PHARMACOGNOSTIC EVALUATION AND CHRYSAZIN QUANTITATION OF Xyris indica FLOWERING HEADS

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Abstract: Flowering heads of Xyris indica L. (Xyridaceae) from 15 different sources in Thailand were investigated for pharmacognostic specification. Chrysazin (1,8-dihydroxyanthraquinone) content in benzene extract of X. indica flowering heads was determined by TLC-densitometry using petroleum ether and ethyl acetate (8:1) as mobile phase and scanning at 430 nm. The results showed the contents of loss on drying, total ash, acid-insoluble ash, water soluble extractive matter and ethanol soluble extractive matter and water as 6.899 ± 0.165, 2.497 ± 0.033, 0.409 ± 0.027, 6.592 ± 0.474, 4.030 ± 0.486 and 11.121 ± 1.132 % dry weight respectively. TLC fingerprint revealed clear fluorescent spot under UV 365 nm. Chrysazin content was 0.022 ± 0.008 % dry weight. The method validation of TLC densitometry of chrysazin in X. indica flowering heads showed the linearity of 15.0-75.0 µg/spot with R²=0.9997, LOD and LOQ were 5.48 and 16.62 µg/spot, respectively. % recovery was in range of 100.97-110.29 % and precision of 0.70-2.98 % RSD. % RSD of robustness was 4.44 %. Antimicrobial activities of petroleum ether and ethanol extracts of X. indica flowering heads were performed by agar well diffusion and broth microdilution method. The ethanol extract inhibited the growth of tested gram positive and gram negative bacteria except P. aeruginosa while the petroleum ether extract were only active against B. cereus and B. subtilis. The ethanol extract showed the lowest MIC on S. aureus (125 µg/ml). The present study is useful for identification and standardization of X. indica crude drug.

Introduction: Herbal medicines have been used in medicine from time immemorial for the treatment or prevention of diseases. Over the last few decades, many people have been turning back to herbal medicines for remedy that are more harmonious with the human metabolism, easily accessible, cheap and safe than synthetic drugs as well as remain the most common source of antimicrobial agents.1,2 To be accepted as safe herbal medicines, herbal medicines have to be tested before taken by people. Most of the known side effects reported for herbal drugs are extrinsic to the preparation and are related to several manufacturing problems.3 From this issue, WHO published the guideline called Quality Control Method for Medicinal Plant Materials to ensure the quality of medicinal plant products. The Kingdom of Thailand has its own system of traditional medicine called “Traditional Thai medicine” (TTM). The historical evidence shows that Thai people began to use herbal medicines before the Sukhothai period. TTM has many herbal medicines use to treat the ailments. One of them is Xyris indica L. (Xyridaceae), a perennial herb which is grass-like with yellow flowers packed between yellowish brown spike bracts. Its Thai name is Kra thin thung and used to treat ringworm, constipation and flatulence. The phytochemical studies of X. indica flowering heads have shown two isocoumarins; xyridin A and xyridin B, two sterols; stigmasterol and spinasterol and three anthraquinones; chrysazin as a main compound, 3-methoxychrysazin and 3-hydroxychrysazin.4-6 Although X. indica have been used for a long time, there is no report on standardization of this plant. Thereby, this study aims to assess the pharmacognostic evaluation of X. indica flowering heads and analyze chrysazin content by thin layer chromatographic (TLC) densitometry to standardize and control quality of raw
material and examine antimicrobial activity by agar well diffusion method and broth microdilution method for evidence based efficacy of this crude drug.

Methodology:

Materials and chemicals
X. indica flowering heads were collected from 15 different sources in Thailand and authenticated by Ruangrungsi N. Voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University. The standard chrysazin was purchased from Sigma-Aldrich Co., USA. All chemicals and reagent were analytical grade.

Determination of loss on drying
Placed 3 g of the ground sample in a pre-weighed crucible. The sample was dried by heating at 105°C until the constant weight, cooled in a desiccator and weighed. The loss of weight was calculated in a percentage with reference to the dried sample.

Determination of total ash
Placed 3 g of the ground sample in a pre-weighed crucible, incinerated at by gradually increasing the heat to 500°C until it was white, cooled in a desiccator, weighed immediately and calculated the content of total ash in a percentage with reference to the dried sample.

Determination of acid-insoluble ash
Added 25 ml of hydrochloric acid (70 g/l) in the crucible containing the total ash and boiled gently for 5 minutes. The insoluble matter was collected on an ashless filter-paper, transferred the filter paper containing the insoluble matter to the original crucible, incinerated at 500°C until the constant weight, cooled in a desiccator, weighed immediately and calculated the content of acid-insoluble ash in a percentage with reference to the dried sample.

