INTRODUCTION

The public warnings issued by the United States Food and Drug Administration (USFDA), the Health Canada and the Medicine Controls Agency (United Kingdom) advised consumers to immediately discontinue use of botanical products containing aristolochic acid (AA). These alerts came from the information that AA and its derivatives were revealed to be nephrotoxic, carcinogenic, and mutagenic. Therefore, the use of botanical products containing AA are no longer permitted in many countries. For these reasons, there are considerations for detection of AA analogues in suspected Thai herbal products before use.

AA analogues are mixtures of structurally related nitrophenanthrene carboxylic acids, found primarily in the plant family Aristolochiaceae including Aristolochia and Asarum genera. Among AA analogues, AA-I is a predominant constituent of Aristolochia spp. and was found to be the most toxic compound in terms of cytotoxicity to renal cell lines. In mice study, the strongest nephrotoxic effect was exerted by AA-I.

In Thailand, Krai-krue is identified as roots of Aristolochia pierrei Lec., together with Aristolochia tagala Cham. This crude drug is commonly available in Thai herbal dispensaries. In addition, Krai-krue is widespread used as an herbal constituent in Thai traditional herbal recipes. In this study, Krai-krue was identified as the root of Aristolochnia gafa Cham. (ATC). Information from the List of Herbal Medicinal Products A.D. 2006 indicates...
four Thai traditional recipes containing Krai-krue including Homnawakod, Dhatbanjob, Prasakanplu and Kheawhom recipes. Homnawakod was the first recipe we selected for this pioneer work. In general, Homnawakod is used for treatment of nausea, vomiting, and vertigo in the elderly. Precautions were reported in pregnant women and pollen allergy patients. Homnawakod has been widely used in Thailand for a long time, with no report for undesirable symptoms.

Up to now, there are many chromatographic methods for determination of AA-I, such as thin layer chromatography (TLC),18 high performance liquid chromatography (HPLC) equipped with UV absorption detection,19-25 capillary electrophoresis (CE) with UV detection,22,24 HPLC-MS/MS26 and ultra-high performance liquid chromatographic tandem mass spectrometry (UHPLC-MS/MS),27 etc. Among these methods, ultra high performance liquid chromatography equipped with UV absorption detection (UHPLC-UV) is our choice, since it doesn’t require sophisticated and specialized equipment. UHPLC-UV showed many advantages compared to the conventional HPLC method, including reduced run time, less solvent consumption and increased peak capacities.26 This work focuses on developing a simple and reliable method for quantitation of AA-I in ATC and Homnawakod recipes using UHPLC-UV.

**MATERIALS AND METHODS**

**Plant materials**

All plant materials were purchased from several trusted local suppliers and authenticated by experienced Thai traditional practitioners. Krai-krue was later identified as Aristolochia tagala Cham., (ATC). Then they were prepared by the GMP certified Manufacturing Unit of Herbal Medicine and Products, Center of Applied Thai Traditional Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. ATC were separated from foreign matters, cleaned, dried and ground. The production of Homnawakod powder (a traditional recipe) started with separating all raw materials from foreign matters. Then they were cleaned and dried. Each raw material was accurately weighed according to the master formula. Then they were ground, sifted and mixed together. Finished Homnawakod powder was packed into clean bottles and labeled. The test specimens were kept in a dry place, at room temperature.

**Chemicals and reagents**

HPLC-grade acetonitrile (ACN) was purchased from LEDA (Spain). Analytical grade acetic acid was obtained from Merck (Germany). Standard AA-I was purchased from Sigma Chemical Co. (St. Louis, USA). HPLC quality water was purified using a Milli-Q water system from Millipore (France).

**Instrument and experiment**

Analyses were carried out using a Water Acquity UHPLC system (Water, Milford, MA, USA) equipped with a binary solvent delivery system, an auto sampler and DAD-UV detector (Acquity UPLC photodiode array detector). The separations were performed on a reverse phase column (BEH C18, 1.7 µm, 2.1×100 mm, Water, Milford, MA) which was maintained at 30°C. In this study, various ratios of the mobile phase compositions and the detection wavelengths were observed to obtain the suitable detection conditions. The experiments were performed under isocratic elution which consisted of 2% acetic acid: ACN at 62:38 (v/v) at a flow rate was 0.4 mL/min. The injection volume for all samples was 2 µL. The DAD-UV detector was scanned from 230 to 450 nm. The quantitation wavelength was set at 395 nm.

