High performance thin layer chromatography fingerprint analysis of guava (*Psidium guajava*) leaves

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Abstract. High-performance thin layer chromatography (HPTLC) fingerprint analysis is commonly used for quality control of medicinal plants in term of identification and authentication. In this study, we have been developed HPTLC fingerprint analysis for identification of guava (*Psidium guajava*) leaves raw material. A mixture of chloroform, acetone, and formic acid in the ratio 10:2:1 was used as the optimum mobile phase in HPTLC silica plate and with 13 bands were detected. As reference marker we chose gallic acid (R_f = 0.21) and catechin (R_f = 0.11). The two compound were detected as pale black bands at 366 nm after derivatization with sulfuric acid 10% v/v (in methanol) reagent. Validation of the method was met within validation criteria, so the developed method could be used for quality control of guava leaves.

1. Introduction  
Guava (*Psidium guajava*) leaves known medicinal plant especially for diarrhea treatment and widely distributed in Indonesia. Guava leaves are commonly used in Indonesia to treat a cough, toothache, digestive problems, skin problems, laryngitis, anorexia, rheumatic, epilepsy, diabetes, hypertension, etc. [1]. A chemical compound present in plants could have different in the concentration because affected by several factors, such as differences in geographical origin, time of harvest, climate, drying processes, and other factors [2].

The efficacy of medicinal plant is affected by its chemical composition and concentration, so identification of medicinal plant is needed to prevent any chance of substitution or to mix with another plant. As we know, the quality evaluation of the medicinal plants usually based on the detection or quantitation of some active compound or reference marker. Unfortunately, the presence of reference marker did not always guarantee we used the exact medicinal plant. Substitution or mixing with other closely-related medicinal plants could be occurs. Another approach to the development of quality control method of the medicinal plant by using fingerprint analysis [3,4].

Fingerprint analysis is an effective method to control and evaluate the quality of raw materials and herbal medicinal product. This approach is also able to monitor the quality of the medicinal plants because able to show the overall composition of the chemical components and their relative concentrations. HPTLC fingerprint analysis becomes the most widely used for quality control of...
medicinal plants and their related products because of its simplicity and reliability. Also, the result from this method could be directly evaluated by visual inspection [5]. The main application of HPTLC fingerprint analysis is for identification and authentication of medicinal plants [6-8]. In this work, we have developed an HPTLC fingerprint analysis for identification and authentication of guava leaves that could be used as a quality control method of its raw material.

2. Materials and Methods

2.1 Materials
Guava leaves were obtained from Cimanggu and Dramaga, Bogor and also from Conservation and Cultivation Unit of Tropical Biopharmaca Research Center, Bogor Agricultural University, Indonesia. Gallic acid (≥97.5%) and (+)-catechin (99.0%) were purchased from Sigma-Aldrich (Missouri, USA). HPTLC silica gel 60 F$_{254}$ methanol, formic acid, acetone, chloroform, and sulfuric acid were obtained from Merck (Darmstadt, Germany).

2.2 Sample and standards preparation
Dried powder of guava leaves (1 g) was mixed with 10 mL methanol and extracted in an ultrasonic bath for 30 minutes at room temperature, and then the extract was filtered. Gallic acid and catechin solutions were prepared by dissolving 1.25 mg of standards in 5 mL methanol to obtain concentration about 250 µg/mL.

2.3 HPTLC fingerprint analysis
Methanol extract of guava leaves (5 µL) and standards solution (2.5 µL) were applied to HPTLC plate as 8 mm bands, at least 4 mm apart, 10 mm from lower edge and at least 15 mm from left and right edge of the plate using TLC semiautomatic sampler Linomat 5 (CAMAG, Muttenz, Switzerland). The plate was developed to a distance of 8 cm in a 20 cm × 10 cm twin trough chamber (CAMAG, Muttenz, Switzerland). As the optimum mobile phase, we used a mixture of chloroform, acetone, and formic acid in the ratio 10:2:1.

2.4 Derivatization and evaluation
Sulfuric acid 10% (v/v) reagent was prepared by mixing 10 mL sulfuric acid with an ice-cooled of 90 mL methanol. Documentation of developed plate used CAMAG Reprostar 3 (CAMAG, Muttenz, Switzerland) with WinCATS software. Before derivatization, plate was documented and evaluated under UV light at 366 nm. For derivatization, the plate sprayed with reagent then heated at 100 oC for 5 minutes. Derivatized plate was also documented and evaluated under UV 366 nm.

2.5 Method validation
Validation of the HPTLC fingerprint analysis of guava leaves was evaluated following the guidelines described by Reich and Schibli [5] by determining the stability of analyte on the plate, in solution, and during chromatography, the stability of derivatized zones, specificity, repeatability, intermediate precision, and robustness.

3. Results and Discussions

3.1 Method Development

3.1.1 Optimization of mobile phase composition. Optimization of the mobile phase for HPTLC fingerprint analysis of guava leaves as an initial step in the method development began using ten single solvents with different degree of polarity. A mixture of chloroform and acetone was chosen because they gave good separation with the highest number of bands compared to other solvents (data not shown). We added formic acid in the mixture of chloroform and acetone to increase the polarity of the mobile phase to increase the R$_f$ of marker compounds (gallic acid and catechin). The optimum
mobile phase composition was found in the ratio 10:2:1 of chloroform, acetone, and formic acid respectively.

Figure 1 showed the HPTLC fingerprint chromatogram using the optimum mobile phase with detection under 366 nm UV light after sprayed with sulfuric acid 10%. Gallic acid and catechin were separated well and also we obtained another 13 bands in the fingerprint chromatogram. The $R_f$ of gallic acid was 0.21 while catechin was 0.11 and both bands detected as pale black bands.

