Separation of Flavonoids in The Extract *Polyalthia longifolia* (Sonn.) Thw. Leaves from Indonesia and The Philippines

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Abstract. *Polyalthia longifolia* (Sonn.) Thw. is a plant that has many benefits on health because every part of this plant contains secondary metabolites. Flavonoid class is one of the secondary metabolite that contained in the leaves. Flavonoids has pharmacological potencies or therapeutic effects. The objective of this research was to observe how many compound of flavonoid from the ethanolic extract *Polyalthia longifolia* (Sonn.) Thw. leaves, that obtained from Indonesia and The Philippines by using Thin Layer Chromatography and Liquid Chromatography-Mass Spectroscopy. Two leaves powder of *Polyalthia longifolia* (Sonn.) Thw. from Indonesia and The Philippines were extracted by using 70% of ethanol, respectively. The dry extract was obtained by rotary evaporation. The flavonoids of both dry extracts that obtained were analyzed by phytochemical assay. The separating of flavonoid class of both extract were analyzed by TLC and LC-MS. Based on the phytochemical screening, both of the ethanol extract of *Polyalthia longifolia* (Sonn.) Thw. from Indonesia and The Philippines showed that positive flavonoids. The result of TLC separation contained of flavonoids based on the yellowish-green, yellow, green, until blue spots under UV exposure. LC-MS separation showed that the both of extracts revealed the presence of flavonoids including to flavonols and cyanidine, with the similar pattern of LC-MS, respectively. The presence of flavonoids were quercetin with RT 5,39 and 5,05, respectively, and molecular weight m/z = 302,5 – 303,5. Rutin showed the single peak with RT 2,61 and 2,62, respectively, and molecular weight m/z = 610,5 – 611,5. Then, leucocyanidin with RT 5,16 and 5,15, and molecular weight m/z = 306,5 – 307,5. Beside that both of extracts also contained analogues compound. In conclusion, the both of ethanolic extract of *Polyalthia longifolia* (Sonn.) Thw. contain flavonoid class such as rutin, quercetin and analogues compounds.

1. Introduction

Glodokan tiang or *Polyalthia longifolia* (Sonn.) Thw. (Family Annonaceae) is a plant that is widely spreaded in tropical and sub-tropical countries in South Asia [1] and Southeast Asia, including Indonesia and the Philippines. This plant has potential as herbal medicines such as antiplasmodial [2], anti-inflammatory [3], antimicrobial [4], antifever, anti-diabetes and anti-hypertension. In addition, this plant is used as ornamental trees that effectively reduce noise pollution [4].
Previous studies reported that this plant contains alkaloids, sesquiterpenes, diterpenoids, flavonoids, and saponins [5]. Based on [6], stem bark also contains proantocyanidin. The content of this secondary metabolite which provides benefits to this plant. Several types of flavonoid compounds are detected in the leaves of this plant, such as quercetin and rutin, and some analog compounds [7]. Flavonoids are a large group of antioxidant compounds named as polyphenols consisting of anthocyanidins, biflavones, catechins, flavanones, flavones, and flavonoids. Quercetin is included in the flavonol compound group, which is distributed with quercetin and glycoside content of approximately 60-75% of the total flavonoids. Flavonoids consist of aromatic compound with antioxidant activity. This activity will prevent body tissue damage on the degenerative disease patient.

Based on the benefit of flavonoid compound, this study will separate the flavonoid compounds from ethanol extract *Polyalthia longifolia* (Sonn.) Thw. leaves from Indonesia and the Philippines. Then, both of extract obtained will analyzed its flavonoid by using Thin layer Chromatography (TLC) and would be confirmed by Liquid Chromatography Mass Spectroscopy (LC-MS).

2. Materials and Methods

2.1. Plant material

The leaves of the plants, especially Indonesia variant, were collected from area Malang, Indonesia at August until September. The dried leaves from The Philippines were deposited at Institute Biosains, University of Brawijaya, Malang, Indonesia. Based on the taxonomical identification, the leaves that obtained were Polyalthia longifolia (Sonn.) Thwaites.