**Sample preparation**

Homnawakod powder and ATC powder were dissolved in 100% methanol at concentrations of 200 mg/mL and 3.66 mg/mL respectively. The extractions were performed by mixing for 30 minutes and ultrasonication for 30 minutes. After being centrifuged at 15,000 rpm for 10 minutes at 4°C, supernatants were filtered through the 0.2 µm PVDF (polyvinylidene fluoride) membrane filters for analysis.

**Validation of the UHPLC method**

**Calibration curve, LOD and LOQ**

Standard AA-I (2.5 mg) was accurately weighed and dissolved in methanol in a 25 mL volumetric flask to make a stock solution (100 µg/mL). The stock solution was filtered through 0.2 µm PVDF membrane filters. Working standard solutions were prepared from the stock solution by further dilution with the appropriate volume of methanol. In this study, the standard curve was established based on eight concentrations (2, 2.5, 5, 10, 20, 30, 40 and 50 µg/mL).

**Precision**

Precision was evaluated with three concentrations of standard solutions under the selected condition five times in a day for intra-daily precision. For inter-daily precision, the measurements were performed three times a day on three consecutive days. All of the measurement precisions were expressed as RSD.

**Repeatability**

Six independently prepared sample solutions of ATC 3.66 mg/mL and Homnawakod 200 mg/mL were analyzed and the results were used for calculation of repeatability.

**Recovery**

The recovery was performed by adding three known amounts of the standard solution. The spiked samples were then extracted, processed, and quantified.

**RESULTS**

Homnawakod is composed of 56 kinds of herbal raw materials. Among these, there is 3.66 mg of ATC in Homnawakod 200 mg. Therefore, determinations of AA-I were performed in 3.66 mg/mL of ATC and 200 mg/mL of Homnawakod. After being extracted by methanol and injected in UHPLC, the chromatogram of ATC and Homnawakod has been shown in Fig 1.

In the present study, the various percentages of mobile phase comprising of ACN and 2% acetic acid were observed to optimize the separation and sensitivity. As the percentages of organic solvent increased, the signal of the AA-I was increased. When the percentage of organic solvent was too high, the peak of AA-I and other compounds were not fully separated, especially in the Homnawakod recipe. Fig 1A, B showed the UHPLC chromatograms of standard AA-I, an extract of ATC at 395 nm, respectively. Using the Homnawakod recipe as a sample, we found that the maximum response with resolution 1.62 of AA-I peak was reached when the percentage of ACN was 38% (Fig 1C). Since AA-II may be found in the traditional recipes containing Aristolochia spp., to confirm whether this compound interfered with the developed method, we injected the mixed standard of AA-I and AA-II using this
condition. The result showed separation of the peak AA-I and AA-II with a resolution value more than 2 (Fig 2A, B).

Selections of the detection wavelength were performed by observation of the signal in various wavelengths. Although AA-I can be detected at 254 nm, it was interfered by some compounds in Homnawakod. In order to avoid those interferences, the detection wavelength was changed to 395 nm. The maximum absorption wavelengths of AA-I were 249.4, 321.3 and 395.2 nm (Fig 1D). When comparing the absorbance spectrum of AA-I, an almost perfect match was obtained from ATC (Fig 1E), whereas some differences were obtained from Homnawakod especially between 230-300 nm (Fig 1F).

The linearity of the plot concentrations (X, µg/mL) versus peak areas (Y) of AA-I was investigated in the range of 2-50 µg/mL. The regression equation and correlation coefficient ($R^2$) of AA-I were as follows: $Y = 9646.802X + 1610.814$ ($R^2 = 0.9999$). The result showed a satisfactory linearity. LOD and LOQ, were calculated as the amount of the compounds needed to produce a signal-to-noise ratios (S/N) of about 3 and 10, were determined to be 0.8 and 2.0 µg/mL, respectively.

For validation of the assay, the results of precision, repeatability and recovery rates are shown in Tables 1 and 2. The precision RSDs of this method were 0.24-0.51 for intra-day and 0.89-1.37 for inter-day. These results represent high precision of the method. The RSDs of the repeatability test were not more than 1.86 and 0.45 for ATC and Homnawakod, respectively. The results showed satisfactory recovery of AA-I in ATC with 101.7%. In contrast, unsatisfactory recovery of AA-I in Homnawakod was shown at 73.82%.

