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Research Article

CHROMATOGRAPHIC FINGERPRINTING AND CLUSTERING OF *PLANTAGO MAJOR* L. FROM DIFFERENT AREAS IN INDONESIA

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ABSTRACT

Plantago major L. is a ubiquitous herbaceous plant with many medicinal activities which has been extensively used in Indonesian traditional medicine. This plant grows at wide range of environment, has several subspecies and varieties, but could not be distinguished morphologically. It is important to develop an effective method for identification and quality assurance of *P. major*, thus the final product has reproducible quality. In this research, a chromatographic fingerprint method was developed for exploring and establishing the variation of chemical substances among different samples of *P. major* collected from 15 areas in Indonesia. The LC (liquid chromatography) data showed considerable variation of chemicals among *P. major* samples. Three chemo-types were visually developed from the LC profiles. The hierarchical clustering analysis also concluded that the samples were divided into three major clusters. Furthermore, the bio-active marker aucubin in this herb was quantitatively determined by a validated LC analysis. Chemo-type II and III were identified as "compound 1"-rich and aucubin-relatively rich chemo-types. These conclusions provide an important basis to establish good agriculture practice and select geo-authentic crude drug for *P. major* in Indonesia. The validated method was concluded to be suitable for fingerprint analysis for the quality control of *P. major*.

Keywords: *Plantago major*, Chromatographic fingerprinting, Clustering analysis, Aucubin, Column liquid chromatography

INTRODUCTION

Development of herbal-based drugs meet several obstacles, one of them is how to ensure the consistency or uniformity of their chemical contents. The chemical constituents of the herbal medicine products may vary depending on harvest seasons and time, plant origins, drying processes and other factors. Medicinal plants collected from different locations and environment may vary in types and levels of chemical components. Therefore, affect their efficacy.

P. major has several subspecies and varieties which could not be distinguished morphologically because of many intermediate forms. In Indonesia, *P. major* grows at a very wide range of regions, from 0 up to 3,300 m above sea level. However, most of them grow at 700 m above sea level or more[1,2]. Their habitats include grasslands, agricultural areas, sides of roads and river side, forests and others, mainly on open fertile and rather hard land[2,3].

P. major contains various chemical compounds, one of them is iridoid glycosides[4]. Previous chemo-taxonomic study concluded that aucubin (Fig. 1) is a typical iridoid compound found in all genus of Plantago, including *P. major*[5-9]. Aucubin has shown wide pharmacological properties, as: hepatoprotective, anti-toxic, anti-inflammatory, antioxidant, anti-aging, anti-osteoporosis, neurotrophic, removal uric acid and diuretic[10-15].

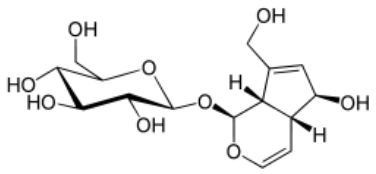

Fig. 1:Chemical structure of aucubin

Adequate identification and quality assurance of crude drug or extract are very important requirement in ensuring the reproducibility of drug. Therefore, support its safety and efficacy. Apart from the extraction process, the quality of extract is determined by the quality of crude drug used. In addition to subspecies and varieties of plants, plant constituents are also influenced by some environmental factors namely: soil (pH, nutrient content and water), climate (temperature, humidity, rainfall, wind speed) and biotic factors (competition with other plants, herbivores and parasites). Previous studies showed that chromatographic fingerprinting analysis is rapid, efficient, and appropriate method for authentication and quality assurance of herbal medicines. This method has been adopted by France, Germany, Britain, India, Japan, WHO, and Chinese SFDA to evaluate the quality of medicinal plants, such as *Centella asiatica, Salvia miltiorrhiza, Crocus sativus*, and *Flemingia philippinensis*[16-20].

In this paper, for the first time an LC method was developed for fingerprinting of *P. major* collected from different locations in Indonesia. In addition, the concentration of aucubin in 15 samples of this plant were compared.

MATERIALS AND METHODS

Plant Materials, Chemicals and Solvents

P. major were collected from 15 different regions in Indonesia (Table 1). Plants were uprooted and washed with tap water. Next, the flowers, petioles and roots were separated from the leaves. A whole plant was left for species determination. Voucher specimens of all the samples, morphologically authenticated by Professor Sutarjadi, were deposited at Center of Information and Development of Herbal Medicine, The University of Surabaya, Indonesia. Then, all of samples were dried (under indirect sunlight) and powdered (Mesh 20) before being stored at desiccator for use.

