Classification of Andrographis paniculata extracts by HPLC fingerprint analysis and chemometrics

CURRENT STATUS: UNDER REVIEW

BMC Research Notes  BMC Series

Mohamad Rafi  mra@apps.ipb.ac.id
Institut Pertanian Bogor

Corresponding Author

ORCID: 0000-0002-5225-8703

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Institut Pertanian Bogor

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DOI: 10.21203/rs.2.18140/v1
SUBJECT AREAS
Forensic Medicine

KEYWORDS
A. paniculata, classification, HPLC fingerprint analysis, principal component analysis
Abstract

Objective: Andrographis paniculata is widely used in Indonesia traditional medicines called jamu as an antidiabetic. The concentration of some chemical compound will be related to the level of therapeutic effect of A. paniculata and the solvent concentration for extraction affects the number of extracted chemical compound. Quality control method is needed to ensure the consistency level of chemical compound in A. paniculata. High-performance liquid chromatography fingerprint analysis combined with chemometrics was used for evaluation of sambiloto extract according to different solvent extraction. In addition, determination of the andrographolide (major bioactive compound in A. paniculata) and α-glucosidase inhibition activity were also performed.

Result: Fingerprint chromatogram of A. paniculata extract with different solvent concentration have similar pattern with several typical peaks appear on each extract, only differ in the peak area and intensity value. Classification of each A. paniculata extract was done by using HPLC fingerprint and principal component analysis. Based on this classification, each extract is grouped in to their respective solvent extraction. The highest andrographolide content and α-glucosidase inhibition activity were in 50% ethanol extract and the lowest were in the water extract. HPLC fingerprint analysis could be used for identification of A. paniculata extract based on solvent extraction.

introduction

Andrographis paniculata is one of the most usable medicinal plant in Indonesia for antidiabetic. This plant known as king of bitter because the taste is very bitter.
Major biological activity of *A. paniculata* is antidiabetic [1] and also reported to have antiangiogenetic [2], antibacterial [3], anticancer [4], antiinflammation [5, 6], antimalaria [7], antioxidant [8], and hepatoprotective activity [9]. As we know, biological activity of medicinal plant like *A. paniculata* comes from its bioactive compound. In *A. paniculata*, diterpene lactone group, such as andrographolide, dehydroandrographolide, neoandrographolide, dan deoxyandrographolide, etc was the major compound class. Andrographolide was found in high content compared to the other diterpene lactone in *A. paniculata* [10]. Flavonoids also present in *A. paniculata* such as andrographidine, apigenin, luteolin etc. [11].

The composition and concentration of the chemical compounds in the plant will be affected by some factors, for example, genetics, environmental growth condition, harvest and postharvest, etc. The type of solvent extraction and its concentration play an important factor in the extraction of a bioactive compound that will be related to the level of biological activity because these differences will affect the amount of extracted bioactive compounds [12].

Quality control of medicinal plant is needed to ensure the consistency of its biological activity and related to its bioactive compound level. There are two approach mainly used for quality control of medicinal plant extract namely marker and fingerprint analysis [13]. The two approach has advantages and could be used in tandem to obtain good evaluation for quality control of medicinal plant. So, in this study we used the two-approach combined with chemometrics for classification of *A. paniculata* extracts.

Several previous studies have reported the *A. paniculata* extract in the composition and levels of chemical compounds and certain biological activities. However, no one has reported for *A. paniculata* extracted with different concentrations of solvents
associated with levels of its marker compounds (andrographolide), chemical fingerprinting and inhibition of α-glucosidase. So, we took this opportunity to investigate the effect of solvent extraction concentration on the chemical compound extracted in A. paniculata using HPLC and inhibition of α-glucosidase.

