FINAL REPORT

DOCTORATE RESEARCH GRANT 2009

**Metabolic and DNA fingerprinting of *Sauropus androgynus*,
In Food, Foodstuff And Food Supplement,
as a Lactagogum For Increasing Human Breast Milk Production**

Dr. Oeke Yunita, S.Si., M.Si., Apt.

**Home institution: Faculty of Pharmacy,
UNIVERSITY OF SURABAYA**

**Doctorate research at: Postgraduate Program,
AIRLANGGA UNIVERSITY, Surabaya**

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FORM 3A

REPORT – COVER PAGE

Type of report (circle one)	1. Progress Report. Design, Protocol, Instrument & progress report 2. Final Report. Publishable manuscript(s)*, final activity report & Budget report 3. Last 20% finance report
Proposal Code	D0042009
Name of principal investigator (PI)	**Dr. Oeke Yunita, S.Si., M.Si., Apt.**
Name of institution	University of Surabaya
Title of proposal	Metabolic and DNA Fingerprinting of *Sauropus androgynus*, in Food, Foodstuff and Food supplement, as a Lactagogum for Increasing Human Breast Milk Production
Duration of the funded project (start and end date)	Start date : January 1, 2010 End date : June 30, 2012
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Date submission of report	13 March 2012

* Please submit all manuscripts that you have drafted.

Name of Advisor **Prof. Dr. rer. nat. Mochammad Yuwono, MS.** *Name of PI :* **Dr. Oeke Yunita, S.Si., M.Si., Apt.**

Signature *Signature*

FORM 3A

REPORT – COVER PAGE

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Name of principal investigator (PI)	**Dr. Oeke Yunita, S.Si., M.Si., Apt.**
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Date submission of report	10 March 2012

* Please submit all manuscripts that you have drafted.

Name of PI : **Dr. Oeke Yunita, S.Si., M.Si., Apt.**

Signature

FORM 3D

FINAL REPORT

Dr. Oeke Yunita, S.Si., M.Si., Apt.

Home institution: Faculty of Pharmacy,
UNIVERSITY OF SURABAYA

Doctorate research at: Postgraduate Program,
AIRLANGGA UNIVERSITY, Surabaya

2012

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EXECUTIVE SUMMARY

Sauropus androgynus (Indonesian name: katuk) is traditionally used by Indonesian people to increase and accelerate human breast milk production. There are many publications that show the lactagogum (agent for increasing breast milk production) effect of this plant. There are many products at the market, contain extract of the *S.androgynus* produced by pharmaceutical manufacture which are claimed has a function as lactagogum.

Despite its important effect on the breastfeeding program, there are also many investigations that reveal the side effect of this plant in Taiwan and Japan. In these countries, people use this plant for reducing body weight. After a wide-spread, prolonged and unregulated use of this plant, a few patients have died and many have developed protracted chronic respiratory failure.

This research will perform chemical assessment, by metabolic fingerprinting with LC-MS and GC-MS, and genetic assessment, by DNA fingerprinting with RAPD method, for ensuring the safety of *S.androgynus* as a lactagogum, which is used in food, foodstuff and food supplement.

The metabolic profiles of *S.androgynus* in food, foodstuff and food supplement will be compared each other for obtaining the specific chemical compound (s) which assumed cause the side effect of *S.androgynus*. For completing this assumption, DNA patterns in food, foodstuff and food supplement, will be compared for obtaining the possibility of different variety or cultivar (s) of *S.androgynus* within the products.

The research in this proposal is the early research for research project which has general aim to ensure the safety of *S.androgynus* which is used as a lactagogum at Indonesia.

Expected output of this research will enhance the researcher's capacity on fingerprinting of the metabolites and DNA. The dissemination of this research could also strengthen the education and research capacity of the researcher's institution, so it could develop the scientific community network. Research output could also contribute recommendations for policy decision at Indonesia, about the safety of herbal preparations.

From this research, the researcher could also build a system for safety or quality control of plant which are used as herbal medicine and herbal supplement. This system will give a feasibility to control the product and process quality in the herbal medicine industry or herbal supplement industry, in the production chain, beginning from raw material, semi-finished product until finished product.

I. INTRODUCTION

In the battle to eradicate poverty in developing countries and in situations of disaster or food insecurity, one small step would ensure the food security is breastfeeding every newborn. Breastfeeding would provide the best nutrition, the greatest infection protection, the most illness prevention and the greatest food security and psychological protection for the infant (Lawrence, 2007).

Except the benefits for individual health, breastfeeding provides significant social and economic benefits to the nation, including reduced health care costs and reduced employee absenteeism for care attributable to child illness. The significantly lower incidence of illness in the breastfed infant allows the parents more time for attention to siblings and other family duties and reduces parental absence from work and lost income. The direct economic benefits to the family are also significant, by saving money per child for infant formula purchases for the first of year after birth (American Academy of Pediatrics, 1997).

Promotion of breastfeeding is a global priority to ensure food security and good health for infants in the first 6 months of life. In 2002, The World Health Organization (WHO) based on a systematic review of scientific evidence, recommended exclusive breastfeeding to newborns from birth through to 6 months of age and that breastfeeding should be extended to 2 years of age along with appropriate complementary foods (Lee *et al*., 2007).

The potential risk factors leading to lower breastfeeding rate and early cessation of breastfeeding were found to increase with rapid urbanization and modernization of a society, women having full time work, which increasing their workloads physically and emotionally, and lacking a role model from mothers or mothers-in-law. This may be attributable to the fact that in metropolitan areas, the use of infant formula is regarded as elite, sophisticated, affordable and convenient (Beasley and Amir, 2007; Lee *et al.*, 2007).

The Maternal and Child Health Study (2001) showed that only 47.5 % infants 0 – 3 months and 14.2 % infants 4 – 5 months were exclusively breastfed and this percentage getting lower at 2008 until only 14 % infants 0 – 6 months were exclusively breastfed (Supraptini *et al*., 2003; Media Indonesia, 2008).

The survey reported that 38 % of mothers stop giving mother's breast milk because of the lack of the mother's breast milk itself (Sa'roni *et al*., 2004).

Sauropus androgynus (Indonesian name: katuk) is traditionally used by people to increase and accelerate mother's breast milk production. Extract of *S.androgynus* leaves had shown lactagogum effect at the dosage 631.6 mg/kg rat's body weight or 900 mg/day for human. Infusum of *S.androgynus* leaves can increase the production of breast milk on mice, but isolate from ether phase and ether-petroleum extract did not increase the production of

breast milk significantly. Extract of the *S.androgynus* leaves (900 mg each day) during 15 days, can increase the mother's breast milk production up to 50.7 %, start on the second or third day postpartum, compared with placebo. There are many products contain extract of the *S.androgynus* produced by pharmaceutical manufacture which are suspected has a function as lactagogum, for increasing breast milk production (Sa'roni *et al*., 2004; Azis and Muktiningsih, 2006).

S.androgynus was first introduced in Taiwan at 1988 and was prepared in some restaurants only as a fried dish. It was cultivated locally on a commercial scale and uncooked *S.androgynus* juice was widely advertised as a 'natural diet vegetable containing large amounts of nutrients and good for rapid weight reduction' (Ger *et al*., 1997; Kao *et al*., 1999).

Since 1994 an endemic chronic obstructive pulmonary disease (COPD) has developed in Taiwan after a wide-spread, prolonged and unregulated use of a body-weight reducing vegetable, *S.androgynus*. Typically, the patients are young or middle aged females with real or self-assumed weight problems, but without any previous history of respiratory ailments. All conventional treatments for COPD, including steroids and bronchodilators, have been ineffective in the patients with *S.androgynus* -induced lung disease. A few patients have died, but many have developed protracted chronic respiratory failure. Because of the chronic debilitation and ineffective conventional treatments, single lung transplants were performed as the last report in patients at the National Taiwan University Hospital. Some cases suffering from temporary insomnia, difficulty breathing or death after ingestion of the *S.androgynus* vegetable, have also been reported to the National Poison Center of Taiwan since August 23, 1995 (Lin *et al*., 1996; Chang *et al*., 1998).

