Pharmacognostic evaluation and chrysazin quantitation of *Xyris indica* flowering heads

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**ABSTRACT**

**Objectives:** The present study aimed to establish quality specification of *Xyris indica* L. flowering heads. The pharmacognostic parameters were investigated. Chrysazin contents were analyzed by TLC image analysis using ImageJ software compared to TLC-densitometry. **Methods:** *X. indica* flowering heads from 15 different sources in Thailand were collected. Morphological and physicochemical parameters were characterized. Chrysazin was successively extracted and determined by TLC image analysis using ImageJ software and TLC-densitometry. **Results:** Macroscopic study was illustrated as whole plant drawing. The microscopic study showed fragment of corolla, seeds, pollen grain and staminode. The pharmacognostic parameters revealed that the loss on drying, total ash, acid-insoluble ash and water content should be not more than 6.90, 2.50, 0.41, and 11.12 of % dry weight respectively while water and ethanol-soluble extractive values should be not less than 6.59 and 4.03 of % dry weight respectively. TLC fingerprint revealed clearly chrysazin yellow fluorescent band at 365 nm. Chrysazin quantitation by TLC image analysis and TLC densitometry were developed and validated. Chrysazin content was 0.022 ± 0.001 % dry weight by both methods. There was no statistically significantly difference between these methods. **Conclusion:** This study provided pharmacognostic specification and chrysazin content of *X. indica* flowering heads that can be used for basic quality control and standardization of plant material. TLC image analysis using ImageJ software showed reliable and convenient for analysis of chrysazin content in this crude drug. **Keywords:** *Xyris indica*, Pharmacognostic specification, Chrysazin, Quantitative analysis, Antimicrobial activities

**INTRODUCTION**

Herbal medicines have been used for the treatment or prevention of diseases from time immemorial in all cultures. Over the last few decades, many people have been turning back to herbal medicines for remedy. However, adulteration and misidentification of crude drug still exist. Thus, development of standardization and quality control of herbal medicines are needed to be prioritized at the earliest stage.

*Xyris indica* L. (Xyridaceae), a perennial herb which is grass-like, known locally as Kra thin thung, is widespread species in Thailand and a native plant of east India. *X. indica* flowering heads used as crude drug in traditional Thai medicine Figure 1 (left). It has been used to treat ringworm, constipation and flatulence. The phytochemical studies of *X. indica* flowering heads showed two isoquomarns; xyridin A and xyridin B, two sterols; stigmasterol and spinasterol and three anthraquinones; chrysazin, 3-methoxychrysazin and 3-hydroxychrysazin[1–3]. The previous antimicrobial studies revealed that 3-hydroxychrysazin showed good antifungal activity against *Trichophyton mentagrophytes* and *T. rubrum*[3] and Δ2-Hydroxyxyridin showed strong effect than standard drug against *Aspergillus niger*[4].

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Chrysazin Figure 1 (right), the main component in X. indica flowering heads was chosen as quantitative marker in this plant. It had been used as a laxative and as a natural colorant. However, the studies in experimental animals indicated that chrysazin is reasonably anticipated to be a human carcinogen\cite{5}. Thus, chrysazin content in this crude drug should be concerned for safety especially use as oral medicine.

Although X. indica has been used for a long time, there have been no reports on standardization of this plant. Therefore, this study aimed to assess the pharmacognostic characters of X. indica flowering heads and determine the chrysazin content by TLC image analysis using ImageJ software and TLC-densitometry for standardization of X. indica crude drug.

**MATERIALS AND METHODS**

**Plant materials**

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, Thailand. The samples were dried in hot air oven at 45°C and ground to powders.

**Plant extraction**

The ground samples (5 g) were exhaustively extracted with benzene by soxhlet apparatus. The extracts were filtered. The filtrate was evaporated to dryness and re-dissolved in ethanol to obtain a concentration of 5.0 mg/ml for analysis of the chrysazin content.

**Standard chrysazin**

Standard chrysazin (purity 96 %) was purchased from Sigma-Aldrich Co., USA. The stock solution of chrysazin (0.5 mg/ml) was prepared in 95 % ethanol and diluted to obtain the series of standard solutions with concentration of 15, 30, 45, 60 and 75 μg/ml.

**Pharmacognostic evaluations**

The pharmacognostic evaluations were performed according to WHO guideline quality control methods for medicinal plant materials\cite{6}. Briefly, macroscopic examination of plant material was illustrated by including shape, size, colour, odour and taste. Microscopic examination of ground sample was observed cell and tissue structures under microscope. Three grams of ground sample were heated at 105°C until constant weight for determination of loss on drying then ignited in an incinerator at 500°C until it was white, cooled and weighed to calculate total ash. The remaining ash was boiled gently with 25 ml of hydrochloric acid (70 g/l), filtered and ignited at 500°C until constant weight, cooled and weighed to calculate acid-insoluble ash. Determination of extractive values was performed with ethanol and water. Water content was determined by azeotropic distillation. TLC fingerprint of ethanolic extract was performed using silica gel 60 GF\textsubscript{254} as stationary phase and a mixture of petroleum ether and ethyl acetate (8:1) as mobile phase. The plate was visualized under UV light at 254 nm, 365 nm and by staining with 5% potassium hydroxide in methanol.
**Figure 2:** Macroscopic, microscopic characteristics and TLC fingerprint of *Xyris indica* L.
Chrysazin quantitative analysis