Determination of water soluble extractive value
Macerated 5 g of the ground sample with 70 ml of distilled water in a closed conical flask for 24 hours, shaked frequently during 6 hours and allowed standing for 18 hours and filtered. Washed the marc with water, pooled and adjusted the final volume of the filtrate to 100 ml. Twenty milliliters of the filtrate was transferred to a pre-weighed small beaker, evaporated to dryness on a water bath, dried at 105°C until the constant weight, cooled in a desiccator, weighed and calculated the content of extractable matter in a percentage with reference to the dried sample.

Determination of ethanol soluble extractive value
This method proceeded as determination of water soluble extractive value. The solvent was changed to ethanol.

Determination of water content
Placed 50 g of the ground sample in the flask of azeotropic apparatus and added water saturated toluene 200 ml in it. The flask was heated until no more water distilled over then removed heat. The receiving tube was allowed cooling in room temperature. After the toluene and the water layers in tube were separated completely, the volume of water distilled over was read and calculate the content of water in a percentage with reference to the dried sample.

Thin layer chromatographic fingerprint
Concentrated 20 ml of the filtrate from determination of ethanol-soluble extractive value to 1 ml. Spotted 3 μl onto Silica gel 60 GF254 TLC plate and developed the 10 cm TLC plate using a mixture of petroleum ether and ethyl acetate (8:1). After development, the plate was visualized under UV light at 254 nm, 365 nm and 5% potassium hydroxide staining.

Preparation of standard solution
The stock solution of chrysazin (0.5 mg/ml) was prepared in ethanol and diluted to obtain the series of standard solutions with concentration of 15.0, 30.0, 45.0, 60.0 and 75.0 μg/ml.
Preparation of extracts of X. indica flowering heads
The ground sample (5 g) was exhaustively extracted with benzene by soxhlet apparatus. The extract was filtered. Then, the solvent was evaporated. The yield was recorded. Each extract was dissolved in ethanol to obtain a concentration of 5 mg/ml (source 2 - 5,10 - 15) and 8 mg/ml (source 1.6 - 9). This concentration was used for TLC densitometry.
For antibacterial tests, the ground sample was continuous macerated with petroleum ether and ethanol respectively. Then, filtered and concentrated to dryness. The yield was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 200 mg/ml for agar diffusion.

TLC densitometry of chrysazin
Spotted 3 µl of the extract and 3 µl of standard solution onto the silica gel 60 GF<sub>254</sub> TLC plate in triplicate and developed the 10 cm TLC plate using a mixture of petroleum ether and ethyl acetate (8:1). After development, the plate was scanned by densitometer (CAMAG, USA) under wavelength of maximum absorbance at 430 nm for quantitative analysis of the spot on TLC plates. The peak area of each spot was obtained from the densitometer and calculated the content of chrysazin in all samples by calibration curve. The calibration curve of chrysazin was shown with plotting peak area versus concentrations of chrysazin applied.

Method validation
Linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, precision and robustness were performed according to the ICH guideline.

Antimicrobial activity testing
Microorganisms

Preparation of the inoculums
Bacterial strains were maintained on Mueller Hinton agar (MHA). They were inoculated at 37°C for 24 hours. The turbidity of the culture was adjusted with 0.85 % NaCl sterile at 625 nm to match 0.5 McFarland standards (1 x 10<sup>8</sup> CFU/ml).

Determination of zone inhibition
Antibacterial testing was done by a modified agar well diffusion method.<sup>7,8</sup> A 100 µl volume of the suspension mixed with sterile seeds agar and poured on sterile base agar. The plates allowed drying at room temperature. Agar wells were cut by a 6 mm cork borer.<sup>9,10</sup> Twenty microliters of plant extracts (200 mg/ml) were added in each well. DMSO was used as a negative control while ampicillin sodium and amikacin sulfate were used as positive control. The plates were incubated at 37°C for 24 hours. Then the inhibition zone diameters were measured.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)
A modified broth microdilution method was performed.<sup>11</sup> Into sterile 96-well microplate, 50 µl of microbial suspended in Mueller Hinton broth (MHA) was added to the wells containing 50 µl of plant extract (0.39-200 µg /ml), 50 µl positive controls (0.019-10 µg/ml) or 50 µl negative control and incubated at 37 °C for 24 hours. The lowest concentration that inhibited the growth of the microorganisms being tested as detected by lack of visual turbidity, matching with negative control was defined as MIC. The broth from wells with no turbidity were streaked onto agar plates and incubated at 37 °C for 24 hours. The least concentration with no microbial growth observed on the plate was considered as MBC.

