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Metabolite Profiling of Three *Curcuma* Species (Zingiberaceae) Based on H-NMR Spectroscopy

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DOI:10.20961/alchemy.15.1.23914.79-88

Received 12 September 2018, Accepted 21 January 2019, Published 01 March 2019

ABSTRACT

Genus *Curcuma*, Zingiberaceae, is a typical medicinal plant in tropical region especially in Indonesia. It has been studied to have antioxidant, antimicrobial, antitumor, anti-inflammatory and anticancer activities. However, little knowledge of the metabolic profile both primary and secondary metabolites have been reported. Thus, this study aims to investigate metabolic profiling both primary and secondary metabolites simultaneously in the *Curcuma* species based on proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. The present work applied metabolomic study which measured the qualitative and quantitative characteristic metabolites. The *Curcuma* species, *Curcuma aeruginosa* Roxb., *Curcuma xanthorrhiza* Roxb., and *Curcuma longa* L., collected from Nguter, Sukoharjo, Indonesia. Two-dimensional (2D)-NMR techniques were applied to further identify a number of different types of compounds. Multivariate data analysis such as Principal Component Analysis (PCA) applied to reveal differences among species. A clear difference occurred among 3 Curcuma species. Primary metabolites responsible for the discrimination are alanine (*C. xanthorrhiza* Roxb. was 3.78 times higher than in *C. longa* L), sugars (*C. xanthorrhiza* Roxb. were 6.03 and 3.81 times higher in *C. aeruginosa* Roxb. and *C. longa* L. respectively). Besides, secondary metabolites which differed among 3 species are curcumin (*C. xanthorrhiza* Roxb. were 38.25 and 25 times higher than in *C. aeruginosa* Roxb.) and xanthorrhizol (*C. longa* L. were 62 and 44.4 times higher than in *C aeruginosa* Roxb.).

Keywords: *Curcuma aeruginosa; Curcuma xanthorrhiza, Curcuma longa,* ^{1}H-NMR, metabolomics, PCA, Secondary metabolites

INTRODUCTION

The Zingiberaceae contains more less 50 genera and 1000 species. Genus *Curcuma*, such as turmeric (*C. longa* L.), temulawak (*C. xanthorrhiza* Roxb.) and temu ireng (*C. aeruginosa* Roxb.) is one of genus widely used as medicinal herbs in Indonesia. It is

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reported to have numerous uses in health as an antioxidant (Jantan *et al.*, 2012), antimicrobial (Mary *et al.*, 2012), antitumor (Yue *et al.*, 2010 and Choi *et al.*, 2004) and anti-inflammatory (Lantz *et al.*, 2005). The bioactivities of herbal medicine are determined by the chemical compounds, both secondary and primary metabolites.

The major secondary metabolites of genus *Curcuma* are curcuminoids and terpenoids. The former consists of curcumin, demethoxycurcumin, and bisdemethoxycurcumin (Hadi *et al.*, 2018 and Asghari *et al.*, 2010). The main terpenoids in genus *Curcuma* are monoterpene and sesquiterpene (Li *et al.*, 2011). Those are germacrone, 1,8-cineol, ar-turmerone, β-sesquiphellandrene (Lee *et al.*, 2014), curcuminol, ar-curcumin (Li *et al.*, 2011) and xanthorrhizol (Jantan *et al.*, 2012). Metabolite profiling of *C. aromatica* and *C. longa* L. grown in Korea resulted in sugars (glucose, sucrose and fructose) and essential oils (eucalyptol, curdione, and germacrone) as primary and secondary metabolites, respectively were significantly different between two species (Jung *et al.*, 2012). However, limited studies are in metabolites profiling in other species of genus *Curcuma*. Therefore, knowledge of a simultaneous analysis of both primary and secondary metabolites in other species of genus *Curcuma* need to be more studied.