Aucubin (purity > 98%) was purchased from Fluka (Germany), water was prepared by degassing of water for injection (Ikapharmindo Putramas, Indonesia), acetonitrile and methanol were purchased from Mallinckrodt (USA).

Preparation of Plant Extract

Twenty five mg powder of each sample was extracted twice with 2 ml methanol under ultrasonic for 5 min, respectively. The extracts were adjusted to 5.0 ml with methanol. Finally, the solutions were filtered through a filter membrane (0.45 μm, Whatman) prior to HPLC analysis.

Instrumentation and Chromatographic Conditions

Chromatographic analysis was conducted with a Waters 1525 Binary HPLC Pump including 2 pumps and uv-vis detector. Separation was achieved on chromatography using a eurospher 100-5 C-18, with guard 250 mm x 4.6 mm ID (Knauer GmbH-Germany). The mobile phase consisted of acetonitrile and water (7:93). The flow rate was kept constant at 0.6 mL min^{-1} and the run time was 10 min. The detector wavelength was set at 204 nm and the injection volume was 20 μl.

Table 1:Geographical locations of *P. major* collected from different locations in Indonesia

Location	Population Code	Location (Latitude, Longitude)		Time of Collection
Kediri	KDR	7°48'36"S,	112°00'36"E	August, 2011
Madiun	MDN	7°37'30"S	111°30'54"E	July, 2011
Surabaya	SBY	7°17'24"S	112°43'36.48"E	August, 2011
Mojokerto	MJK	7°40'12"S	112°36'36"E	August 2011
Malang	MLG	7°59'6"S	112°38'6"E	July, 2011
Salatiga	SLTG	7°20'24"S	110°29'42"E	July, 2011
Tegal	TGL	6°20'24"S	109°08'6"E	July, 2011
Purwokerto	PWK	7°26'2.4"S	109°14'57.12"E	July, 2011
Banyumas	BYM	7°31'48"S	109°18'18"E	July, 2011
Cilacap	CLC	7°43'48"S	108°59'24"E	July, 2011
Cakranegara	CKR	8°35'42"S	116°08'42"E	July, 2011
Suranadi	SRN	8°34'S	116°06'E	February, 2012
Bonjeruk	BJR	8°39'28.8"S	116°13'33.6"E	February, 2012
Telaga Waru	TGW	8°37'19.2"S	116°07'4.8"E	February, 2012
Praya	PRY	8°42'S	116°14'E	February, 2012

Precision, Repeatability and Stability

The precision was determined by replicating injection of the same sample (sample KDR) solution for five times. The repeatability test was analyzed by injecting five independently prepared samples (sample KDR). The stability test was determined by four injections with one sample (sample TGW) solution during 24 h. The RSD of relative retention times and relative peak areas of each test were calculated.

Calibration, Linearity, Recovery

Standard stock solution of aucubin was prepared by dissolving certain amount of aucubin in 10 mL methanol to obtain a concentration of 210 mg L^{-1}. The concentrations of aucubin reference standard used for calibration were 2.1, 5.25, 10.5, 26.25, 52.5, 63, 105, 157.5 and 210 mg L^{-1}. Three injections were performed for each dilution. The standard curve was calibrated using the linear regression equation derived from the peak areas.

Recovery was assessed by the method of standard additions. Sample (20, 25, and 30 mg) were spiked by addition of 1.5 ml of stock solution, then extracted, processed, and quantified as described above. Experiment was repeated for three times. Recovery was calculated by comparing the concentration of aucubin resulted from measurement to the actual concentration.

Quantification of Aucubin

The concentrations of aucubin in different samples were calculated according to the regression parameters derived from the standard curve. Each sample was prepared in three replications and each replication was injected in three times.