A new outbreak of *S.androgynus* -associated bronchiolitis obliterans (BO) also occurred in Japan. In Japan, cultivation of *S.androgynus* started in 1996 and recently it has also been used for weight control by young and middle-aged women, similar to Taiwan (Oonakahara *et al*., 2005).

To further evaluate the association between *S.androgynus* and BO syndrome, a hospital-based case-control study was conducted at Veteran General Hospital-Kaohsiung between April and September 1995. The result of this study revealed that a larger total amount of *S.androgynus* compsumtion, preparation of *S.androgynus* food without cooking and ingesting *S.androgynus* food prepared by a vendor were the significant risk factors associated with BO syndrome (Ger *et al*., 1997).

In early August 1995, the Bureau of Food Sanitation of the Republic of China urged the public to stop consuming *S.androgynus* until epidemiologic and/or animal studies confirmed its safety (Ger *et al*., 1997).

The side effects of *S.androgynus* at Taiwan and Japan, which was associated with BO syndrome will decrease the selling value of of *S.androgynus* as a lactagogum in Indonesia,

which is frequently used by Indonesian people as food, foodstuff and food supplement. Therefore this research will do early research on metabolic and DNA fingerprinting of *S.androgynus* in food, foodstuff and food supplements in Indonesia. This research is only one small part of the general research which its objective is to ensure the safety of *S.androgynus* as a lactagogum, for maintaining food security through increasing human breast milk production.

Research Question

General Question

How can we assure the safety of *Sauropus androgynus*, which is consumed by Indonesian people as food, foodstuff and food supplement, for increasing human breast milk production ?

Research Question

1. How is the classification of the metabolic profiles and DNA patterns of *Sauropus androgynus* which are cultivated at several different geographic areas on East Java?
2. How is the metabolic profiles of *Sauropus androgynus* which are consumed by Indonesian people as uncooked and cooked food with various process of cooking and food storage?
3. How is the metabolic profiles of *Sauropus androgynus* which are consumed by Indonesian people as food supplement which contain *S.androgynus* extract?

Objectives

The aim of this research is to obtain metabolic profiles and DNA patterns of *S.androgynus*, in food, foodstuff and food supplement. The metabolic profiles of *S.androgynus* in food, foodstuff and food supplement will be compared each other for obtaining the specific chemical profiles which will be tested for the toxicity effect of *S.androgynus*, in the next research. For completing this assessment, DNA patterns in food, foodstuff and food supplement, will be compared for obtaining the possibility of different variety or cultivar (s) of SA in the SA product within.

In the present study, the Internal Transcribed Spacer (ITS) sequences of *S.androgynus* nuclear ribosomal DNA (rDNA) were amplified by Random Amplified Polymorphic DNA (RAPD) method to explore the original plant species from which they were derived. The metabolomic study was performed by High Performance Liquid Chromatography-Photodiode Array (HPLC-DAD) and Gas Chromatography-Mass Spectrometry Detector (GC-MSD). We then observed the influence of several cooking process and food storage towards metabolic profiles of *S.androgynus*. Commercial food supplement contain *S.androgynus* extracts also being studied and compared with fresh leaves extract.

II. METHODOLOGY

This research project had been conducted for exploring the metabolic profiles and DNA patterns of *Sauropus androgynus* as a food, foodstuff and food supplement.

2.1 Sampling Method

Sauropus androgynus were collected during June – July, 2010, from several locations at six areas with different geographic conditions (Surabaya, Trenggalek, Bojonegoro, Purwodadi-Purwosari, Batu). Almost all samples were obtained from individual gardens except sample from Batu was obtained from Balai Materia Medika. All the samples were authenticated by the Center of Information and Development of Traditional Medicine (PIPOT), Faculty of Pharmacy, University of Surabaya, East Java, Indonesia.

Leaves of *Sauropus androgynus* were collected, washed free of dirt, mopped dry and quickly stored at -80 ºC until used.

2.2 DNA Fingerprinting of *Sauropus androgynus*

2.2.1 DNA Isolation

Genomic DNA isolation was modified from Yunita (2010). Fresh leaves 1.0 g were ground and 500 µl of β-mercaptoethanol and 3500 µl of 2X CTAB solution (2% CTAB, 0.1 M Tris-HCl, 1.4 M NaCl) were added. The mixture was incubated at 55 ºC for 40 min with occasional inversion and then cooled at room temperature and further incubated at 55 ºC for 15 min. A 3500 µl of chloroform : isoamyl acetate (24:1 v/v) was added, incubated at room temperature for 30 min and then shaken for 10 min. After centrifugation at 4000 rpm for 30 min, 500 µl of the upper phase was subsequently mixed with 3500 µl phenol : chloroform : isoamyl acetate (25:24:1 v/v). 500 µl of the upper phase was mixed with 3500 µl of chloroform : isoamyl acetate (24:1 v/v) and then was mixed and centrifuged 4000 rpm for 30 min. Upper phase was mixed with 50 µl CTAB 10 % (65 ˚C) and 500 µl 65 ˚C ppt buffer (1% CTAB, 0.05 M Tris buffer pH 8, 0.01 M EDTA pH 8), then the DNA-CTAB complex was formed at room temperature for 15 min. The mixture was centrifuged at 13,000 g at 4 ºC for 15min and DNA pellet was mixed with 500 µl of 1 M NaCl-TE and incubated at 55 ºC until DNA dissolved. Isopropanol 500 µl was added in this mixture. After centrifugation 13000 g at 4 ºC for 15min, the DNA pellet was washed twice with 600 µl of 70 % ethanol and DNA was dissolved in 75 µl water free nuclease and kept at – 20 ºC until used.

2.2.2 Amplification of DNA Fragment by PCR

The isolated DNA, was then amplified with primer ITS Y-5 (5’ TAGAGGAAG GAGAAGTCGTAACAA 3’) and ITS Y-4 (5’ CCCGCCTGACCTGGGGTCGC 3’). The

DNA was pre-denatured at 95 ºC for 2 min, cycled 35 times at 95 ºC for 30 sec, 57 ºC for 1 min and 71 ºC for 2 min in a thermocycler. The final extension cycle allowed an additional incubation for 5 min at 71 ºC, as in Yunita (2010). The amplification reaction of the ITS region by RAPD primer, was modified from Verma et al. (2004), while the mixture contained 12.5 µl GoTaq® Green Master Mix (Promega) which contain GoTaq® DNA polymerase supplied in 2X Green GoTaq® Reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP and 3 mM $MgCl_2$.; 3.5 µl RAPD primer and 9 µl PCR result from PCR with ITS primers method in 25 µl reaction volume. The mixture was cycled 44 times at 94 ºC for 1 min, 35 ºC for 1.5 min and 72 ºC for 1.5 min in a thermocycler. The final extension cycle allowed an additional incubation for 5 min at 72 ºC.

2.2.3 Visualization of DNA Pattern by Electrophoresis

Amplification products were separated by electrophoresis through 1.5 % agarose gels in 0.5 X TBE buffer, visualized and imaged after staining with ethidium bromide.

2.2.4 Statistical Analysis

Each amplification product (band) was considered to be a RAPD marker. Number one will be attributed to band presence and zero to absence. The binary data set was used to calculate the similarity index and to assemble the corresponding similarity matrix. The matrix obtained was used to generate a dendogram. All the analysis were performed with the aid of the *SPSS 11.5 for Windows* computer program.

2.3 Metabolic Fingerprinting of *Sauropus androgynus*

2.3.1 Preparation of Methanol Extract

Leaves of *Sauropus androgynus* were collected, washed free of dirt, mopped dry and quickly stored at -80 ºC until used. 500,0 mg of mature-leaves were mixed and grounded in a mortar and pestle and 5,0 ml 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 3000 g for 1 min and filtered. Before being analysed with GC-MSD, HPLC-DAD or LC-MS, sample extract was evaporated with nitrogen gas and diluted with methanol, respectively.

2.3.2 Food Preparation from *Sauropus androgynus*

The leaves of *Sauropus androgynus* from one location (Surabaya) had been processed into food with variation methods, such as uncooked and cooked; various duration of cooking processes (boiled 5 min, 10 min and 15 min) and duration of food storage (6 hours, 15 hours and 24 hours).