Metabolomics is a set of identification and quantification of all metabolites present in biological samples. It applies a technique of chemical analysis which is unbiased, rapid, reproducible and stable during the time. Moreover, it requires simple sample preparation. Nuclear magnetic resonance (NMR) has been widely used to do metabolic profiling with multivariate data analysis such as principal component analysis (PCA). This combined analysis has been reported several studies green tea (Tarachiwin *et al.*, 2007), grapes (Ali *et al.*, 2011b) and *Angelica gigas* (Kim *et al.*, 2011). A powerful technique for characterization different species (Choi *et al.*, 2005).

In this study, we aimed to investigate a metabolic characterization of three species in genus *Curcuma* (Zingiberaceae). The species are *C. longa* L., *C. aeruginosa* Roxb., and *C. xanthorrhiza* Roxb. To elaborate the differences, ^{1}H-NMR coupled with 2D NMR techniques combined with PCA was applied.

EXPERIMENTAL DETAIL

Methanol d-4 (CD_3OD) 99.95% (Merck); potassium phosphate (KH_2PO_4) (Merck); 0.01% TSP (Chem Cruz); buffer D_2O, NaOD 1 M (Santa Cruz Biotechnology). All solvent used are NMR-deurated specification.

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Rhizome from 3 species in genus *Curcuma* were *C. longa* L., *C. xanthorriza* Roxb., and *C. aeruginosa* Roxb. They were collected at Tanggungrejo Village, Nguter District, Sukoharjo Regency at 8 months planting. Each species has three replicates. All samples were cut and ground into fine powder. It kept at -40 °C and freeze-dryed.

Extraction and NMR Measurements

An extraction process referred to the study by Kim *et al.* (2010). 30 mg of freeze-dryed plant material was added into 2 mL eppendorf tube. 630 µL of CD_3OD and 270 µL of buffer KH_2PO_4 with 0.01% of TSP diluted in D_2O were added into the sample. The mixture was vortexed for 1 minute at room temperature then sonicated for 20 minutes. The mixture was centrifuged for 10 minutes at 13,300 rpm at room temperature (28 °C). Supernatant (600 µL) from centrifugation process was finally transferred into 5 mm NMR tube.

^{1}H-NMR and 2D-NMR were recorded at 25°C on a 400 MHz Agilent P. Deurated water were used as the internal lock. Each ^{1}H-NMR spectrum scanned 128 times with the following parameters: relaxation delay = 2.0 s and pulse width (PW) = 90° (6.80 µs). The residual H_2O signal was suppressed using a pre-saturated sequence with low power selective irradiation at the H_2O frequency during the recycle delay. Pre-processed steps such as phasing, baseline correction and calibrated to TSP at 0.00 ppm using XWIN NMR. NMR spectra were examined using 2D NMR consist of ^{1}H-^{1}H J-resolved and ^{1}H-^{1}H Correlation Spectroscopy (COSY), then compared with literature.

Data analysis and statistical methods

^{1}H-NMR spectra were reduced to ASCII files. Spectral intensities were scaled to internal standard and reduce to integrated regions of equal width (0.04) corresponding to the region of δ_H 0.0 – 10.0. Residual signal of D_2O and CD_3OD at the region of 4.85-4.95 and 3.2-3.4 were excluded from the analysis, respectively. Bucketing was performed by AMIX software (Bruker) with scaling on internal standard. Data from ^{1}H-NMR spectroscopy was analyzed with multivariate statistical analysis using SIMCA-P version 15.0. Multivariate statistical analysis was done using Principal Component Analysis (PCA) method based on species differences.

Data analysis was done using The One-way Analysis of Variance (ANOVA) with significant level 95%. Post hoc test, LSD, was done to determine which groups differ significantly.