### DISCUSSION

Several HPLC-UV methods have been developed to identify AA-I in plants. The analytical time of those methods were all longer than 15 min. Study using UHPLC-MS/MS method could determine AA-I within 10 min. By using the presented UHPLC-UV method, AA-I could be determined within 6 min. Therefore, our developed UHPLC-UV method shows advantage in analytical time. Since, AA-I was identified by comparisons of the retention time (RT) and absorbance spectrum profiles of the samples with standard AA-I as well as spiking the samples with AA-I for the accuracy test. We are certain for the identification of AA-I in ATC. In this study, the content of AA-I in ATC was 0.237 %, whereas the unsatisfactory results of the absorbance spectrum and recovery of Homnawakod were found. The undesirable results may came from the complex recipe of Homnawakod with comprises multiple components blended together. The complex chromatogram pattern originated from a lot of interfering compounds. This problem can be found when a sample extract is applied directly to HPLC apparatus without a clean-up step, especially with the complex recipes. Removal of many interfering compounds is necessary for detection and accurate measurement of AA-I in the Homnawakod recipe. Our unpublished study investigated whether anion-exchange resin would clean-up Homnawakod sample before injection into the UHPLC system. The result showed a decrease of the interfering peaks with similar absorbance spectrum patterns compared with standard AA-I. Therefore, a clean-up step is crucial for identification and quantitation of AA-I in a Homnawakod sample.

A rapid and reliable UHPLC-UV method for quantitation of AA-I in ATC was established in this study. By using an isocratic mobile phase composed of 2% acetic acid

### TABLE 1. Intra-daily and inter-daily Precisions of Aristolochic acid I

<table>
<thead>
<tr>
<th>Chemical compound</th>
<th>Concentration (µg/mL)</th>
<th>Intra-daily Contents (µg/mL ± S.D.)</th>
<th>RSD (%)</th>
<th>Inter-daily Contents (µg/mL ± S.D.)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>3.83 ± 0.02</td>
<td>0.45</td>
<td>3.94 ± 0.05</td>
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<tr>
<td>Aristolochic acid I</td>
<td>25</td>
<td>24.42 ± 0.13</td>
<td>0.51</td>
<td>25.64 ± 0.29</td>
<td>1.12</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>50.41 ± 0.12</td>
<td>0.24</td>
<td>50.06 ± 0.44</td>
<td>0.89</td>
</tr>
</tbody>
</table>
and ACN, AA-I could be determined within 6 min. RSDs of intra-day and inter-day precision were not more than 1.37. The result showed satisfactory recovery of AA-I in ATC with 101.7%. Due to its rapidity, high accuracy and precision, the developed method could be directly used as a screening method to analyze ATC samples from several sources. Whereas, quantitation of AA-I in Homnawakod sample require clean-up step before analysis by UHPLC-UV apparatus.

ACKNOWLEDGMENTS

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REFERENCES


TABLE 2. Repeatability and recovery tests of Aristolochic acid I.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Original (µg/mL)</th>
<th>Found (µg/mL)</th>
<th>Recovery (± SD)</th>
<th>Mean recovery (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATC</td>
<td>8.67 ± 0.16</td>
<td>1.86</td>
<td>5.0</td>
<td>13.86 ± 0.26</td>
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<tr>
<td>Homnawakod</td>
<td>5.78 ± 0.03</td>
<td>0.44</td>
<td>4.0</td>
<td>14.86 ± 0.13</td>
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<tr>
<td>9.00 ± 0.03</td>
<td>0.45</td>
<td>7.6</td>
<td>12.55 ± 0.17</td>
<td>1.35</td>
</tr>
<tr>
<td>7.58 ± 0.03</td>
<td>0.44</td>
<td>10.0</td>
<td>74.44</td>
<td></td>
</tr>
<tr>
<td>ATC</td>
<td>7.32 ± 0.13</td>
<td>1.73</td>
<td>8.7</td>
<td>16.19 ± 0.30</td>
</tr>
<tr>
<td>Homnawakod</td>
<td>6.90 ± 0.03</td>
<td>0.45</td>
<td>7.6</td>
<td>12.55 ± 0.17</td>
</tr>
<tr>
<td>7.58 ± 0.03</td>
<td>0.44</td>
<td>10.0</td>
<td>74.44</td>
<td></td>
</tr>
</tbody>
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113