2.2. Sample preparation and extraction

The leaves were air dried and powdered. The extracts of both of powder leaves were prepared by maceration using 70% ethanol for 3 days with occasional stirring, after that the filtrates were obtained by filtration using Whatman filter No. 1, respectively. The ethanol was evaporated by rotary vacuum evaporator at 50 °C to obtain the dry extract. The dry extract were stored at 4°C until use.

2.3. Identification of flavonoids compound

One milliliter of extract 5 % was diluted in the 50% hot methanol 2 mL. Then, it added some magnesium powder and 4-5 drops of HCl. Identification of the flavonoid in the both of extracts were showed red-orange solution.

2.4. Identification of flavonoids by Thin Layer Chromatography (TLC)

The dry extracts were dissolved in 70% ethanol (10 mg/mL), three mobile phases were prepared in the chamber that consist of (I) Toluene : Ethyl Acetate : Formic Acid (5 : 4 : 0.2); (II) ethyl acetate : methanol : water (5 : 1 : 5); (III) n-Butanol : Acetic acid glacial : water (5 : 1 : 4), and those were saturated in 30 min. Thin layer chromatography was performed according [5], with modifications. On 3 x 10 cm plates pre-coated with silica gel G, the samples of extract were spotted on the lower left of the TLC plat. Then, the plats were run one dimensionally in the mobile phases at room temperature. Identification of the flavonoids in the extracts was done under UV light after the application of ammonia.

2.5. Identification of Flavonoids by Liquid Chromatography-Mass Spectroscopy (LC-MS)

PL ethanol extract samples were analyzed using LC-MS to obtain qualitative data. The results of LC-MS obtained by chromatogram and peak molecular weight of compounds contained in both extracts. The operational conditions of LC-MS equipment were adjusted for running. The column used was Hypersil Gold (50 mm x 2.1 mm x 1.9 μm). UHPLC brand ACELLA Thermo Scientific type 1250. Elution was carried out with mobile phase A consisting of 0.1% formic acid in aquabidest and mobile phase B consisting of 0.1% formic acid in acetonitrile, with linear gradient and flow rate of 300 μL/minute. The column was controlled at 30 °C and the autosampler compartment was set for C16. Mass Spectroscopy was used MS/MS Quadrupole Mass Spectrometer TSQ QUANTUM ACCESS MAX from Thermo Finnigan with ESI (Electrospray Ionization). The ionization source controlled by
TSQ Tune software which was operated by Negative ionization mode. The determination of targeted compounds was carried out by the SRM (Selected Reaction Monitoring) method.

3. Results and Discussion
3.1. Sample preparation
The leaves were air dried and powdered to get high surface area, that it can be increasing the powder contact with solvent. Extraction was done through maceration or soaking methods using 70% ethanol. The maceration method was chosen because of the easy and simple tools used. This method can maximize solvent contact and materials and can be used to extract substances that are not heat resistant. The extracts of Indonesian and Filipino PL extract obtained were 26,80 % and 23 %.

3.2. Identification of flavonoids
Identification of flavonoids was done by addition of Hydrochloric acid (HCl) solution and magnesium (Mg) powder to produce a red-orange solution. Based on this analysis, the two ethanol extracts of the leaves of *Polyalthia longifolia* (Sonn.) Thw. contain flavonoids by producing a yellowish-orange solution after adding Mg powder and HCl. The ethanol extract of *Polyalthia longifolia* leaves from two countries contain flavonoids which were have biological activities such as antimicrobial, antiradical and have cytotoxic activity against cancer cells [7].

