High perfomance liquid chromatography fingerprint analysis for quality control of brotowali (*Tinospora crispa*)

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**Abstract.** Brotowali (*Tinospora crispa*) is widely used in Indonesia as ingredient of herbal medicine formulation. To ensure the quality, safety, and efficacy of herbal medicine products, its chemical constituents should be continuously evaluated. High performance liquid chromatography (HPLC) fingerprint is one of powerful technique for this quality control process. In this study, HPLC fingerprint analysis method was developed for quality control of brotowali. HPLC analysis was performed in C18 column and detection was performed using photodiode array detector. The optimum mobile phase for brotowali fingerprint was acetonitrile (ACN) and 0.1% formic acid in gradient elution mode at a flow rate of 1 mL/min. The number of peaks detected in HPLC fingerprint of brotowali was 32 peaks and 23 peaks for stems and leaves, respectively. Berberine as marker compound was detected at retention time of 20.525 minutes. Evaluation of analytical performance including precision, reproducibility, and stability prove that this HPLC fingerprint analysis was reliable and could be applied for quality control of brotowali.

1. **Introduction**

Brotowali (*Tinospora crispa*) is a medicinal plant that commonly used as ingredient of antidiabetic formula. This medicinal plant contains several types of secondary metabolites, including quaternary alkaloids [1], diterpenoid lactones, flavanoid glycosides, steroid, sesquiterpenoids, phenolic, and polysaccharide [2]. Those secondary metabolites are expected to be related with pharmacological activities of brotowali as antihyperglycemic [3], antioxidant, antiproliferative [4], antimalarial [5], antimicrobial [6], and anti-inflammatory [7].

The content of chemical constituents, including secondary metabolites, in brotowali could be affected by some factors, such as cultivating area, climate (temperature, humidity, light, and wind), geography, harvest time, processing methods, and storage. Since the secondary metabolites of brotowali (individual or synergistic) expected to be correlated with its pharmacological activities, evaluation of chemical constituents of brotowali could be performed as quality control of brotowali as raw material of herbal medicine.

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The pattern-oriented approach such as fingerprinting is a useful technique for quality control of herbal medicine and its raw materials. A comprehensive profile of all chemical constituents of a herbal medicine could be exhibited in fingerprints profile, make it convenient for identification and authentication of raw materials and herbal products [8,9]. Chromatography fingerprint analysis has been developed as a method of quality control and also recommended by the World Health Organization (WHO), the Food and Drug Administration (FDA), European Medicines Agency (EMA), and the Chinese State Food and Drug Administration (CSFDA) [10]. High performance liquid chromatography (HPLC) is an analytical technique that is widely used for quality control method based on chromatography. HPLC has several advantages in terms of analysis, which is more efficient, high sensitivity, high accuracy, good repeatability, and can identify chemical compounds in a material in all measurements [11].

In this study, HPLC fingerprint analysis of brotowali was developed using reverse phase chromatography system. Berberine was used as marker compound since this compound is one of the important quaternary alkaloids in brotowali. Berberine was reported as inhibitors of acetylcholinesterase [12] and as antidiabetic [13].

2. Research Method

2.1. Material
Brotowali (leaves and stems), binahong (Anredera cordifolia) leaves, green betel (Piper betle) leaves were obtained from Tropical Biopharmaca Research Center garden located at Cikabayan, Bogor, Indonesia. Berberine with the purity of ≥90% (Sigma-Aldrich, Missouri, United States), ethanol, HPLC grade methanol, acetonitrile HPLC grade, formic acid (Merck, Darmstadt, Germany), and aquadest.

2.2. Sample and Standard Preparation
500 mg of dried and powdered sample were sonicated with 5 mL of methanol for 1 hour at room temperature. After filtration through a 0.22 μm membrane filter, the sample solution diluted to 10 mL with methanol before injected into HPLC system. Berberine solution was prepared in methanol at concentration of 100 ppm. In other flask, sample solution was spiked with 1 mL of berberine standard (100 ppm) and diluted to 10 mL with methanol.

2.3. Chromatographic condition
Chromatographic separation was carried out at HPLC Shimadzu LC-20 with photodiode array detector (PDA). A 20 μL of sample solution and the standard was injected into the HPLC system. Acetonitrile (ACN) and formic acid 0.1 % was used as mobile in HPLC analysis.

2.4. Method Validation
Analytical performance of the method for chromatographic fingerprint analysis was evaluated by determining the precision, repeatability, stability, and specificity. The precision, repeatability, and stability are expressed as relative standard deviation (RSD) of the relative retention time (RRT) and relative peak area (RPA).

3. Result and Discussions

3.1. Optimization of HPLC Conditions
Quality control methods based on HPLC fingerprints need to be optimized in order to obtain a good separation between the peaks and to give an informative results. In this study, optimization of HPLC condition was conducted through investigating the influence of mobile phase type, mobile phase composition, and column temperature. Acetonitrile (ACN) and formic acid 0.1 % was used as mobile phase at the flow rate of 1 mL / min. Detection was conducted using photodiode array detector (PDA).
Evaluation of optimum condition of HPLC was performed based on number of peak, resolution (R≥1.5), and signal to noise ratio (S/N≥3).