Data Analysis

Four characteristic peaks in the chromatograms were selected and the peak of aucubin at retention time 4.9 min was used as a reference. Relative retention time (RRT, the ratio between retention time of characteristic peaks to that of reference peak) and relative peak area (RPA, the ratio between peak area of characteristic peaks to that of reference peak) of each characteristic peak to reference were calculated in the chromatograms. The hierarchical clustering analysis (HCA) of 15 samples was performed based on the variation patterns of four chemical constituents of each sample using SPSS 17.0 software (SPSS Inc., USA).

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions

In addition to iridoid glycosides, *P. major* also contains various chemical compounds classified to the group of flavonoids, terpenoids, alkaloids, cafeic acid derivatives, polysaccharides, fats, vitamins and organic acids[4]. Among others iridoid glycosides, aucubin seemed to be the most important iridoid glycosides and could be used as a marker in chemo-taxonomic study of *P. major* species[5-9].

Previous study showed that HPLC and HPTLC were mainly used for determining the aucubin contents in Plantago species[9,21,22]. In this paper, an HPLC metabolite fingerprinting method was used and developed for quality control of *P. major*. Reversed phase liquid chromatography with C18 column and acetonitrile-water mobile phases were chosen to separate *P. major* substances. The optimized mobile phases systems are shown in Table 2. The acetonitrile-water (7:93) system was the final choice. Iridoid glycosides including aucubin are polar compounds and they have good solubility in polar system, hence solution rich in water had better resolution and could improve the peak form of aucubin.

Table 2. The mobile phases were used in optimization

Mobile Phases	Elution Mode
Acetonitrile-methanol (1:99)	Isocratic
Acetonitrile-methanol (2:98)	Isocratic
Acetonitrile-methanol (3:97)	Isocratic
Acetonitrile-methanol (5:95)	Isocratic
Acetonitrile-methanol (10:90)	Isocratic
Acetonitrile-methanol (20:80)	Isocratic
Acetonitrile-methanol (30:70)	Isocratic
Acetonitrile-water (5:95, 20:80, 90:10, 5:95)	Gradient
Acetonitrile-water (7:93)	Isocratic

To gain optimal extract, a preliminary study chose methanol as extraction solvent compared to n-hexane and acetone. In addition, extraction method (ultrasonic) and extraction time (2 x 5 min) was also selected. In the present study, the maximum absorbance of aucubin was observed on the uv-vis spectrophotometer. The detector wavelength of 204 nm was selected, thus more detectable peaks could be observed and the baseline was well improved around 204 nm on the chromatographic profiles.

Method Validation

For chromatographic fingerprinting, we selected aucubin as target compound. Aucubin showed many pharmacological properties[10-15] and easily analyzed[9,21,22]. Therefore, it was predicted as a good analytical marker as well as bio-active marker. Prior to determination of aucubin concentration and chromatographic fingerprinting of *P. major*, the method was validated. The validation parameters consisted of precision, repeatability, stability, calibration, linearity and recovery.

Precision, Repeatability and Stability Test

Precision testing was analyzed by replicate injection of the same sample (sample KDR) solution for five times consecutively in a day. The results are shown in Table 3. The RSD of RRT and RPA did not exceed 0.64 and 6.34%, respectively, which is indicative of the good reproducibility.

Table 3:Results of relative retention times and relative peak areas of precision test, repeatability test and stability test of *P. major* populations from 15 locations in Indonesia

Peak No	Relative Retention Time Mean (RSD%)			Relative Peak Area Mean (RSD%)		
	Rpt	Rrt	Rst	Rpt	Rrt	Rst
1	0.74 (0.23)	0.73 (1.29)	0.71 (1.38)	2.13 (4.84)	2.53 (11.42)	1.44 (2.95)
2 (r)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)
3	1.45 (0.39)	1.45 (9.21)	1.46 (0.56)	0.11 (6.34)	0.10 (4.13)	0.061 (2.84)
4	1.84 (0.64)	1.84 (0.43)	1.87 (0.40)	0.06 (5.78)	0.09 (9.24)	0.37 (4.50)

Rpt, Rrt, and Rst represent the results of relative retention times and relative peak areas of precision test, repeatability test, and stability test on LC fingerprint of *P. major* populations from different locations in Indonesia, respectively

The repeatability was evaluated by analyzing five independently prepared samples (sample KDR, 25 mg) of *P. major*. The results are shown in Table 3. The RSD of RRT and RPA were not more than 9.21 and 11.42%, respectively. These results suggested the method was feasible and rational for four analytes.

