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^{1}H NMR Fingerprinting of Medicinal Herbs Contain Chemical Drug Material Allopurinol

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ABSTRACT

A study to differentiate the pure medicinal herbs from the mix medicinal herbs with chemical drug material has been done. For this purpose, we conducted fingerprinting of commercial medicinal herbs and chemical drug material allopurinol using ^{1}H-NMR followed with chemometrics analysis. Nine commercial traditional herbal medicines claimed for rheumatic were used as samples as well as allopurinol as the chemical drug standard. Extraction of samples was done by ultrasonicator for 15 min in methanol-d4 containing 0.01% TMSP as an internal standard. Each type of herbal medicine was prepared in three replicates. The phytochemical analysis was done by 500 MHz JEOL NMR. The chemometrics analysis was done using SIMCA software following the ^{1}H NMR spectra processing with MNOVA software. All spectra showed no contamination with allopurinol. The specific signals of allopurinol at aromatic regions were confirmed not present when the spectra were stacked together. Hence, the result of OPLS-DA analysis convinced that the herbs were clearly separated the medicinal herbs into 3 classes. *Jamu* 1 is separated from others showed very high intensity of several signals which may indicate an addition of chemical medicines but not allopurinol. The clear separation of other two groups may corresponds to the similarity of ingredients. These results also showed that most of traditional medicines which produced by small industries, the traditional medicines contain no active pharmaceutical ingredients (allopurinol) indicating a high safety of Indonesia traditional medicines.

Key words: ^{1}H NMR; traditional medicine; chemometrics; OPLS-DA.

INTRODUCTION

The herbal medicine is present in several countries such as China (Chen *et al.*, 2017), Korea (Chen *et al.*, 2014), India (Sen and Chakraborty, 2017) and Indonesia. In Indonesia, this traditional herbal medicine is known as *jamu*. The industries producing *jamu* are ranging from small-scale to large-scale industries. Based on Indonesian Medicinal Herbs Index there are 5000 kinds of medicinal herbs have already known and studied to benefit of curing various diseases (Elfahmi, Woerdenbag and Kayser, 2014). Many efforts have been put to support the *jamu* into conventional medicines due to the increasing trends of consuming *jamu* to cure diseases. However, several reports about adulteration, and contamination in *jamu* indicating that safety is still become a serious issue. The contaminants were reported as heavy metals, pesticide residues (Harris *et al.*, 2011; Tripathy *et al.*, 2015; Shaban, Abdou and Hassan, 2016), mycotoxin, PAH (Poly Aromatic Hydrocarbon) (Tripathy *et al.*, 2015; Wang *et al.*, 2015) and also chemicals drugs (Rahmatullah and Fikri, 2018). One of problem with chemical drug contamination is on the unmonitored dosage consumed by the patient due to the unclear amount that had been added as the ingredient. This type of contamination may occur in the *jamu* produced by small industry. As it gives faster healing response, thus people tend to satisfy and consume it again when they encounter the same problem. Generally, people do not have knowledge about the danger and effect of taking this mixed herbal-drug medicines in the long term (Anonim, 1999)

According to the Deputy for the Supervision of Traditional Medicines, Cosmetics and Complementary Products, chemically drugs often mixed in *jamu* are drugs for allergy, fever, rheumatic paints, and anti-inflammatory drugs. *Jamu* mixed with chemical drugs usually illegally distributed without a registration number from the National of Food and Drug Control or it has a fictitious registration number (Candra, 2012). Various side effects from light to serious ones such as organ failure can be observed as a result of long period consumption of *jamu*-chemical drug mixed medicines.

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Allopurinol used for treating gout and hyperuricemia (Lefort *et al.*, 2014) as 1,5-Dihydro-4H-pyrazolo [3,4-d]-pyrimidin-4-one (Nourudin W Ali, Nada S Abdelwahab, 2014) is an official drug from British and United States. This drug was indicated and found as an additive material in herbs medicine (*jamu*) in Indonesia (Rahmatullah and Fikri, 2018). Allopurinol as a drug has already validated using TLC-densitometric and RP-HPLC method analysis (Breithaupt H, 1981; Ali, 2014). However these detection methods need the availability of standard compounds every time test which cost a lot of money.

