INTRODUCTION

Validation is a concept to ensure that a method used to determine the quality of the medicinal ingredients and the right and available drug preparations proven effectiveness and reproducibility [1].

The large number of medicinal plants in Indonesia encourages the pharmaceutical industry to develop the new and advance formulations to be able to increase the bioavailability of medicinal plants based product. The most important thing in the formulation of a phytopharmaceutical product is that the product contain standardized phytochemical from medicinal plants. *Andrographis paniculata* (Burm f.) Ness. or locally known as Sambiloto in Indonesia has been traditionally used to treat various diseases. This plant has been shown to display anti-diabetic activity [2-5]. This plant also display potent antioxidants activity [6-8]. *Phyllanthus niruri* L. or locally known as Meniran in Indonesia also has been used widely in India, China and Malaysia. *Phyllanthus niruri* is ethnobotanically used to treat hepatitis, dysentery and rheumatism. The activity of *Phyllanthus niruri* as a source of antioxidants has been scientifically shown [9], in addition to other activities such as antibacterial, anti-diabetic and anticancer [10-12].

Previous studies have been carried out to validate the content of andrographolide from *A. paniculata* extract. Da'i et al. [13] studied the validated method to determine *A. paniculata* polyherbal mixture using RP-HPLC. Manasa and Suneetha [14] developed the method to determine the content of andrographolide levels using a UV-Vis spectrophotometer. The result of this study showed good validation results for andrographolide levels in polyherbal mixture.

The purpose of this study was to validate the method for determination of andrographolide levels in nanoemulsion preparations containing a combination of *Andrographis paniculata* and *Phyllanthus niruri*. The validation was done by determine the parameters such as linearity, intra and interday precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ) in the range of 30-80 µg/mL. The accuracy (recovery) varied in the range of 97.15 to 104.42 %.

Percentage of relative standard deviation (% RSD) for precision and intermediate precision value were 3.23 and 3.02 % with LOD value 211 µg/mL and the LOQ 705 µg/mL. As a conclusion, this method is suitable to detect andrographolide content in herbal nano-preparation.

Keywords: *Andrographis paniculata*, *Phyllanthus niruri*, Spectrophotometry, Andrographolide, Nanoemulsion.
Andrographis paniculata and Phyllanthus niruri extract using a UV-Vis spectrophotometer.

**EXPERIMENTAL**

Dried herb of Andrographis paniculata was bought from the local herbal shop, Solo, Indonesia, while Phyllanthus niruri was purchased from the wet market (Pasar Gede) Solo, Indonesia. P. niruri was then dried under indirect sunlight for 3 days.

**Sample extraction:** Dried Andrographis paniculata and Phyllanthus niruri plants were grounded into powder using dry blender to get a uniform size. To eliminate chlorophyll, the samples were then soaked with petroleum ether (Sigma-Aldrich) in the ratio of 1:5 for A. paniculata and 1:7 for P. niruri. Then the mixture was filtered and the residue was dried for overnight. Dried residue were macerated using 70% ethanol (Merck) for 5 days, while stirring occasionally. Then the filtrate was evaporated with a vacuum rotary evaporator (Buchi) until thick extract was obtained.

**Preparation of nanoemulsion:** Nanoemulsion samples were prepared by mixing SNEDDS (self nanoemulsion drug delivery system) into water with a water and SNEDDS ratio (2:1). Then the solution was homogenized using a magnetic stirrer for 10 min at medium speed without heating.

**Stock and working solutions:** A solution of andrographolide stock was prepared at concentration of 500 μg/mL (solution A). For accuracy study, a solution of andrographolide stock (in methanol) was prepared at concentration of 5 mg/mL (solution B). Solution C was prepared by mixing 1% picric acid in water and 10% NaOH in water. Ratio of picric acid and NaOH was 8:2. All the solutions were prepared fresh prior to experiments.

**Optimization of experimental conditions**

**Determination of operating time:** The operating time was determined by mixing 0.4 mL of solution A with 2.5 mL of solution C then made up the volume of 5 mL with methanol. The solution is read after every 2 min at 481 nm.

**Determination of maximum wavelength:** The maximum wavelength is determined by mixing 0.4 mL of solution A with 2.5 mL of solution C then made up the volume of 5 mL with methanol. The solution is read after every 2 min at 481 nm. The solution is read again after incubation for 22 min. From the results, maximum wavelength (λmax) was found to be 479 nm.

**Andrographolide standard curve:** Andrographolide concentration was prepared at different concentrations from 30 to 80 μg/mL. Then each concentration was analyzed using a UV-Vis spectrophotometer at operating time and maximum λmax = 479 nm. The absorbance obtained is a linear regression equation using a UV-Vis spectrophotometer.

**Determination of validation parameters**

**Intra-day and inter-day precision:** Intra-day precision was performed by reading six replicates at the same concentration in the same day. Inter-day precision was made by conducting experiment at different days. The acceptance criteria is the RSD value less than 5%.

