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The diversity of leaves and asiaticoside content on three accessions of *Centella asiatica* with the addition of chicken manure fertilizer

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Abstract. *Mumtazah HM, Supriyono, Widyastuti Y, Yunus A. 2020. The diversity of leaves and asiaticoside content on three accessions of Centella asiatica with the addition of chicken manure fertilizer. Biodiversitas 21: 1035-1040. Centella asiatica* (L.) commonly known as an essential medicinal herb. This plant has high diversity, especially in leaf morphology. The main objective of this research was to determine the morphology of leaves and the effect of chicken manure dosage on the increase of asiaticoside content. The leaves of three accessions characterized, and then *C. asiatica* planted. The research design used a completely randomized design (CRD) factorial, with the first factor is accession (C) with 3 levels (C1: accession 1, C2: accession 2, C3: accession 3). The second factor was chicken manure with 4 levels dosage (P1: 0 ton ha-[1], P2: 10 tons ha-[1], P3: 15 tons ha-[1] and P4: 20 tons ha-[1]). Asiaticoside analysis used the TLC method with chloroform: methanol: water (65:25:4) as mobile phase. The result from this research showed that the diversity in leaf morphology of three accessions is in leaf size and petiole length. Accession 3 has the widest leaf size (7.3 cm) and the longest petiole (9.8 cm), accession 2 with a leaf width of 5.8 cm and petiole length in 8.5 cm, while the accession 1 has the least leaf width (5.3 cm) and the length of the petiole is the shortest with 7.3 cm. Observation results of asiaticoside showed that the highest content of asiaticoside produced by accession 3 (0.19%), then accession 1 with a content of 0.15%, while accession 2 showed the lowest asiaticoside content (0.13%). The treatment of chicken manure addition significantly increase asiaticoside content in accessions 1 and 3, but in accession 2 it was not significantly increased. The highest content of asiaticoside (0.34%) were in accession 3 with the addition of chicken manure 20 tons ha-1 (P4).

Keywords: Accession, asiaticoside, *Centella asiatica*, chicken manure, diversity

INTRODUCTION

Information about the diversity of medicinal plants in Indonesia needs to continually enriched. Indonesia has a lot of natural resources, one of them is in medicinal plants (Elfahmi et al. 2014). People often use *Centella asiatica* (L.) as medicine or consume it as an additional vegetable. This plant easily cultivated and propagated vegetatively.

Centella asiatica has diverse variations in morphology and leaf size. Morphological variation of this plant noticed in the shape and color of the leaves and the length of petiole. Nandini (2008) has carried out morphological characterization on 17 accessions of *C. asiatica*. The results showed that the plant has a variety of leaf colors, including light green, yellowish-green and dark green. The differences in morphology are also shown from the results of research by Bernawie (2013), which states that there are differences in the characteristics and size of *C. asiatica* morphology that grow in different environments. Morphological differences influenced by genetic factors, environmental factors, and the interaction of both (Benito et al. 2016).

Centella asiatica is known as Gotu kola was originated from Sri Lanka, North Australia, and Malaysia (Brinkhaus et al. 2000). The major pharmaceutical value of this plant is the content of asiaticoside, which is the main component in *C. asiatica* (Hashim et al. 2011; Chong and Aziz 2011). Gotu kola can enhance memory and help improving memory systems for those who experience deterioration in brain function. A study proves that this plant can raise IQ, improve mental abilities, enhance nerve function (Roy et al. 2013; Ghosh et al. 2017), antidepressant, antifungal, antioxidant, and anti-inflammatory (Park et al. 2017). China has been using Centella as a tonic, while in Malaysia is used to treat bronchitis, ulcers, asthma, and kidney disorders (Chandrika and Kumara 2015).

The treatment of nutrient addition with various doses of chicken manure was an effort to increase asiaticoside content in *C. asiatica*. Siddiqui et al. (2011) state that the higher the dose of organic manure given, the higher the asiaticoside produced by Centella. The study of asiaticoside content is needed to meet pharmaceutical needs.