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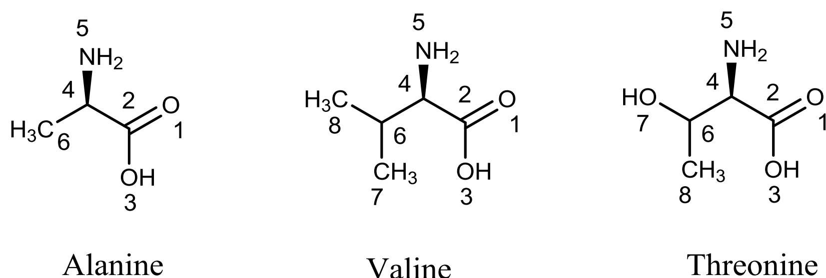
RESULTS AND DISCUSSION

^{1}H-NMR spectra in general divided by three regions, amino acids and organic acids (δ_H 0.5–2.5) ppm, sugars (δ_H 2.5–5.5) ppm, and aromatic compounds (δ_H 5.5–8.0) ppm. To identify metabolite in ^{1}H-NMR, 2D ^{1}H-^{1}H *J*-resolved and correlation 2D-NMR COSY were used to provide additional information on each signal.

Amino acids identified were alanine, valine and threonine. Alanine proton signal identified at δ_H 1.49 ppm (d, *J* =7.13 Hz, H-6) and δ_H 3.76 ppm (q, *J* = 6.36 Hz, H-4). Correlation of signals of H-6 and H-4 was observed in the COSY spectrum. Signals at δ_H 1.02 ppm (d, *J* = 7 Hz, H-8), δ_H 1.07 ppm (d, *J* = 7 Hz, H-7), and δ_H 3.79 ppm (m, H-6) were identified as valine. Signals at δ_H 1.33 ppm (d, *J* = 6.82 Hz, H-8), δ_H 3.53 ppm (d, *J* = 4.72 Hz, H-4), and δ_H 4.04 ppm (m, H-6) were associated to threonine. Correlation between signals at δ_H 1.33 ppm (H-8) and δ_H 3.53 ppm (H-4) with δ_H 4.04 ppm (H-6) was observed in the COSY spectrum.



Sugars regions were identified as α-glucose, β-glucose, fructose and sucrose. Signal of δ_H 5.17 ppm (d, *J* = 3,94 Hz), δ_H 4.56 ppm (d, *J* = 8.24 Hz), and δ_H 5.41 ppm (d, *J*= 3,96 Hz) were identified as α-Glucose, β-glucose and sucrose, respectively. Formic acid was identified by proton signal at δ_H 8.49 ppm singlet.

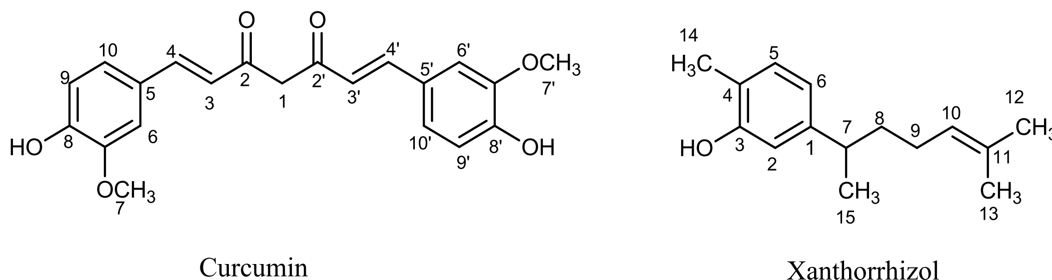


Figure 1. Secondary metabolite structures

Phenolic and terpenoid as major compounds were curcumin and xanthorrhizol identified in genus *Curcuma*, respectively (Figure 1). Signals at δ_H (ppm): 3.92 (s, H-7/7'), 6.64 (d, *J* = 15.68 Hz, H-3/3'), 7.57 (d, *J* = 15.68 Hz, H-4/4'), 6.89 (d, *J* = 8.59 Hz, H-

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9/9’), and 7.15 (dd, *J* = 8.21; 3.16 Hz, H-10/10’) were identified as curcumin. ^{1}H-^{1}H COSY spectra showed a signal correlation between H-3/3’ and H-4/4’. Besides signal H-9/9’ and H-10/10’ were correlated in ^{1}H-^{1}H COSY spectra. Signals at δ_H(ppm): 1.87 (s, H-12), 1.93 (s, H-13), 2.00 (s, H-14), 1.22 (d, *J* = 7.06 Hz, H-15), 2.69 (q, *J* = 7.35 Hz, H-7), 7.23 (d, *J* = 3.05 Hz, H-2), 7.57 (dd, *J* = 8.21; 3.16 Hz, H-6), and 6.89 (d, *J* = 8.59 Hz, H-5) were assigned as xanthorrhizol. Correlation between H-15 and H-7 were shown in ^{1}H-^{1}H COSY spectra. Besides, both signals at H-5/H-2 and H-6 were correlated in ^{1}H-^{1}H COSY spectra.