2.3.3 Preparation of Food Supplement based on *S.androgynus* Extract

Two or three tablets (caplets) of *S.androgynus* were weighed and crushed. After a proper homogenization, 500.0 mg of powdered food supplement (tablets, caplets or capsules) which contained *S.androgynus* was added with 5.0 ml of methanol. The mixture was homogenized by vortex and sonicated and mixed thoroughly. The mixture was then be centrifuged at 3000 g for 5 min and filtered through a 0.45 µm nylon membrane prior to analysis.

2.3.4 Optimization of the Analysis Condition on HPLC

Various eluting conditions for separations had been tried and a gradient of several solvents will be employed to achieve well separation. Several kinds and compositions of mobile phase will be used to find the optimum condition with several acid / base compounds for regulation the separation pH.

2.3.5 HPLC-DAD and LC-MS Condition

Metabolomic study was performed on an Agilent High Performance Liquid Chromatography Instrument (HPLC 1100 Series) (analytical column: Merck Lichrospher® 100, RP-18, 5µm, 250.0mm X 4.0mm, 5µm); injected sample volume: 20.0 µl; mobile phase: methanol (A) and water (B) using a gradient program of 90% (B) in 0-15 min; 40% (B) in 15-20min; 20% (B) in 20-25min and 90% (B) in 25-30min; flow rate:1.0ml/min; temperature: 30 ˚C, measured at λ 214nm, 254nm, 280nm, 360nm, and 370nm). Further analysis of chemical identity was performed on a Waters Liquid Chromatography-Mass Spectrometry Instrumen (analytical column: *Xterra*® MSC18 5.0 µm; 2.1mm x 100 mm), with the modified method as described above.

2.3.6 GC-MSD Condition

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 microlitre of the extract was injected into a HP-5 capillary column (30 m x 250 µm i.d, 0.25 µm film thickness; 5 % polyxylosane; Agilent J&W scientific, Folsom, CA) 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 28.5 min. Helium was the carrier gas with a flow rate set at 1.3 ml 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.

2.3.7 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 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.

2.3.8 Multivariate Statistics

All the GC-MSD raw data were exported into Microsoft Office Excel 2007 in a table which contained the resulting three-dimentional 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 utilizes *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 estimage linkages between different classes within the data set. Euclidean distance on the PCs with Ward's linkage methods was used to derive a similarity matrix, which was processed by agglomerative or divisive clustering algorithms to construct a dendogram.

III. RESULTS AND DISCUSSION

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 in Table 1.

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

LOCATION	COORDINATE	SAMPLE CODE	ALTITUDE*) (meter amsl)	TEMPERATURE (˚C)	RELATIVE HUMIDITY (%)
Surabaya	7° 14' 57" South, 112° 45' 3" East	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	7° 9' 0" South, 111° 52' 0" East	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	8° 2' 52" South, 111° 42' 31" East	T I	120	24.1-33.3	31-82
		T II	120	24.1-33.3	31-82
Purwodadi	7° 48' 7" South, 112° 44' 10" East	PWD	320	29.1-33.5	64-73
Purwosari	7° 46' 13" South, 112° 44' 28" East	PWS I	220	29.7-32.3	66-69
		PWS II	240	29.2-33.5	64-73
Batu	7° 52' 12" South, 112° 31' 42" East	B I	840	25.7-33.7	39- 74
	7° 52' 12" South, 112° 31' 42" East	B II	840	25.7-33.7	39- 74

*) relative altitude, compared to Surabaya

All plant samples were collected and authenticated as *Sauropus androgynus*, based on their morphological structures, as seen on figure 1. 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 complete analysis about *S.androgynus*. All leaves were harvested in the morning, before 12.00 p.m, 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.

Fig. 1. *Sauropus androgynus* (Indonesian names: katuk)

3.2 DNA Isolation

Genomic DNA from accessions of *S.androgynus* was isolated using the method as in Yunita (2010), for isolation the DNA of *Piper betle*. The modified method was performed in this research including the usage of phenol for lowering secondary metabolic contamination at DNA isolation process.

The DNA purity and concentration of isolated DNA from fresh leaves from many locations show many variations but proved amenable to PCR amplifications, with OD 260/ OD 280 ratio > 1.8 and range DNA concentration between 406 – 2731 ng/µl.

The isolated DNA of fresh leaves from Surabaya Timur (ST), was used for optimization the amplification process with ITS primers and screening for RAPD primers.

For amplification purpose, the amount of fresh leaves for DNA isolation is about 1.0 g because this amount of plant materials would give large yields without decrease their DNA purity. Large yield is very important in RAPD method because this method will need many sample DNAs.

3.3 PCR-RAPD on ITS region

Our preliminary work had revealed that amplification the DNA of *S.androgynus* with the RAPD primers directly often resulted the smear band (data not shown), but after amplification of the ITS region of *S.androgynus*, we could get the distinct and clear bands on agarose gel after staining with ethidium bromide. Yunita (2010) assumed that the DNA genome is too long and complex so that the RAPD primers could anneal to many sites at the DNA so that the yield of amplification is too low; this was shown with the smear band at agarose gel after electrophoresis method.

The isolated DNA of fresh leaves of *S.androgynus*, then was amplified with ITS primers by Polymerase Chain Reaction (PCR) Method. These primers amplify the entire ITS region of *S.androgynus'* DNA.

ITS regions of DNA were subsequently amplified with twenty decamer primers by PCR-RAPD method for preliminary research to obtain the best primers which gave result clear and sharp profiles of DNA banding pattern after gel electrophoresis process.

Three primers (OPF-07, OPF-12,OPF-15) resulted clear and sharp profiles of DNA banding pattern after PCR-RAPD method on ITS region of *S.androgynus'* DNA, as shown on figure 2.

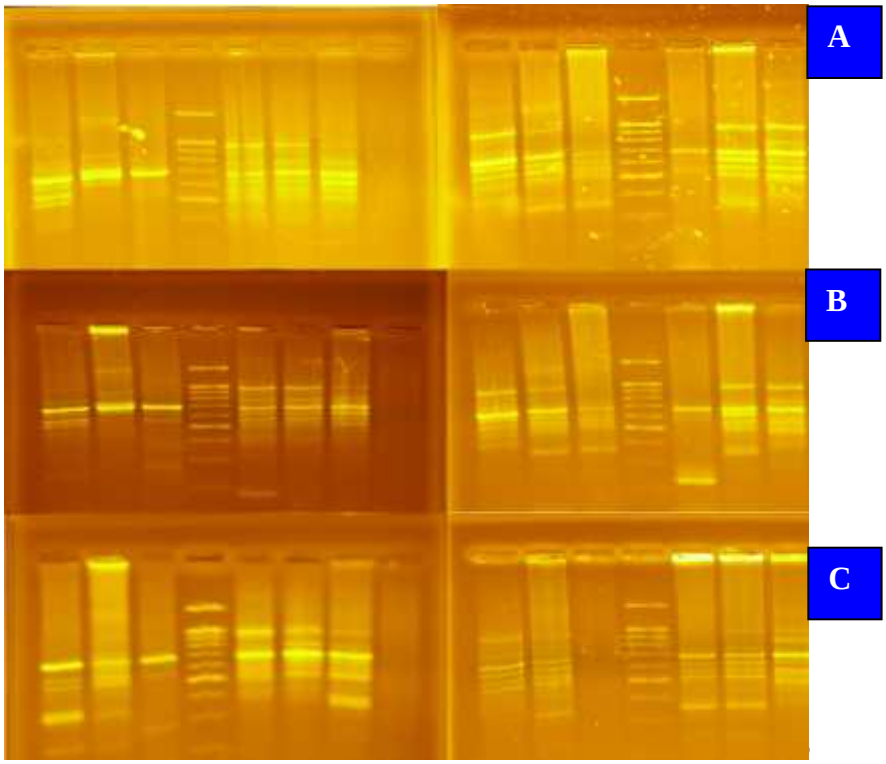

Fig 2. RAPD profiles from ITS regions on DNA of *S. androgynus* accessions, with primers OPF-7 (A), OPF-12 (B), OPF-15 (C). 1:SB; 2:SP, 3:ST, 4:marker 100 bp ladder, 5:T-I; 6:T-II, 7: B-I, 8:blank, 9:BJ-I, 10:BJ-II, 11: BJ-III, 12: marker 100 bp ladder, 13: PWS-I, 14:PWS-II, 15:PWD

After visualizing by UV light transluminator, BioDocAnalyze Biometra helped to measure the weight of DNA bands comparing with the marker 100 bp ladder. The measurement result showed the monomorphic band, 600 – 700 kb in samples of *S.androgynus*. In the next research, this band could be investigated further for obtaining specific biomarkers of *S.androgynus*.