To meet the escalating needs of *C. asiatica* (Widiyastuti et al. 2016), we need to set a standard for every accession to determine which accession promising to developed. During this time, the fulfillment of *C. asiatica* herb needs comes from wild plants that have not been

standardized. Development of *C. asiatica* on a large scale arranged with quality cultivation efforts (Vinolina 2018). This study aimed to determine the diversity of leaves morphology, asiaticoside content, and efforts to increase the content of asiaticoside in three *C. asiatica* accessions as an initial step to determine which plants potential to develop.

MATERIALS AND METHODS

Morphology identification

The three accessions used collected from the Center for Research and Development of Medicinal Plants and Traditional Medicines (B2P2TOOT), Tawangmangu, Indonesia. Accession 1 came from Tawangmangu, Central Java, Indonesia, accession 2 came from Malang, East Java Indonesia, and accession 3 came from Bogor, West Java, Indonesia. Morphological character identification arranged by a non-experimental descriptive method. Variables observed were characteristic of leaves and petiole of leaves. Quantitative measurements carried out using a ruler.

Experimental design

The planting of *C. asiatica* used a complete randomized design (CRD) factorial, with the first factor is accession (C) consisted of 3 levels (C1, C2, C3). The second factor was chicken manure with 4 levels dosage; P1: 0 ton ha^{-1}, P2: 10 tons ha^{-1}, P3: 15 tons ha^{-1} and P4: 20 tons ha^{-1}, so that 12 treatment combinations obtained. An analysis of the chicken manure nutrient content arranged before planting. Chicken manure analysis carried out at the Soil Chemistry Laboratory, Faculty of Agriculture, Sebelas Maret University, Surakarta, Indonesia. Planting carried out in Jumantono experimental field belongs to the Faculty of Agriculture Universitas Sebelas Maret in April to June 2019 for 3 months and harvested.

Herb extract analysis

Extracts of *C. asiatica* herbs by the maceration method using 70% ethanol. *C. asiatica* herbs that have been dried, mashed and placed 10 grams into a bottle. Weighed 10 grams and placed them into a bottle. Samples have homogenized by adding 50 ml of 70% ethanol and macerate for 72 hours. Macerated extracts filtered and placed in Petri dishes and evaporated on a water bath to produce crude extracts. Herb extracts analysis obtain based on the difference value of Petri dish containing *C. asiatica* herb extract and initial weight of the Petri dish, multiplied by 100% (Research and Development Center for Medicinal Plant and Traditional Medicines 2018).

Asiaticoside analysis

Sample preparation

Centella asiatica herb was blended to a powder then weighed as much as 100 mg, put in the bottle. *C. asiatica* extracts as obtained by adding 10 ml 90% ethanol to each bottle, sonicated for 15 minutes and macerated for 24 hours so that the *C. asiatica* extract formed (Research and Development Center for Medicinal Plant and Traditional Medicines 2018).

TLC Analysis

Standard asiaticoside solution weighing 0.5682 mg dissolved in 3 ml of 90% methanol. TLC analysis was done using solution chloroform: methanol: water (65:25:4). The mobile phase solution was poured into the chamber and left overnight. The stationary phase used was the silica gel GF254, which was an aluminum plate. Standard solutions of 1 µL, 2 µL, 3 µL, 4 µL, and 5 µL were consecutively dotted on a silica gel, then followed by bottling a sample solution taken from *C. asiatica* extract, which had been obtained as much as 5 µL. Silica gel was eluted in the chamber then sprayed with Liebermann-Bourchard reagents, then the silica gel was dried. The quality of asiaticoside was seen through the results of visualization under UV light 366 nm and UV-Vis spectrophotometer λ 230 nm (Research and Development Center for Medicinal Plant and Traditional Medicines 2018). The data obtained in the form of retention factor (Rf). The asiaticoside content of each sample calculated by the formula by Fitrianda et al. (2011) below:

$$\text{Content (\%)} = \frac{Au}{Ap} \text{ x } \frac{Cp}{Cu} \text{ x f x } 100$$