Table 1. ^{1}H-NMR chemical shifts (δ) and coupling constants (Hz) of compounds identified in genus Curcuma (*C. aeruginosa, C. xanthorrhiza*, and *C. longa*) using 1D and 2D NMR spectra. It dissolved in CD_3OD-KH_2PO_4 in D_2O, pH 6.0

Metabolites	**Chemical shift (δ) and copling constants (Hz)**
Alanine	1.49 (d, *J* = 7.13 Hz, H-6), 3.76 (q, *J* = 6.36 Hz, H-4)
Threonine	1.33 (d, *J* = 6.82 Hz, H-8), 3.53 (d, *J* = 4.72 Hz, H-4)
Valine	1.02 (d, *J* = 7.00 Hz, H-8), 1.07 (d, *J* = 7.00 Hz, H-7)
α-Glucose	5.17 (d, *J* = 3.94 Hz, H-1), 3.49 (dd, *J* = 9.72; 3.7 Hz, H-2), 3.74 (t, *J* = 9.72 Hz, H-3), 3.42 (t, *J* = 9.62 Hz, H-4)
β-Glucose	4,56 (d, *J* = 8.24 Hz, H-1), 3.19 (t, J = 8.55 Hz, H-2)
Fructose	3.99 (dd, *J* = 12.27; 3.11 Hz, H-4), 4.16 (d, *J* = 8.43 Hz, H-3)
Sucrose	3.42 (t, *J* = 9.22 Hz, H-10), 3.74 (t, *J* = 9.30 Hz, H-11), 3.49 (dd, *J* = 9.62; 4.33 Hz, H-12), 5,41 (d, *J* = 3.96 Hz, H-7), 3.65 (s, H-13)
Cholin	3.32 (s), 3.49 (dd, *J* = 9.62; 4.33 Hz)
Formic Acid	8.49 (s, H-2)
Curcumin	3.92 (s, H-7/7’), 6.64 (d, *J* = 15,68 Hz, H-3/3’), 7.57 (d, *J* = 15,68 Hz, H-4/4’), 6.89 (d, *J* = 8.59, H-9/9’), 7.15 (dd, *J* = 8.21; 3.16 Hz, H-10/10’)
Xanthorrhizol	1,87 (s, H-12), 1.93 (s, H-13), 2.00 (s, H-14), 1.22 (d, *J* = 7.06 Hz, H-15), 2.69 (q, *J* = 7,35 Hz, H-7), 7.23 (d, *J* = 3.05 Hz, H-2), 7.57 (dd, *J* = 8.21; 3.16 Hz, H-6), 6.89 (d, *J* = 8.59 Hz, H-5)

Multivariate data analysis particularly PCA was used to identify differences in data (Jung *et al.,* 2012). PCA is an unsupervised and clustering method to reduce the dimensionality of multivariate data. The principal components (PCs) are shown in a graphical form as a score plot. It is useful to identify any groupings in the data set. Loading plots are shown from coefficients by which the original variables must be multiplied to obtain the PCs (Kim *et al.,* 2010). Loading plots are useful to detect the spectral areas responsible for the separation of the data.

Scoring plot of PCA showed 63.1% separation in PC1; *C. xanthorrhiza* Roxb. and *C. longa* L. extracts were grouped in positive quadrant while *C. aeruginosa* Roxb. extract was grouped in negative quadrant (Figure 2A). PCA loading plot resulted in signals

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responsible for the separation (Figure 2B). Amino acids such as alanine, valine, and threonine showed to have higher signals at *C. aeruginosa* Roxb.. On the other hands, sugars (α-glucose, β-glucose, fructose and sucrose), phenolic (curcumin), and terpenoid (xanthorrhizol) compounds found to be discriminating for *C. xanthorrhiza* Roxb. and *C. longa* L.