Examination of RAPD polymorphisms of the leaves samples by the use of arbitrary primers, was accomplished by primer OPF-07, OPF-12 and OPF-15. The results of statistical analysis indicate that RAPD patterns among the samples had high similarity (0.786-0.895), as indicated in figure 3.

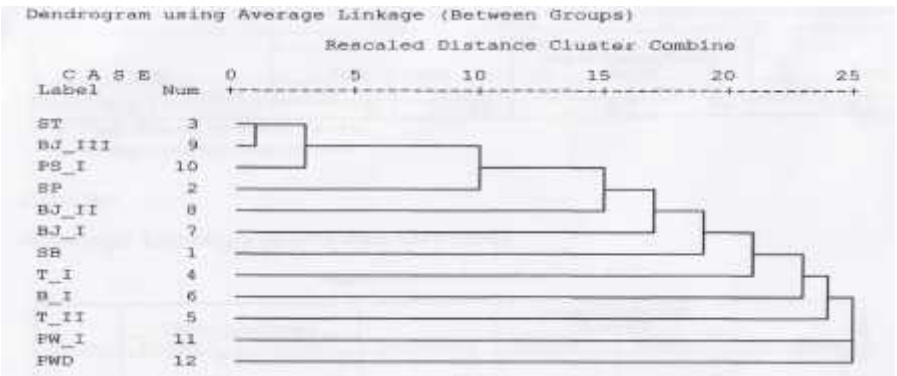

Fig. 3. Dendogram showing diversity of *Sauropus androgynus*' samples based on RAPD of ITS regions

A dendogram of DNA banding pattern from *S.androgynus* samples revealed that genetic assessment by DNA fingerprinting, could distinguish *S.androgynus* cultivars more precisely than morphological assessment. There is no literature review could distinguish *S.androgynus* cultivars based on their morphological characteristics. All *S.androgynus* samples were authenticated by Center of Information and Development of Traditional Medicine at species level and they could not be differentiated at variety and locality level. Mandal et al. (2007) stated that molecular identification could support the identification based on morphology for correctly designating different accessions of the same species. This method could support the assessment of raw materials in traditional medicine industry for assuring the quality of raw material and product.

DNA banding pattern of *S.androgynus* from Surabaya Timur (ST) and Bojonegoro (BJ-III) show the highest similarity, although the samples were collected from two locations with different geographical conditions, such as relative altitude, temperature and relative humidity.

Despite the highest similarity, sample from Purwodadi (PWD) show the lowest similarity of DNA banding pattern comparing with other samples.

This research showed that environmental conditions might not be the major factor that influence the differences between accessions. The slight differences on DNA banding patterns of samples might be more influenced by different genetically accessions of *S.androgynus* samples collected from several locations. Asmarayani and Pancoro (2005) also stated that human interference in plant domestication results in botanical evolution diversity.

For the next research, DNA fingerprinting of this plant must be done on the same genetically plant accession, which are cultivated on several locations with different geographical conditions, to analyze the influence of environmental conditions on genetic diversity of *S.androgynus*.

3.4 Optimization of the Condition Analysis on HPLC

Methanol was chosen as the extraction solvent for developing *S.androgynus* fingerprints because it could enhance the extraction efficiency and a variety of compounds with different polarity can be coextracted effectively. Besides, the interference from sugars in the raw herbs could also be minimized by extraction using methanol (Lu et al., 2005; Qian et al., 2006).

The extraction methods of *S.androgynus* were also optimized in our study, before analyzing the extract with HPLC-DAD and LC-MS. Two different extraction methods, such as maceration and ultrasonic extraction, were explored, and each extract was analyzed by HPLC, as showed at fig. 4.

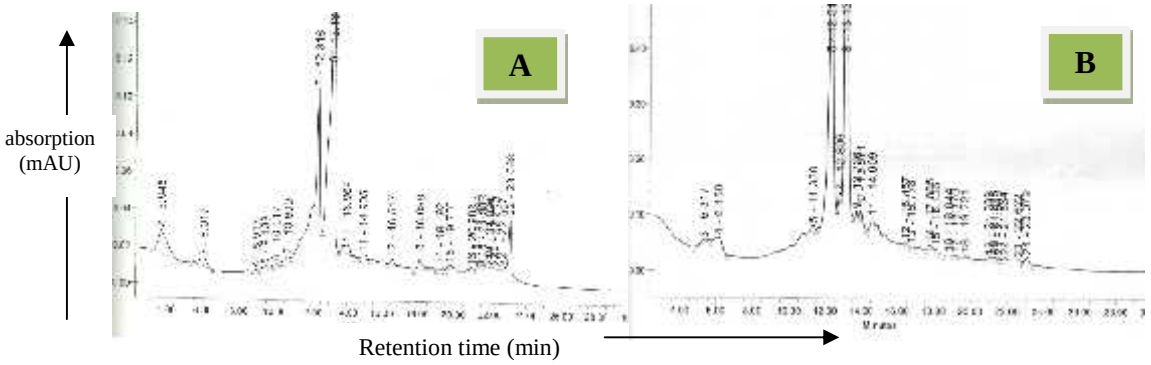

Fig. 4. HPLC metabolic profile of *S.androgynus* methanol extract using (A) ultrasonic extraction, 60 minutes; (B) maceration, 24 hours

In a full-scan experiment, chromatogram of *S.androgynus* extracted by using ultrasonic wave shows more components information and better separation than *S.androgynus* extracted by maceration. Despite the chromatogram profiles, from time and economic point of view, extracting the sample using ultrasonic takes only 60 min and therefore was chosen for all further analysis.

Various mobile phase for the separations were tried, such as acetonitrile and 0.1% H_3PO_4 (45:55); acetonitrile and 1 % formic acid; methanol and acetic ammonium (80:20) and also methanol and water, was employed to achieve well separation. It was shown at fig. 5 that mobile phase contain methanol and water could give better separation and more peaks in the chromatogram profile.

