Thin Layer Chromatography and Total Flavonoid Contents of Iler Leaves (Plectranthus Scutellarioides) Under Drought Stress Treatment

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Abstract. Iler (Plectranthus scutellarioides) contain secondary metabolites flavonoid that widely used in the treatment of hemorrhoids, antioxidants, and tuberculosis. The objectives of this study were to increase total flavonoid content of Plectranthus scutellarioides treated with several level of drought stress. The study was conducted at the screen house, Jumantono belong to Sebelas Maret University Surakarta, July to October 2018 and flavonoids analysis was carried out at the Center for Research and Development of Traditional Medicinal Plants and Medicines (B2P2TOOT), Central Java. The research method used a completely randomized design with 4 accessions as the first factor (coarse purple, fine purple, green, and red leaves) and drought stress watered once a day, watered every 2 days, watered every 3 days, and watered every 4 days as the second factor. The data obtained were analyzed by analysis of variance (ANOVA) followed by Duncan Test (DMRT) 5%. Total flavonoid content was estimated spectrophotometrically using AlCl3, analysis of TLC was done by using TLC densitometric method. The highest total flavonoid content performed by fine purple leaves and watered once a day with 11.044. Coarse and fine purple leaves formed the more number in TLC with 10 spots that indicates the more active compound formed.

1. Introduction
Natural products derived from plants for the treatment of diseases have proved that nature stands a golden mark to show the relationship between the interrelationship between man and his environment, also had been one of the important sources of remedial agents for thousands of years [1,2]. Iler is a plant that comes from the Lamiaceae family. In Indonesia, this plant is commonly known by the local name Jawer Kotok. In ethnopharmacology it has been used in various treatments such as postpartum, dermatitis, abdominal pain, cough, pain in muscles, asthma, digestive disorders and others. Based on pharmacognostic studies and their traditional uses, P. scutellarioides has therapeutic potential in the development of herbal medicines [3]. Patterns, shapes, and colors Iler varied, but which is efficacious as a medicine are leaves which are brownish red and purplish red. The potentiality of plants to act as
therapeutic agents is based on its phytoconstituents such as phenols, flavonoids, tannins, alkaloids, proteins, amino acids, and steroids [4].

Flavonoids, a group of natural substances with variable phenolic structures, are found in fruits, vegetables, grains, bark, roots, stems, flowers, tea and wine [5], containing two aromatic benzene rings that are connected by 3 carbon atoms, or a phenylbenzopiran (C6-C3-C6). According to previous assessment, approximately 9000 different flavonoids have already been reported from plant sources, but no doubt many more remain to be discovered [6]. Flavonoid known by their antioxidant properties and one of the most important sources for humans is the diet [7] and protect human from many diseases such as cancer, arthritis, emphysema, retinopathy, neurodegenerative cardiovascular diseases, atherosclerosis and cataracts [8].

Drought stress is one of the consequences of climate change that has a negative impact on crop growth and yield [9]. In such cases, plants use various strategies to overcome water deficits in order to survive, divided into two categories: avoiding drought and tolerance to drought [10], depends on drought pressure (intensity and duration) and the ability of plants to carry out molecular, biochemical, and physiological modifications [11]. Antioxidant activity is increasing with the increasing level of drought stress given [12]. In Arabidopsis, flavonoid responses to drought stress are dynamic to different levels of water deficit [13].

Among widely used chromatographic methods modern thin-layer chromatography is not only the simplest to perform but is also considered as respectable analytical method in various phases of drug discovery and development processes such as monitoring of synthesis, identification of bioactive substances from various natural sources and their isolation and purification, determination of lipophilicity and other physico-chemical parameters, quantitative structure-activity relationship studies, bioautography, as well as qualitative and quantitative analysis of drugs and their metabolites [14].

Changes in amount of flavonoids under different drought conditions in Iler (Plectranthus scutellarioides) have not been well investigated. The purpose of this study was to find out whether there was an interaction between the accession treatment with several levels of drought stress on the growth and total flavonoid content of Iler plants also to analyze its TLC profile.

2. Methodology
2.1 Time and location of research
The experimental study was conducted at the screen house, Jumantono belong to Faculty of Agriculture, Sebelas Maret University Surakarta, from July to October 2018 and flavonoids analysis was carried out at the Center for Research and Development of Traditional Medicinal Plants and Medicines (B2P2TOOT), Central Java.

