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Standardization and Validation Method of the Assay of Andrographolide Content of Ethanol (70%) Extract of A. Paniculata Nees. Using TLC (Thin Layer Chromatography)-Densitometric Method

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Abstract
Traditional medicine has a lot of history in the development of health care in the world. A. paniculata where people often refer to it as "king of bitter" is often used as an herbal dietary supplement product due to many of its activity in the treatment of disease. To be a traditional medicine preparation that produced on an industrial scale, the A. paniculata ethanol extract should be standardized in advance to ensure its quality. Ethanol (70%) usually use in industrial production scale, because the remaining ethanol from the maceration process is more than using ethanol with other concentrations and reusable. TLC (Thin Layer Chromatography)-Densitometric analysis method is an analytical method that has many advantages such as it is economical advantages for many pharmaceutical labs, it is rapid, relatively simple, and samples don't need to be very pure in comparison with HPLC. Therefore, in this article review will provide the explanation of the process of standardization and the validation method of the assay of andrografolida content of ethanol (70%) extract of A. paniculata Nees. using TLC-Densitometric Method.

Keywords : *A. paniculata Nees., standardization, validation method, TLC-Densitometric.*

I. INTRODUCTION

Traditional medicine has a lot of history in the development of health care in the world. Basically traditional medicine is made through the recipe formulas of our ancestors where the formula used the medicinal plant material. The World Health Organization (WHO) estimates that three fourth of the world population presently use herbal medicines and approximately 120 active compounds from plant isolates are widely used in modern medicine today.[7,30] One plant that has the property as a medicinal plant is *A. paniculata* where people often refer to it as "king of bitter" because of its bitter and characteristic taste.[21] *A. paniculata* is often used as an herbal dietary supplement product with the aim of improving the quality of life and preventing diseases of elderly people.[16,21] *Andrographis paniculata* (Burm. f.) Wall. ex Nees (family: *Acanthaceae*) is a herbaceous plant that grows in many South Asian countries and also used as a traditional medicine in China, Hong Kong, Philippines, Malaysia, Indonesia and Thailand.[21,28]

A. paniculata is an upright herb, which grows naturally in the lowlands to an altitude of ± 1600 dpl. *A. paniculata* habitats are open places such as fields, roadsides, cliffs, ducts or rivers, bushes, under the stands of teak or bamboo trees. *A. paniculata* plants have adaptability to the local ecological environment. These plants are found throughout the archipelago as they can grow and multiply on various topography and soil types. It grows well in rainfall 2000-3000 mm/year, air temperature 25-32 °C and humidity required between 70-90%. *A. paniculata* has single leaves, ovoid, crossed opposite, base and pointed tip, flat edge, length ± 8 cm, width ± 1.7 cm. *A. paniculata* stems with wood, cross section, base of round stem. The yellow stems are rectangular but after the old becomes round. *A. paniculata* has many branches, monopoidal, and rectangular (kwadrangularis) with enlarged nodes. The flowers on the *A. paniculata* are clustered bunches in the armpits of the leaves and the ends of the stems, the lanceolate petals, the five share, the green attached base, the stamens two, the round, the purple round purple head, the short pistil, the brownish purple pistil, the oval crown, base attached, the inside is purple-stained white, the red haired outer part. In young *A. paniculata* fruit is green but after dark becomes black, it consists of 11-12 seeds. While the root is a taproot.[1,19]

In *A. paniculata* there are several secondary metabolites which are usually used as the active ingredient of treatment ie diterpenes, lactones, and flavonoids. Flavonoids mainly exist in the root, leaves, and in aerial parts contain alkanes, ketones, and aldehydes. Andrographolide, 14-deoxy-11, 12-didehydroandrographolide-2, 14-deoxyandrographolide-3, 14-deoxyandrographolide-4, 14-deoxy11-oxoandrographolide-5, neoandrographolide-7, 14-deoxyandrographiside-95 are reported to be contained in *A.paniculata* at significant quantities. [1,17,21] There are

many activities in the treatment of diseases possessed in *A.paniculata*, such as antimicrobial activity[25], traditionally used for sluggish liver as antidote in case of colic dysentery and dyspepsia[12], as a domestic remedy in the treatment of colic pain[23], loss of appetite[23], irregular stools and diarrhea[23], liver protection[22], anticancer[15], and antimalarial activities[20], inhibition of replication of the HIV virus[31], antibacterial activity[31], antihyperglycemic effects[31], suppression of various cancer cells[31], antiinflammatory properties[30], and many more of treatment of diseases activities[21]. In China, the herb derived from the leaves or aerial parts of *A. paniculata* is known as Chuanxinlian, Yijianxi or Lanhelian. It has activities as an antipyretic, detoxicant, anti-inflammatory, and detumescent, and is thought to remove "pathogenic heat" from the blood, pharyngolaryngitis, diarrhea, dysentery, cough with thick sputum, carbuncle, sores, and snake bites.[1]

