* and *Curcuma xanthorrhiza* adulterated with *Zingiber cassumunar* using partial least square–discriminant analysis (PLS-DA)

Observation	Weight	Y1	Pred (Y1)	F (Adulterated)	F (*C. xantho-rrhiza*)	F (*Z. cassu-munar*)	P (Adulterated)	P (*C. xantho-rrhiza*)	P (*Z. cassu-munar*)
Zingeber cassumunar (Zs)10%	1	Adulterated	Adulte-rated	1.108	-0.009	-0.100	0.615	0.201	0.184
Zs 25%	1	Adulterated	Adulte-rated	1.026	-0.013	-0.013	0.586	0.207	0.207
Zs 40%	1	Adulterated	Adulte-rated	1.067	-0.010	-0.057	0.600	0.204	0.195
Zs 50%	1	Adulterated	Adulte-rated	0.872	0.048	0.080	0.529	0.232	0.239
Zs 75%	1	Adulterated	Adulte-rated	0.875	-0.009	0.134	0.529	0.219	0.252
Zs 100%	1	*Z. cassu-munar*	*Z. cassu-munar*	0.044	-0.004	0.961	0.224	0.214	0.562
C. xanthorrhiza 100%	1	*C. xantho-rrhiza*	*C. xantho-rrhiza*	0.007	0.997	-0.004	0.214	0.575	0.211

F = scores during Partial Least Square–Discriminant Analysis (PLS-DA).

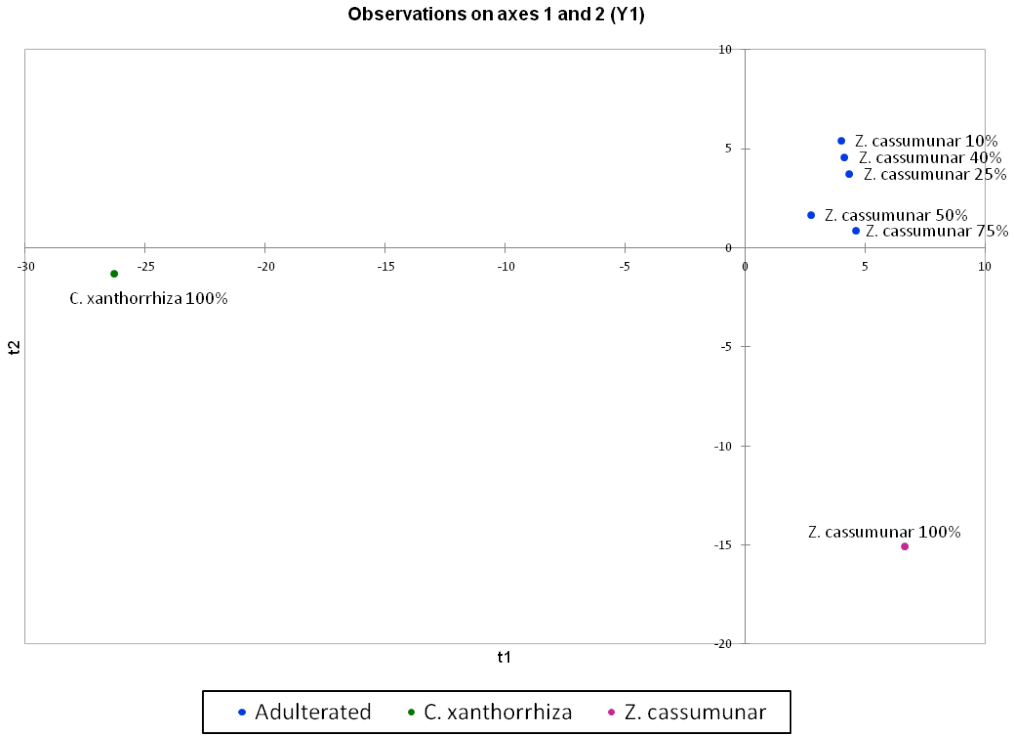


Fig. 4: PLS-DA score plot of *Curcuma xanthorrhiza* and *Curcuma xanthorrhiza* adulterated with various concentrations of *Zingiber cassumunar* as adulterants

PLS-DA model provided better class separation of pure *C. xanthorrhiza* from the adulterated *C. xanthorrhiza* than that provided by PCA model. The efficiency of PLS-DA in classifying and discriminating the samples can be assessed through the confusion matrix as shown in table 2. The confusion matrix presented the percentage of the observations that have been well classified. This is equal to the ratio of the number of well-classified observations over the total number of observations. In this study, the percentage of well-classified observation is equal to 100%.