Aside the previously mentioned method, identification of purity of the herbal medicine added with active pharmaceutical ingredients can be performed with several analytical methods and instruments such as GC-MS (Heyman and Meyer, 2012; Khan *et al.*, 2017), LC (Dona *et al.*, 2016), LC-MS (Chan *et al.*, 2010), UPLC (Kim *et al.*, 2011) and ^{1}H-NMR (Heyman & Meyer, 2012; Kim et al., 2011). ^{1}H-NMR is the most appropriate instrument to use, because it non-destructive to the sample and gives more detailed information about the structure of secondary metabolites in natural product (Pauli, Jaki and Lankin, 2005; Preto *et al.*, 2013; Simmler *et al.*, 2014). NMR profiling has been successfully applied to identify the characteristics of tomatoes (Hohmann *et al.*, 2014), as well as to distinguish between the herbs and drug material (Lefort *et al.*, 2014). In addition, NMR also has a high degree of accuracy and repeatability compared to other instruments (Malz and Jancke, 2005). Although NMR allows us to detect the present of chemical drugs from the mixture by the signals markers of the corresponding compound, Rundlöf et al., (2014) emphasis the inclusion of standards compound for an accurate and precise results. In this current study we performed NMR fingerprinting to nine commercial *jamu* claimed for rheumatic for the possible contamination with allopurinol.

Multivariate analysis is also needed in metabolomics study due to its robustness to discriminate the different characters of sample as well to identify which characters contribute to the discrimination. The tools that commonly used such as Principal Component Analyisis (PCA), Hierarchical Cluster Analysis (HCA), Partial Least Squares- Discriminant Analysis (PLS-DA) and Orthogonal Projections to Latent Structures-Discriminant Analysis (OPLS-DA) have been applied to differentiate between Korean and Chinese medicines, the *Pueraria thomsonii* (Chen *et al.*, 2014), Danggui (Zhang *et al.*, 2016) herbs and Jacobaea plants (Nuringtyas *et al.*, 2012)

METHODOLOGY

Materials

Materials used in this experiments were nine traditional herb medicines with rheumatic claimed collected from stalls or small shops selling jamu in Yogyakarta, Indonesia. Allupurinol USP reference standard from Sigma, methanol-d4 with deuteration degree min. 99.8 % for NMR spectroscopy and internal standard TMSP (Tri Methyl Sylil Phospate) from Merck.

The instrument used in this experiments were NMR JEOL ECZ-R 500MHz with *Superconducting Magnet* (SCM) 11.74 Tesla (resonance frequency ^{1}H- 500MHz), spectrometer FT (*Fourier Transform*) ZETA, Auto-tuning 5 mm Royal probe and VT (*Variable Temperature*) till 180ºC, analytical balance (OHAUS), sonicator (Elma S 30 H), microsentrifuge (MPW 55) ,vortex (Thermo Scientific) and micropipette (Socorex, 100-1000 ul).

Methods

Medicinal herbs extraction

Dried medicinal herbs powder in 50 ml tube dried using *freeze-dryer* 24 hour. An approximately of 50 mg dry sample was added in 2 ml eppendorf containing 1 ml methanol-d4 (Merck) with 0.1% w/w TMSP [*3-(trimethylsilyl)propionic-2,2,3,3-d4*] . *A*fter that, it was vortexed in the room temperature (20 – 25 ^{o}C) for 1 min, then ultrasonificated for 15 minute in the same condition (Kim, Choi and Verpoorte, 2010).

Then the tube was sentrifugated using microtube sentrifuge. Supernatan put in 1,5 ml eppendorf then 700 µl supernatan transferred in 5 mm NMR for proton analysis (^{1}H-NMR).

Allopurinol extraction

An amount of 5 mg Allopurinol standard was solved in 1 ml methanol-d4 (Merck) homogenously then was vortexed for 1 min in room temperature. After that, the mixture was sonicated for 15 min followed with sentrifugation for 10 min with 10.000 rpm to obtain the supernatant for NMR analysis.

