Biomass, sinensetin content, and α-glucosidase inhibition activity of *orthosiphon aristatus* were influenced by fertilization and harvest

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Abstract. *Orthosiphon aristatus* has been proved as a diabetic remedy due to its anti-hyperglycemic activity. One of its phenolic compounds, sinensetin, has a capability to inhibit α-glucosidase enzyme activity. As cultivation techniques play roles in the medicinal properties of plants, this study was aimed to determine the best fertilization and harvest method to obtain the high yield of dry leaves, sinensetin content and α-glucosidase inhibition activity. The experiment was conducted at Leuwikopo Experimental Farm IPB University, from October 2014 until April 2015 and used a split-split plot design with three replications. The treatments were the technique of organic fertilizer application (whole and split) as the main plot; harvest interval (3, 5 and 7 weeks) as subplot; and cutting height (10, 20 and 30 cm above ground level) as sub-sub plot. The results showed that the whole application of 10 ton ha⁻¹ organic fertilizer produced the higher weight of *O. aristatus* dry leaves than split application. The crop produced a high weight of dry leaves with the 3- and 5-week harvest interval but produced high sinensetin content with the 7-week harvest interval. *O. aristatus* harvested by cutting at 30 cm above ground level produced the highest weight of dry leaves, sinensetin content and α-glucosidase activity inhibition.

1. Introduction

*Orthosiphon aristatus* is one of Lamiaceae family which spread widely in Africa and Southeast Asia. The dried leaves are usually used as a herbal herb known as Java Tea. It has anti-inflammatory, analgesic, antioxidant, anti-hypertensive, anti-proliferative, anti-sebum, antibacterial, diuretic and anti-obesity activities as well as anti-hyperglycemic activity [1]. Romarinic acid and some phenolic compounds such as sinensetin, eupatorin, 3'-hydroxy-5,6,7,4'tetramethoxyflavone were found in *O. aristatus* leaves [2]. Seven triterpene compounds (ursolic acid, oleanolic acid, betulinic acid, hydroxy botulinic acid, maslinic acid, α-amyrin, β-amyrin) [3] and essential oils were also isolated from its leaves [1].

Sinensetin is one of the abundant phenolic compounds found in *O. aristatus* leaves. It was confirmed that both sinensetin and 50% ethanolic extract of *O. aristatus* leaves can inhibit the activity of α-glucosidase enzyme [4]. *O. aristatus* leaves aqueous extract also can inhibit α-amylase and α-glucosidase enzyme [5]. The α-glucosidase is one of the carbohydrate hydrolyzing enzymes responsible to break down carbohydrate into absorbable monosaccharide. Delaying its activity can reduce postprandial glucose and become a good treatment for people with diabetes type 2 [6].
The quantity and quality of bioactive compounds may be influenced by farming practices, such as fertilization and harvest. As perennial and multi-harvested plant, *O. aristatus* needs special attention for fertilization and harvest management. Organic fertilizer is preferred for medicinal plant cultivation [7] but its slow release character needs to be considered. The dosage and time of fertilizer application for the multi-harvested plants may be different [8] because the ratooning system may need multi-timing fertilization. The right time of medicinal plant harvest is when the accumulation of bioactive compounds is maximum [9]. Phenolic compounds increase at the initial flowering or full flowering [10,11].

The existing cultivation standards of *O. aristatus* in Indonesia are mostly applicable to produce high biomass. On the other hand, a specific cultivation technique is required to grow a medicinal plants that produce high bioactive compounds. The aim of this study was to obtain the best fertilization method, harvest interval and cutting height of *O. aristatus* to obtain a high yield of dry leaves, sinensetin content and α-glucosidase inhibition activity.

2. Materials and Methods

The field experiment was conducted at the rainy season (>200 mm rainfall per month) from November 2014 until April 2015 at Leuwikopo Experimental Farm, Bogor Agricultural University, Indonesia, located at 6.56° S, 106.72° E and 250 m above sea level. Soil pH was 6.90. Nitrogen level, available P2O5 and K2O in the soil were 0.13%, 170 ppm, and 213 ppm, respectively.