Where:
Au : Uptake of the test solution
Ap : Uptake of the comparative solution
Cu : Concentration of the test solution
Cp : Concentration of the comparative solution
F : Dilution factor

Data analysis

Research data variables include leaf morphology, yield extract, and asiaticoside content of *C. asiatica*. Morphological data in the form of qualitative data were analyzed descriptively, while quantitative data were analyzed using SPSS (Statistical Product and Service Solution) version 16.0. The research data were analyzed using the Analysis of Variants (ANOVA) followed by the Duncan Multiple Range Test (DMRT) level of 5%.

RESULTS AND DISCUSSION

The morphological character of *Centella asiatica* L. leaf

Centella has morphologically varied (Nandini 2008; Roskov et al. 2013), including variations in leaf diameter and petiole length. Morphological character analysis carried for three accessions concerning leaf size, petiole length, and color of the petiole. The smallest leaf width and petiole size are at accession 1, followed by accession 2, while accession 3 is the longest and widest leaf. The color of the leaf petiole is purplish-green to reddish-green (Table 1).

The morphological characters of leaf (Figure 1 and Figure 2) from three accessions are kidney shape, green, 2.5-4.2 cm long, 5.3-7.3 cm wide, rounded serrated leaf tip, wavy serrated leaf edge, serrated leaf base, curved leaf base, palmate leaf venation, single leaf, slippy and visible surface texture, thin leaf flesh like paper, and the length of the petiole is 7.3-9.8 cm.

Table 1. Morphological character of 3 accessions from *Centella asiatica* leaf

Character observation	Results of observation		
	Accessions 1	Accessions 2	Accessions 3
Habitus/physique	Herb	Herb	Herb
Leaf morphological character			
Leaf shape	Kidney	Kidney	Kidney
Leaf color	Dark green	Dark green	Dark green
Leaf length	2.5	3	4.2
Leaf width	5.3	5.8	7.3
Leaf tip shape	Rounded serrated	Rounded serrated	Rounded serrated
Leaf edge shape	Wavy serrated	Wavy serrated	Wavy serrated
Leaf base shape	Curved	Curved	Curved
Tipe of leaf venation	Palmate	Palmate	Palmate
Tipe of leaf arrangement	Single	Single	Single
Leaf surface texture	Slippery	Slippery	Slippery
Bottom surface texture	Rough	Rough	Rough
Leaf flesh	Thin like paper	Thin like paper	Thin like paper
Petiole length	7.3	8.5	9.8
Color of the petiole	Purplish green	Reddish green	Reddish green

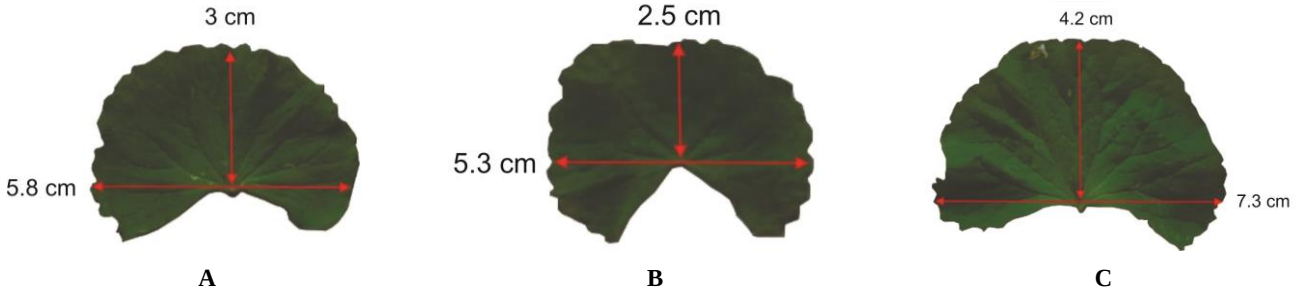

Figure 1. Leaf morphological characteristics of three *Centella asiatica* accessions: A. Accessions 1, B. Accessions 2, C. Accessions 3