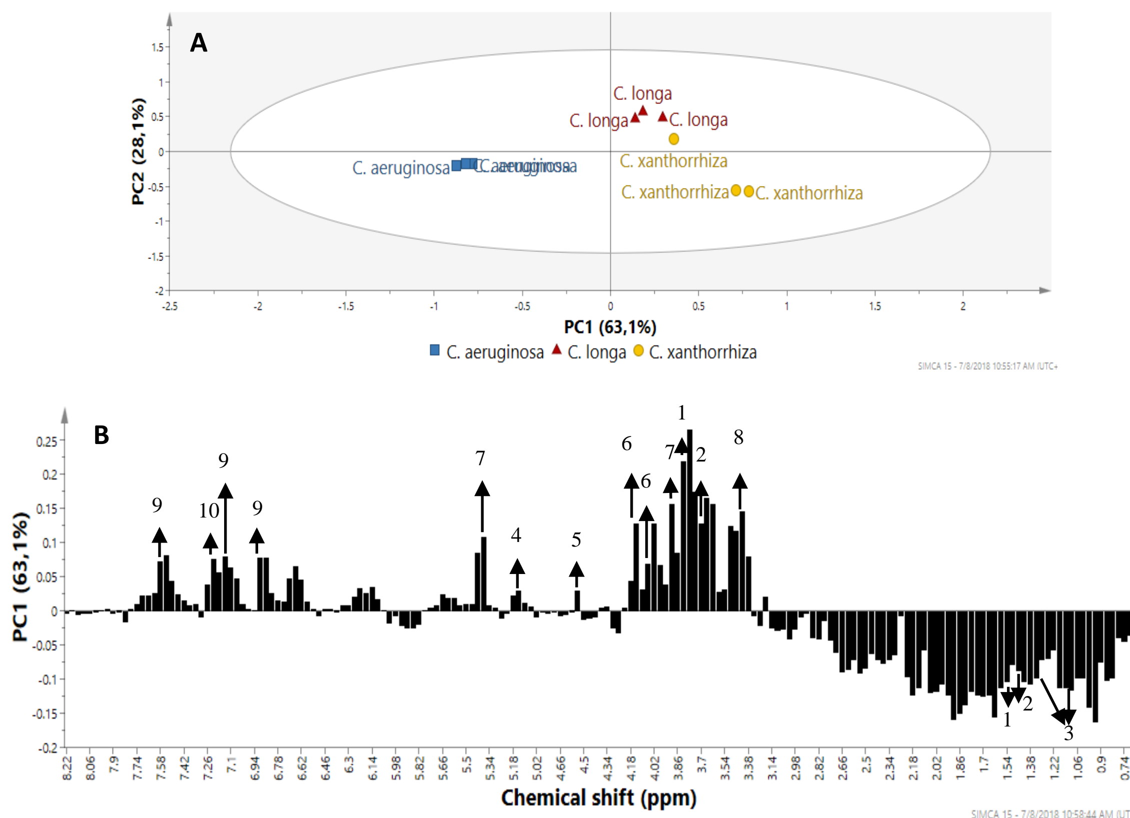

Figure 2. Scoring plot (A) and loading plot (B) of PCA in genus Curcuma. Metabolites identified are (1) alanine, (2) threonine, (3) valine, (4) α-glucose, (5) β-glucose, (6) fructose, (7) sucrose, (8) choline, (9) curcumin, (10) xanthorrhizol.