Planting material was stem cutting (2 nodes or 10-15 cm long) of white flower variety of *O. aristatus*. Stem cuttings were planted in planting tray filled with a mixture of topsoil, manure and rice husk (1:1:1; v/v/v). Four-week-old seedlings were transplanted into 2.1 m x 1.8 m x 0.25 m field plot with 30 cm x 30 cm spacing. As much as 1-ton dolomite/ha was applied at the same time with fertilization treatment. A full dose (10 t ha⁻¹) of manure was applied as a whole application while only half dose (5 t ha⁻¹) was applied as a split application. The remaining dose (5 t ha⁻¹) was added after the second harvest in each harvest interval treatment by top dressing between plant rows. Weed and pest control were conducted manually if needed. The first harvest was carried out simultaneously at one week after flowering (8 weeks after transplanting/WAT). The next harvests followed the harvest interval schedule as treatments. Fertilization and harvest schedule are shown in (Table 1). The leaves, stems, and flowers were weighed before drying. Fresh leaves were air-dried at room temperature before using an oven to preserve its color. Water content should be no larger than 14%. Observations were done at 23 WAT.

Table 1. Fertilization and harvest schedule of *Orthosiphon aristatus* according to harvest interval

| Year | 2014 | 2015 |
|------|------|------|
| Month| Nov  | Dec  | Jan  | Feb  | Mar  | Apr  | NH |
| HI (weeks) | 0 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 |
| WAT (week after transplanting) | X | X | X | X | X | X | 6 |
| 3 | V | X | X | X | X | X |
| ½V | X | X | ½V | X | X | X |
| 5 | V | X | X | X | X | X | 4 |
| ½V | X | X | ½V | X | X | X |
| 7 | V | X | X | X | X | X | 3 |
| ½V | X | X | ½V | X | X | X |

Note: HI: harvest interval; NH: number of harvest; X: harvest at three cutting heights (10 cm, 20 cm and 30 cm above ground level); V: 10 t ha⁻¹ of organic fertilizer
2.1. Sinensetin Assay

Sinensetin assay was conducted at Biopharmaca Study Center Laboratory, Kencana Park, Bogor, Indonesia. The tested samples were selected from each two significant treatments by dry leaves production due to the limitation during the assay. Dry leaves of *O. aristatus* were ground into powders. The dry leaves were collected from all harvests except the first harvest. Sinensetin assay followed Akowuah *et al.* [2] with moderate modification. One g of each sample (dry weight) was extracted with 100 ml of methanol (Merck) for 4 hours with continuous stirring. The extracts were filtered through filter paper (Whatman No. 1) under vacuum and then evaporated using a rotary evaporator until it remained approximately 5 ml and diluted into 10 ml.

As much as 1 ml of methanol extract was diluted with methanol: distilled water (6:4) until 5 ml and then filtered through Whatman filter paper 0.45 μm. HPLC analysis was performed using a Hitachi UV-VIS L-2420 system equipped with a UV detector. A Linchrocart R125-4 was used as a column. The temperature was maintained at 25°C, with an injection volume of 20 μl volume and flow rate 1 ml min⁻¹. A wavelength of 340 nm was used. Sinensetin marker and samples were separated with methanol: distilled water: tetrahydrofuran (45/50/25; v/v). Sinensetin content was calculated as followed:

\[
\text{content (mg/g)} = \frac{\text{sample area} \times \text{marker area} \times \text{marker concentration (ppm)} \times \text{sample volume (ml)} \times \text{dilution factor}}{\text{sample weight (g)} \times 1000}
\]

2.2. α-glucosidase Assay

The α-glucosidase was carried out at Biochemistry and Chemistry Laboratory, Department of Food Science and Technology, Bogor Agricultural University. Testing for α-glucosidase inhibition activity used the procedure of Phan *et al.* [12] with modification by Widyawati [5]. The ground sample (0.5 g) was extracted with 20 ml of distilled water. Then, the extracts boiled for 5 minutes, cooled, and filtered with filter paper under vacuum. The extracts were centrifuged at 4°C with 2500 rpm for 15 min. Frozen storage was recommended for further use.

The buffer used in this assay was sodium phosphate buffer (0.1 M, pH 6.9). As much as 0.5 unit ml⁻¹ α-glucosidase enzyme (Sigma-Aldrich; catalogue no. G5003) solution was prepared with phosphate buffer. The assay continued with preparation of four mix solutions (blank, control A, control B, and sample solution). The blank solution contained 200 μl buffer, control A contained 100 μl buffer and 100 μl enzyme solution, control B contained 100 μl buffer and 100 μl sample extract solution while sample solution contained 100 μl enzyme and 100 μl sample extract solution. Each solution was pre-incubated at 37°C for 10 min. After pre-incubated, 100 μl of 0.025 M p-nitrophenyl-α-D glucopyranoside (pNPG) (Sigma-Aldrich; catalogue no N1377) in above buffer solution was added to initiate the colorimetric reaction. The solutions were incubated at 37°C for 20 min. As much as 350 μl of 2 M sodium carbonate solution was added into each solution to stop the reactions followed by the addition of 1 ml of distilled water. Absorbance was recorded at 410 nm and the inhibition percentage was calculated as followed:

\[
\text{inhibition (%) } = \frac{\text{(control A – blank)} - \text{(sample – control B)}}{\text{(control A – blank)}}
\]

