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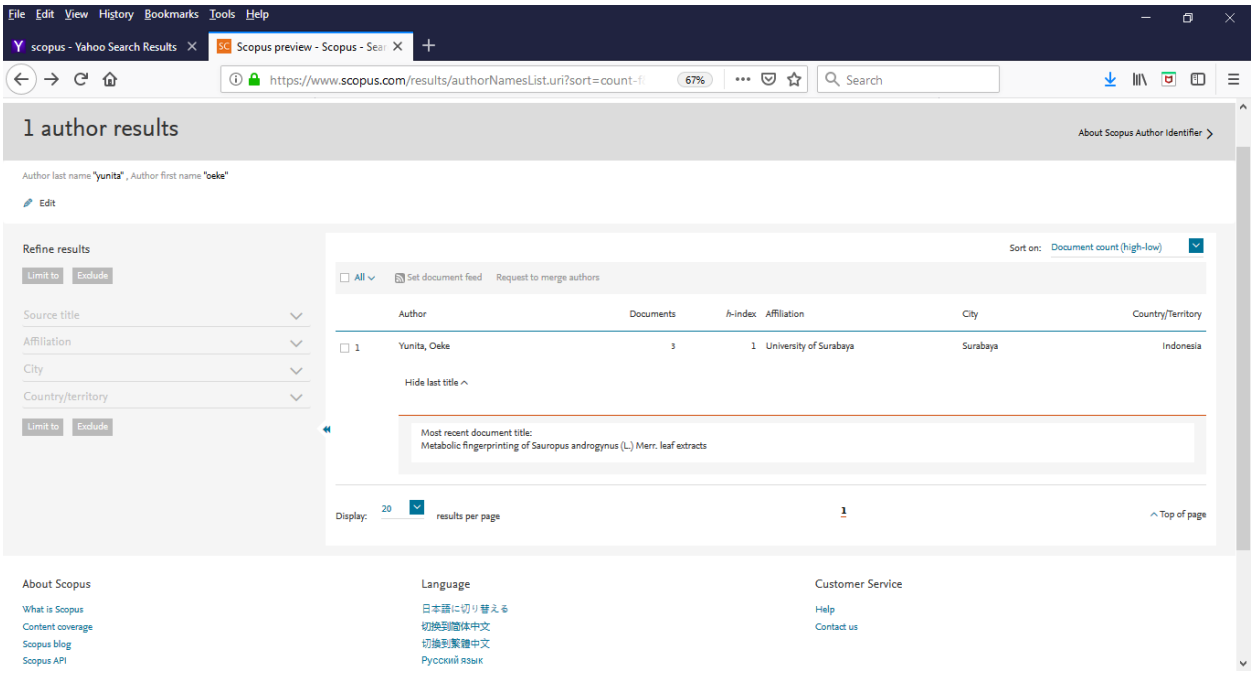