Multivariate Data analysis

The spectra obtained from NMR were preprocessed using MNOVA software for phasing, baseline correction and internal standard TMSP signal correction at 0.00 ppm. The spectra Were then subjected for binning using the default MNOVA software with 0.04 ppm width. The data was then subjected for multivariate analysis using SIMCA software (Sartorius) version 14.

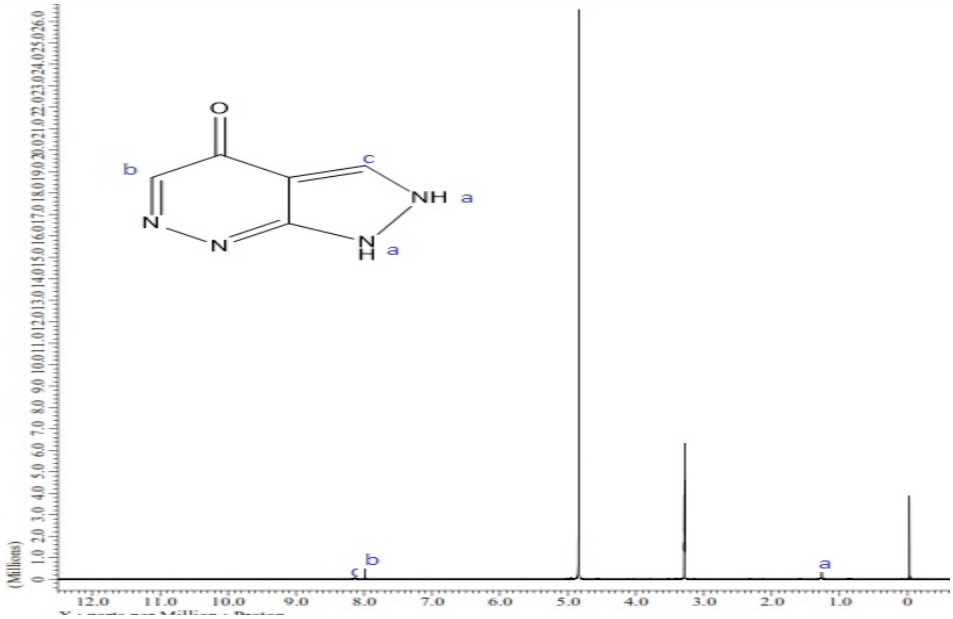

Figure 1. ^{1}H-NMR Spectra of Allopurinol standard

RESULTS AND DISCUSSION

Identification of signal marker for Allopurinol

The ^{1}H-NMR spectra of allopurinol showed the specific three signal markers that can be used as allopurinol identification, including 1.28 ppm that confirm the proton from amine (-NH) from pyrimidine, the second and third peaks were observed in the aromatic regions as singlet 8.00 ppm and broad singlet 8.15 ppm. Proton that was bond with carbon in pyrimidin ring found at peak in 8.00 ppm while the 8.15 ppm corresponds for proton attached at the carbon in pyrazole ring (Refat, Mohamed and Fathi, 2010) (Figure 1).

^{1}H-NMR Spectrometry and chemometrics supervised pattern recognition of OPLS-DA for authentication of *jamu*

The spectra from ^{1}H-NMR of *jamu* is normally divided into three region including 8-6 ppm, 6-3 ppm and 3-0 ppm which corresponds to aromatics, sugars and terpene or amino acids regions, respectively. ^{1}H-NMR spectra of jamu is derived from a mixture of many herbs extract, thus within the spectra we observed many overlapping and crowded signals especially in the sugar and the terpene regions. However, we can still can analyzed and recognized the patterns that developed by the combination of proton signals of *jamu.* For this, we did chemometric analysis in order to solve and manage the data. We applied multivariate data analysis including PCA prior to OPLS-DA to ensure the good models. Firstly, we did PCA analysis for the total of eighteen ^{1}H-NMR spectra of nine brand of *jamu*. The automated principal component analysis of PCA resulted high Q2 of 0.65 explaining the high quality of the corresponding model. However, the score plot of PCA did not show a clear separation. Interestingly we observed that *jamu* 1 is quite well separated from others at the negative area of PC2. In addition, we also observed that the rest of the spectra tend to be grouped into two in which sample 7, 6 and 3 were clustered at positive area of PC2. To improve the separation, we then conducted the OPLS-DA. The OPLS-DA resulted 2 principal component with Q2 value of 0.637. The scoreplots of OPLS-DA underline the result of PCA in which three groups were observed (Figure 2a). The first group consisted of *jamu* 1 was far separated from the other groups by PC1. The second and third group were separated from each other by PC2. The second group consisted of *jamu* 2, 4, 5, 8 and 9 while the third group consisted of *jamu* 3, 6 and 7. The validation of the OPLS-DA model was done by permutation test and CV-ANOVA. The permutation test showed that the calculated models had lower values of Q2 and R2 compared to our model (Figure 2b). The *p* value of CV-ANOVA was very significant ($p = 8.84 \cdot 10^{-8}$) indicating a good model. The grouping of samples observed in OPLS-DA models may indicating that *jamu* in the same group contains similar pharmaceutical ingredients on it. We checked the NMR spectra and stack spectra of each group to do the comparison of NMR profile. This model obtain the high value of R2X (0.786) and R2Y (0.912) that showed the model is fit.

