Characterization of Marker Compounds in *Curcuma zanthorrhiza* Using NMR

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Abstract. *Curcuma zanthorrhiza* is a plant from the family Zingiberaceae that commonly grows in Indonesia. Traditional medicine, spices of food, beverages, cosmetics, and coloring agents, especially food coloring use the plant quite often. The experiments used a complete random factorial design with 3x3 replicates. The treatments consisted of Curcuma varieties (V) from Malang, Blora, and Sukoharjo, drought stress (K) expose was daily watering plants as the control (K1), one every two days watering plants (K2), and one every three days watering plants (K3). The research aimed to determine the response of Curcuma in agronomic characters and metabolite compounds during drought stress treatments. The drought stress (K) showed significant results on Curcuma plant height, in which three-day watering negatively affected the plant height. Sukoharjo variety with every-day watering produced the best rhizome weight compared to two other varieties. Secondary metabolites of xanthorrhizol and curcumin identified with Nuclear Magnetic Resonance (NMR) analysis from the Sukoharjo variety ginger rhizome showed a relative concentration of 1.25±0.46 and 1.53±0.54, respectively.

1. Introduction

*Curcuma zanthorrhiza* is a plant from the Zingiberaceae that grows in Indonesia known as temulawak, Java ginger, Javanese ginger, or Javanese turmeric is a plant species, belonging to the ginger family. The demand for *C. zanthorrhiza* reaches 3000 tons/year and continues to increase every year. This plant is widely used as traditional medicine, food spices, beverages, cosmetics and coloring agents, particularly for food. For health, *C. zanthorrhiza* has the potential as an anti-inflammatory, anti-immunodeficiency, antiviral, antibacterial, antifungal, antioxidant, anticarcinogenic and anti-infectious agents [1-3].

Curcuminoids are secondary metabolites of diferuloylmethane which are widely reported as active compounds in *C. zanthorrhiza* [4].

The two secondary metabolites have been identified by previous studies on ginger. The results showed that the curcumin content was higher than xanthorrhizol content, the main compound in *C. zanthorrhiza*. This finding is similar to that in *C. zanthorrhiza* from Thailand in which xanthorrhizol could not be identified [5]. The content of secondary metabolites is strongly influenced by various environmental factors including climate (such as light, air temperature, and humidity). The physicochemical properties of soil and water availability in the soil also affect the rooting environment. Local farmers have difficulty supplying *C. zanthorrhiza* as a raw material for the medicinal industry due to secondary metabolite requirement, especially the curcumin content and material quality. Therefore, standardizing *C. zanthorrhiza* quality is essential. The *C. zanthorrhiza* grew in Java and Madura produce the highest curcumin compared to *C. zanthorrhiza* of other regions. Thus, this study was the starting point to
cultivate *C. zanthorrhiza* that produces high and stable curcumin necessary for raw material in the medical industry [6].

Metabolomics is a holistic method that provides an overall picture of both primary and secondary metabolites in a biological system [7]. Many different sensitive methods can be used to analyze plant metabolites, one of which is Nuclear magnetic resonance (NMR). It has been used extensively as a tool to analyze the chemical profile of a plant with the support of Principal Components Analysis (PCA) [8]. Recently, a combination of NMR and PCA is widely applied to analyze the chemical content profile of some plants [9,10]. This method has proven to be an appropriate tool for characterizing the chemical contents of several species [11,12] and cultivars [13]. However, the literature on metabolomic studies using NMR has not been reported.

2. Methods

The first year study was carried out in the experimental garden of the Faculty of Agriculture, Sebelas Maret University, Surakarta. Profiles of primary and secondary metabolites of Curcuma cultivars from each region were carried out using NMR at the UNS MIPA Central Laboratory. Curcumin marker compounds which have high curcumin content were carried out using SIMCA-P software, version 12.0 Umetrics, Umea, Sweden.