2.2 Materials and equipment
The tools used are 7 liters of plastic bucket polybags, meter, stakes, raffia ropes, lux meters, rotary evaporators, analytical scales, mortars, 0.05 mm sieves, test tubes, Erlenmeyer flasks, kjeldahl flasks, steth, ovens, water baths glass cups, desiccators, distillation machines, digesting machines, dropper pipettes, flacons, small tubes, filter paper, funnels, measuring flasks, Duran bottles, micropipets, blue tips, timers, and a set of UV-Vis spectrophotometer. The materials used are Iler plants, coral reefs (dark brown mediteran), manure, soil samples, concentrated H2SO4, Se, CuSO4, Na2SO4, 4% H3BO4, Conway indicator, HCl, 50% NaOH, methanol, AlCl3 ethanol, acetic acid 95%, liquid paraffin, aquabidest, aquadest and quercetin as a comparison standard.

2.3 Research design
The research design used was a Completely Randomized Design (CRD) consisting of 2 factors. The first factor is accession of Iler (A) plants with 4 levels (A1, A2, A3, A4). The second factor is drought
stress (C) with 4 levels, C1: watered once a day; C2: watered every 2 days; C3: watered every 3 days; C4: watered every 4 days. Each treatment was repeated 5 times each to get 80 experimental units. The data obtained were analyzed by analysis of variance (ANOVA) and followed by Duncan Test (DMRT) 5%.

Accession used is obtained from several location which have been localized in Research and Development Center for Medicinal Plant and Traditional Medicines (B2P2TOOT), Tawangmangu, Central Java. Four accession of Coleus showed in figure 1, 2, 3 and 4 i.e coarse purple leaves (Figure 1), fine purple leaves (Figure 2), green leaves (Figure 3), and red leaves (Figure 4).

Five (5) weeks old seedlings were used and transplanted to screen house belong to Faculty of Agriculture Universitas Maret in Jumantono, Karanganyar, and Central Java. *Coleus* was planted in plastic bucket filled with soil from Karangpandan (soil type: dark brown Mediteran) and cattle manure with composition 2:1 as much as 5 liters. Determination of field capacity (FC) is done to determine the volume of watering that is by the gravimetric method. The various drought stress treatment were watered once a day (C1), watered every 2 days (C2), watered every 3 days (C3), and watered every 4 days (C4). The planting media is doused with water until saturated (initial volume), under the bucket a place is provided to hold water droplets. Then leave it for 24 hours or until the water does not drip, then count the amount of water dripping in the water reservoir (final volume). The difference between the initial volume and the final volume is the amount of water given to plants with 100% field capacity.
2.4 Flavonoid and TLC analysis

Leaves were dried to constant then crushed in mortar as much as 5 gram, put in the flakon. Ethanol to 50 ml were added, then homogeneous shaked, and leave for 3 x 24 hours. Samples were filtered with a funnel and filter paper until no dripping, droplets accommodated in 50ml measuring cup, oven, and let stand 1 x 24 hours. Analysis of TLC was done using n-hexane solution: ethyl acetate (6:4), extract of coleus leaves as much as 2 mg added and stirred until dissolved. Sonification was done for 15 minutes. The solution was taken as much as 2 µ and attached to the plate. The saturated plate was inserted into the chamber. Finally, the observation was done under UV light with λ 366 nm, Rf value of the spot was calculated using the formula Rf = Distance traveled by solute/ Distance traveled by the solvent. Analysis of total flavonoid content was carried out by the AlCl3 method using a UV-Vis spectrophotometer by Lamaison and Carnet with slight modifications. 100 mg of coleus leaf extract was dissolved in 10 ml ethanol, sonified for 15 minutes and deposited overnight. Then the sample was taken as much as 4 ml and then evaporated into the oven at 50°C until dry and dissolved in 8 ml of methanol, then sonified for 15 minutes and deposited overnight. After that, the flavonoid test was carried out with a spectrophotometer using wavelength and operating time which previously obtained with a blank solution containing 0.2 ml of sample and 4.8 ml of aquabidest and the sample solution containing 0.2 ml of sample, 1 ml of AlCl3 and 3.8 ml of aquabidest. Before reading, the solution was incubated for 15 minutes. The average calculation is obtained from three measurements. Total flavonoid levels are stated as equivalent to quercetin (µg / µL), Flavonoid levels can be calculated using the formula: F = C x V x FP x 10^-3 x 100% /M. Description: F: Number of flavonoids in the method of AlCl3, C: Equality Quercetin (µm / mm), V: Total volume of ethanol extract (ml), FP: Dilution factor, M: Sample weight (mg) [15]