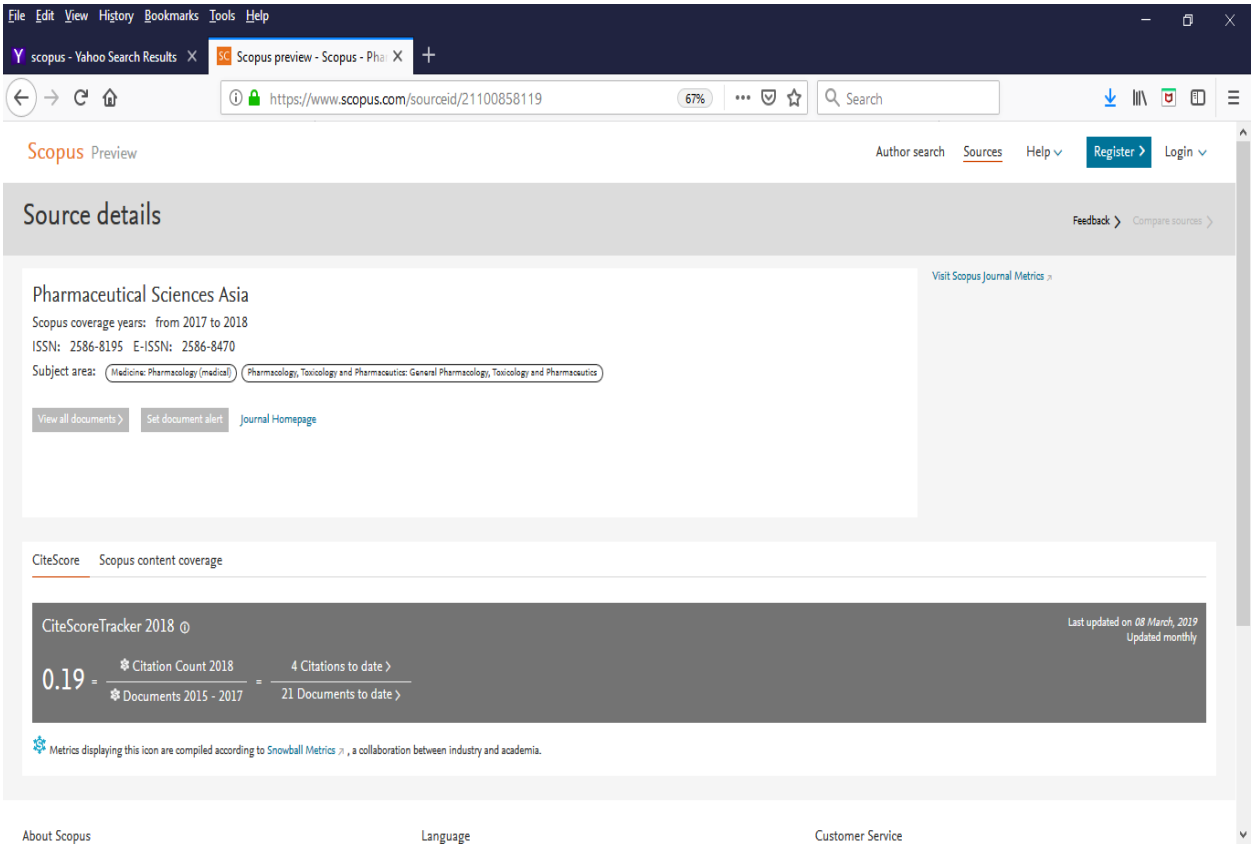

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Research Article

Metabolic fingerprinting of *Sauropus androgynus* (L.) Merr. leaf extracts

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Gas chromatography; Metabolomics; *Sauropus androgynus*

ABSTRACT

The leaves of *Sauropus androgynus* (L.) Merr. have been traditionally used in Malaysia, Thailand and Indonesia as food and herbal product for various medicinal purposes. It is worth noting that different concentrations of metabolites in herbal medicine, cultivated in several different geographic locations, may result in different therapeutic effects. The present study was aimed to identify the metabolic profiles of *S. androgynus* extracts. This is the first report on metabolomic study of *S. androgynus* leaf extracts from six different geographic locations. *S. androgynus* leaf extracts from twelve samples were subjected to study the metabolic fingerprinting using Gas Chromatography - Mass Spectroscopy (GC-MS) method. *S. androgynus* data from various locations were grouped and classified by Principle Component Analysis (PCA) and Cluster Analysis, respectively. The samples from Purwosari alone was merged into one group or a cluster and the other samples were merged into the other cluster, while samples from East Surabaya and Trenggalek showed the close relationship in score plot of PCA and in the dendrogram. Metabolic fingerprinting of *S. androgynus* by GC-MS shows clearly that *S. androgynus* leaf extracts from several various different geographic locations contain many biologically active compounds in various concentrations.

1. INTRODUCTION

Recently, there is an increasing interest in using metabolomic approaches to obtain comprehensive chemical signatures for quality control of herbal medicines due to the challenges and complex interactions between external factors and the herbal's physiological and metabolic systems. Metabolic fingerprinting, as one of major area in metabolomic, is continuously being applied to new areas of research such as drug discovery from natural resources and quality control of herbal material (authentication). This approach could give comprehensive analysis about plant metabolites and discover the differences between samples. Plant metabolites may vary depending on harvest seasons and cultivation sites. The metabolites in a plant species also varies from grower to grower and crop to crop. Metabolic fingerprinting could sort datasets into categories so that conclusions can be drawn about the classification of individual samples[1-3].

Gas Chromatography – Mass Spectrometry (GC-MS) has been described as the gold standard for applied technology in metabolomics, because its application is wide ranging in metabolomics, special for analysing volatile and low molecular weight metabolites. GC-MS is a combined system where volatile and thermally stable compounds are first separated by GC and then eluting compounds are detected traditionally by electron–impact mass spectrometers[1,4]. Metabolic fingerprinting using GC-MS technique was used by several researchers for analysing the metabolic profiles of many plants as raw materials and herbal preparations. Some researcher had developed GC-MS fingerprint method for fresh and injection of *Houttuynia cordata*, respectively[5,6]. Quality of several herbal preparations such as Pu-Erh Green Tea also analysed by GC-MS fingerprint method[7]. The method of GC–MS combined with multivariate analysis was a powerful tool to evaluate the quality of *Cyathula officinalis*[8] and discriminate multi-origin Chinese herbal medicines according to species and medicinal parts, tuberous roots (*Curcumae Radix) and rhizomes (Curcumae Rhizoma and Curcumae* longae Rhizoma) derived from four *Curcuma species (e.g., C. wenyujin, C. kwangsiensis, C. phaeocaulis and C. longa)*, which will be helpful for ensuring their quality, safety and efficacy[9].