The experiments used a basic randomized complete design. Drought Stress Treatment (K) consisted of three levels, i.e., daily watering plants as the control (K1), one every two days watering plants (K2), and one every three days watering plants (K3). The watering volume was 100 ml. The stress treatment was carried out for two months and was done after the plants were aged 4. When the plants were four months, all of them were treated with standard treatment and regular watering every morning with the same amount (100 ml). Curcuma rhizome was grown in a 10 kg polybag (black silver plastic). Each treatment was repeated ten times.

2.1 Observation of agronomic components

Observation of agronomic components included (1) plant height by measuring the length of the stem base to the highest tip of the leaf, (2) number of bulbs by counting the number of rhizomes formed including the main rhizome, (3) rhizome weight after being cleaned from attached soil remnants. Agronomic observation data were analyzed using a two-way variant analysis, followed by a midpoint test of the Least significant Difference (LSD).

2.2 Extraction and Measurement of secondary metabolites using NMR

The harvested rhizome samples were cleaned, cut into small pieces, and blended to a small size. Blended rhizomes were put into pots of ointment and placed into a deep freezer (-20 °C) for 3 x 24 hours. The frozen rhizome was then moved to a freeze dryer at a temperature of -108 °C for 4 x 24 hours (Kim et al., 2005). The dried samples were then ground to a fine powder. A fine powder of 30 mg was put into a 2 ml Eppendorf tube then added with 270 µl of phosphate buffer mixture (KH2PO4) in deuterium oxide containing 0.01% TSP and 630 µl CD3OD with a ratio of 3:7. The mixture was vortexed (1 minute, room temperature), authenticated (20 minutes, room temperature) and centrifuged (13,300 rpm, 28 °C, 10 minutes). The supernatant obtained from the centrifugation was transferred to a new Eppendorf tube for measurement.

A supernatant of 600 µl was put into 5 mm NMR tube for 1H-NMR analysis. The 1H-NMR spectra were recorded at 25 °C using Agilent P 400 MHz 1H-NMR spectroscopy. 3-(Trimethylsilyl)propionic acid-d4 sodium salt (TSP) was used as an internal standard. The sample was read on spectral width -1.0 to 11.0 ppm. Measuring 1H-NMR spectra were 128 scans with the following parameters: presaturation delay 2 seconds, acquisition time 3,408 seconds, relaxation delay 2 seconds, observe pulse = 6, 8 µs (90°). Presaturation was used to suppress H2O residues. Spectra results were manually carried out baseline correction and calibration using TSP on 0.00 ppm chemical shift. The 2D NMR J-resolved spectra were performed with 8 scans per 64 increments with 1-second relaxation delay and 0.625 seconds acquisition time. Spectra J-Resolved tilted 45° and symmetrized, then calibrated using TSP at a chemical shift of 0.00 ppm. The Homonuclear Correlation
Spectroscopy (COSY) was performed with 1-second relaxation delay, the spectral width of 512 Hz in F1 and 4807.7 Hz in F2. All spectra were carried out manually by baseline correction and internal standard calibration (TSP = 0.00 ppm).

3. Results

Testing of the average values of plant height, rhizome weight, and number of tillers are shown in Table 1.

Table 1. Testing of the average value (curcuma variety from Malang: M, Sukoharjo: S, Blora: B, every day watering as the control: K1, every two days watering: K2, every 3 days watering: K3)

| Treatment | Plant height (cm) | Rhizome Weight (g) | Number of Rhizomes |
|-----------|------------------|--------------------|-------------------|
| M         | 311 c            | 3.85 a             | 17 a              |
| S         | 320 b            | 4.32 cb            | 47 c              |
| B         | 306 a            | 4.08 b             | 29 b              |
| K1        | 361 c            | 4.26 cb            | 46 c              |
| K2        | 298 b            | 4.12 b             | 29 b              |
| K2        | 280 a            | 3.87 a             | 18 a              |

![Figure 1](image-url)  

Figure 1. Spectra proton xanthorrhizol on C. xanthorrhiza Roxb. (A) $^1$H-$^1$H J-resolved. (B) Spectra $^1$H-$^1$H COSY shows the correlation between protons H-15 and H-7 (1), the correlation between protons H-5 and H-6 (2).
The identification and quantification of secondary metabolites with NMR are as follows. The xanthorrhizol proton signal in the $^1$H-$^1$H J-resolved and $^1$H-$^1$H COSY spectra of $C. xanthorrhiza$ is shown in Figure 1. $^1$H-$^1$H J-resolved and $^1$H-$^1$H COSY curcumin from $C. xanthorrhiza$ extract is presented in Figure 2. Figure 3 represents the quantification of xanthorrhizol and curcumin in the Sukoharjo ginger rhizome.