2.3. Statistical Analysis

Split-split plot design with three replications was used in this study. The main plot was the methods of fertilizer application (whole and split application). Harvest intervals (harvest every 3, 5 and 7 weeks) and cutting heights (10 cm, 20 cm and 30 cm above ground level) were subplot and sub-sub plot, respectively. The observed data were analyzed using Microsoft Excel 2010 and Statistical Analysis System (SAS) v.9 Portable. Analysis of Variance (ANOVA) at *p* ≤ 5% was adopted to check the significant differences of mean values and Duncan Multiple Range Test (DMRT) at *p* ≤ 5% was used to analyze the level of differences.
3. Results and Discussion

3.1. Yield

The increasing dry leaves yield by plant age occurred at three harvest intervals (Figure 1a-1c). The whole application of fertilizer tended to increase 14% higher total dry weight of *O. aristatus* dry leaves than that of split application (Table 2). The results indicated that half dose (5 t ha\(^{-1}\)) of organic fertilizer at the earlier phase cannot fulfil the demand of *O. aristatus* growth.

Table 2. Total fresh and dry leaves, water content and leaf proportion of *Orthosiphon aristatus* as influenced by fertilization methods, harvest interval, and cutting height

| Treatments          | Total fresh leaves (t ha\(^{-1}\)) | Total dry leaves (t ha\(^{-1}\)) | Water content from fresh leaves (%) | Leaves/total biomass proportion (DW) (%) |
|---------------------|-----------------------------------|---------------------------------|-------------------------------------|----------------------------------------|
| **Fertilization (A)** |                                   |                                 |                                     |                                        |
| Whole               | 20.59 a                           | 3.04 A                          | 85.20                               | 63.07 a                                |
| Split               | 17.90 a                           | 2.67 B                          | 84.99                               | 63.92 a                                |
| **Harvest interval (B)** |                                   |                                 |                                     |                                        |
| 3-week              | 20.12 A                           | 2.95 A                          | 85.25                               | 75.26 A                                |
| 5-week              | 19.69 AB                          | 2.98 A                          | 84.84                               | 62.58 B                                |
| 7-week              | 17.93 B                           | 2.62 B                          | 85.20                               | 52.65 C                                |
| **Cutting height (C)** |                                   |                                 |                                     |                                        |
| 10 cm               | 18.32 B                           | 2.64 B                          | 85.49 A                             | 64.44 A                                |
| 20 cm               | 19.19 AB                          | 2.81 B                          | 85.23 A                             | 62.67 B                                |
| 30 cm               | 20.23 A                           | 3.10 A                          | 84.56 B                             | 63.38 AB                               |
| A*B                 | n.s                              | n.s                             | n.s                                 | n.s                                    |
| A*C                 | n.s                              | n.s                             | n.s                                 | n.s                                    |
| B*C                 | n.s                              | n.s                             | n.s                                 | n.s                                    |
| A*B*C               | n.s                              | n.s                             | n.s                                 | n.s                                    |

Note: Total: total yield until 23 weeks-old (3- and 5-week harvest interval) and 22 weeks old (7-week harvest interval); DW: dry weight; Data are the mean, mean separation by DMRT (p < 0.05); n.s: value not different along the column.

Harvest at 10 cm cutting height only resulted in the highest dry leaves yield at first harvest. At the next harvests, 30 cutting height treatment produced higher dry leaves than the two other treatments (Figure 1d-1f). A similar result was also found on *Pennisetum galacum* forage yield [13]. Production of total fresh and dry leaves and organs proportion were also affected by cutting heights (Table 2). The 30 cm cutting height treatment increased total fresh and dry weight, namely 10% and 17% higher than that of 10 cm, respectively. Less of sources, the presence of leafy portion and the active growth condition from leftover parts retarded the growth of the crop for the low cutting height at first harvest [14]. The water content of fresh leaves also influenced the total dry leaves (Table 2).