3.3. Thin Layer Chromatography analysis
Thin-layer chromatography separation was a separation process based on the distribution of active compounds in two phases, the mobile phase (consist of a mixture of solutions that have a similar polarity with the compound to be separated) and the stationary phase was a silica gel coated plate (usually gives luminescence under UV light). The best separation product was showed by spots without tailing and no overlapping spots. Separation of flavonoid compounds using the TLC method was carried out with 3 mobile phases or eluent, and produced spots which can be luminescence under UV (ultraviolet) lamps at wavelengths of 254 nm and 366 nm. Table 1 showed the TLC results of the flavonoid compounds from the ethanol extract of leaves of *Polyalthia longifolia* (Sonn.) Thw from Indonesia and the Philippines.

| Source Plant | Mobile Phases | Spot Assumption | Rf Number | Spot Appearance under UV |
|--------------|---------------|-----------------|-----------|--------------------------|
| **Indonesia** | Ethyl Acetate : Methanol : Water (5 : 1 : 5) | 11 | 7 | 0.05; 0.37; 0.58; 0.80 | Green; Yellow; Yellowish orange; Yellowish green |
| | | | | | |
| The Philippines | | | | | |
| **Indonesia** | Toluene : Ethyl Acetate : Formic Acid (5 : 4 : 1) | 12 | 7 | 0.05; 0.56; 0.82; 0.88 | Yellowish green; Green; Yellow; Yellowish orange; Dark Green under UV |
| The Philippines | | | | | |

Table 1. TLC Separation of Flavonoids Compound.
Based on previous study, flavonoids on the TLC system would show yellowish green [5], dark chocolate under UV$_{254}$ [10], yellow and blue [8]. Besides that, positive flavonoid showed the fluorescence spot yellow, green, and blue [9]. The first eluent (EA: MeOH: W-5: 1: 5) was a polar solvent and it was capable of producing yellowish-green stains under UV$_{366}$. Retention factor (Rf) from P. longifolia leaves ethanol extract from Indonesia and the Philippines were 0.67 and 0.73, respectively, which were assumed to be rutin and analogs. According to Sampath and Vasanthin [5] flavonoid rutin, as standard compound, showed Rf 0.676 with a yellowish-green color under UV lamps. Secondary eluent (Toluene: EA: Acid Format-5: 4: 1) was a polar mobile phase and produces 12 spots from both of P. longifolia leaves ethanol extract, Indonesia and the Philippines. The result of this study was flavonoids spots showed on P. longifolia leaves from the Philippines, that showed spot at Rf 0.66 with a dark color under UV$_{254}$ light. According to [10], flavonoid of quercetin from P. longifolia plants showed at Rf 0.62 with dark brown spot under UV$_{254}$ light. The third eluent (nButOH: AAG: W-4: 1: 5) was a polar solvent that was able to provide good separation of polar compounds in the TLC. According to [8] separation by TLC using this mobile phase would show a clear separation between O-glycosides and C-glycoside flavones that were not hydrolyzed (mid low Rf) and aglycone (high Rf), while the expected color was light brown, bright yellow, and yellow-green. The result of separating using the third eluent showed yellow and green spots that suspected as flavonoids. Figure 1 shows the best separating of the second mobile phase.

| Indonesia      | n-Butanol : Acetic Acid Glacial : Water (4 : 1 : 5) | 6   | 2   | 0.33; 0.76 | Dark green under UV$_{254}$ | Greenish; Yellowish green |
|----------------|-----------------------------------------------|-----|-----|------------|-----------------------------|----------------------------|
| Philippines    |                                               | 7   | 4   | 0.10; 0.51; 0.77; 0.82 | Dark green; Violet; Yellow |

Figure 1. Separating pattern of flavonoid by TLC using Toluene: Ethyl Acetate : Formic Acid (5:4:1). (A) P. longifolia from Indonesia, (B) P. longifolia from the Philippines.

3.4. Liquid Chromatography-Mass Spectroscopy analysis

Liquid Chromatography-Mass Spectroscopy (LC-MS) was an advanced procedure to analysis a compound that confirmed from TLC result. In the LC-MS system, the separated compound, from LC, would go into MS system, then identified based on molecular mass. The molecular mass of flavonoids compound would be identified by ESI/MS, then showed as fragments with m/z (M$^+$). There were six target compounds that detected by LC-MS analysis. Table 2 showed the flavonoid compounds and also analog compounds that was contained in the Polyalthia longifolia leaves ethanol extract from Indonesia and The Philippines based on LC-MS separation.
Table 2. Flavonoid compounds from *Polyalthia longifolia* leaves ethanol extract from Indonesia and The Philippines based on LC-MS.