Materials and methods

Materials and chemicals

*Andrographis paniculata* was collected from *Pusat Studi Biofarmaka Tropika* medicinal plant garden in 2019. Andrographolide (purity > 99.8 %) was obtained from ChromaDex Inc. (Santa Ana, USA), ethanol, acetonitrile HPLC grade, and formic acid were from Merck (Darmstadt, Germany). Filtration of sample solutions was using Whatman membrane filters (0.22 μm pore size; PTFE; P/N E252, Buckinghamshire, England). Alpha-glucosidase and p-nitrophenyl-α-D-glucopyranoside (PNG), phosphate buffer (pH 7), dimethyl sulfoxide (DMSO), and Na₂CO₃ were obtained from Sigma Aldrich (St Louis, USA).

*Sample preparation and extraction*

We used three months old of *A. paniculata* and before the extraction process, the sample was prepared by dry sorting and washed with water to clean the sample. After that, the sample was dried and pulverized. About 10 gram of *A. paniculata* powdered sample was added 100 mL of solvent extraction and soaking it along with continuously stirring for 6 hours and without stirring for 12 hours. The solvents used for extraction were water, ethanol, 30%, 50%, and 70% ethanol. We collected the filtrate, concentrated with a rotary evaporator, and further dried with the freeze-drying process.
**α-glucosidase inhibition activity**

About 10 mg of *A. paniculata* extract was dissolved using 1 mL DMSO. We added 50 µL phosphate buffer 0.1 M (pH 7), 25 µL PNG 10 mM and 25 µL of α-glucosidase (0.04 u/mL) to the 10 µL of sample solution and incubated for 30 minutes at 37°C. The reaction was stopped by adding 100 µL Na₂CO₃ 0.2 M. Enzymatic hydrolysis of the substrate to produce p-nitrophenol was monitored at 410 nm in microplate reader (Epoch-BioTek. Winooski, USA). We also prepared the blank and measured each sample extract in triplicate analysis.

**HPLC Conditions**

HPLC conditions was followed the method developed by Song et al. [10] with some modifications. An HPLC LC-20 AD equipped with a Shimpack VP ODS C18 column (150 nm x 4.6 mm i.d.) (Shimadzu, Kyoto, Japan) was used for the separation of compounds in *A. Paniculata*. The mobile phase used consists of 0.1% formic acid in acetonitrile (A) and 0.2% formic acid in water (B) in gradient elution program of 10-30% (A) for 0-30 min, 30-85% (A) for 30-55 min, and 85% (A) for 55-60 min. The mobile phase was filtered using the Whatman filter membrane 0.45 µm and sonicated for 30 minutes before use. We used the flow rate of 1 mL/min, 20 µL for injection volume, and monitored the separation at 254 nm.

**Preparation of sample solutions**

The sample solution was prepared by weighing 10 mg of dried extract, diluted with 5 mL 50% methanol (HPLC grade), and sonicated for 1 hour. After that, the sample solutions were diluted with 10 mL of 50% methanol and filtered through a 0.45 µm
membrane filter before injected into the HPLC system. Five different sample solutions were prepared and injected to the HPLC.

**Determination of andrographolide**

Andrographolide content was determined in each extract. A series of a standard solution with five concentrations of andrographolide was made in the range of 10-140 μg/mL to construct a calibration curve. Quantification of andrographolide is using the calibration curve with triplicate measurement.

**Classification of A. paniculata extracts**

Principal component analysis (PCA) was used for the classification of A. paniculata extracts. We used the Unscrambler X version 10.1 (CAMO, Oslo, Norway) for running the PCA model using peak area of 8 major peaks in each extract.

**results and discussion**

**Extraction and inhibition of α-glucosidase activity**

The extraction of A. paniculata was done by maceration at room temperature. From the result obtained, the yield of extraction with different concentrations of ethanol and water solvents was different slightly, as shown in Table 1. The highest yield when we extracted A. paniculata using 50% ethanol, while the lowest yield with water indicating that different concentrations of solvent extraction will affect the level of metabolite extracted.