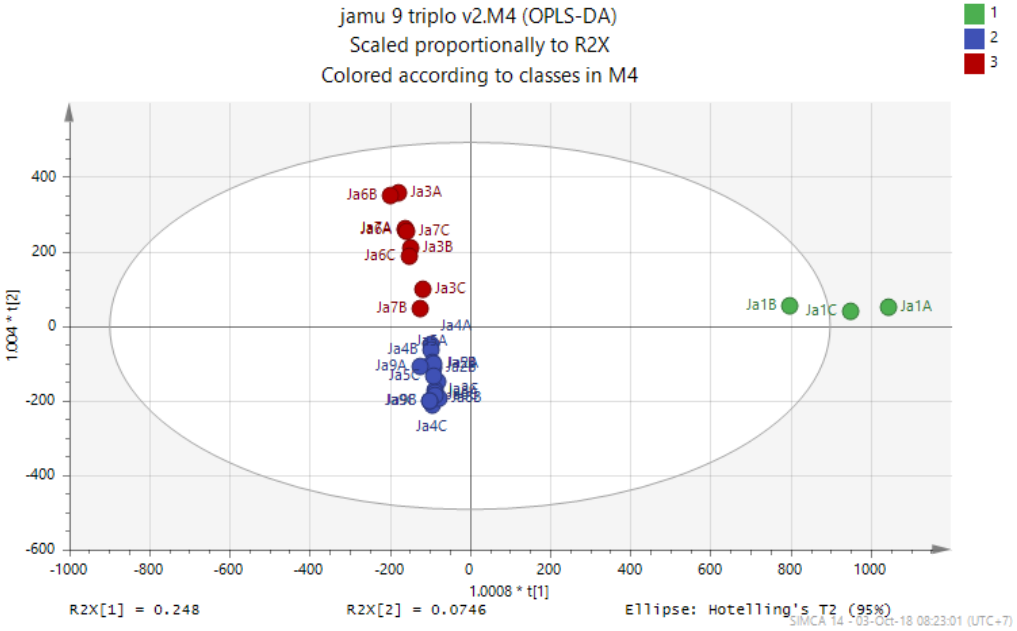

Figure 2a. OPLS-DA score plot of 9 *jamu*

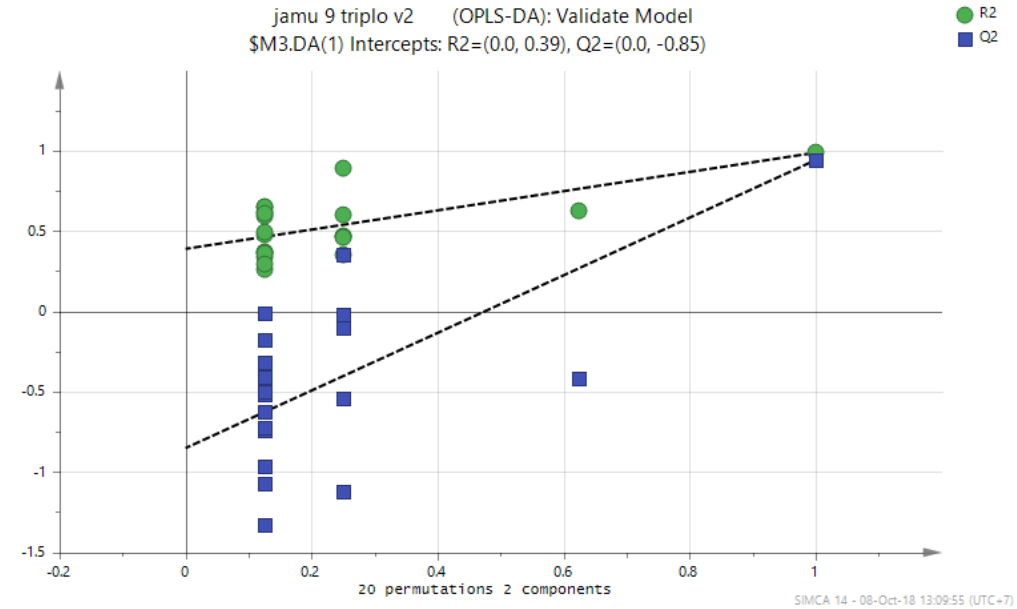

Figure 2b. Summary fit OPLS-DA of 9 *jamu*

The model also give the high value of Q2 (0.818) it shown the goodness of predictivity of the model. The value of R2 and Q2 > 0.5 showed that the model is fit and good predictivity (Windarsih, 2018). Pareto scalling conducted to separate the predictive and non-predictive variation on OPLS-DA model (Zhang *et al.*, 2016). The permutations in figure 2b showed the intersection of Q2 and R2 confined the validity of this model.

Detection of allopurinol in *jamu*

To identify and mark the contaminant of allopurinol inside it we stacked the ^{1}H-NMR spectra of the active pharmaceutical ingredients (allopurinol) and *jamu* 1 (Figure 3).

From the Figure 3, it shown if there is no indication of allupurinol contamination in *jamu* 1. The characteristic peaks from allopurinol do not appear in ^{1}H-NMR spectra of medicinal herbs