The average yield of dry leaves in 3, 5-, and 7-week harvest interval treatment were 0.08-0.7, 0.5-1, 0.8-1.3 t ha\(^{-1}\) respectively (Figure 1a-1f). The 3-weeks interval harvest treatment produced least dry leaves at each harvest but produced the highest total dry leaves due to the higher number of harvests (Table 2). More frequent harvests increased the total yield. The same response also found in *Artemisia annua* production [15].
Figure 1. Dry leaves yield in three harvest intervals of Orthosiphon aristatus influenced by fertilization method (a, b, c) and cutting height (d, e, f). ▲ = whole application, ■ = split application, ● = 10 cm, × = 20 cm, ▬ = 10 cm.

The growth stage of plants at harvest time was different among three harvest interval treatments. Plant with 3-week harvest interval was harvested during the vegetative stage while plant with 5-weeks interval treatment was at early inflorescence and those with 7-week harvest interval treatment were at

5-week interval

7-week interval

The growth stage of plants at harvest time was different among three harvest interval treatments. Plant with 3-week harvest interval was harvested during the vegetative stage while plant with 5-weeks interval treatment was at early inflorescence and those with 7-week harvest interval treatment were at
full blooming. Different growth stages were the reason why the organ proportions were significantly affected by harvest interval (Figure 2a). The 3-week harvest interval produced the highest leaves proportion of harvested biomass and the smallest flower proportion. This indicated that intensive harvest could maintain the vegetative stage of *O. aristatus*. The longer harvest interval is conducted, the less leaves and the more branches and flowers are harvested. These results confirmed *Phalaris arundinacea* L. [16]. Roots, leaves and stems or branches are competitive assimilation utilization areas during the vegetative stage, meanwhile, during the generative stage, reproduction utilization areas become stronger and restrict assimilation distribution to the leaves, stems/branches and roots [17].

Leaf proportion from harvested biomass was also significantly influenced by cutting height (Figure 2b). Harvest with 10 cm cutting height resulted in more leaf than 20 cm cutting height but statistically had a similar proportion as of 30 cm cutting height. Meanwhile, the 20 cm cutting height resulted in a higher proportion of branch than the 30 cm cutting height. Characteristic differences of harvested biomass caused these different proportions of organs. The 10 cm height cutting harvested short unbranched and less flowering shoots as resulted in a higher proportion of leaf. The 20 and 30 cm cutting height harvested both branching and flowering shoots, but the leaves of the 20 cm cutting height were less than the leaves of the 30 cm cutting height, therefore the branch proportion was higher with 20 cm cutting height. The shoots harvested from the 30 cm cutting height also had more flowering branches than the 20 cm cutting height.

### 3.2. Sinensetin Content

Sinensetin content in this study ranged 0.06-0.08%, it was assayed with 100% methanolic extract. The previous studies showed that *O. aristatus* had high variation sinensetin content (0.008-2.99%) due to the different assay methods, sample selections, cultivation practices, and accessions or varieties of the crop [2, 18, 19, 20].

Sinensetin content and yield estimation in this study were significantly influenced by all treatments except fertilization method (Table 3). Several researches also demonstrated that fertilization gave various effect on the chemical content of medicinal plant [7]. The longer harvest interval increased sinensetin content. The 7-week harvest interval produces 27% higher sinensetin content than that of the 3-week treatment. The increase in cutting height also increased sinensetin content. This pattern
might be related to the plant stage when harvested. The plant with the 7-week harvest interval and the 30 cm cutting height treatment were in the flowering stage. Some phenolic compounds highly accumulate during the flowering stage [10, 11, 21, 22, 23]. This was also likely related to the proportion of mature leaves. The longer harvest interval increased the proportion of *O. aristatus* mature leaves [24] and the mature leaves of *O. aristatus* contained higher sinensetin content than the young ones [18].

**Table 3.** Sinensetin content, sinensetin yield, α-glucosidase inhibition activity and IC50 of *Orthosiphon aristatus* leaves as influenced by fertilization method, harvest interval, and cutting height

| Treatments | Sinensetin content (%) | Sinensetin yield (kg/ha) | α-glucosidase inhibition activity (%) | IC50 (mg/ml) |
|------------|------------------------|--------------------------|--------------------------------------|-------------|
| **Fertilization (A)** | | | | |
| Whole      | 0.074                  | 2.13                     | 62.86                                | 22.15       |
| Split      | 0.067                  | 1.58                     | 74.30                                | 17.23       |
| **Harvest interval (B)** | | | | |
| 3-week     | 0.062 ^B               | 1.73 ^B                  | 69.70                                | 19.95       |
| 7-week     | 0.079 ^A               | 1.98 ^A                  | 67.46                                | 19.44       |
| **Cutting height (C)** | | | | |
| 10 cm      | 0.060 ^B               | 1.43 ^B                  | 62.40 ^B                             | 21.23       |
| 30 cm      | 0.081 ^A               | 2.28 ^A                  | 74.76 ^A                             | 18.16       |
| A*B        | n.s                    | n.s                      | n.s                                  | n.s         |
| A*C        | n.s                    | < 0.05                   | n.s                                  | n.s         |
| B*C        | n.s                    | n.s                      | n.s                                  | n.s         |
| A*B*C      | n.s                    | n.s                      | n.s                                  | n.s         |