The stability of sample was determined by analyzing the same sample (sample TGW) every 6 h on a single day. During this period, the solution was stored in refrigerator. The results are shown in Table 3. The RSD of RRT and RPA were less than 1.38 and 4.50%, respectively. The similarity of these results indicated that the samples were not degraded during this period.

Calibration, Linearity and Recovery

Calibration curve of aucubin was designed from nine different concentrations (x) of aucubin against its peak area (y). Aucubin was accurately measured and dissolved in methanol to create stock solution. This solution then diluted to nine concentrations of aucubin. Calibration curve was conducted with linear regression analysis (Table 4). Plotting for aucubin showed good linearity (r^2 > 0.999) within the test ranges investigated. The limit of detection (LOD = 8.3911 mg L^{-1}) and the limit of quantification (LOQ = 27.9703 mg L^{-1}) were evaluated on the basis of signal-to-noise ratios of 3 and 10, respectively. Moreover, the recovery of aucubin (three independently prepared samples, sample CLC) was 104% and the RSD was 2.88%.

Quantitative Analysis and Chromatographic Fingerprint

The chromatographic fingerprints of *P. major* from different populations showed variation of chemical compounds which were especially in quantification (Fig. 2). One peak was identified by using external standard. In this study, according to the contents and biological activities of major constituents in *P. major*, the peak of aucubin was selected as reference peak. The level of aucubin in different samples varied significantly, from 0.44 to 1.72%, as listed in Table 4. LC fingerprint of *P. major* showed 3-4 peaks which could be selected as marker peaks in the fingerprint. These peaks can serve as characteristic peaks for identification of ''unknown'' samples and can distinguish three chemo-types based on chromatogram profiles.

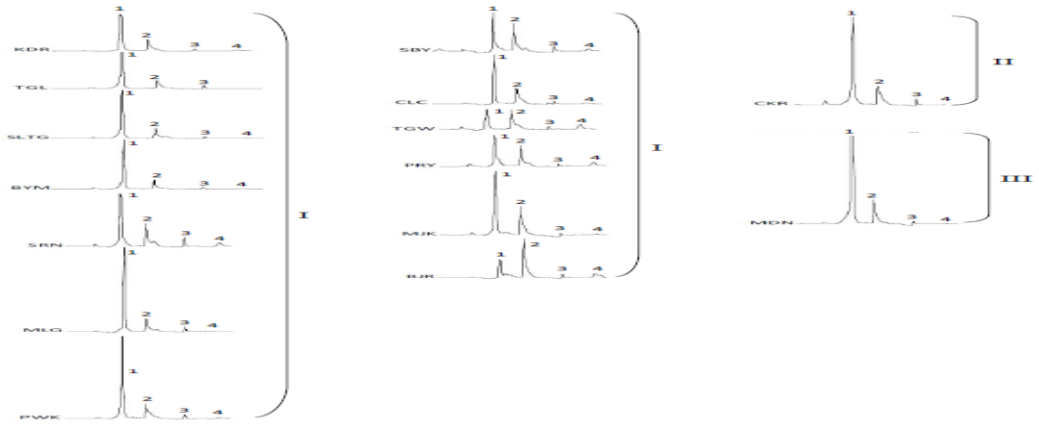

Fig. 2:Original liquid chromatography profiles of methanol extracts, showing three chemotypes identified based on 15 *P. major* samples representing different populations (Peak 2 = aucubin)

Table 4:Contents of aucubin in different *P. major* populations

Region	Population Code	Regression equation[a]	Content (%)[b]
Kediri	KDR	y = 15431.2712 x + 692001.3808	0.70 (8.57)
Madiun	MDN	r^2 = 0.9994	1.16 (1.31)
Surabaya	SBY		1.10 (6.36)
Mojokerto	MJK		1.67 (4.50)
Malang	MLG		0.77 (9.37)
Salatiga	SLTG		1.54 (1.98)
Tegal	TGL		0.59 (9.73)
Purwokerto	PWK		0.61 (8.98)
Banyumas	BYM		0.71 (14.5)
Cilacap	CLC		1.63 (8.31)
Cakranegara	CKR		0.87 (7.68)
Suranadi	SRN		0.44 (5.68)
Bonjeruk	BJR		1.72 (1.21)
Telaga Waru	TGW		0.75 (8.53)
Praya	PRY		1.01 (14.38)