Standardization is a quality assurance program for production and manufacturing of herbal drugs.[3,13] Standardization of herbal medicines can also describe from the process of prescribing a set of inherent characteristics, constant parameters, definitive qualitative and quantitative values that carry an assurance of quality, efficacy, safety and reproducibility.[13,14] There are six major methods for evaluation and standardization of herbal drugs ie the methods and the evaluation parameters are including authentication, organoleptic evaluation, microscopy evaluation, chemical evaluation, physical evaluation, and biological evaluation.[10,13] In Indonesian Herbal Pharmacopoeia, standardization of medicinal plants consists of two parameters: the specific parameters and nonspecific parameter. Standardized test conducted on the specific parameter is organoleptic test and the assay, whereas the nonspecific parameter is the test of total ash, acid insoluble ash content, and moisture content.[5]

Literature review revealed that various methods have been reported for analysis of active compounds including TLC (Thin Layer Chromatography)[2,9], HPLC (High Performance Liquid Chromatography)[9,11,27], HPTLC (High Performance Thin Layer Chromatography)[9,24], UV-Vis (Ultra Violet-Visible Spectroscopy)[9], and GC-MS (Gas Chromatography-Mass Spectrometry)[9]. The developed TLC method has several advantages over other available methods such as ability to analyze several samples simultaneously in parallel, as well as using small quantities of solvents as a mobile phase which reduces time and cost of analysis.[4,18] A TLC method could facilitate the investigation of drugs, especially for the analysis of *A. paniculata* and its derivatives, which don't present any UV chromophores, therefore sophisticated detection techniques are required. Moreover, another strong argument may exist for application of TLC because of its economical advantages for many pharmaceutical labs. It is rapid, relatively simple, and samples need not be very pure in comparison with HPLC.[9,29]

Ethanol (70%) extract of *A. paniculata* usually use in industrial production scale, because the remaining ethanol from the maceration process is more than using ethanol with other concentrations and can be reused in the next maceration process. In order to become a traditional medicine preparation that can be produced and marketed into the community, the preparation of *A.paniculata* ethanol extract should be standardized and validated in the assay of andrographolide content levels using TLC (Thin Layer Chromagraphy)-densitometric method analysis. Therefore, standardization process of the preparation of etanol (70%) extact of *A. paniculata* must be done to fulfill the requirement.

II. METHODS

A. Extraction Method

The extraction process was performed by maceration method. 1 kg of the dried and powdered whole plat of *A. paniculata* was pulverized into powder then weighed 1 kg, soaked in 10 L ethanol (70%) and agitated for 24 h. After 24 h, the ethanol was collected and each plant powder was re-macerated twice with 5 L of ethanol (70%). All collected ethanol was filtrated using buchner funnel and concentrated to 20% of its original volume using rotary evaporator set at 40 °C. Following this, the concentrated extract was dried in an oven at 39 °C to form crude extracts.[26]

B. Standardization

The standardization process was performed based on the two required stages: the determination of the specific parameters (organoleptic and the determination of active compounds) and the non-specific parameters (determination of total ash content, the determination of the acid insoluble ash content, and the determination of water content) of the extract.[26]

C. Organoleptic

Using the senses to describe shapes, colors, smells, and flavors.[5]

D. Assay of active compound

TLC-densitormetric method was used for determination of active compounds process. The process of determining the content of andrographolide used the mobile phase chloroform : ethanol (9:1).[26] The preparation of the test solution was done by dissolving 500 mg of extract in 25 mL of ethanol in the test tube, mixed using vortexer tool for 10-15 minutes then continued to ultrasonic process for 10 minutes. Strain into a measured flask 25 mL, add ethanol through the filter until the mark. Making standard solution was done by dissolving (andrographolide) approximately 10 mg in 10 ml measuring flask, then mixed using vortexer tool for 10-15 minutes until dissolved. Made at least 8 different concentration solutions based on the optimization of the assay. Strip separately each 10 µL replication test solution four times and each standard solutions on the plate silica gel 60 F245 20 x 10 cm, then eluation with corresponding mobile phase. After eluation process was done, the absorption of the spots were measured at maximum absorption wavelength 232 nm in the densitometer. After the data collected from the analysis results and after the calculation was done, andrographolide content contained in extract ethanol (70%) *A. paniculata* expressed in units %w/w.