Figure 2. Leaves petiole of three *Centella asiatica* accessions; C1: Accession 1, C2: Accession 2, C3: Accession 3. Bar = 1 cm

Herb extract

Ethanol extraction of *C. asiatica* produced a blackish green extract. The analysis results of the extract presented in Table 2. Based on data analysis, there is an interaction between the accession and treatment of chicken manure on the extract. The treatment level of chicken manure addition significantly affected the yield extract of accessions 1 and 3. In accessions 2 the treatment level of chicken manure addition did not significantly affect the yield extract. A greater yield content may not necessarily have a greater active (asiaticoside) ingredient.

Asiaticoside content

Based on the standard of Ministry of Health the Republic of Indonesia (2008), asiaticoside content for medicinal plants is 0.07%. In conditions without fertilization, that is in treatment C11, C2P1, and C3P1, the content of the three *C. asiatica* accessions meet the standards of Indonesian Herbal Farmakope. Accession 1 (C1P1) showed an asiaticoside content 0.15%, accession two (C2P1) was 0.13%, and accession 3 (C3P1) bestowed with 0.19%.

Table 2. Extract yields of *Centella asiatica* from 3 accessions that resulted from nutrient additional treatment

C*/P	P1	P2	P3	P4
C1	1.150 a	1.220 b	1.313 c	1.330 d
C2	1.253 bc	1.356 def	1.393 ef	1.403 f
C3	1.193 ab	1.320 cd	1.513 f	1.520 g

Note: Numbers followed by different letters in the same row show significant differences in DMRT level of 5%. C1: Accession 1, C2: Accession 2, C3: Accession 3, P1: without manure (0 tons ha^{-1}), P2: 10 tons ha^{-1}, P3: 15 tons ha^{-1}, P4: 20 tons ha^{-1}

Table 3. Retention factor of three *Centella asiatica* accessions that resulted under UV light λ366 nm

Sample	Rf
Standard	0.27
C1P1	0.27
C1P2	0.27
C1P3	0.27
C1P4	0.27
C2P1	0.27
C2P2	0.27
C2P3	0.27
C2P4	0.27
C3P1	0.27
C3P2	0.27
C3P3	0.27
C3P4	0.27

Note: C1P1 (Accession 1 without manure), C1P2 (Accession 1 supplemented with chicken manure 10 tons ha^{-1}), C1P3 (Accession 1 supplemented with chicken manure 15 tons ha^{-1}), 4: C1P4 (Accession 1 supplemented with chicken manure 20 tons ha^{-1}), 5: C2P1 (Accession 2 without manure), 6: C2P2 (Accession 2 supplemented with chicken manure 10 tons ha^{-1}), 7: C2P3 (Accession 2 supplemented with chicken manure 15 tons ha^{-1}), 8: C2P4 (Accession 2 supplemented with chicken manure 20 tons ha^{-1}), 9: C3P1 (Accession 3 without manure), 10: C3P2 (Accession 3 supplemented with chicken manure 10 tons ha^{-1}), 11: C3P3 (Accession 3 supplemented with chicken manure 15 tons ha^{-1}), 12: C3P4 (Accession 3 supplemented with chicken manure 20 tons ha^{-1})

Table 4. Asiaticoside content of three *Centella asiatica* accessions that resulted from the treatment of nutrient supplementation

C*/P	P1	P2	P3	P4
C1	0.1567 bc	0.190 cd	0.2333 e	0.3033 f
C2	0.1367 ab	0.110 a	0.1000 a	0.1300 ab
C3	0.1933 cd	0.1900 cd	0.2133 de	0.3400 g