3. Results and Discussion

3.1 Dry Matter Distribution of Plants

Plant dry matter reflects the accumulation of organic compounds that have been successfully synthesized by growth from inorganic compounds [16]. Treatment of accession and drought stress had a significant effect on dry matter of plant, but there were no interaction between treatments presented in Table 1 below.

| Accession | Leaves (gr) | Root and stem (gr) | Total |
|-----------|-------------|--------------------|-------|
| 1         | 15.65 a     | 23.00 a            | 38.65 a|
| 2         | 15.50 a     | 25.95 a            | 41.45 a|
| 3         | 12.70 a     | 30.00 a            | 42.70 a|
| 4         | 19.80 b     | 33.00 a            | 52.80 b|
| Drought Stress (%) | Leaves (gr) | Root and stem (gr) | Total |
| C1        | 20.00 b     | 31.40 a            | 51.40 b|
| C2        | 15.90 a     | 29.40 a            | 45.30 a|
| C3        | 14.25 a     | 25.75 a            | 40.00 a|
| C4        | 13.50 a     | 25.40 a            | 38.90 a|

Note: Numbers followed by different letters in the same column showed significant differences in DMRT level 5%.

From table 1 can be seen that dry matter of plants decreases as the drought stress is given. Water deficits affect the partitioning of dry matter to above- and below-ground plant parts [17]. Plants that are tolerant of drought stress can prevent a sharp decline in production and are able to maintain their productivity relatively the same as plants in normal conditions [18]. Research data showed similar pattern with [19] explanation that explained that drought stress affects the growth, dry mater and harvestable yield
in plants, also mention that timing, duration, severity and speed of development undoubtedly have pivotal roles in determining how a plant responds to water deficit. Thus the character of total dry matter can be concluded that accession 4 is the most adaptive accession to drought stress, and watered once a day (C1) yielded the best result.

3.2 Rendemen Percentage

Iler leaves have the potential to be developed as medicinal raw materials, so quality, safety, and usefulness must be improved through research and development. One of the extract quality parameters is the extract yield produced. The rendemen is a comparison of the amount (quantity) of oil produced from the extraction of aromatic plants. The higher the value of the rendemen percentage indicates the value of the extract produced is more and more [20]. There were interaction in rendemen percentage of leaves, with highest value found in accession 1 (coarse purple leaves) and drought stress watered every 3 days with 8.28% as presented in table 2.

Table 2. Rendemen Percentage of Iler leaves

| A*C  | A1     | A2     | A3     | A4     | Average |
|------|--------|--------|--------|--------|---------|
| C1   | 7.04 c  | 7.02 b  | 8.08 d  | 5.1 a  | 6.81    |
| C2   | 7.06 b  | 6.96 a  | 8.04 d  | 7.74 c  | 7.45    |
| C3   | 8.28 d  | 6.96 a  | 8.22 c  | 7.92 b  | 7.845   |
| C4   | 8.06 c  | 8.02 b  | 8.26 d  | 6.04 a  | 7.595   |
| Average | 7.61 | 7.24  | 8.15  | 6.7   |

Note: Numbers followed by different letters in the same column showed significant differences in DMRT level 5%.

The increase in rendemen can be done with two approaches, the cultivation process and in the processing [21]. Based on table 2 above we can see that watered every 3 days for accession 1 can increase the rendemen percentage of Iler leaves. The quality and quantity of rendemen is influenced by the origin / simplisia, one of which is the location of the original plant. Location is an external factor, namely the environment in which plants react can be in the form of energy (weather, temperature, light) and matter (water, organic and inorganic compounds).

3.3 Total flavonoid content

For the analysis of total flavonoids, absorbance measurements were carried out in the standard solution which will be used as a comparison to the measurement of total flavonoids in the sample. The absorbance measurements were carried out using a UV-Vis spectrophotometer with a maximum absorption of 440 nm.

On the measurement of total flavonoid absorptions for determining the quercetin calibration curve at wavelength of 440 nm, the regression equation \( y = 0.0162x + 0.1206 \) is obtained. The standard solution of flavonoids is a linear relationship between absorbance and concentration in absorbance measurements with a correlation coefficient of 0.9943. The value (r) that approaches one indicates that the regression equation is linear.