S. androgynus is mostly grown in South Asia and Southeast Asia which has antioxidant capacity and nutritive values[10]. It is commonly used in Indonesia as food and herbal supplement for increasing human breast milk production[11,12]. This leaf extract from East Java, Indonesia, was less cytotoxic to human mesenchymal stem cell culture derived from bone marrow with an IC50 of 2450 mg L^{-1} [13] and leaf juice of *S. androgynus* did not change the physical condition of female wistar rats after acute toxicity test[14].

Some analytical method including thin layer chromatographic (TLC)-densitometry, high-performance liquid chromatography (HPLC), *liquid chromatography-mass spectrometry* (LC-MS) and *nuclear magnetic resonance* (NMR) methods were developed for qualitative analysis of metabolites in *S. androgynus*. This plant has various nutritive value and biologically active constituents such as vitamin (α-carotene, β-carotene, vitamin C, vitamin E), phytosteroid, phenolic compound, quercetin and kaempferol[10, 15-21].

S. androgynus from several different geographic locations at East Java, Indonesia were previously distinguished by several DNA-based methods such as random amplified polymorphic DNA (RAPD)[22] and sequencing of internal transcribed spacers (ITS)[23]. The results had shown that these DNA markers are sufficient to distinguish *S. androgynus* from several locations. The obtained results also showed that the ITS sequence provides a good and reliable indicator for geographical origin[23].

The present study was aimed to identify the metabolic profiles of *S. androgynus* extracts from several different geographical locations at East Java, Indonesia for ensuring the quality of plant-based pharmaceutical products generated from this species. To the best of our knowledge, this is the first report showing the metabolomic study of *S. androgynus* from different geographical locations for obtaining new active compounds and identifying correlation between active compounds with the protein target in the systems biology in the future research.

2. MATERIALS AND METHODS

2.1. Materials

The fresh leaves of *S. androgynus* were obtained from six areas with different geographic conditions at East Java Province, Indonesia. All twelve samples were authenticated by the Center of Information and Development of Traditional Medicine, Faculty of Pharmacy, University of Surabaya, East Java, Indonesia. High performance liquid chromatography (HPLC) grade water and methanol were obtained from Merck. All other chemicals were of analytical grade.

2.2. Preparation of methanol extract

Leaves of *S. androgynus* were collected, washed free of dirt, mopped dry and quickly stored at -80ºC until used. Five hundred miligram of mature-leaves were mixed and grounded in a mortar and pestle and 5.0ml of methanol was added. The mixture was homogenized by vortex and sonicated for 20 minute and mixed thoroughly. The mixture was then be centrifuged at 3000g for

1min and filtered. Before being analysed with GC-MS, sample extract was evaporated with nitrogen gas and diluted with methanol.

2.3. GC-MSD conditions

Metabolomic study was performed on an Agilent gas chromatography instrument (Agilent 6890) coupled to a Agilent mass spectrometry detector(5973 network), NIST mass spectrometer database (Wiley, W8N05ST.L) and a GC-MS solution workstation. One micro litre of the methanol extract was injected into a HP-5ms capillary column (30m x 250µm i.d, 0.25µm film thickness; (5%-Phenyl)-methyl polysiloxane; Agilent) in the splitless mode. The injection temperature was set to 270˚C; and the ion source temperature was adjusted to 250˚C; Initial GC oven temperature was 80˚C to 180˚C at a rate of 5˚C min^{-1}, then the GC oven temperature was raised to 250˚C with 4˚C min^{-1} during 28.5min. Helium was the carrier gas with a flow rate set at 1.3ml min^{-1}. The measurements were made with electron impact ionization (70eV) in the full scan mode (m/z 30-550). Blank samples were analyzed in order to detect possible contamination resulting from the reagents, sample preparation, or the instrument. The validation of the analytical method was carried out with sample solutions. The instrument/injection precision (repeatability) was obtained by analyzing the variations of relative retention time and relative peak area of six injections.

2.4. Metabolic Profile Data Processing

Chromatogram acquisition, automated peak deconvolution and library searches were performed using Agilent GC-MS workstation in combination with the Automated Mass Spectral Deconvolution and Identification System (AMDIS 32, version 2.69, 2010). Few modifications on the parameters settings of the AMDIS software were done to permit the best signal/noise ratio. Compounds were tentatively identified by comparing their mass spectra with those of the National Institute of Standards and Technology (NIST) library. The libraries were created after analyses and deconvolutions of the raw data. Each sample was analyzed and the data were save individually as a *.msl library. Thus all samples produced several individual libraries that could be combined and used for statistical analysis.