| Prediction compound                          | Source plant       | M⁺       | Retention time (RT) |
|---------------------------------------------|--------------------|----------|---------------------|
| **Quercetin (C_{15}H_{10}O_{7})**           | Indonesia          | 302,5 – 303,5 | 5,39               |
|                                              | The Philippines    |          | 5,05               |
| **Rutin (C_{27}H_{36}O_{15})**              | Indonesia          | 610,5 – 611,5 | 2,61               |
|                                              | The Philippines    |          | 2,62               |
| **Vicenin-2 (C_{27}H_{36}O_{15})**          | Indonesia          | 595,5 – 596,5 | 2,80               |
|                                              | The Philippines    |          | 2,80               |
| **Quercetin-3-O-glucoside** (C_{12}H_{20}O_{12}) | Indonesia      | 464,5 – 465,5 | 2,69               |
|                                              | The Philippines    |          | 4,88               |
| **Quercetin-O-O-galloyl hexoside (C_{30}H_{30}O_{16})** | Indonesia | 614,15 – 615,5 | 5,57               |
|                                              | The Philippines    |          | 5,57               |
| **Leucocyanidin-(+)2,3,4-cis-3,4,5,7,3’,4’-hexahydroxyl flavan (C_{15}H_{14}O_{7})** | Indonesia | 306,5 – 307,5 | 5,16               |
|                                              | The Philippines    |          | 5,15               |

Based on LC-MS identification, the extract of *P. longifolia* from Indonesia and the Philippines founded quercetin. Based on the table, quercetin was found at m/z 302,5–303,5 as [M+H]^+ and rutin at m/z 610,5 – 611,5 as [M+H]^+. The similar compounds were characterized too from both of extracts. This study showed Vicenin 2, one of 4 analogue compounds of flavonoids at m/z 595,5 – 596,5 [M+H]^+. Quercetin derivative vicenin 2 was showed at m/z 594,8 (M) which usually following molecular peaks at m/z 611,7 [M+O^+], the addition of oxygen ion, and m/z 649,2 with addition three water molecules, [M+3H_{2}O]. Quercetin-3-O-glucoside with m/z 464,5 – 465,5 as [M+H] and Quercetin-O-O-galloyl hexoside with m/z 614,5 – 615,5 as molecular ion, were detected. The molecular ion of Quercetin-O-O-galloyl hexoside was identified as M-31. That was mean MS spectra of those compounds was obtained another fragments which referred as additional of formic acid, elimination of CO_{2}, elimination of hexoside, and additional of acetate ion [10]. Leucocyanidin-(+)2,3,4-cis-3,4,5,7,3’,4’-hexahydroxyl flavan was another compound that inculding to the flavonoid class. Based on the LC-MS study, it was observed at m/z 306,5 – 307,5 as molecular ion [M+H]. Figure 2 showed the structure of flavonoid compounds.

LC-MS analysis usually revealed the identity of compounds based on fragmentation behavior with process of protonation which throughout dehydration, loss or additional of some functional groups, and C-ring fission. Pattern of fragmentation was associated with the applied collision energy. If the collision energy is less than main fragment in the MS, the spectra produced was (M + H)^+. However, by enhancing collision energy, a complete fragmentation of the protonated molecule can be obtained [10].
Figure 2. Structure of Flavonoid Compound. (A) Rutin, (B) Quercetin, (C) Vicenin-2, (D) Quercetin-3-O-glucoside, (E) Quercetin-O-O-galloyl hexoside, (F) Leucocyanidin-(+)2,3,4-cis-3,4,5,7,3’,4’-hexahydroxyl flavan.

4. Conclusion
In conclusion, the ethanol extracts of Polyalthia longifolia (Sonn.) Thwaites. from Indonesia and the Philippines revealed the present of flavonoid compounds. Based on LC-MS analysis, the both of extracts contain of quercetin, rutin, and the analogue compounds such as Vicenin-2, Quercetin-3-O-glucoside, Quercetin-O-O-galloyl hexoside, and Leucocyanidin-(+)2,3,4-cis-3,4,5,7,3’,4’-hexahydroxyl flavan.

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