Interaction also found in total flavonoid content while the best result performed in accession number 2 (fine purple leaves) and stress level 3 (watered every 3 days) with 11.044. The average value of each treatment is presented in table 3.
Table 3. Total Flavonoid Content

| A  | A1      | A2      | A3      | A4      | Average |
|----|---------|---------|---------|---------|---------|
| C1 | 6.255 d | 6.122 c | 2.666 a | 5.101 b | 5.0360  |
| C2 | 6.793 d | 5.558 c | 2.758 a | 4.281 b | 4.8476  |
| C3 | 4.916 c | 11.044 d| 2.942 a | 3.846 b | 5.6871  |
| C4 | 5.187 b | 8.527 c | 2.615 a | 9.454 d | 6.4457  |
| Average | 5.788 | 7.813 | 2.745 | 5.670 |  |  |

Note: Numbers followed by different letters in the same column showed significant differences in DMRT level 5%.

For accession 1, the highest flavonoid compound presented by C2 (watered every 2 days). Accession 2 under mild drought stress (watered every 3 days), the amount of total flavonoids was significantly higher than its amount under control conditions and other stress level, the same as accession 3. For accession 4, the highest flavonoid compound presented by C4 (watered every 4 days). Changes in flavonoid metabolism in response to different drought conditions are dynamic and depend on the intensity and duration of drought stress [22].

3.4 TLC (Thin Layer Chromatography)

Thin layer chromatography (TLC) is one of the easiest and most versatile methods of doing this because of its low cost, simplicity, quick development time, high sensitivity, and good reproducibility. According [23], thin layer chromatography can be used to: Monitor the progress of a reaction, identify compounds present in a given substance, and determine the purity of a substance. The behavior of an individual compound in TLC is characterized by a quantity Known as Rƒ and is expressed as a decimal fraction. The results of TLC (Figure 5) were examined under UV light at the wavelengths by 255 and 366 nm.

![Figure 5. TLC on UV Light with λ 366 nm](image)

The Rƒ is calculated by dividing the distance the compound traveled from the original position by the distance the solvent travelled from the original position (the solvent front). Rƒ values can be used as evidence in identifying compounds. If the identification of the value of Rƒ has the same value then the compound can be said to have the same or similar characteristics to the comparison [24]. The Rƒ value results are shown in table 4 below.
| Rf | Colour | Rf | Colour | Rf | Colour | Rf | Colour |
|----|--------|----|--------|----|--------|----|--------|
| 1.3 | Red | 1.3 | Red | 1.3 | Red | 1.4 | Red |
| 1.5 | Light Red | 1.5 | Light Red | 1.5 | Light Red | 1.6 | Red |
| 2.7 | Black | 2.7 | Black | 2.7 | Black | 2.7 | Black |
| 3.2 | Light Black | 3.2 | Light Black | 3.2 | Light Black | 3.3 | Light Red |
| 4.4 | Light Red | 4.4 | Light Red | 4.4 | Light Red | 4.4 | Light Red |
| 4.9 | Light Red | 4.9 | Light Red | 4.9 | Light Red | 4.9 | Light Red |
| 5.8 | Red | 5.6 | Light Red | 5.6 | Red | 5.6 | Red |
| 6.3 | Red | 6.1 | Light Red | 6.1 | Red | 6.1 | Red |
| 6.9 | Light Red | 6.7 | Light Red | 6.7 | Light Red | 6.7 | Light Red |
| 7.3 | Red | 7.1 | Red | 7.1 | Red | 7.1 | Red |

Table 4. The result of Rf analysis of TLC

The average of spots formed in accession are 10, accession 2 formed 10 number of spots, accession 3 formed 9 number of spots, while accession 4 formed 9 number of spots. The difference in the number of spots formed between accession indicates that iler accession contain different amounts of active
compounds. The same Rf value at each level of drought stress in each accession shows that the application of drought stress to the iler plants does not affect the amount of active ingredients contained in the leaves of the Iler plant. The clearer spot color is formed, it was indicating that the quality of the active compounds contained in the sample is good.

4. Conclusion
The best agronomy properties performed by accession number 4 (red leaves) with average dry matter 52.80 gram and drought stress level 1 (watered once a day) with 51.40 gram. The highest rendemen percentage found in accession number 1 (coarse purple leaves) and drought stress level 3 (watered every 3 days) with 8.28%. The highest total flavonoid content performed by accession number 2 (fine purple leaves) and stress level 3 (watered every 3 days) with 11.044. Accession number 1 (coarse purple leaves) and 2 (fine purple leaves) formed the more number in TLC with 10 spots that indicates the more active compound formed.

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