2.5. Multivariate statistics analysis

All the GC-MSD raw data were exported into Microsoft Office Excel 2007 in a table which contained the resulting three-dimensional matrix involving peak index (RT-m/z pair), sample names (observations) and peak area percent were introduced into The Unscramble software package (Unscramble-X® ver 10.1, CAMO software, AS), which utilized *Principle Component Analysis* (PCA) to display natural separation among the *S. androgynus* samples by visual inspection of 3-D score plots. Furthermore, hierarchical cluster analysis (HCA) was applied in the software package to estimate linkages between different classes within the data set. Euclidean distance on the PCs with average linkage methods was used to derive a similarity matrix, which processed by agglomerative or divisive clustering algorithms to construct a dendrogram.

3. RESULTS

3.1. Sampling

S. androgynus were collected from six areas with different geographic conditions at East Java, such as Surabaya, Trenggalek, Bojonegoro, Purwodadi, Purwosari and Batu, as summarized and described in Table 1 and Fig. 1. All plant samples were collected and authenticated as *S. androgynus*, based on their morphological structures. This research used mature leaves, which were characterized by dark green color, because this leaves contain more secondary metabolites than young leaves, therefore could give more comprehensive analysis about *S. androgynus*. All leaves were harvested in the morning, before 12.00, when the photosynthetic process was still happening. After harvesting, all leaves were transported into the laboratory in the cold condition (-20˚C) for preserving their freshness and keep at -80˚C until used.

Table 1. Sampling Condition of S. androgynus from East Java

LOCATION	SAMPLE CODE	ALTITUDE*) (meter amsl)	TEMPERATURE (˚C)	RELATIVE HUMIDITY (%)
Surabaya	ST	0	29.3-30.0	73-74
	SP	0	25.7-33.7	47-74
	SB	0	27.8-30.5	50-74
Bojonegoro	BJ I	50	24.8-30.0	52-66
	BJ II	50	32.8-33.6	54-55
	BJ III	80	32.1-35.4	48-57
Trenggalek	T I	120	24.1-33.3	31-82
	T II	120	24.1-33.3	31-82
Purwodadi	PWD	320	29.1-33.5	64-73
Purwosari	PWS I	220	29.7-32.3	66-69
	PWS II	240	29.2-33.5	64-73
Batu	B I	840	25.7-33.7	39-74

*) relative altitude, compared to Surabaya

Figure 1. Location of six sampling areas of *S.androgynus* at East Java, Indonesia[24]

3.2. Metabolomic study of S. androgynus methanol extract

Methanol extract of *S. androgynus* leaves was subjected to GC-MSD analysis and the chromatogram was showed in Fig. 2. According to the comparison of the recorded mass spectra with the MS library (NIST), the main peaks of chromatograms were identified about 25 compounds are listed in Table 2., with their relative retention times compared to n-hexadecanoic acid (palmitic acid) as a relatively stable compound with a high area percent.

Table 2. Chemical Compound in *S. androgynus* Methanol Extract (Reliability to MS standard or NET>80)

No	COMPOUND	RRT	No	COMPOUND	RRT
1	Pyrimidine, 4-methyl-	0.09	14	2-furancarboxaldehyde,5-(hydroxymethyl)-	0.31
2	Pyrazine, 2,5-dimethyl-	0.11	15	1,2,3-propanetriol, monoacetate	0.32
3	Butyrolactone	0.11	16	acetophenone, 4'-methoxy-	0.39
4	2,3,4-trimethylpyrrole	0.13	17	methyl tetradecanoate (methyl miristate)	0.79
5	Phenol	0.13	18	tetradecanoic acid (myristic acid)	0.82
6	Pyridine,3,4-dimethyl-	0.14	19	hexadecanoic acid, methyl-ester (methyl palmitate)	0.97
7	Pyridine,3,5-dimethyl-	0.13	20	n-hexadecanoic acid (palmitic acid)	1.00*)
8	benzeneacetaldehyde	0.17	21	9,12-octadecadienoic acid (Z,Z)-,methyl ester (methyl linoleic)	1.12
9	Phenyl ethyl alcohol	0.22	22	Phytol	1.13
10	2,5-dimethyl-4-hydroxy-3 (2H)-furanone	0.18	23	isophytol	1.13
11	2-butanone	0.23	24	octadecanoic acid, methyl ester (methyl stearate)	1.15
12	4H-pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-	0.24	25	α-tokoferol (vitamin E)	2.15
13	benzofuran,2,3 -dihydro-	0.30			