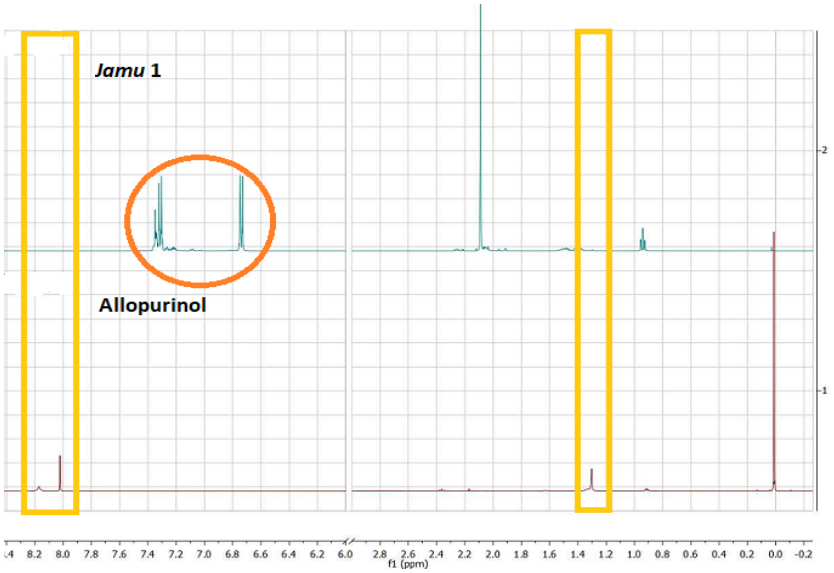

Figure 3. ^{1}H-NMR Stacking spectra of allopurinol standard and *jamu* 1

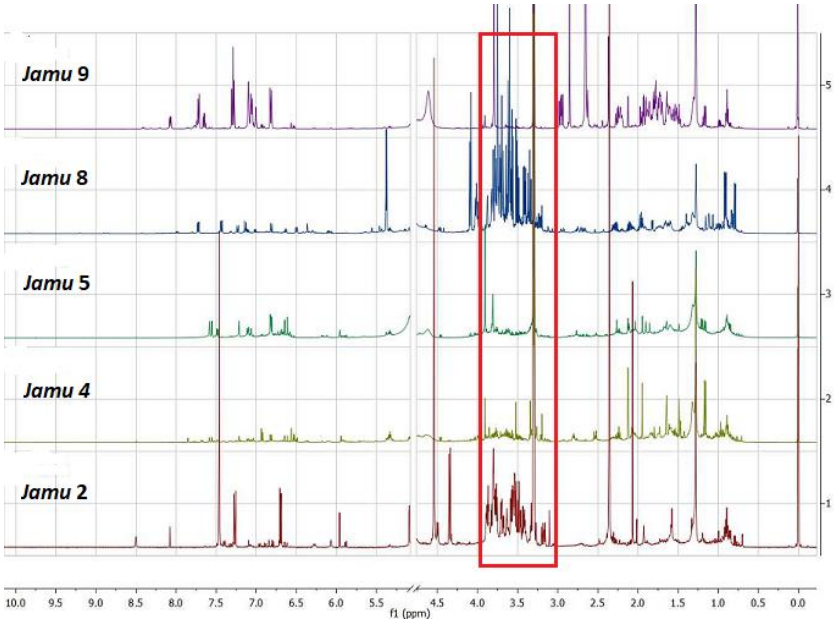

Figure 4a. 1H-NMR of *jamu* from class 2

(*jamu* 1) but there are several peaks in aromatic region from *jamu* 1 that indicated the contamination of other pharmaceutical ingredient.

Figure 4a and 4b shown the stacking of spectra in class 2 and class 3 based on OPLS-DA model result. From figure 4a, the spectra from *jamu* 2,4,5,8 and 9 that looked close in minus area of t2. The high and crowded peak in region 3-4 ppm is sugar. Probably the *jamu* add much sugar on it, beside that several peak also have similar pattern from the herbs. The stacking spectra of class 3 from *jamu* 3, 6 