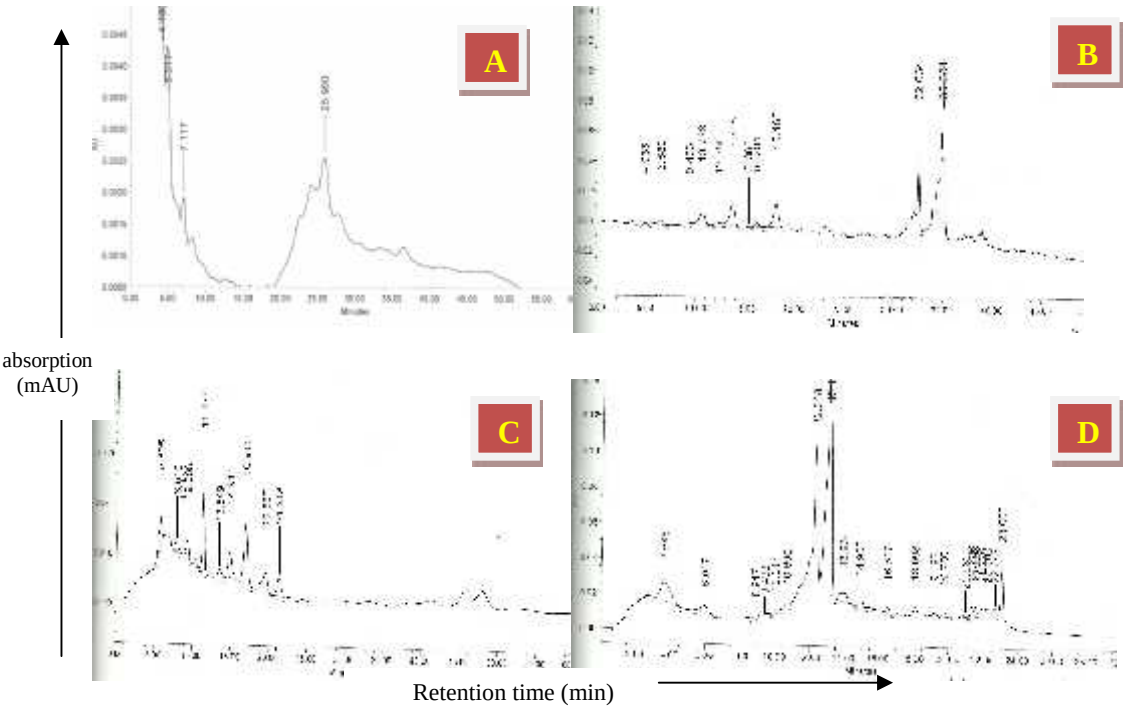

Fig. 5. Chromatogram of *S.androgynus* methanol extract with various mobile phase by HPLC-UV: (A) acetonitrile and 0.1% H_3PO_4 (45:55); (B) acetonitrile and 1 % formic acid; (C) methanol and acetic ammonium (80:20); (D) methanol and water

3.5 Metabolomic study of *S.androgynus* methanol extract

3.5.1 Metabolomic study by HPLC

The optimized of HPLC condition was utilized to reveal the fingerprint of *S.androgynus* leaf extract collected from several different locations at East Java. Photodiode array detector (DAD) was used in the current study. The detection wavelength at 254 nm gave the best abundance for target compounds within the chromatographic windows, as described in fig. 6.

The results indicated that their chromatographic patterns were generally consistent among all samples, especially at retention times under 25 minutes, although the absorption intensity of some peaks was different. This indicated that there was resemblance in terms of chemical constituents of *S.androgynus* sample from several locations with different geographic condition at East Java. More samples are needed to obtain a more representative population.

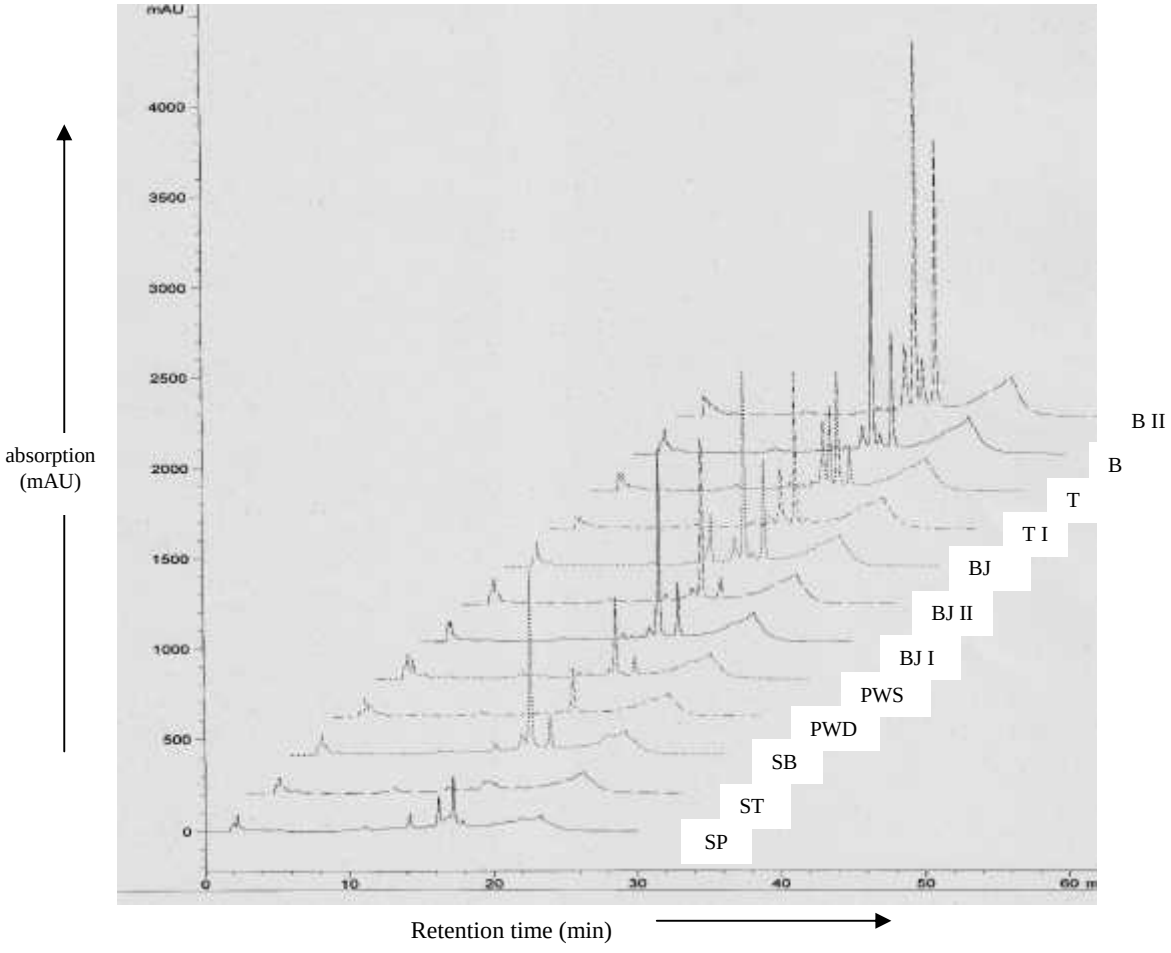

Fig. 6. HPLC Fingerprint of 12 Samples of Fresh *S.androgynus* from various areas at East Java : SP = Surabaya Pusat (Center Surabaya), ST = Surabaya Timur (East Surabaya), SB = Surabaya Barat (West Surabaya), PWD = Purwodadi, PWS = Purwosari, BJ I = Bojonegoro I, BJ II = Bojonegoro II, BJ III = Bojonegoro III, T I = Trenggalek I, T II = Trenggalek II, B I = Batu I, B II = Batu II

Nevertheless, the results indicated that ST had unique chromatogram profile at retention time (Rt) 18-24 min that had not been similar with the other sample, indicated that sample ST had unique chemical contents compared with the others.

In order to identify structures of the major chemical components in *S.androgynus*, the sample was analyzed by Liquid Chromatography Mass Spectrometry (LC-MS) techniques. ESI in both negative and positive modes were performed. By studying on the characteristic mass spectra of these peaks and comparing with the UV and ESI-MS spectra of authentic compounds, no Papaverine HCl was identified, as also stated by *Bureau of Food Sanitation* (Ger *et al*., 1997) and Yu *et al*. (2007). Papaverine HCl is a compound which was reported that assumed can cause respiratory side effects after *S.androgynus* consumption (Kao *et al*., 1999).

3.5.2 Metabolomic Study by GC-MSD

Methanol extract of *S.androgynus* leaves was subjected to GC-MSD analysis, which resulted the chromatogram in fig 7. According to the comparison of the recorded mass spectra with a 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 stable compound with a high area percent.