**Linearity:** Linearity is performed by standard addition method by adding 3 series of concentration levels, 80% (596 μg/mL), 100% (746 μg/mL), 120% (895 μg/mL). Each series of levels were replicated thrice. The acceptance criteria is the recovery value in range 95-105%.

**Limit of detection (LOD) and Limit of quantification (LOQ):** Andrographolide standard curve was prepared using different concentrations (30-80 μg/mL).

**RESULTS AND DISCUSSION**

**Optimization of wavelength and operating time:** Andrographolide in polyherbal formulation can be determined using spectrophotometry analysis based in the presence of chromophore and auxochrome groups in the compound. Therefore, it is necessary to add picric acid in alkaline condition to form andrographolide complex which is orange in colour (Scheme-I).

From the optimization results a value of λ maximal of 479 nm and operating time for 22 min (Fig. 1) were obtained.

![Graph](image.png)

Fig. 1. Spectra of standard andrographolide absorption; Andrographolide level was analyzed using UV-vis spectrophotometer in a wavelength range of 400-800 nm. OT 22 min with λmax 479 nm

**Linearity:** Linearity between the concentration of andrographolide and absorbance was calculated based on a series of concentrations of ranging from 30 to 80 μg/mL. The results are plotted between absorbance versus concentration and then a linear regression equation is made and the correlation coefficient value is obtained. From the experimental results, the linear regression equation is $y = 1.09 \times 10^2 - 2.07 \times 10^2$ with r value of 0.9945 (Fig. 2) [15].

**Intra-day and Inter-day precision:** Intra-day and inter-day precision of the experiment was conducted to validate the
Fig. 2. Standard curve of andrographolide; The standard andrographolide curves were analyzed using a UV-vis spectrophotometer at a wavelength of 479 nm, 22 min OT, with a range of 30-80 µg/mL. Linear regression $y = 0.0109x - 0.2066$ with $r = 0.9945$

Scheme-I: Mechanism of reaction for the formation of an andrographolide-picric complex

**Accuracy:** Evaluation of accuracy is based on percentage of recovery. The value of % recovery from three levels of concentration between is 97.15 to 104.42 % with % RSD value of 1.99-2.56 % (Table-2).

**Limit of detection (LOD) and Limit of quantification (LOQ):** The LOD and LOQ values are found to be 211.6 and 705.4 µg/mL. These results indicated that UV-Vis spectrophotometer method can detect andrographolide with the lowest concentration of 211.6 µg/mL, besides that it can quantify the andrographolide with the lowest limit of 705.4 µg/mL.

A study conducted by Manasa and Suneetha [14] with different reagents and conditions were almost similar with the
TABLE-1
INTRA- AND INTER-DAY PRECISION OF ANDROGRAPHOLIDE

<table>
<thead>
<tr>
<th>Sample concentration (mg/mL)</th>
<th>Average ± SD</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-day precision</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.451</td>
<td>1.439</td>
<td>3.23</td>
</tr>
<tr>
<td>1.549</td>
<td>1.441</td>
<td>4.21</td>
</tr>
<tr>
<td>1.508</td>
<td>1.508</td>
<td>3.06</td>
</tr>
<tr>
<td>Inter-day precision</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.489</td>
<td>1.486 ± 0.047</td>
<td>3.06</td>
</tr>
<tr>
<td>1.453</td>
<td>1.453</td>
<td>3.21</td>
</tr>
<tr>
<td>1.526</td>
<td>1.526</td>
<td>3.06</td>
</tr>
</tbody>
</table>

TABLE-2
ACCURACY RESULTS OF ANDROGRAPHOLIDE

<table>
<thead>
<tr>
<th>Concentration level (%)</th>
<th>Analyte found (mg/mL)</th>
<th>Recovery (%)</th>
<th>Average ± SD</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>1.53</td>
<td>100.5</td>
<td>99.94 ± 2.56</td>
<td>2.56</td>
</tr>
<tr>
<td></td>
<td>1.51</td>
<td>97.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.54</td>
<td>102.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>1.71</td>
<td>104.42</td>
<td>102.19 ± 2.04</td>
<td>1.99</td>
</tr>
<tr>
<td></td>
<td>1.69</td>
<td>101.74</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.68</td>
<td>100.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>1.83</td>
<td>100.45</td>
<td>102.31 ± 2.32</td>
<td>2.27</td>
</tr>
<tr>
<td></td>
<td>1.84</td>
<td>101.56</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.87</td>
<td>104.91</td>
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In conclusion, a fast, simple and inexpensive method for detection and quantification of andrographolide concentration in nanoemulsion preparations containing a combination of Andrographis paniculata (Burm f.) Ness. and Phyllanthus niruri L. has been developed. The linear range of standard curve 30-80 µg/mL with r = 0.9945 and the accuracy range at 97.15-104.42 % with RSD value is 2.27 %. The precision of intra-day and inter-day were 1.48 and 1.49 %, respectively. This validated method is expected to be a reference method for standardization of herbal nanopreparation.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

REFERENCES