and 7 shown in figure 4b. The figure inform the similar pattern of *jamu* in the region of sugar (3-5-5 ppm) also have crowded peak in up field chemical shift that usually from the herbs.

CONCLUSION

Multivariate analysis used OPLS-DA method proves very useful for differentiation of medicinal herbs and predict the contamination of pharmaceutical ingredients (allopurinol) in *jamu*. The combination between ^{1}H-NMR based metabolite profiling and OPLS-DA method analysis is a sophisticated method for autenthication of medicinal herbs and identification of pharmaceutical ingredients contaminant on it.

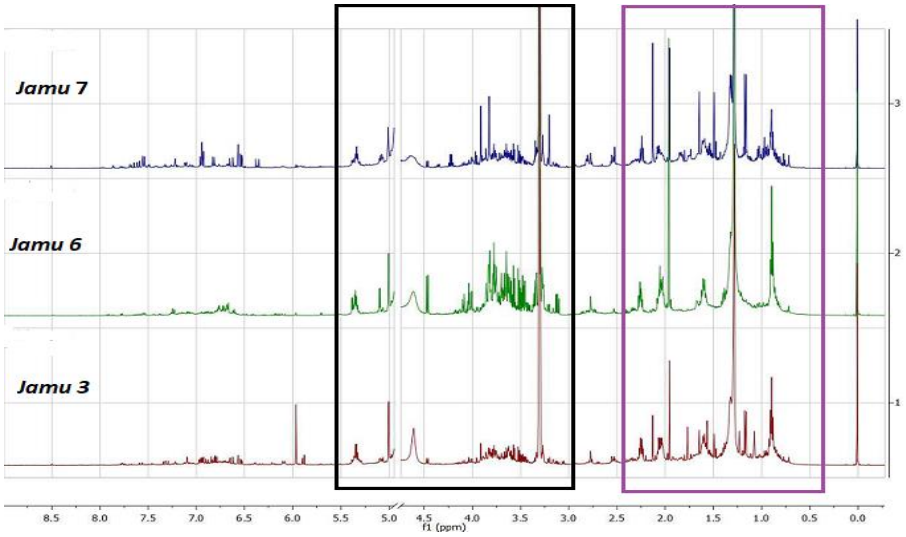

Figure 4b. 1H-NMR Spectra of *Jamu* from class 3

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