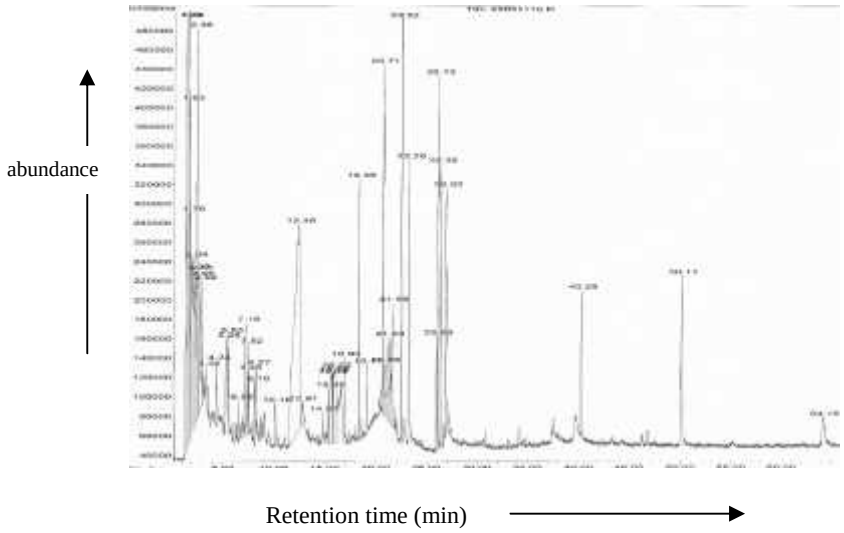

Fig 7. Total ionic chromatogram (GC-MSD) of *S.androgynus* methanol extract obtained with 70 eV using a HP-5MS column (30 m x 0.25 mm) with He gas as the carrier at a flow rate of 1.3 ml min^{-1}

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 dominant components such as phytol and isophytol were also found in the *S.androgynus* methanol extract, as described in table 2.

Table 2 Chemical Compound in *S.androgynus* Methanol Extract (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* (metil miristat)	0,79
5	*Phenol*	0,13	18	*tetradecanoic acid* (asam miristat)	0,82
6	*Pyridine,3,4-dimethyl-*	0,14	19	*hexadecanoic acid, methyl-ester* (metil palmitat)	0,97
7	*Pyridine,3,5-dimethyl-*	0,13	20	*n-hexadecanoic acid* (asam palmitat)	1,00*)
8	*benzeneacetaldehyde*	0,17	21	*9,12-octadecadienoic acid (Z,Z)-,methyl ester* (metil linoleat)	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* (metil stearat)	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

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 a less than 0.1 % and 20 % (n=6). These results indicate that the method is reliable and applicable to the analysis of metabolic profile of *S.androgynus*.

Typical GC/MS chromatograms of samples from different 5 areas at East Java were illustrated in fig 8. 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 were 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 came from the same single species.

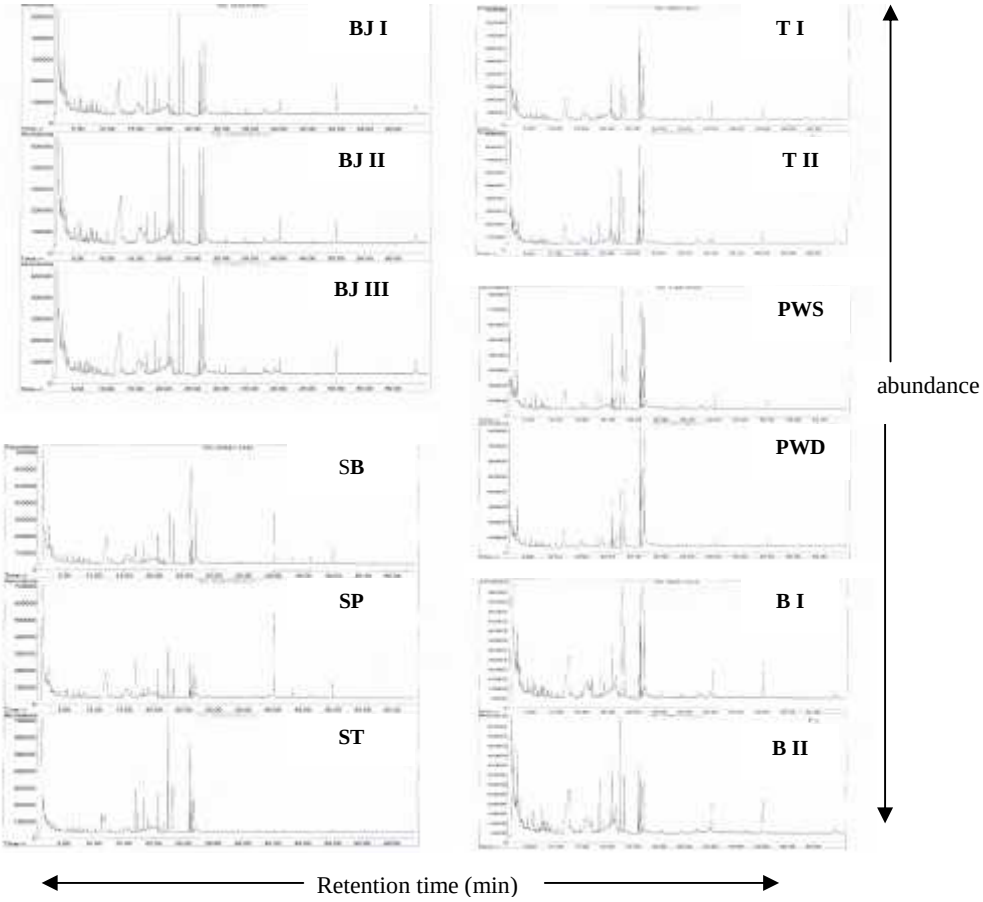

Fig. 8. GC-MSD metabolic profile of 12 samples of *S.androgynus* from East Java: (B) Batu, (BJ) Bojonegoro, (PWD) Purwodadi, (PWS) Purwosari, (SB) West Surabaya, (SP) Center Surabaya, (ST) East Surabaya

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, perform the deconvolution of peaks based on the MS data. The analyses reveal 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 is shown in fig. 9. 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 identifiy the geographical origin of samples. *S.androgynus* from lowland area, such as Surabaya and Bojonegoro are grouped in one cluster, except sample from East Surabaya (ST).

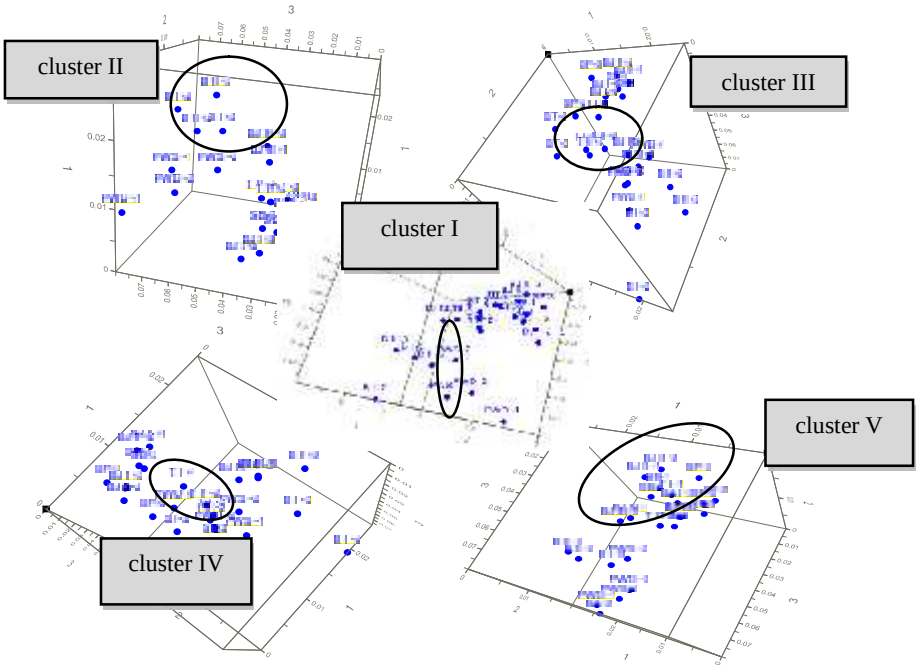

Fig. 9. PCA 3-D score plot of metabolic profile of *S.androgynus* from different areas at East Java, showing the first three principal components: (B) Batu, (BJ) Bojonegoro, (PWD) Purwodadi, (PWS) Purwosari, (SB) West Surabaya, (SP) Center Surabaya, (ST) East Surabaya

Hierarchical Cluster Analysis (HCA) of *S.androgynus* samples by Euclidean distance separated the samples into five clusters (fig. 10.), with one cluster comprising only sample from Purwosari (PWS). Samples from lowland areas, such as Surabaya (except East Surabaya, ST) and Bojonegoro are clustered together in Cluster V. Samples from Batu, highland area, are clustered in Cluster II. In this study, HCA had clustered samples from Trenggalek, TI and TII, into two different clusters, each of them is clustered with samples from Purwodadi (PWD) or East Surabaya (ST) respectively, which suggests that sample T I has similar metabolic profiles with samples PWD and sample T II is similar to sample ST.