![Figure 2](image_url)

**Figure 2.** Proton curcumin spectra on $C. xanthorrhiza$ Roxb. (A) $^1$H-$^1$H J-resolved. (B) Spektra $^1$H-$^1$H COSY shows the correlation between protons H-3/3’ and H-4/4’ (1), the correlation between protons H-9/9’ and H-10/10’ (2)
Figure 3. Quantification of xanthorrhizol and curcumin identified in the extract of C. xanthorrhiza Roxb analyzed by $^1$H-NMR.

4. Discussion

The response of the three Curcuma varieties to water treatment water was varied. Sukoharjo variety showed better plant height, rhizome weight, and rhizomes than Malang and Blora varieties. The K1 demonstrated the most significant influence on plant height, followed by K2 and K3. Everyday water supply produced a positive influence on the plant hight components. The amount of continuously available water in the final phase of Curcuma growth can maintain the soil element supply in the root area by plants; thus, the plant growth process takes place well. The K1 revealed the best effect on the rhizome's western observations, although it was not different from the K2 supply. The Sukoharjo variety produced the largest rhizome weight and the highest number of tillers compared to two other varieties. This is possible because the planting was done in Sukoharjo area so that the variety has a better adaptation than the others. Identification of the main secondary metabolites, namely xanthorrhizol and curcumin, was observed in the rhizome of Sukoharjo Curcuma.

The xanthorrhizol proton signal in C. xanthorrhiza Roxb. can be detected in $^1$H-$^1$H J-resolved at 87H 1.87 ppm (s) (H-12), δH 1.93 ppm (s) (H-13), δH 2.00 ppm (s) (H-14), δH 1.22 ppm (d, J = 7.06 Hz, H-15), δH 2.69 ppm (q, J = 7.35 Hz, H-7), δH 7.23 ppm (d, J = 3.05 Hz, H-2), δH 7.57 ppm (dd, J = 8.21; 3.16 Hz, H-6), and at δH 6.89 ppm (d, J = 8, 59 Hz, H-5). In 1H-1H COSY there was a correlation between protons at δH 1.22 ppm (H-15) and δH 2.69 ppm (H-7), and protons at δH 6.89 ppm (H-5) and δH 7.57 ppm (H-6).

The Curcumin compounds in C. zanthorrhiza Roxb. shown by the proton signal H-7/7' appeared at δH 3.92 ppm as singlet because it did not have a neighboring proton atom. Proton H-3/3' appeared at δH 6.64 ppm (d, J = 15.68 Hz) and the proton signal at H-4/4' appeared at 7H 7.57 ppm (d, J = 15.68 Hz) $^1$H-$^1$H COSY data on H-3/3' correlated with δH7.57 ppm (H-4/4'). The proton signal H-9/9' appeared at δH 6.89 ppm (d, J = 8.59 Hz), the signal appeared as a doublet with J 8.59 Hz because protons at H-9, 9' had one neighboring proton ortho position. The proton H-10/10' signal appeared at 15 7.15 ppm (dd, J = 8.21; 3.16 Hz). These signals appeared as large and small double lobes because the protons H-10/10' had each of the neighboring protons in the ortho and meta positions. Proton on H-9/9' in $^1$H-$^1$H COSY correlated with protons on H-10/10'.

5. Conclusion

Based on the data obtained from agronomic components and metabolites using NMR, it can be concluded that drought stress treatment (K) showed significant results on the height of C. zanthorrhiza plants. The C. zanthorrhiza plant watered once every two days negatively affected plant height. Sukoharjo variety watered everyday produced the best rhizome weight compared to two other varieties. The NMR-based metabolomic method can be used as a differentiator for the presence of metabolites in C. zanthorrhiza.
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