Results, Discussion and Conclusion: The pharmacognostic parameters of *X. indica* flowering heads were presented in Table 1. The constant numbers from the studies could be used to standardize and control quality of this crude drug.
TLC fingerprint revealed clearly separated spot of chrysazin as the major compound, appearing dark spot at 254 nm and yellow spot at 365 nm with hRf value of 59. The compound turns into pink spot with 5% potassium hydroxide. The picture of X. indica dried flowering heads, structure of chrysazin and TLC fingerprint are shown in Figure 1. This method can be used to identify the authenticity of herbal extracts.

To analyze the amount of chrysazin in 15 samples of X. indica flowering heads, the ground samples were exhaustively extracted with benzene as selective solvent by soxhlet apparatus. The percent yields were varied from 2.26 to 6.91% w/w.

For the TLC densitometry, the developed TLC plate gave a good separation for chrysazin. The yellow colored band of chry

### Table 1. The pharmacognostic parameters of Xyris indica flowering heads

<table>
<thead>
<tr>
<th>Parameter (% by weight)</th>
<th>Mean ± SDa</th>
<th>Range (Mean ± 3SD)</th>
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</thead>
<tbody>
<tr>
<td>Loss on drying</td>
<td>6.899 ± 0.165</td>
<td>6.403 - 7.395</td>
</tr>
<tr>
<td>Total ash</td>
<td>2.497 ± 0.033</td>
<td>2.399 - 2.595</td>
</tr>
<tr>
<td>Acid-insoluble ash</td>
<td>0.409 ± 0.027</td>
<td>0.329 - 0.490</td>
</tr>
<tr>
<td>Water-soluble extractive</td>
<td>6.592 ± 0.474</td>
<td>5.170 - 8.014</td>
</tr>
<tr>
<td>Ethanol-soluble extractive</td>
<td>4.030 ± 0.486</td>
<td>2.573 - 5.487</td>
</tr>
<tr>
<td>Water content</td>
<td>11.121 ± 1.132</td>
<td>7.725 - 14.518</td>
</tr>
</tbody>
</table>

a The parameters were shown as grand mean ± pooled SD. Samples were collected from 15 difference sources in Thailand. Each sample was tested in triplicate.

TLC densitometric method for quantitative analysis was validated including linearity, LOD, LOQ, accuracy and precision to confirm that the analytical procedure employed reliable and accurate information. For linearity, the calibration curves between peak area and concentration were plotted and showed good linearity relationships over the range from 15.0-75.0 µg/spot (R²=0.9997) (Figure 2). The LOD and LOQ values were at 5.48 and 16.62 µg/spot, respectively. The precision of this method was studied using three concentration of standard chrysazin at 10.0, 25.0 and 45.0 µg/spot. The results showed that repeatability and intermediate precision of the method was satisfactory as % RSD less than 10 (Table 2). The robustness studied by changing composition of mobile phase (petroleum ether: ethyl acetate 8:1, 8.1:0.9, 8.2:0.8, 7.9:1.1, 7.8:1.2) was 4.44 % RSD of peak area. The accuracy was examined from %recovery by spiking known amount of chrysazin in a sample. The recovery values were in the range of 100.97-110.29 % (Table 3). These results exhibited an acceptable accuracy of the method. The chrysazin contents in dried flowering heads found in the range of 0.013-0.039 % w/w (0.022 ± 0.008 % w/w). Previous study of chrysazin isolation from dried leaves and stems of Xyris semifusca by means of steam distillation followed by column chromatography on silica gel reported 50 mg of chrysazin as dark orange crystals from 30 g of plant materials. 12 Chrysazin also occurs naturally in Cassia, Aloe, Rheum and Rhamnus species. 13

TLC-densitometry is widely used for quantitative analysis of major compound in medicinal plants. For example, this method was selected for quantitative determination of arbutin in cowberry leaves (Vaccinium vitis-idaea L.). 14 It was not only used to analyze in single herb but also used to determine in formulation. The amount of phyllanthin and gallic acid from polyherbal hepatoprotective formulation could be determined by TLC-densitometry in accepted range of method validity. 15 TLC-densitometry is shown to be efficient and reliable for quantitative analysis of chrysazin in X. indica as well.
Table 2. Repeatability and intermediate precision of chrysazin (n = 3)

<table>
<thead>
<tr>
<th>Sample conc. (µg/spot)</th>
<th>Repeatability (% RSD)</th>
<th>Intermediate precision (% RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>2.77</td>
<td>2.98</td>
</tr>
<tr>
<td>25</td>
<td>0.87</td>
<td>1.17</td>
</tr>
<tr>
<td>45</td>
<td>0.76</td>
<td>0.70</td>
</tr>
</tbody>
</table>