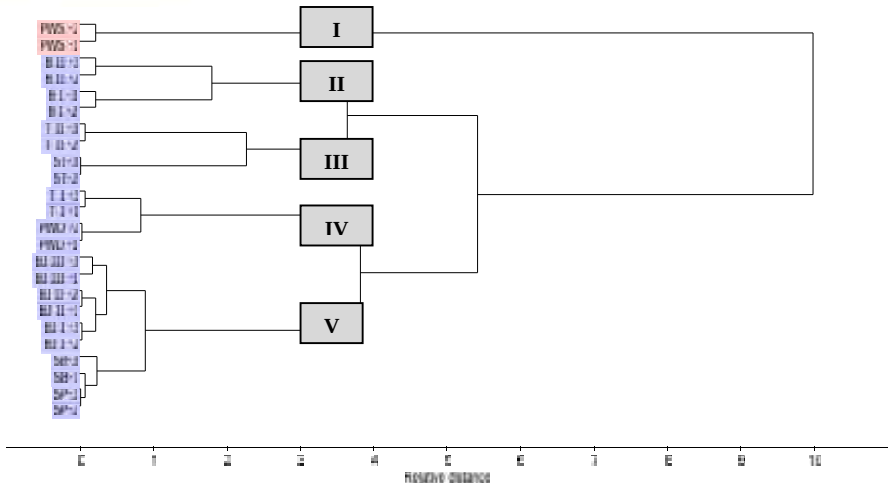

Fig. 10. Dendogram showing the Hierarchical Cluster Results for the Metabolic Profiling of *S.androgynus* from 12 locations, using Euclidean Distance and Average Linkage : (B) Batu, (BJ) Bojonegoro, (PWD) Purwodadi, (PWS) Purwosari, (SB) West Surabaya, (SP) Center Surabaya, (ST) East Surabaya

From the PCA-Bi Plot which combine the score plot and loading plot on fig.11, the following substances with the greatest influence on the model were extracted, which can be seen as characteristic metabolites for all samples from different geographic areas. PC1 and PC2 could explain 53 % and 21 % of sample diversity, respectively.

This study found seven characteristic compound 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 stated in fig. 11. These compounds had relatively higher area percent than all compounds in *S.androgynus* samples.

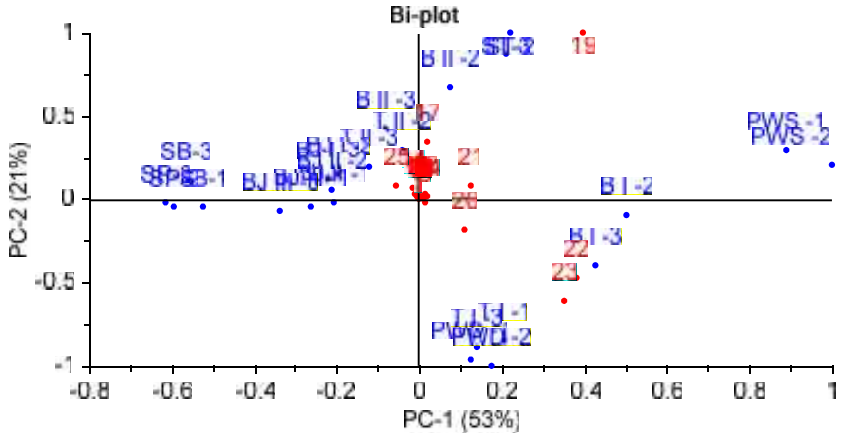

Fig. 11. PCA-Bi Plot for all samples of *S.androgynus*, which 7 characteristic compounds such as methyl miristate (17), methyl palmitate (19), palmitic acid (20), methyl-linoleic (21), phytol (22), isophytol (23) and vitamin E (25) : (B) Batu, (BJ) Bojonegoro, (PWD) Purwodadi, (PWS) Purwosari, (SB) West Surabaya, (SP) Center Surabaya, (ST) East Surabaya

Although there are 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 reaction of *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 (Yunita and Sulisetiorini, 2011). Accordingly, it could be assumed that difference of metabolic profile among the samples was caused not only by different environment condition, but also by different of gene characteristic of samples.

Metabolomic study of *S.androgynus* by GC-MSD shows that it consisted of several major compounds such as fatty acids, diterpen 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 are the dominant compounds, as stated by Agustal et al. (1997), Ching and Mohamed (2001), Bağci (2007) and Ogunlesi et al. (2009).

S.androgynus from several areas were determined by GC-MS 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 those of the other samples were merged into the other cluster. Dissimilarity of PWS from other samples could be caused by the difference of area peak percent among the metabolites in PWS samples. GC-MSD analysis had shown that several metabolites in PWS had area peak 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) show the closely relationship in score plot of PCA and in the dendogram. We assumed that their closely relationship is due to the same environment condition. On the other hand, samples from Trenggalek I (T I) was grouped in the same cluster with samples from Purwodadi (PWD), is due to similarity of environment condition at the two areas, while they were cultivated at mountainous area.

The influence of food processing of *S.androgynus* were studied in this research, as described in fig. 12 , such as cooked *S.androgynus* by boiling 40 ˚C 15 min and 100 ˚C 5min. Food storage of cooked *S.androgynus* during 15 hours was also studied for observing the deterioration of food during the storage time.