Note: Numbers followed by different letters in the same row show significant differences in DMRT level of 5%. C1: Accession 1, C2: Accession 2, C3: Accession 3, P1: without manure, P2: 10 tons ha^{-1}, P3: 15tons ha^{-1}, P4: 20 tons ha^{-1}

Based on Table 4, there is an interaction between accession with the treatment of chicken manure on the production of asiaticoside content. Significant interaction was shown in accession 1 and 3 with the treatment of chicken manure 20 tons ha^{-1}(P4). In accession 2, the content of asiaticoside did not show significant results. The highest content of asiaticoside was in the accession 3 treated with doses of chicken manure 20 tons ha^{-1}(P4) which was 0.34%. Accession 1 with the treatment of chicken manure dose 20 tons ha^{-1}(P4) showed an asiaticoside content of 0.30%, while accession 2 showed the lowest content compared to two other accessions which were 0.13%.

Discussion

Centella asiatica originates from tropical Asia and is often found in open areas such as fields, paddy fields, road edges, and grasslands (PIER 2014). *C. asiatica* is classified as an annual herbaceous plant and blooms throughout the year. This plant habitat is generally in tropical and sub-tropical regions (USDA-ARS 2014). These plants also include herb plants that grow spread and branch out to form new plants that are clumped (Campbell et al. 1999; Zambesiaca 2014).

Centella asiatica has high morphological variations. In this study, accession 1 has the smallest leaf size with a diameter of 5.3 cm, while accession 2 has wider leaf width than accession 1 with a diameter of 5.8 cm. Accession 3 is a type of gotu kola wide leaf with a diameter of 7.3cm. Besides that, the diversity that can be observed is the color of the petiole. The color of petiole in accession 1 is purplish-green, while accession 2 and 3 with reddish-green petiole. Bernawie and Mardiana (2008) research results that have characterized *C. asiatica* of 16 accessions originating from Sumatra, Java, Bali, and Papua show data that the largest leaf size is CASI 002 of 3.12cm. This shows that the 3 accessions of *C. asiatica* studied have a larger leaf width. In contrast to the results of the study Vinolina et al. (2012) who have conducted a survey and identified the morphology of *C. asiatica* leaves in Sumatra. Data obtained from the 6 accessions were found, has a leaf width between 3-9.2cm. The longest petiole at accession 3in this study is 9.8cm, then accession 2 is 8.5cm and the shortest petiole is accession 1 at 7.8cm. Similar to the results of van Steenis (1997) research states that the petiole of *C. asiatica* length around 5-15cm.

The extract represents the number of bioactive compounds that are soluble in ethanol. The treatment of increasing the dose of chicken manure addition is in line with the increase in the yield of *C. asiatica* herb extract (Armando 2009). Then followed by an analysis of asiaticoside to determine total asiaticoside content at each accession and treatment.

Analysis of *C. asiatica* is important to provide information on the content of asiaticoside. TLC analysis in Centella will contribute to evaluate the quality of medicine compounds (Chaturvedi et al. 2011). The TLC method developed by Bele and Khale (2011), generally uses 2 variables, namely the mobile phase in the form of liquid and the stationary phase using silica gel, cellulose, or alumina. In asiaticoside analysis, a standard solution is used as a comparison with sample measurements.

Based on the results of the visualization, the value of the Retention factor (Rf) obtained. The results of visualization under UV light, the Rf values of all samples were similar to the standard (Table 3). Rf value used as evidence in identifying a compound. If the Rf value of the sample shows the same value as the standard Rf, the compound can be declared to have the same character as the standard (Gritter et al. 1968). Absorbance measurements performed using a UV-Vis spectrophotometer with an absorption level of 230 nm. Chromatogram results from TLC analysis showed peak and area value of the samples. Furthermore, the asiaticoside content of each sample calculated and presented in Table 4.