E. Determination of total ash content

Three replications were performed. Refined 3 g of etanol (70%) extact of *A. paniculata*, then put into the crucible silicate, gently heat until the charcoal is exhausted, cooled and then calculated using the formula[5] :

$$= \frac{((Crucible + ash\ extract)\ -\ (Empty\ crucible))}{Weight\ sample\ (g)} x100\%$$

F. Determination of acid insoluble ash content

Three replications were performed. Ash collected from the determination of total ash content was dissolved in 25 mL dilute hydrochloric acid 2 M for 5 minutes. Collect the acid-insoluble parts, strain through the ash-free filter paper, wash with hot water, heat in the crucible until the weight is fixed. The acid soluble ash content was calculated using the formula[5] :

$$= \frac{((Crucible + ash\ extract)\ -\ (Empty\ crucible))}{Weight\ sample\ (g)} x100\%$$

G. Determination of water content

Three replications were performed. Added 5 g of etanol (70%) extact of *A. paniculata* and 200 mL toluene to the measuring flask, attach the tool set. Heat the flask for 15 minutes, after 15 minutes set the distillation at ± 2 drops/sec and then increase the distillation speed to 4 drops/sec. After all the water was distilled, refinish for 5 minutes. If water and toluene were separated, measure the volume of water by the formula [5] :

$$= \frac{Water\ volume\ (mL)}{Weight\ sample\ (g)}\ x\ 100\%$$

H. Validation of the assay andrographolide content

Specificity: This specificity test is a validation method to see the similarity between the standard stain and the sample stain in the klt plate used in the assay method. The similarity is seen from the correlation between spectra produced by each stain (peak identity). From the value of the correlation (peak identity) it can then be concluded that there is a standard compound in the sample identified. Spectra used as a reference usually comes from the standard mid-level of the linear standard curve level curve.[6,8]

I. Detection limits (LOD/LOQ): Determine the S/N price by measuring the largest flame of shell fluctuations at 20 times peak width at ½ height of the analytical peak (N) and peak height of the analyte (S). Made several concentrations of standard solutions ie 0.03, 0.04, 0.05, 0.10, 0.15, and 0.20 ppm. The following equations were applied in order to calculate the LOD and LOQ value respectively where σ–standard deviation of response, S–slope of calibration plot[6,8] :

$$\text{LOD} = 3.3 \text{ x } \sigma/S \quad \text{Eq. 1}$$
$$\text{LOQ} = 10 \text{ x } \sigma/S \quad \text{Eq. 2}$$

Linearity: Liniaerity was done with making a series concentration of mixed etanol (70%) extact of *A. paniculata*. The concentrations were used ie 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, and 0,35 ppm for process of determining the content of andrographolide; Then calculated data result of liniertias include correlation coefficient (r), y intercept, slope of regression line.[6,8]

Accuracy: Accuarcy was done with making 3 contents of andrographolide solution with concentration 80%, 100%, and 120% replicated 3 times. Determination of contents of 100% obtained from the respective contents of andrographolide obtained during the assay of andrographolide in etanol (70%) extact of *A. paniculata*. For process of determining the content of andrographolide, the standard concentrations of 80%, 100%, and 120% were respectively ie 0.2 ppm, 0.25 ppm, and 0.3 ppm Then the results calculated as a percentage of recovery (% recovery).[6,8]

Precision: Precision was done by making 6 concentrations of standard with the same concentration. For process of determining the content of andrographolide concentration, the standard concentration was 0.25 ppm and for determining content of stigmasterol was 0.3 ppm. Then the results calculated as standard deviation (SD) or relative standard deviation (RSD).[6,8]

III. CONCLUSION

The standardization and the validation method of the assay of andrografolida content of ethanol (70%) extract of *A. paniculata* Nees. must be done to fulfill the requirement of traditional medicine product and TLC-Densitometric method is the best method of analysis not only in the standardization process but also in validation method of the assay of andrografolida content.

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