RRT = relative retention time, compared to palmitic acid

*) compound used as reference standard

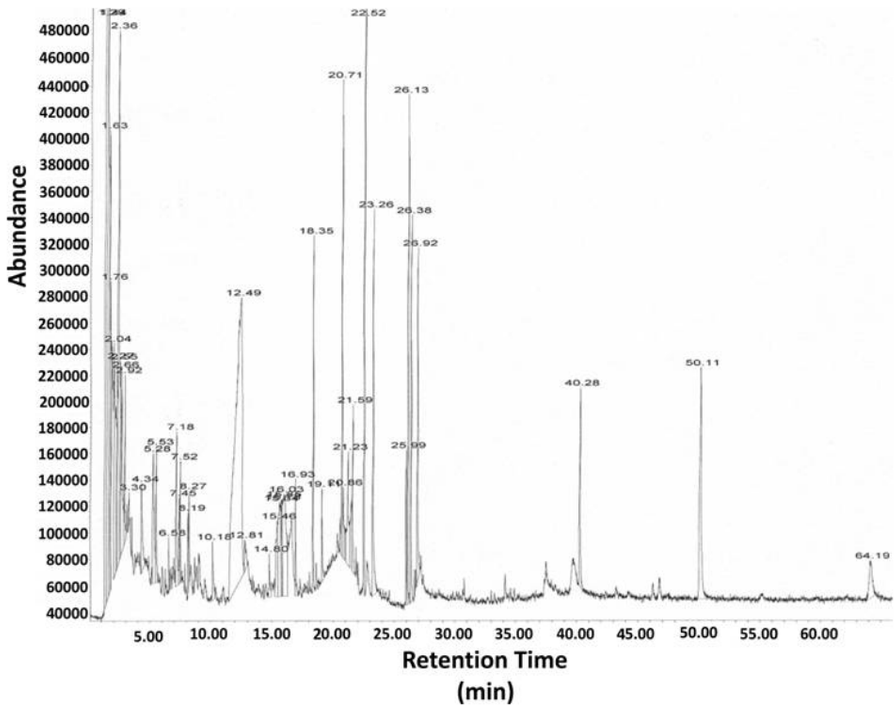

Figure 2. Total ionic chromatogram (GC-MSD) of *S. androgynus* methanol extract obtained with 70 eV using a HP-5MS column (30m x 0.25mm) with He gas as the carrier at a flow rate of 1.3ml min^{-1}

The largest part of the metabolic profile of *S. androgynus* was composed of fatty acids and their esters, such as myristic acid, palmitic acid, methyl-linoleic, methyl-stearic. Other major components such as phytol and isophytol were also found in the *S. androgynus* methanol extract.

Reproducible chromatographic profile of *S. androgynus* could be showed by conducting a method validation on the developed GC-MS analysis. Method precision was investigated by repeatedly analyzing the same set of samples, with the values of relative standard deviations (RSDs) for relative retention time (RRT) and relative peak area (RPA), respectively, reported less than 0.1% and 20% (n=6). These results indicate that the method was reliable and applicable to the analysis of metabolic profile of *S. androgynus*.

Typical GC-MS chromatograms of twelve samples from different six areas at East Java were illustrated in Fig 3. The total ion chromatograms (TIC) of *S. androgynus* samples are showed in this figure, which the x-axis indicates time and the y-axis indicates the total ion signals. Single mass spectra had been obtained from every time window and all signals were plotted as a function of time. Visual inspection of these spectra indicated that their chromatographic patterns were generally consistent to one another, although there are some variations in peak abundance because the raw materials were originated from the same species.