The optimum chromatographic separation was achieved using linear gradient elution of acetonitrile (ACN) and 0.1% formic acid in water with 5-10% ACN for 0-5 minutes, 10-60% ACN for 5-30 minutes, and 60-95% ACN for 30-55 minutes. Detection wavelength for fingerprint analysis of brotowali was set at 254 nm since most of organic compounds such as aromatic compounds absorbed radiation at this wavelength [8]. Wavelength at 254 nm provided straight baseline and higher sensitivity than other wavelengths. HPLC chromatogram of brotowali stem and leaves at optimum HPLC condition was shown in Figure 1. The optimum of chromatographic conditions produced a peak with the resolution of each peak greater than 1.5 and S/N greater than 3. About 32 peaks for stems and 23 peaks for leaves were detected and could be used as fingerprint of brotowali.

3.2. Method Validation
The precision, repeatability, and stability were expressed as relative standard deviation (RSD) of the relative retention time (RRT) and relative peak area (RPA). The precision was evaluated based on injection precision. The injection precision was determined by five replicate injection of the sample solution in the same day. The repeatability was assessed by analyzing five independently prepared samples of brotowali (stems and leaves). The RSD of RRT and RPA at injection precision test were less than 1.7% and 4.5%, for stems and leaves respectively. Meanwhile, the RSD of RRT and RPA at repeatability were less than 2.6% and 4.6%, for stems and leaves respectively.

The sample stability was determined through injection of a sample solution at 0, 3, 6, and 24 hours after it was prepared. The sample solution was stored in the refrigerator at the temperature of 5-10 °C. The RSD of RRT and RPA in stability test of stems and leaves were less than 5%, indicated that the sample relatively stable for 24 hours storage. Analytical performance of developed HPLC fingerprint analysis of brotowali showed a reliable result, indicated that the proposed method could be used as quality control method of brotowali.

In this study, we also evaluated the specificity of the optimum HPLC fingerprint analysis method. The specificity was evaluated through comparing the HPLC fingerprint profile of brotowali with binahong and green betel leaves. The leaves of those two medicinal plants are almost similar with brotowali leaves. In addition, the dried leaves of each sample were almost indistinguishable in shape and color. The HPLC fingerprint profile of brotowali was clearly distinguishable from HPLC fingerprint profile of those two other leaves (Figure 2). The differences in HPLC fingerprint profile of three samples indicated that the type and content of chemical constituents in each samples were also
different. Accordingly, the developed HPLC fingerprint method was specific for brotowali.

![HPLC chromatogram](image)

**Figure 2.** HPLC chromatogram of brotowali stem (a) and leaves (b), binahong leaves (c), and green betel leaves (d) using the optimum HPLC condition

3.3 Identification of Berberine in Brotowali

Berberine is one of the important quaternary alkaloids in brotowali (Figure 3). Identification of berberine in stems and leaves of brotowali was conducted by using the developed HPLC conditions. Berberine in sample was identified by comparing the retention time of sample and berberine standard. Berberine in standard solution was identified at retention time of 20.525 minutes (Figure 4a). Peak at the same retention time was undetectable in stem and leaves extract chromatogram (Figure 4b and 4d). Peaks at retention time of 20.5 were detected in stem and leaves extract after addition (spike) of berberine standard, indicated that berberine was present in very low concentration in both sample [14].

![Berberine structure](image)

**Figure 3.** Berberine structure
Berberine was reported as anticancer [15], antimalarials [16], antibacterial, antipyretic, anti-inflammatory [17], and antidiabetic [18]. Although berberine has some pharmacological activity, Indonesian Food and Drug Administrator [19] issued regulations on the prohibition of production and circulation of traditional medicines and health supplements containing berberine. Berberine cause irritation of the kidneys and exhibited nephrotoxic effect.

Figure 4. HPLC chromatogram of 100 ppm berberine standard (a), brotowali before (b) and after (c) spike with berberine standard, brotowali leaves before (c) and after (d) spike with berberine standard

Figure 5. HPLC chromatogram of 50% etanol extract of brotowali stem (a), leaves (b), and water extract of brotowali stem (c) and leaves (d)
Berberine which is contained in the brotowali should be eliminated in order to avoid its negative side effect. Choosing the extraction solvent was believed to be one of the most effective effort to eliminate the berberine. In this study, we also evaluated the influence of different extraction solvents on the berberine content in the extracts. Water and 50% of ethanol were used as extraction solvent of brotowali. Each extract was analyzed using optimum HPLC condition. The 50% ethanol extract showed higher berberine peak than water extract (Figure 5) both for stem and leaves, indicated that water was recommended as extraction solvent for brotowali since the extracted berberine content was lower in water extracts than in 50% ethanol extracts.

4. Conclusion
High performance liquid chromatography fingerprint analysis has already developed for quality control of brotowali. The optimum mobile phase for brotowali fingerprint analysis was acetonitrile (ACN) and 0.1% formic acid in gradient elution with 5-10% ACN for 0-5 minutes, 10-60% ACN for 5-30 minutes, and 60-95% ACN for 30-55 minutes and detection was conducted at wavelength of 254 nm. The analytical performance of developed method met the criteria for parameter of precision, repeatability, stability, and specificity. Berberine as marker compound of brotowali was detected at retention time of 20.525 minutes. Water was recommended as extraction solvent for brotowali in order to minimize the berberine content in the extract.

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