Tabel 5. Correlation between variables

	Correlation
Asiaticoside	1
Accession	0.075 ns
Chicken manure	0.470**
Yield	0.265 ns

Note: The number followed by the sign (**) is significantly different from the control based on the test results tα = 0.01

Asisticosida is the main compound in Centella which belongs to terpenoids (Chong and Aziz 2011; Hashim 2011). Asiaticoside has a property to accelerate wound healing (Zhao and Li 2018), to improve memory (Prakash 2017) and as an antidepressant (Kalshetty et al. 2012). (Ceremuga et al. 2015) stated that asiaticoside isolated from Centella originating from Sumatra, Indonesia showed significant antidiabetic activity in rats.

Bernawie and Mardiana (2008) have analyzed the content of asiaticoside on 16 accessions of *C. asiatica* originating from Sumatra, Java, Bali, and Papua, with asiaticoside content ranged from 0.15-1.49%. It is different from the results of Vinolina et al. (2013) which has analyzed 6 accessions of *C. asiatica* from various regions in Sumatra. The results of the analysis showed varying levels, which ranged from 0.24-2.38%. The result from this study showed that asiaticoside content on 3 accessions of *C. asiatica* showed varying levels which ranged from 0.10-0.34%.

The treatment of supplementing nutrients in the form of chicken manure increased the content of asiaticoside in Centella. However, the response from each accession in responding to the treatment will show a different result. The result of this study showed that accession 1 and 3 showed positive responses, but not with accession 2. Variation in the morphological and secondary metabolites content caused by genetic in each accession and environmental factors (Yang et al. 2018; Panichayupakaranant et al. (2001)

Chicken manure contains high contents of nitrogen and phosphorus (Singh 2012) which are related to the process of formation of amino acids, carbohydrates, and proteins (Mokhele et al. 2012). Organic manure is material that contains macro and micronutrients. It can use to improving soil organic matter, soil microbes, and soil structure (Prasetya and Anderson 2011).

Based on the results of data analysis, there is a relationship between the content of asiaticoside with the treatment of chicken manure (Table 5). Chicken manure has the highest N and P elements compared to other manure (Singh 2012). Phosphorus is an element that is rich in energy and becomes a constituent of primary and secondary metabolites. The P content greatly influences the formation of asiaticoside. Asiaticoside is synthesized by means of mevalonic acid pathway. Phosphorous elements influence the formation of enzymes that play a role in the formation of precursor compounds of asiaticoside (Thimmappa et al. 2014). Kim (2010) has identified genes that play a role in the biosynthesis of asiaticoside in Centella, one of which is the role of phosporus. Phosphor plays a role in the formation of isoprene units which are the smallest units of asiaticoside, besides that Phosphorus is also part of nucleotides (RNA and DNA), which play a role in photosynthetic reactions and various other metabolisms (McCauley et al. 2017).

There is a positive relationship between asiaticoside content with nitrogen and phosphorus content. Siddiqui et al. (2011) state that the higher nitrogen and phosphorus elements received by Centella, the higher asiaticoside produced. Gusmaini (2016) states that the increase in the availability of P elements to a certain extent will be followed by increasing production of secondary metabolites, especially terpenoids. The results of Vinolina's research (2018) showed that the application of P fertilizer up to a dose of 50 tons ha-1 was able to increase asiaticoside content in *C. asiatica*. Sutardi (2011) and Hartoyo (2012) stated that the addition of P element can increase asiaticoside content in *C. asiatica*.

In conclusion, the diversity of leaves that characterizes on three accessions of *C. asiatica* is the width of leaf and length of petiole. The narrowest leaf and shortest petiole is accession 1, meanwhile, accession 3 has the widest leaf and longest petiole. The content of asiaticoside of three accessions is above the FHI standard. Accession 3 shows the highest asiaticoside content with 0.19%. The chicken manure treatment at 20 ton ha-1 can increase the asiaticoside content in accession 1 (0.30%) and accession 3 (0.34%).

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