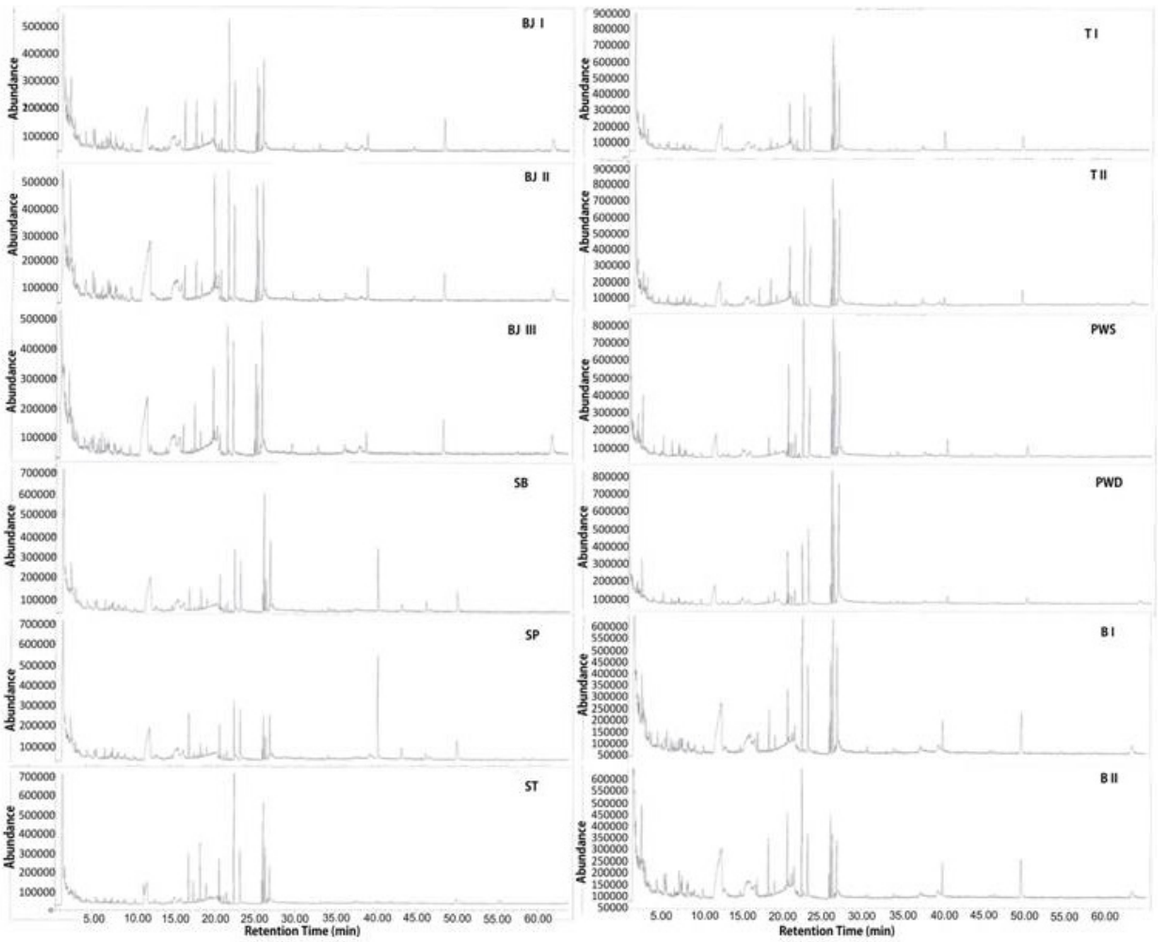

Figure 3. GC-MS metabolic profile of 12 samples of *S. androgynus* from East java (B) Batu, (BJ) Bojonegoro, (PWD) Purwodadi, (PWS) Puwosari, (SB) West Surabaya, (SP) Center Surabaya, (ST) East Surabaya

The output data set was organized in a three-dimensional matrix encompassing relative retention times, sample locations (observations) and peak area percentage (variables). Raw data file from the chromatogram could be read with AMDIS and based on user selected parameters, performed the deconvolution of peaks based on the MS data. The analyses revealed that although some components could be identified in several samples, the majority of the products were present in only few ones, resulting in some degree of uniqueness of the profiles, allowing for their distinction.

The quantitative data of GC-MS analyses were exported to the software Unscramble-X® ver 10.1. The data set was pre-processed by standardization to give all variables the same variance. Then PCA was used to transform the original measurement variables into new variables called Principle Components (PC). The scores scatter plot of the first three PCs calculated using all integrated peaks was shown in Fig. 4. Several samples are noted in five clusters in 3-D score plot, while this movable modelling could show clearly the relationship among the samples in each cluster better than in 2-D score plot between PC 1 and PC 2. It was noticeable that the samples were clustered in different domains, which represented the similarities and differences of different source of samples. The PC scores were used to identify the geographical origin of samples. *S. androgynus* from lowland area, such as Surabaya and Bojonegoro were grouped in one cluster, except sample from East Surabaya (ST).