The α-glucosidase inhibitory activity was determined in order to evaluate the effect of different solvent extraction to its biological activity. The assay principle is, α-glucosidase will hydrolyze glucose in the substrate p-nitrophenyl-α-D-
glucopyranoside to α-D-glucose and p-nitrophenol and inhibitory activity was measured based on p-nitrophenol produced. Table 2 showed the α-glucosidase inhibitory activity in each extract with inhibition activity was high in 50% ethanol extract follow by 70%, 30% ethanol, water and ethanol. The result showed that combination of water and ethanol could extract more polar and semipolar compounds that are predicted to have α-glucosidase inhibition activity.

HPLC fingerprint and andrographolide content

Each extract of A. paniculata was analyzed using HPLC to know their differences in the composition of metabolite extracted using different solvent extraction. Figure 1 showed the fingerprint chromatogram of A. Paniculata extracts. About 23 peaks were detected in all extract with the percentage of the area more than 5%. Peak 15 (andrographolide) is the major peak in A. paniculata because the intensity and peak area are highest in all extracts. The fingerprint chromatograms obtained in all samples have a similar pattern with peak number 2, 7, 8, 10, 11, 13, 15, 21 are appear in all sample extracts. The differences mostly come from the peak height and area because each solvent used for extraction have different polarity and ability for extracting the chemical compounds.

Another differences also showed in typical peaks that appear in each extract, such as peak 12 and 22 only appear in ethanol extract. So, this indicates the peak is typical for the fingerprint pattern of the extract. Also, peak 1 appears in 30%, 50% ethanol, and water extracts. It is also can be seen that the more polar extraction solvents will give more detected peaks. We found in Figure 1 water extract gives more detected peaks compared to other extracts. This result is following the previous study that more addition of water in ethanol, which means more polar
solvent extraction, the yield is increased [14].

Andrographolide is one of the primary bioactive compounds present in A. paniculata. We have determined this compound in five extracts used in this study to see which extract has a higher andrographolide level. The andrographolide levels in each sample extract are shown in Table 1. Based on the result obtained, the highest andrographolide levels were found in the 50% ethanol extract, while the lowest level is in the water extract. The andrographolide content was shown in the following order, 50% ethanol > ethanol > 70% ethanol > 30% ethanol > water. These results indicating the amount of andrographolide extracted depends on the polarity of the solvent extraction. As we know from earlier study, andrographolide has a lactone ring and this ring is very vulnerable, reactive and easily rearranged. The opening of the lactone ring in andrographolide is the initial stage to begin the decomposition process. In water, ring opening will happen through hydrolysis, whereas in alcohol will happen through trans-esterification mechanism. The hydrolysis is estimated to be faster than trans-esterification. Therefore, the rate of andrographolide decomposition depends on the type of solvent. According to research conducted by Kumoro et al. [14], the addition of water will lead to the conversion of andrographolide into deoxyandrographolide through the hydrolysis process, so will reduce the andrographolide levels in the sample.

Classification of A. paniculata extract

The HPLC fingerprint chromatogram for A. paniculata extracts used in this work has a similar chromatogram pattern, only differ in the peak height and area correspond to the level of chemical extracted by different solvent extraction. To differentiate based on HPLC fingerprint chromatogram only will not easy, so we need an aid from
chemometrics analysis. We used principal component analysis (PCA) for classification or grouping the extract according to its solvent extraction. The peak area of 8 major peaks (Peak 2, 7, 8, 10, 11, 13, 15, 21) were used as a variable. Before subjected to the PCA, the variable was pretreated using autoscaling method. Pretreatment of the data is an important step before the chemometric analysis to get a good result because the quality of input data greatly affects the quality of the output of the analysis. The common autoscaling method is applied by using standard deviation as a scaling factor and producing good analytical output using PCA chemometric analysis techniques [15].