Relative concentration of alanine, α-glucose, sucrose, fructose dan choline showed significant differences among species (F = 5,8; df = 2; *p* = 0,040; F= 8,953; df = 2; *p* = 0,016; F= 14,496; df = 2; *p* = 0,002; F= 17,863; df = 2; *p* = 0,003; F= 27,636; df = 2; *p* = 0,001, respectively). Alanine relative concentration in *C. xanthorrhiza* Roxb. were 3.78 times and 1.3 times higher than in *C. longa* L. and in *C. aeruginosa* Roxb. Relative concentration of α-glucose in *C. xanthorrhiza* Roxb. was 6.2 times higher than in *C. aeruginosa* Roxb., while relative concentration in *C. xanthorrhiza* Roxb. was 3.9 times higher than in *C. longa* L. Sucrose relative concentration in *C. xanthorrhiza* Roxb. was 6.08 times higher than in *C. aeruginosa* Roxb., whilst relative concentration in C. *xanthorrhiza* Roxb. was 3.6 times higher than in C. *longa* L. Fructose relative concentration in *C. xanthorrhiza* Roxb. were 5.8 times and 3.93 times higher than in *C.*

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aeruginosa Roxb. and *C. longa* L respectively. Relative concentrations of choline in *C. xanthorrhiza* Roxb. were 4.09 and 3.03 times higher than in *C. aeruginosa* Roxb. and *C. longa* L., respectively. Primary metabolites such as alanine, α-glucose, sucrose, fructose (Figure 3) differed significantly between *C. aromatica* and C. longa grown in Korea (Jung *et al.*, 2012).

Secondary metabolite concentrations of curcumin and xanthorrhizol also showed significant differences among species (F= 13,735; df = 2; p = 0,006 and F= 15,297; df = 2; p = 0,004, respectively). Those compounds (Figure 3) were not present in *C. aeruginosa* Roxb.. Curcumin relative concentration in *C. xanthorrhiza* Roxb. was 38.25 times higher than in *C. aeruginosa* Roxb., while curcumin relative concentrations in *C. longa* L. were 62 times and 1.62 times higher than in *C. aeruginosa* Roxb. and *C. xanthorrhiza* Roxb. respectively. Xanthorrhizol relative concentration in *C. xanthorrhiza* Roxb. was 25 times higher than in *C. aeruginosa* Roxb., whereas xanthorrhizol relative concentration in *C. longa* L. was 44.4 times and 1.78 times higher than in *C. aeruginosa* Roxb. and in *C. xanthorrhiza* Roxb. respectively. Curcumin and xanthorrhizol in *C. xanthorrhiza* Roxb. and *C. longa*. reported present in *C. xanthorrhiza* Roxb. (Rafi *et al.*, 2015; Lechtenberg *et al.,* 2004). Xanthorrhizol was the major compound in *C. xanthorrhiza* Roxb. (Ab Halim *et al.*, 2012). However, Jarikasem *et al.* (2003) reported that xanthorrhizol was not identified in *C. xanthorrhiza*. This difference might be due to the rhizome age which has the optimum abundance at 12 months planting (Endrasari and Mas'adi, 2011). Curcumin in *C. longa* was two times higher than in *C. xanthorrhiza* Roxb.. Curcumin has been reported to have the highest abundance 3.6% in *C. longa* L. than in *C. xanthorrhiza* Roxb. (2.3%) (Jantan *et al.*, 2012).