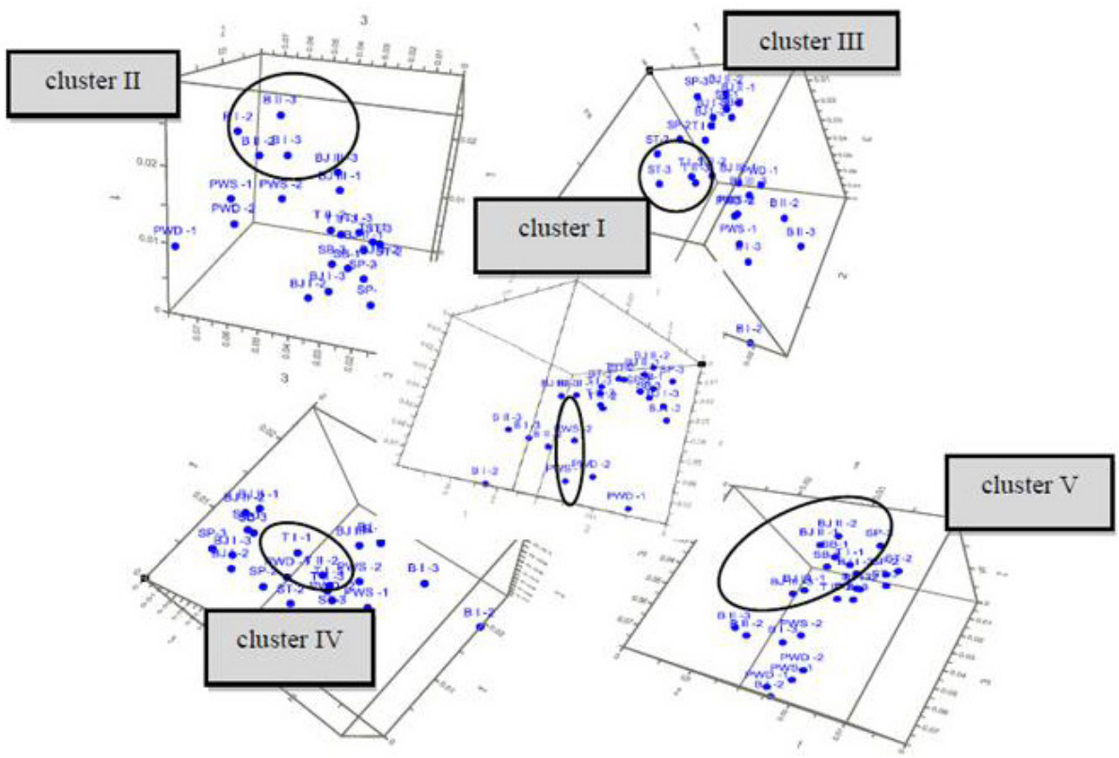

Figure 4. PCA 3-D score plot of metabolic profile of *S. androgynus* from different areas at East Java, showing the first three principal components: (B) Batu, (BJ) Bojonegoro, (PWD) Purwodadi, (PWS) Purwosari, (SB) West Surabaya, (SP) Center Surabaya, (ST) East Surabaya

Hierarchical Cluster Analysis (HCA) of *S. androgynus* samples by Euclidean distance separated the samples into five clusters (Fig. 5A.) with one cluster comprising only sample from Purwosari (PWS). Samples from lowland areas, such as Surabaya (except East Surabaya, ST) and Bojonegoro were clustered together in Cluster V. Samples from Batu, highland area, were clustered in Cluster II. In this study, HCA had clustered samples from Trenggalek, TI and TII, into two

different clusters, each of them was clustered with samples from Purwodadi (PWD) or East Surabaya (ST) respectively, which suggested that sample T I had similar metabolic profiles with samples PWD and sample T II was similar to sample ST.

From the PCA-Bi Plot which combined the score plot and loading plot on Fig.5B., the following substances with the greatest influence on the model were extracted, which could be seen as characteristic metabolites for all samples from different geographic areas. PC1 and PC2 could explain 53% and 21% of samples diversity, respectively.

This study found seven characteristic compounds for all samples such as methyl miristate (17), methyl palmitate (19), palmitic acid (20), methyl-linoleic (21), phytol (22), isophytol (23) and vitamin E (25), as being stated in Fig. 5B. These compounds had relatively higher area percent than all compounds in *S. androgynus* samples.