Note: Yield was from multiple harvest within 23 weeks; IC50: concentration of compound that reduced enzyme activity by 50%; Data are the mean, mean separation by DMRT (p < 0.05); n.s: value not different along the column

High biomass content does not always ensure high sinensetin yield. Sinensetin yield involves both sinensetin content and total dry leaves. In this study, the 3-week harvest interval produced higher biomass but contained less sinensetin content than that of the 7-week treatment. The interaction between the fertilization method and cutting height demonstrated that the crop under whole fertilizer application obtained the highest sinensetin yield with 30 cm cutting height treatment.

3.3. α-glucosidase Inhibition

The fertilization method and harvest interval did not affect α-glucosidase inhibition activity in this study but cutting height strongly did. Harvest at 30 cm height above ground resulted in higher α-glucosidase inhibition activity of *O. aristatus*. The higher cutting height left more leftover parts above ground, particularly leaves. It is known that leaves play an important role in photosynthesis and provide more sources in secondary metabolites synthesis. Moreover, leaves also serve as storage organs of those secondary metabolites.

A negative and weak relationship (r = -0.058) was observed between sinensetin content and α-glucosidase inhibition activity in this study although the previous study demonstrated that pure sinensetin (0.31-2.5 mg ml-1) has inhibition activity (32-89%) [4]. This could be due to solvent difference during assay namely methanol for sinensetin assay and water for α-glucosidase activity inhibition assay. In practice, *O. aristatus* is commonly used as an herbal tea or in a simple way, brewing the dried leaves into hot water. This approach was applied in this study. Methanol or ethanol had higher extraction ability than water [25]. It was reported that sinensetin content extracted from *O. aristatus* aqueous extract (0.03%) was smaller than methanolic extract (0.08%) [26]. *O. aristatus* dry leaves extracted with 96% ethanol, 70% ethanol, 50% ethanol and 100% water contained 1.9%, 1.3%, 1.1% and 0.3% sinensetin, respectively [27]. However, aqueous extract of *O. aristatus* in this study (sinensetin content ranged 0.06-0.08%) can inhibit α-glucosidase activity ranged from 62.40-74.76% (Table 3). This likely related to the involvement of other bioactive compounds of *O. aristatus*, mostly
phenolic compounds. Some previous studies reported that phenolic (include flavonoids) compounds also had inhibition role for α-glucosidase activity or provide antidiabetic properties in some plants [28, 29, 30, 31]. The findings from the current experiment showed that further studies are needed to confirm other possible bioactive compounds that play a role in the inhibition activity.

IC50 value of *O. aristatus* with aqueous extract ranged from 17.23-22.15 mg/ml (Table 3). The high IC50 value indicated a low inhibition activity of *O. aristatus*. This value was much higher than IC50 value of acarbose (1.93 mg/ml) and 50% ethanolic extract of *O. aristatus* (4.63 mg/ml) [4] but slightly different compared to the aqueous extract of same *O. aristatus* accession (14.97 mg/ml) in the previous study [5].

4. Conclusion

The fertilization and harvest method influenced the biomass production, sinensetin content and α-glucosidase inhibition activity of *O. aristatus*. In the present study, organic fertilizer application did not significantly affect its medicinal properties, but it affected the dry leaves yield. The whole application (10 t ha⁻¹) of organic fertilizer at transplanting date produced higher dry leaves yield than split application. Results also showed that there were two recommendations for harvest interval of *O. aristatus* : 1) The cultivation of *O.aristatus* emphasizes on biomass production (dry leaves) as herbal tea can harvest the plant shoot every 3 or 5 weeks, but concerning harvest labor cost, the 5-week harvest interval is more recommended. 2) The cultivation of *O.aristatus* emphasizes sinensetin yield (for example at pharmaceutical manufacturing in sinensetin extraction), the 7-week harvest interval is recommended. Meanwhile, in cutting height treatment, the 30 cm above ground level produced not only high biomass and sinensetin yield but also high inhibition of α-glucosidase inhibition activity.

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