Table 3. Recovery of chrysazin (n = 3)

<table>
<thead>
<tr>
<th>Chrysazin added (µg)</th>
<th>Chrysazin found (µg)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>26.03</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>35.71</td>
<td>100.97</td>
</tr>
<tr>
<td>25</td>
<td>46.69</td>
<td>109.28</td>
</tr>
<tr>
<td>45</td>
<td>64.40</td>
<td>110.29</td>
</tr>
</tbody>
</table>

The results of antimicrobial assay indicated that the ethanol extract inhibited the growth of tested gram positive and gram negative bacteria except *P. aeruginosa* while the petroleum ether extract were only active against *B. cereus* and *B. subtilis*. DMSO as negative control showed no activity on all of the tests. The ethanol extract displayed large inhibition zone on *B. cereus* and *B. subtilis* that were shown in Figure 3. The ethanol extract showed the lowest MIC on *Staphylococcus aureus* (125 µg/ml). The MIC and MBC showed that both of extracts were bacteriostatic agent (Table 4). The previous study found that the isolated compounds (chrysazin, 3-methoxychrysazin, 3-hydroxychrysazin) of *X. indica* flowering heads exhibited antibacterial activity on selected bacteria. Furthermore, 3-Hydroxychrysazin showed good antifungal activity with MIC value 0.78 µg/ml against *Trichophyton mentagrophytes* and *Trichophyton rubrum*. The anthraquinone derivatives were also found in *Xyris* species.16

Table 4. Antibacterial activities of petroleum ether and ethanol extracts of *X. indica* flowering heads

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Zone of Inhibition (mm.), MIC and MBC (µg /ml)</th>
<th>petroleum ether extract</th>
<th>Ethanol extract</th>
<th>Ampicillin sodium</th>
<th>Amikacin sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. cereus</em></td>
<td></td>
<td>7.3±0.6, 250, 2000</td>
<td>13.3±0.6, 500, 2000</td>
<td>40.3±0.6, 12.3±0.6, 500, 2000</td>
<td>30.0±0.0, 2000, &gt;2000</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td></td>
<td>7.3±0.6, 250, 1000</td>
<td>12.3±0.6, 500, 1000</td>
<td>19.3±0.6, 12.50, 12.50</td>
<td>29.3±0.6, 12.50, 12.50</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td></td>
<td>NA</td>
<td>10.3±0.6, 125, &gt;2000</td>
<td>50.3±0.6, 0.19, 6.25</td>
<td>30.0±0.0, 0.19, 6.25</td>
</tr>
<tr>
<td><em>M. luteus</em></td>
<td></td>
<td>NA</td>
<td>11.3±0.6, 500, &gt;2000</td>
<td>46.0±1.0, 0.19, 0.39</td>
<td>30.0±0.0, 0.19, 0.39</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td>NA</td>
<td>9.7±0.6, 2000, &gt;2000</td>
<td>32.0±0.0, 3.12, 3.12</td>
<td>21.0±0.0, 3.12, 3.12</td>
</tr>
<tr>
<td><em>E. aerogenes</em></td>
<td></td>
<td>NA</td>
<td>8.7±0.6, 2000, &gt;2000</td>
<td>18.7±0.6, 50, 100</td>
<td>23.3±0.6, 50, 100</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td></td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>30.7±0.6, NA</td>
</tr>
<tr>
<td><em>S. typhimurium</em></td>
<td></td>
<td>NA</td>
<td>11.0±1.0, &gt;2000, &gt;2000</td>
<td>40.0±0.0, 0.39, 0.78</td>
<td>30.0±0.0, 0.39, 0.78</td>
</tr>
</tbody>
</table>

Means ± SD, NA = no activity. Each sample was tested in triplicate.
Plants remain the most important sources of antimicrobial agents. Studying plant-based antimicrobial properties provides additional information in developing natural antibiotics and discovering the alternative of antimicrobial drugs for the treatment of infectious disease as well as helping solve the problem of drug resistance in human pathogenic microorganism.

Dried flowering heads of *X. indica*

![Structure of chrysazin](image)

Figure 1. Dried flowering heads of *Xyris indica*, structure of chrysazin and TLC fingerprints

![TLC fingerprint of ethanolic extract](image)

Figure 2. 3D TLC-Densitometry chromatogram at 430 nm (Left) The calibration curve of chrysazin (Right)
Figure 3. The inhibition zone of *Bacillus cereus* from ethanol extract (Left)
The inhibition zone of *Bacillus subtilis* from ethanol extract (Right)

References:

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Keywords: *Xyris indica*, pharmacognostic evaluation, chrysazin, TLC densitometry, antimicrobial activity