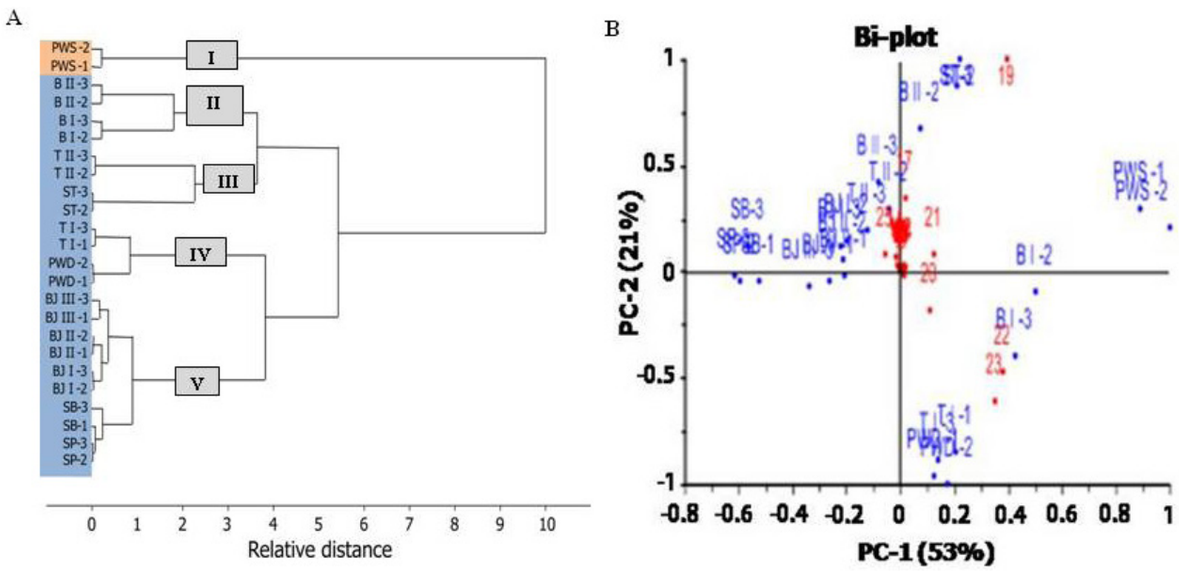

Figure 5. Multivariate Statistical Analysis for the metabolic profile of *S. androgynus* from 12 locations. (A) Dendrogram showing the Hierarchical Cluster Results using Euclidean Distance and Average Linkage, (B) PCA-Bi Plot for all samples of *S. androgynus*, which showed 7 characteristic compounds such as methyl miristate (17), methyl palmitate (19), palmitic acid (20), methyl-linoleic (21), phytol (22), isophytol (23) and vitamin E (25). (B) Batu, (BJ) Bojonegoro, (PWD) Purwodadi, (PWS) Purwosari, (SB) West Surabaya, (SP) Center Surabaya, (ST) East Surabaya

4. DISCUSSION

Although there were no morphological differences between *S. androgynus* samples from different areas at East Java, the chromatograms of different samples were found generally different, with some common characteristics, both in retention times and abundance of components. This clearly indicated that different geographical areas could influence the metabolic process in *S. androgynus,* by changing the metabolic pathway or modifying the enzyme responsible for the metabolic process. Further research had also showed that *S. androgynus* from several different areas on East Java, Indonesia, had different profiles of DNA banding patterns[22]. Accordingly, it could be assumed that difference of metabolic profile among the samples was caused not only by different environment conditions, but also by different gene characteristic of samples.

Metabolomic study of *S. androgynus* by GC-MSD shows that it consisted of several major compounds such as fatty acids, diterpene and vitamin. Some of the detected metabolites can be used as biochemical markers for the identification and differentiation between samples. Twenty five chemical constituents in *S. androgynus* had been identified by matching their mass spectrum with reference at NIST library, while palmitic acid and phytol were the major compounds. Researcher[25]

had reported that *S. androgynus* contained palmitic acid as a major compound, while phytol was a major compound in the plants which grouped in the same family (Euphorbiaceae) with *S. androgynus*[16,26,27].

S. androgynus from several areas were determined by GC-MSD and therefore, grouped and classified by Principle Component Analysis (PCA) and Cluster Analysis, respectively. The sample from Purwosari (PWS) alone was merged into one group or a cluster and the other samples were merged into the other cluster. Dissimilarity of PWS from other samples could be influenced by its specific area percent. GC-MSD analysis had shown that several metabolites in PWS had area percent c.a. 3-5 times bigger than those in other samples (data not shown).

According to their metabolic profiles, samples from East Surabaya (ST) and Trenggalek II (T II) showed the close relationship in score plot of PCA and in the dendrogram. We assumed that their close relationship was due to the same environment condition, while they were cultivated at lowland areas. On the other hand, samples from Trenggalek I (T I) was grouped in the same cluster with samples from Purwodadi (PWD), was due to similarity of environment condition at the two sample cultivation areas, while they were cultivated at mountainous area.

5. CONCLUSION

From this study it is obvious that *S. androgynus* leaf extracts from several various different geographic locations contain many biologically active compounds in various concentrations. This clearly indicated that different geographical conditions could influence the metabolic process in *S. androgynus,* by changing the metabolic pathway or modifying the enzyme responsible for the metabolic process.

Further work is required in order to investigate the metabolic profiles between samples which harvested in different harvesting time and post harvest handling, grown in different soil types, with different plant parts composition, with added adulterance etc. A multidisciplinary approach combining ecology, biochemistry, and molecular biotechnology, would have great potential to unravel the extent to which plant–environment interactions contribute to herbal medicine. Economical development of herbal medicine and quality development of herbal products through metabolomic approach shoud go hand in hand for a better future for herbal medicine.

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Conflict of interest

None to declare

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Ethical approval

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