Samples grouping to its solvent extraction was based on the chemical composition using PCA. This multivariate analysis is work to simplify the observed variables by reducing their dimensions and giving an overview of grouping sample through the principal component (PC) [16]. Figure 2A showed the PCA score plot of the *A. paniculata* extracts. As we can see in the PCA plot, the extracts were grouped according to the solvent extraction. Samples that show the similarity of the profile of the metabolite will be grouped together and the sample that shows the difference will form another group. The two principal components that are most often used in the analysis are the PC1 and PC2. Cumulative percentage of the two PCs used in this study is 89%. According to Varmuza (2001) [17], if the diversity amount of the main components one (PC1) and two (PC2) greater than 70%, the score plot shows good two-dimensional visualization.

PCA biplot is a combination of score plot and loading plot. Loading plot will give us information about how strong each variable affected on the principal component. Figure 2B showed the PCA biplot of *A. paniculata* extract with the most contribution variables affecting its grouping. We found, peak 5 and 7 gives strong contribution
for grouping 50% and 70% ethanol extracts of A. paniculata.

Conclusion

HPLC fingerprint chromatogram of each A. paniculata extract showed similar pattern only differ in the peak height and area of each detected peak. The 50% ethanol extract exhibit higher andrographolide content and percentage of α-glucosidase inhibitory activity compared to the other extract. Combination of HPLC fingerprint with PCA could be classified A. paniculata extract according to its solvent extraction.

limitations

Verification of the modified HPLC method was not performed.

declarations

Abbreviations

HPLC: high-performance liquid chromatography
PCA: principal component analysis
PC: principal component

Ethics approval and consent to participate

There is no human and animal study in this work.

Availability of data and materials

The data could be requested from the corresponding author upon reasonable
request.

**Funding**

This work was supported by *Penelitian Unggulan Perguruan Tinggi* Grant 2018 (No: 1764/IT3.L1/PN/2018) from Ministry of Research, Technology, and Higher Education, Republic of Indonesia and partly supported by the *Riset Kolaborasi Indonesia* Grant 2019 (No: 0847/IT3.L1/PN/2019) World Class University Program of IPB University.

**Acknowledgement**

We would like acknowledged Mrs Laila Wulansari, laboratory technician at the Pusat Studi Biofarmaka Tropika Institut Pertanian Bogor for her contribution in this work.

**Consent for publication**

Not applicable.

**Competing interests**

We declare we don’t have competing interests

**Author’s contribution**

MR, design the research, performed the research, major contributor in writing manuscript

AFD, sample collection, performed the research

UDS, design the research, analyzed the data

RH, performed the research, writing manuscript

IHS, design the research, performed the research
MBA, analyzed the data, writing manuscript

AR, analyzed the data, writing manuscript

BP, writing and checking manuscript

LWL, writing and checking manuscript

All authors have read and approved the final manuscript

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Table 1 Yield, percentage of α-glucosidase inhibitory activity and andrographolide content in *A. paniculata* extract

| Solvent extraction | Percentage of Inhibition (% ± SD)\(^b\) | Andrographolide content (mg/g ± SD)\(^b\) |
|--------------------|------------------------------------------|------------------------------------------|
| Water              | 54.80 ± 4.05                             | 25.18 ± 1.49                             |
| 30% ethanol        | 58.42 ± 2.41                             | 50.29 ± 1.43                             |
| 50% ethanol        | 79.66 ± 6.45                             | 114.56 ± 2.30                            |
| 70% ethanol        | 60.02 ± 0.32                             | 96.48 ± 0.89                             |
| Ethanol p.a.       | 49.58 ± 0.97                             | 102.08 ± 2.73                            |

Average of 2 replication\(^a\), 3 replication\(^b\)

Figures
Figure 1

HPLC Chromatogram of A. paniculata extracts with solvent extraction: (a) ethanol
Figure 2

(A) PCA plot and (B) PCA biplot of A. paniculata extracts; ethanol p.a. ( ), 70% eth