**INTRODUCTION**

Increasing Reactive Oxygen Species (ROS) may decrease the cellular antioxidant system in patients with diabetes mellitus. This condition may lead to the complications of diabetes mellitus. Previous research stated that combination of an oral antidiabetic with antioxidant is potential to treat disease and the complications of diabetes mellitus.  

Various studies have proven that *Andrographis paniculata* have an efficacy as an antidiabetic, and antioxidant activity. Meniran herbs (*Phyllanthus niruri*, L) is one of medicinal plants used in traditional medicine in India, China and Indonesia. *P. niruri* has been used as a laxative, dissolve kidney stones, diuretic, hepatitis, dysentery, and rheumatism. The activity of *P. niruri* as source of antioxidants have been proven, besides other activities such as antibacteria, antidiabetic, and anticancer.

Recently, preparation of self-nanoemulsifying drug delivery system (SNEDDS) containing combination of extract of *A. Paniculata* (APE) and *P. Niruri* (PNE) has been successfully developed. The formulation of nanoemulsion system reduced the dose of the extract, enhanced effectiveness as antidiabetic and antioxidant, and led to improving patient acceptance in consuming traditional medicine. The nanoemulsion system was purposed to be build into Obat Herbal Terstandar (Standardized Herbal Medicine).

According to Indonesian government regulations, Standardized Herbal Medicine (SHM) is a preparation of natural materials that have demonstrated safety and efficacy in preclinical trials with the raw material has been standardized. Traditional medicine formulations as SHM will improve the quality and efficacy of traditional medicines marketed, and also the image and appearance of traditional medicine which is expected to be complied by patients. This regulation is in correlation with WHO guidelines that an herbal product needs to be standardized before releasing it into the market in order to ensure its safety and efficacy.

Standardization of raw material in the SHM play an important role, i.e. to ensure a consistent biological activity and chemical profile of the product, and also as a tool for quality assurance in production and manufacturing process of herbal drugs. Many methods have been introduced for the quantitative analysis of andrographolide. Maiti have introduced the use of spectrophotometry to quantify andrographolide in *A. paniculata* extract, based on red color caused by the complex formation in the addition of NaOH, but this color complex is unstable and easily disappears. Gravimetric method of Pharmacopoeia of India also can be used to quantify andrographolide, and the use of lactone titration introduced by Aromdee, but this method is time consuming. The other method introduced were HPTLC and RP-HPLC with gradient elution system using 1% formic acid and methanol or ACN:methanol and 0.1% phosphoric acid with gradient elution. In this research andrographolide was determined using a mobile phase of acetonitrile and 0.1% phosphoric acid (40:60, flow rate 1 ml/min). This research was conducted to obtain a valid method to determine andrographolide in herbal extracts using RP-HPLC.

**MATERIALS AND METHODS**

**Materials**

The materials used are standard andrographolide (Wako), acetonitrile (grade gradient for liquid chromatography,
Methods

Preparation of sambiloto leaves and meniran herbs extract

Seven hundred grams of dry powder of sambiloto leaves and 480 g of meniran herbs were macerated with petroleum ether as much as 5 times the weight of crude herbs. Subsequently dried pulp was macerated with ethanol 70% as much as 10 times the weight of crude herbs for 5 days, while stirring occasionally. The filtrate was evaporated with a vacuum rotary evaporator to obtain a viscous extracts.

Preparation of stock solutions of andrographolide

Weighed as much as 50 mg of andrographolide thoroughly, then diluted with 10 ml of methanol to obtain a concentration of 0.5 mg/ml (solution A).

Elution optimization

A two hundred micro liter of solution A was inserted into the vial, 800 µL of methanol was added to obtain a final volume of 1.0 mL. Solution of 20 µL was automatically injected and eluted using a mobile phase of acetonitrile:0.1% phosphoric acid (40:60) in isocratic system and a flow rate of 1 mL/min. The detection was made at a wavelength of 230 nm. The optimization resulted an elution time of 10 minutes.

Preparation of calibration curve

A series of andrographolide of 100-600 mg/ml was prepared. The each concentration was analyzed by HPLC system.