Table 2: Confusion matrix of *Curcuma xanthorrhiza* and adulterated *Curcuma xanthorrhiza* with *Zingiber cassumunar* obtained during analysis with partial least square–discriminant analysis (PLS-DA)

from \ to	Adulterated	*C. xanthorrhiza*	*Z. cassumunar*	Total	% correct
Adulterated	5	0	0	5	100.00%
C. xanthorrhiza	0	1	0	1	100.00%
Z. cassumunar	0	0	1	1	100.00%
Total	5	1	1	7	100.00%

C. xanthorrhiza = Curcuma xanthorrhiza; Z. cassumunar = Zingiber cassumunar

CONCLUSION

Metabolite fingerprinting using ^{1}H-NMR spectroscopy and chemometrics proposed in this study could be applied for the authentication of *C. xanthorrhiza* adulterated with *Z. cassumunar*. The combination of chemometrics of PCA and PLS-DA allowed the consistent discrimination and classification of pure *C. xanthorrhiza* and *C. xanthorrhiza* adulterated with *Z. cassumunar.*

ACKNOWLEDGMENT

The authors would like to thank Faculty of Pharmacy, Universitas Gadjah Mada (UGM) and the Ministry of Research and Higher Education, Republic Indonesia for the financial support through Directorate of Research UGM via scheme "World Class research" awarded to Prof. Dr. Abdul Rohman with contract number 1973/UN.1. DITLIT/DIT-LIT/lT/2019.

AUTHORS CONTRIBUTIONS

TW performed research activities and analyzed data. AR, SR and EL designed research, prepared manuscript and made critical thinking on the manuscript.

CONFLICT OF INTERESTS

Declared none

REFERENCES

1. Krishnan P. Metabolite fingerprinting and profiling in plants using NMR. J Exp Bot 2004;56:255–65.
2. Kim HK, Choi YH, Verpoorte R. NMR-based metabolomic analysis of plants. Nat Protoc 2010;5:536–49.
3. Schripsema J. Application of NMR in plant metabolomics: techniques, problems and prospects. Phytochem Anal 2010;21:14–21.
4. Rukayadi Y. *In vitro* anticandidal activity of xanthorrhizol isolated from *Curcuma xanthorrhiza* roxb. J Antimicrob Chemother 2006;57:1231–4.
5. Jantan I, Saputri FC, Qaisar MN, Buang F. Correlation between the chemical composition of *Curcuma domestica* and *Curcuma xanthorrhiza* and their antioxidant effect on human low-density lipoprotein oxidation. Evid Based Complement Alternat Med 2012:1–10. Doi:https://doi.org/10.1155/2012/438356
6. Ramdani ED, Marlupi UD, Sinambela J, Tjandrawinata RR. A new method of xanthorrhizol isolation from the rhizome extract of *Curcuma xanthorrhiza*. Sch Acad J Biosci 2016;4:732–7.
7. Prabaningdyah NK, Riyanto S, Rohman A, Siregar C. Application of HPLC and response surface methodology for simultaneous determination of curcumin and desmethoxycurcumin in curcuma syrup formulation. J Appl Pharm Sci 2017;7:58-64.
8. Prabaningdyah NK, Riyanto S, Rohman A. Application of FTIR spectroscopy and multivariate calibration for analysis of curcuminoid in syrup formulation. J Appl Pharm Sci 2018;8:172-9.
9. Mangunwardoyo W. Antimicrobial and identification of active compound *Curcuma xanthorrhiza* Roxb. Int J Basic Appl Sci 2012;12:69–78.
10. Mukherjee PK. Quality control of herbal drugs: an approach to the evaluation of botanicals. Business Horizons; 2002.
11. Rafi M, Wulansari L, Heryanto R, Darusman LK, Lim LW, Takeuchi T. Curcuminoid's content and fingerprint analysis for authentication and discrimination of *Curcuma xanthorrhiza* from *Curcuma longa* by a high-performance liquid chromatography-diode array detector. Food Anal Methods 2015;8:2185–93.
12. Rafi M, Rohaeti E, Miftahudin A, Darusman LK. Differentiation of Curcuma longa, curcuma xanthorrhiza and zingiber cassumunar by thin layer chromatography fingerprint analysis. Indones J Chem 2011;11:71–4.
13. Windarsih A, Rohman A, Swasono RT. Application of H-NMR metabolite fingerprinting and chemometrics for the authentication of curcuma longa adulterated with curcuma manga. J Appl Pharm Sci 2018;10:174-80.
14. Awin T, Mediani A, Shaari K, Faudzi SMM, Sukari MAH, Lajis NH, *et al*. Phytochemical profiles and biological activities of *Curcuma* species subjected to different drying methods and solvent systems: NMR-based metabolomics approach. Ind Crops Prod 2016;94:342–52.
15. Yuliani F, Riyanto S, Rohman A. Application of FTIR spectra combined with chemometrics for analysis of candlenut oil adulteration. Int J Appl Pharm 2018;10:54-9.
16. Choi HK, Choi YH, Verberne M, Lefeber AWM, Erkelens C, Verpoorte R. Metabolic fingerprinting of wild type and transgenic tobacco plants by 1H NMR and multivariate analysis technique. Phytochem 2004;65:857–64.
17. Rohman A, Lumakso FA, Riyanto S. Use of partial least square-discriminant analysis combined with mid-infrared spectroscopy for avocado oil authentication. Res J Med Plant 2016;10:175–80.