[a] In the regression equation y = bx + a, y refers to the peak area, x the concentration of aucubin (mg L^{-1}), and r^2 is the correlation coefficient of the equation

[b] Measured from 3 dry individuals of *P. major*, members in parenthesis indicate the RSD

The results of hierarchical clustering analysis showed that the samples from various regions could be divided into three groups (Fig. 3). It is important to be noted that the grouping of 15 *P. major* populations was in a good agreement with the visual comparison of their chromatograms, as presented by the chemo-types (Fig. 2). This clustering based on all peaks, not only based on aucubin content. Hence, concentration of aucubin varies in the same group (cluster I). Chemo-type I was identified as "compound 1"-low chemotype. Some of them rich in aucubin content. This chemo-type included all of population except CKR and MDN. Furthermore, chemo-type II (sample CKR) and III (sample MDN) were suggested as "compound 1"-rich and aucubin-relatively rich chemo-types. This indicates that the place of origin significantly influences the content of constituents. This could certainly lead to variation of pharmacological effects. From this finding we could not justified that quality of *P. major* is only influenced by the source of plant since sub species, varieties, and cultivation practices also have possibilities in affecting the quality of plant.

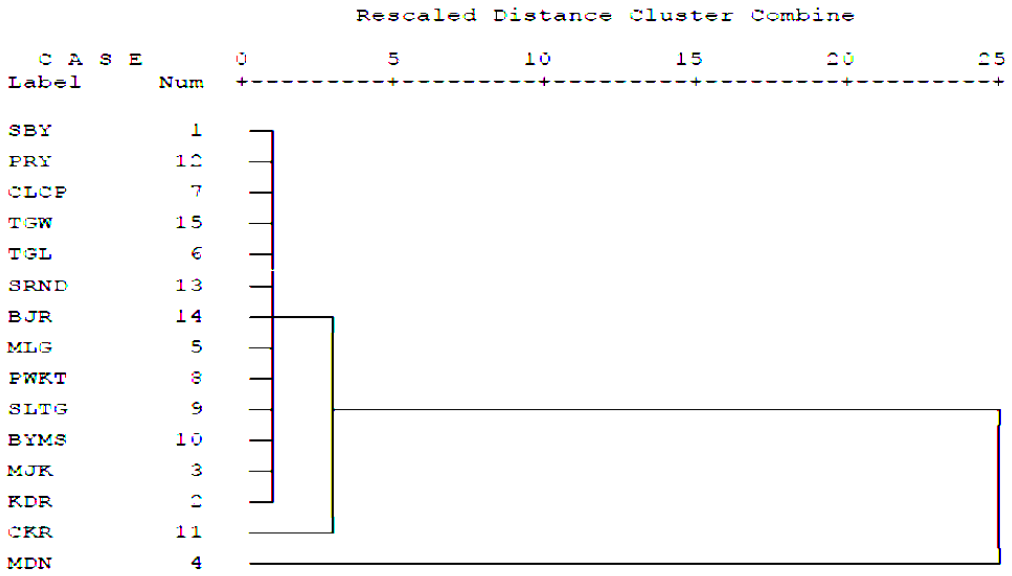

Fig. 3:Hierarchical clustering analysis of 15 *P. major* samples (dendrogram using average linkage between groups) rescaled distance cluster combine

CONCLUSIONS

The bio-active constituent aucubin in *P. major* was quantitatively determined by a validated reverse-phase HPLC analysis. Three chemo-types of *P. major* were visually developed from the chromatographic profiles. The hierarchical clustering analysis further suggested that the samples were divided into three major groups. Chemo-types II and III were suggested as "compound 1"-rich and aucubin-relatively rich chemo-types. These findings provide a solid basis to establish good agriculture practice and select geo-authentic crude drug for *P. major*.

Moreover, the established method was considered suitable for fingerprint analysis to control the quality of *P. major* (RSD of RRT and RPA were not than 15%). This suggested that the method used chromatographic fingerprint combining similarity hierarchical clustering analysis and target peaks quantitative expression may afford consistent discrimination of *P. major* populations based on chemical components profiling as a tool for chemo-taxonomy.

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