Sample preparation

A total of 100.0 mg of extract of sambiloto and 100.0 mg of extract meniran was carefully weighed using a weighing bottle and then diluted with methanol up to 5.0 ml. The solution was treated by ultrasonic for 10 minutes due to degassing and improve the dilution. Sample was filtered and the filtrate was filtered through a 0.45 µm micropore. A 50 µl aliquot was taken and added by methanol up to 1000 µl. Solution was ready to inject into the separation system.

Determination of validation parameter

Validation of the method followed the protocol issued by ICH2 for accuracy, inter and intra-day precision, linearity and LOD-LOQ.

Accuracy

Accuracy parameter performed by standard addition method by adding three levels concentration of standard (80 % (1.03 mg/ml), 100 % (1.3 mg/ml) and 120 % (1.56 mg/ml)). Each series levels were replicated 3 times.

Levels obtained calculated value of recoveries with 95-105 % acceptance condition.

Intra and inter day Precision

Intra-day precision was performed by injection of six replicates at the same concentration and the same day. Inter-day precision was made by injection at 3 different days. The acceptance criterion is the RSD value less than 2.5%.

Linearity

Parameter of linearity was obtained by created 7 series concentration of samples on the range of 70-130%, then calculated the value of R with the terms of acceptance of more than or equal to 0.98.

RESULTS AND DISCUSSION

Optimization of elution using separation system was performed to obtain a separation profile sample and standard of andrographolide. Based on the chromatogram, the retention time of andrographolide standard was 3 minutes, and as well as the sample also showed the peaks in the same retention time (Fig 1). Due to the chromatogram profiles, the separation system is relatively rapid, selective and sensitive. One of the advantages of this research from previous study is the simple combination of mobile phases and the short of elution time.

Calibration curve obtained was \( y = 3 \times 10^8 x + 1 \times 10^6 \) with \( r^2 = 0.975 \). This equation showed that this method has good sensitivity. Evaluation of accuracy is based on percentage of recovery. The results showed that the percentage of recovery and relative standard deviation were met to the requirements. These data indicate that there is no systematic error in the HPLC method to determine andrographolide in the extract matrix.

Table 1: Percentage of recovery, intra and inter-day precision and linearity of Andrographolide (n=3)

<table>
<thead>
<tr>
<th>Level concentration (%)</th>
<th>Amount of added (mg)</th>
<th>Amount of found (mg)</th>
<th>% recovery ± SD</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>1.04</td>
<td>1.03</td>
<td>99.33 ± 1.78</td>
<td>1.79</td>
</tr>
<tr>
<td>100</td>
<td>1.30</td>
<td>1.25</td>
<td>95.69 ± 0.60</td>
<td>0.63</td>
</tr>
<tr>
<td>120</td>
<td>1.56</td>
<td>1.52</td>
<td>97.12 ± 1.79</td>
<td>1.84</td>
</tr>
<tr>
<td>Intra-day precision</td>
<td></td>
<td></td>
<td>6.54</td>
<td></td>
</tr>
<tr>
<td>Inter-day precision</td>
<td></td>
<td></td>
<td>6.32</td>
<td></td>
</tr>
<tr>
<td>Linearity (r)</td>
<td></td>
<td></td>
<td>0.9949</td>
<td></td>
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</table>
Table 1 showed that the precision parameter have larger value of RSD than standard requirement. None the less the precision could be received due to complexity of matrix and heterogeneous sample. In addition the larger value of RSD describes the random error which could be reduced by adding number of replication. Extract was used to assess linearity parameter of this research to obtain lineier responses of method to alteration of sample amount. Linearity assessed was based on coefficient correlation that must be larger than 0.98, due to that the data met the requirement. Chromatogram showing a change in a linear response to changes in the amount of samples (figure 2).

From these results it can be concluded that this method were accurate and precise to determine andrographolide in extract.

CONCLUSION

The lineer range of calibration curve is 0.1-0.6 mg/ml, with \( r^2 = 0.975 \), and the accuracy range at 95.58%-100.7% with RSD value is 1.42\%. The Precision of intra and inter day were 6.54\% and 6.32\%, respectively. This validated method is expected to be a reference method for standardization of herbal nanoemulsion that being developed.

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REFERENCES


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