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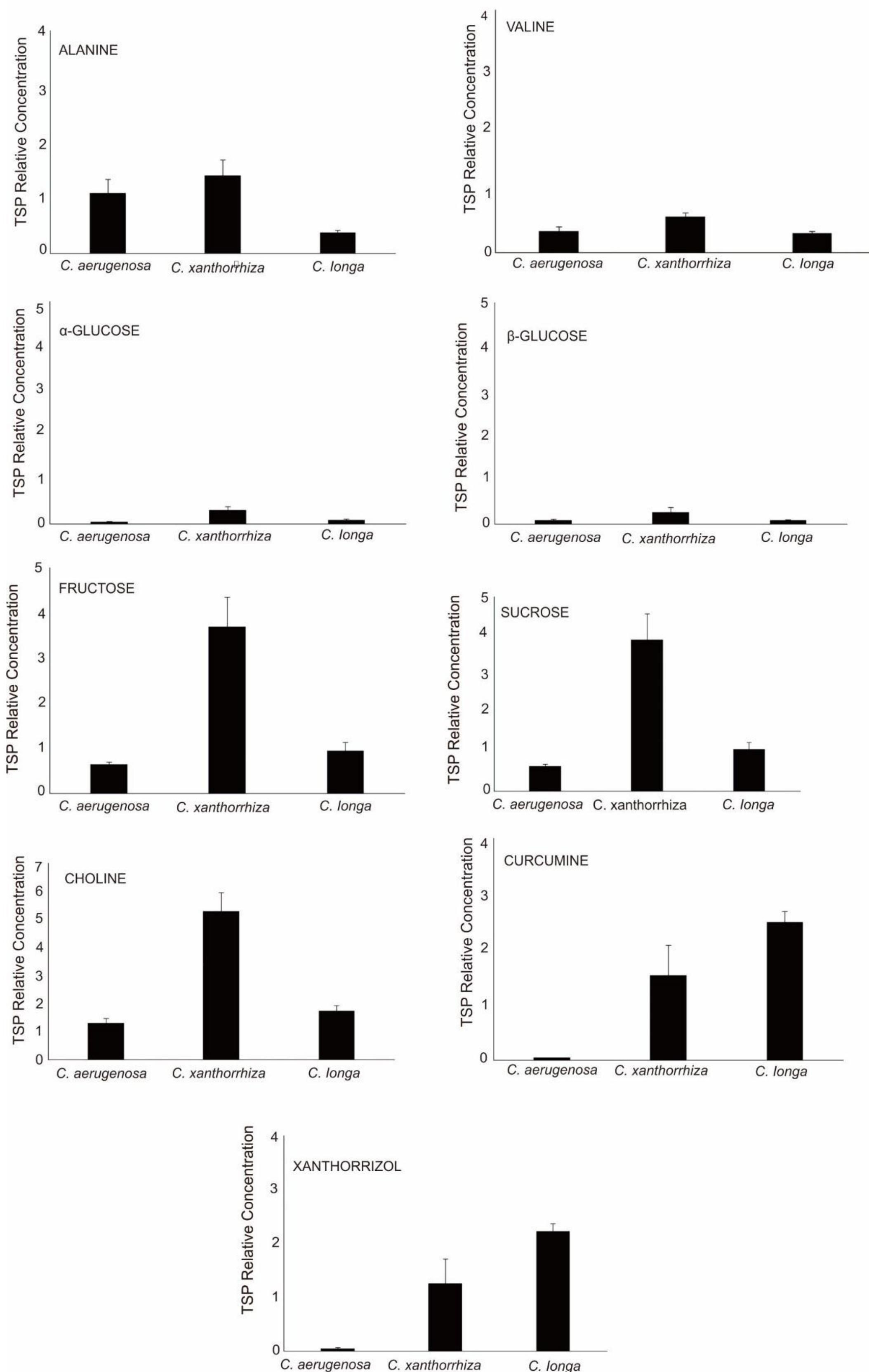

Figure 3. ANOVA metabolite quantification ($p < 0.05$) of *Curcuma* extracts that were analyzed using ^{1}H-NMR. Different letter indicated significant values at 95% confidence level.

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CONCLUSION

Profiling metabolites in three species of *Curcuma* (*C. aeruginosa* Roxb., *C. longa* L., and *C. xanthorrhiza* Roxb.) can be done using metabolomic study based on ^{1}H-NMR. It could differ three *Curcuma* species significantly by either primary and secondary metabolites. The former were alanine, α-glucose, sucrose, fructose and choline, and the latter were curcumin and xanthorrhizol. Those metabolites showed a particular profile which was potentially useful as a marker of species. The study of other *Curcuma* species needs to be done to enlarge the knowledge of *Curcuma* genera metabolite profiling.

ACKNOWLEDGEMENT

The first author thanks to the Natural Medicine research study. This work was supported by PNBP Universitas Sebelas Maret, 2018. The authors declared no potential conflict of interest in this study.

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