Figure 1. HPTLC chromatogram of guava leaves extract. Track 1: gallic acid, 2: catechin, 3: guava leaves methanol extract

Validation of the developed method

The developed method was validated by determining the stability of analyte on the plate, in solution, and during chromatography, the stability of derivatized zones, specificity, repeatability, intermediate precision, and robustness. The focus on validation of the developed HPTLC qualitative method for identification of guava leaves was fingerprint profile, such as $R_f$ value and color bands. The results obtained from the validation step are evaluated according to criteria described by Schibli and Reich [5].

3.1.2 Stability. Stability of analyte on the plate, in solution, and during chromatography were evaluated, and the results should not be different in the position and color of bands. Based on the result obtained in Figure 2, the guava leaves extract is stable on the HPTLC plate and in solution because no differences are seen in zone intensity, and there are no zones that appear or disappear. Therefore, the sample is considered stable for at least 3 hours in solution and on the plate.

Stability of the sample extract during chromatography was investigated by 2-D development. If the sample is stable during chromatography, all components should be detected on the diagonal line connecting the application position and the intersection of the two solvent front. Figure 3 showed the 2-D HPTLC chromatogram, and the sample is stable during chromatography because no zones are located off of the diagonal. Therefore, the sample is considered stable during chromatography.

Figure 2. HPTLC chromatogram which showed stability on plate and in solution. Track: 1: first extract applied on plate for 3 hours prior to chromatography, 2 and 4: second extract applied immediately prior to chromatography, 3: first extract 3 hours in solution and applied immediately prior to chromatography
In the stability of derivatization, if there is no significant change in the image within 30 min meaning the derivatization yields are stable. As can be seen in Figure 4, after derivatization, the chromatogram is stable for at least 1 hours.

Figure 3. HPTLC two-dimensional chromatogram of guava leaves extract which showed stability during chromatography.

Figure 4. HPTLC chromatogram of guava leaves extract stable after derivatization at least 1 hours. Track: 1: after 2 minutes, 2: after 5 minutes, 3: after 10 minutes, 4: after 20 minutes, 5: after 30 minutes, 6: after 60 minutes.

3.1.3 Specificity. The number, color, intensity, and position of the zones in the HPTLC fingerprint chromatogram was evaluated for the specificity. A method will be called specific if the sample tested from different location growth gives similar fingerprint profile, and the reference marker is also detected. The developed method provides similar pattern in the HPTLC chromatogram of guava leaves from different location growth only differ in the intensity of the detected bands, but all of the samples contained gallic acid and catechin as the reference marker (Figure 5).

Figure 5. The HPTLC fingerprint profiles of various guava leaves extract from different location growth. Track: 1: gallic acid, 2: catechin, 3: dried guava leaves from Tropical Biopharmaca Research Center, 4: fresh guava leaves from Cimanggu, Bogor 5: fresh guava leaves from Dramaga, Bogor.
3.1.4 **Precision.** Evaluation in the precision (same day) and intermediate precision (three consecutive days) of the HPTLC fingerprint analysis of guava leaves is determined by the repeatability of the selected bands $R_f$ values for each of the three zones on the three plates. We chose gallic acid, catechin and one compound at the $R_f$ 0.75 as the selected bands for the determination of the developed method precision. Variation in the $R_f$ values from the three selected bands are less or equal than 0.02 and 0.05 respectively (Table 1), so the developed method is precise.

| Compounds      | $R_f$ (repetition) | $\Delta R_f$ |
|----------------|---------------------|--------------|
|                | 1st                 | 2nd          | 3rd          |
| Precision      |                     |              |              |
| Catechin       | 0.13                | 0.12         | 0.11         | 0.02         |
| Gallic acid    | 0.21                | 0.20         | 0.19         | 0.02         |
| $X$ compound   | 0.75                | 0.73         | 0.73         | 0.02         |
| Intermediate precision |        |              |              |
| Catechin       | 0.14                | 0.13         | 0.13         | 0.01         |
| Gallic acid    | 0.21                | 0.20         | 0.19         | 0.02         |
| $X$ compound   | 0.81                | 0.81         | 0.76         | 0.05         |

3.1.5 **Robustness.** In this study, we used two criteria for evaluation of the robustness. The $R_f$ values of the reference marker (gallic acid and catechin) bands must lie within the acceptance criteria with differences in the $R_f \leq 0.05$ when we used two type of chamber (twin through and flat bottom) and two different lengths (7 and 8 cm) for developing distance. From the results obtained in Table 2, the differences of the $R_f$ values is more than 0.05 when we used different chamber type; it means the flat-bottom chamber gives different results so this method should only perform using twin trough chamber. Decreasing in the developing distance from 8 cm to 7 cm did not affect so much in the $R_f$ values of the two reference marker (Table 3).

| Compounds      | $R_f$ (chamber type) | $\Delta R_f$ |
|----------------|----------------------|--------------|
|                | Twin-trough          | Flat-bottom  |              |
| Catechin       | 0.11                 | 0.20         | 0.09         |
| Gallic acid    | 0.21                 | 0.35         | 0.14         |

| Compounds      | $R_f$ (developing distance) | $\Delta R_f$ |
|----------------|-----------------------------|--------------|
|                | 8 cm                        | 7 cm         |              |
| Catechin       | 0.11                        | 0.12         | 0.01         |
| Gallic acid    | 0.21                        | 0.22         | 0.01         |

4. **Conclusion**
The optimum mobile phase for HPTLC fingerprint analysis of guava leaves extract has used a mixture of chloroform, acetone, and formic acid in the ratio 10:2:1. Gallic acid and catechin were used as reference marker with the $R_f$ 0.21 and 0.11, respectively. The developed validated HPTLC fingerprint analysis method could be used as identification and authentication method of guava leaves.
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