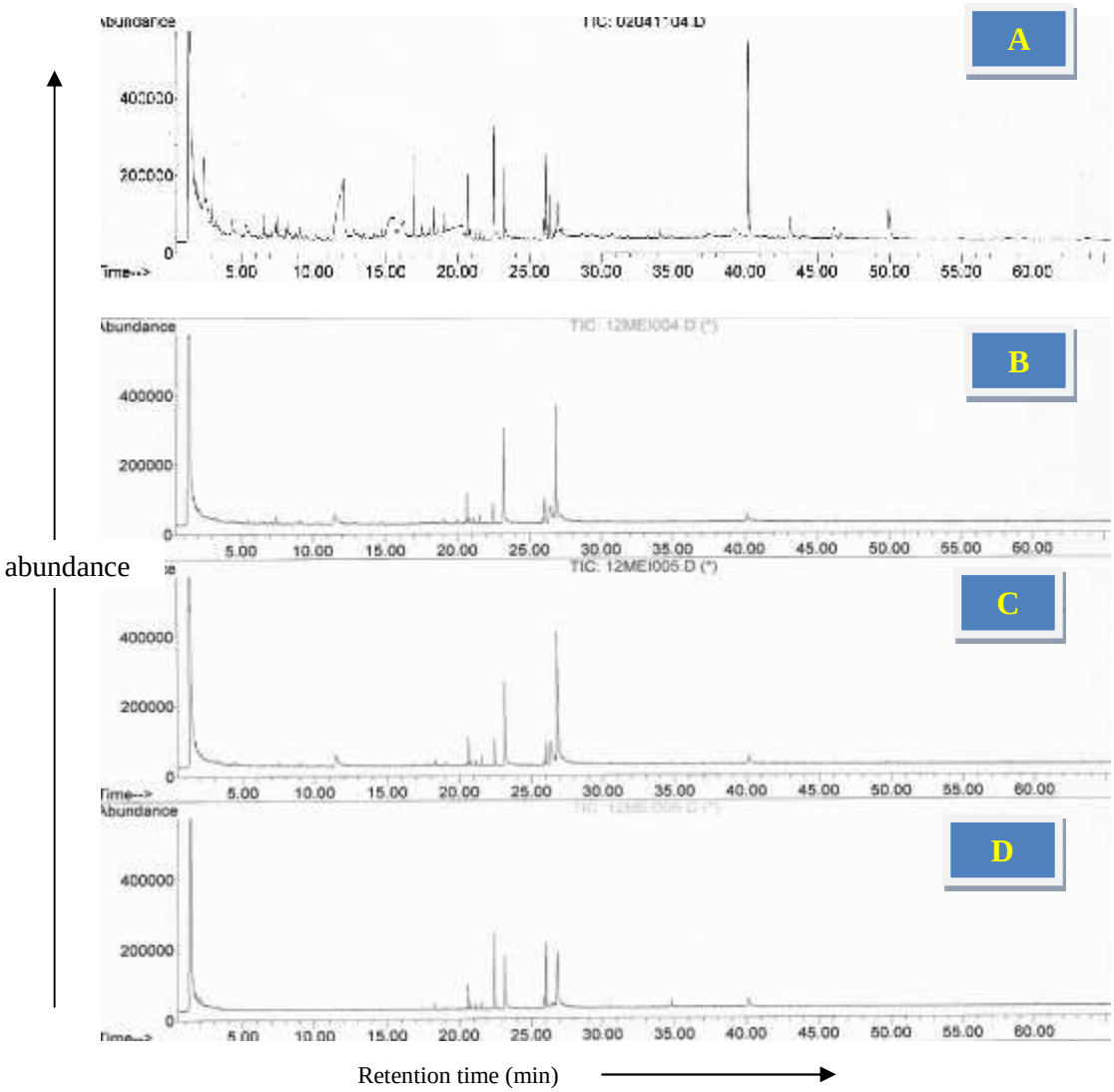

Fig. 12. Fingerprints of the methanol extract and several food preparation and storage of *S.androgynus* by GC-MSD: (A) methanol extract; (B) boiled 40˚C 15 min; (C) boiled 100˚C 5 min; (D) food storage during 15 hours

Metabolic profiles of cooked *S.androgynus* had shown the same pattern (Rt = 20-30 min) with the fresh leaves extract, although the absorption intensity of all peaks had decreased. This result is similar with Rahmat *et al*. (2003) which had found that boiling the leaves may deteriorate the active compounds such as flavonoid, terpenoid, lignin, sulphide, polyphenol, carotenoid, coumarin, saponin, curcumin, vitamins and sterol.

Many brands of herbal supplement products based on *S.androgynus* have been available in the Indonesian markets, therefore chromatographic profile of several commercial products were compared with profile of methanol extract from fresh leaves, as seen in fig. 13.

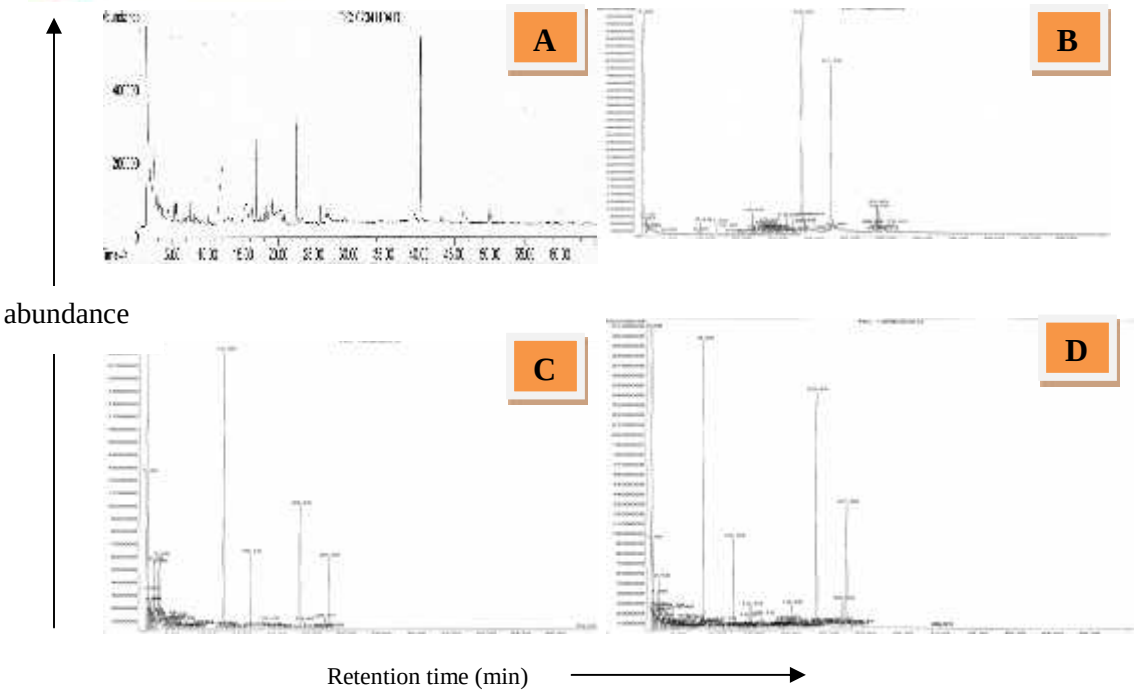

Fig.13. GC-MSD Fingerprints of the methanol extract and commercial product of *S.androgynus*. (A) methanol extract; (B) the tablet BD; (C) caplet LS; (D) capsule AF

Most of the contents in the fresh leaves of *S.androgynus* are generally higher than that of its commercial product. Probably the differences were attributed to the lost or decomposition during the drying process in the formulation technology, as also stated by Meng et al. (2005) about fresh *Houttuynia cordata* dan its dried counterpart.

IV. CONCLUSION AND RECOMMENDATION

Safety assessment is one of the key issues in the modernization of Indonesian herbal supplement. The early investigation at this research is to find the system for controlling the identity of raw material on industry by using molecular study with RAPD method on ITS region of DNA and metabolomic study with HPLC and GC-MSD of *Sauropus androgynus* accessions from several locations, at East Java, Indonesia.

Random Amplified Polymorphic DNA (RAPD) method could generate different DNA fingerprint of *S.androgynus* from several areas at East Java, using ITS region of DNA as DNA template. DNA banding pattern of *S.androgynus* from Surabaya Timur (ST) and Bojonegoro (BJ-III) show the highest similarity, although the samples were taken from two locations with different geographical conditions. Meanwhile, sample from Purwodadi (PWD) show the lowest similarity of DNA banding pattern comparing with other samples. This result showed that environmental condition might not be the major factor could influence the differences between accessions.

HPLC and GC-MSD could generate different metabolic fingerprint of *S.androgynus* from several areas at East Java. The results and conclusion generated from the present developed direct HPLC-DAD fingerprint analysis are generally comparable and consistent with our studies using GC-MSD fingerprint method on *S.androgynus*.

GC-MSD analysis of *S.androgynus* samples could show different metabolic profiles among samples from different geographical areas. Statistical analysis could classify all *S.androgynus* samples into 5 clusters that each *S.androgynus* sample from areas with similar geographical conditions could be grouped in the same cluster, except *S.androgynus* sample from Purwosari (PWS).

Further analysis by GC-MSD on cooked *S.androgynus* and commercial products of *S.androgynus* had showed the decreasing in quality and quantity of several metabolites in *S.androgynus*.

In the next research, in vitro and in vivo toxicity assay of *S.androgynus* will be performed for studying the mechanism of its toxic effect and finding the lethal dose after consumption. Furthermore, the correlation between metabolic profiles of *S.androgynus* with its toxic effect will be examined for finding compound (s) in *S.androgynus* that was highly correlated with its toxicity.

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   - High Performance Liquid Chromatography – Photodiode Array Detector (HPLC-DAD)
   - Liquid Chromatography – Mass Spectrometry (LC-MS)
   - Gas Chromatography- Mass Spectrometry Detector (GC-MSD)
   - Cold refrigerator (-20˚C)
   - Centrifuge
   - Waterbath
   - Vortex

- **Ethical clearance**

According to the Board of Ethical Clearance Commission at Airlangga University, this research did not need the Ethical Clearance, because this research was a descriptive research, not an experimental research, which only used a plant as the research object. This research did not use animal or human as a research subject and did not give an intervention to the research process

- **Financial report**

(written in the separate pages)