**TLC-densitometry of chrysazin**

Three microliters of benzene extract and standard chrysazin solutions were applied on the silica gel 60 GF$_{254}$ plate (Merck, Germany; 20 x 10 cm, 0.25 mm thickness) by Linomat 5 applicator (Camag, Switzerland). The plate was developed to a distance of 8.0 cm in a TLC chamber with petroleum ether and ethyl acetate (8:1), then dried and scanned at 430 nm using TLC scanner 3 (Camag, Switzerland) in operation with winCATS software. The quantitative analysis was performed in triplicate.

**TLC image analysis of chrysazin by ImageJ software**

Developed TLC plate from above was visualized in UV viewing cabinet (Spectroline, USA) under UV 365 nm and photographed by a charge-coupled device camera (Canon Power shot A650). The images saved as JPEG files were opened with ImageJ software (NIH, USA) and applied with smooth function 5 times. Then a rectangular tool was used to crop the band and create plot profiles. After drawing the line under the plot, the peak areas were measured as square pixels by wand tool.

**METHOD VALIDATION**

The analytical procedures were validated according to the ICH guideline in terms of specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ) and robustness.

**RESULTS**

**Pharmacognostic evaluations**

*X. indica* is a perennial herb to 30–60 cm tall. Stems are tufted. Leaves are narrowly linear, acute at apex, 0.4–0.5 × 13–40 cm. The yellow flowers are packed between bracts. Bracts are form a compact head or spike, 0.5–1.4 × 0.5–2.2 cm. The yellowish brown bracts are suborbicular or ovate, 5–8 × 5–7 mm. There are 3 yellow sepals. 2 lateral sepals are boat-shaped, 0.8–1.4 × 5–7 mm. Median sepal is cap-shaped, 2–2.5 × 4–6 mm. There are 3 yellow petals, obovate, serrate at apex, 3–4 × 3–4.5 mm. For stamen, there are 3 fertile stamens with anthers 2 lobed and 3 staminodes with hairy. Ovary is obovoid with 3 styles. Seeds are ovoid. The flowering heads powder was slightly characteristic odour and bitter, astringent taste. Dried flowering heads of *X. indica* was used as crude drug. Macroscopic and microscopic characteristics were shown in Figure 2.

The pharmacognostic parameters of *X. indica* flowering heads were shown in (Table 1). The loss on drying, total ash, acid insoluble ash and water content should be not more than 6.899, 2.497, 0.409 and 11.121 % of dry weight respectively while water-soluble extractive and ethanol-soluble extractive values should be not less than 6.592 and 4.030 % of dry weight respectively. TLC fingerprint revealed clearly separated spot of chrysazin, appearing yellow fluorescent spot at 365 nm with hRf value of 59. The compound turns into pink spot with 5% potassium hydroxide in methanol (Figure 2).

**Table 1: The pharmacognostic parameters of Xyris indica flowering heads**

<table>
<thead>
<tr>
<th>Parameter (% by weight)</th>
<th>Mean ± SD*</th>
<th>Range (Mean ± 3SD)</th>
</tr>
</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-insoluble 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>

*The parameters were shown as grand mean ± pooled SD. Samples were collected from 15 different sources in Thailand. Each sample was tested in triplicate.*
Chrysazin quantitative analysis

TLC was selected for quantitative analysis of chrysazin using the mixture of petroleum ether and ethyl acetate (8:1) as mobile phase. This mobile phase showed good separation for chrysazin on developed TLC plate. The yellow fluorescence spot of chrysazin was clearly detected under UV 365 nm (Figure 3). The percent yield of benzene extracts were 2.26 - 6.91% w/w and the average was 3.55±1.34% w/w. The chrysazin contents in 15 samples of X. indica flowering heads analyzed by TLC-densitometry were between 0.0126–0.0390% w/w (0.0223 ± 0.0011% w/w). The contents by TLC image analysis were between 0.0124–0.0377% w/w (0.0219 ± 0.0007% w/w). The chrysazin contents of both methods were not statistically significantly different (P > 0.05) as determined using paired t-test.

METHOD VALIDATION

The specificity of the TLC method was confirmed by comparing UV/VIS spectrum of the chrysazin peak in the sample with standard chrysazin peak. The result showed the identical absorption spectra with maximum absorbance at 430 nm. The calibration curves between peak area and concentration of standard chrysazin were linear regression over the range of 15.0–75.0 µg/ml. For TLC-densitometry, the equation was y = 98.67x - 147.7 (R² = 0.9997) whereas the equation of TLC image analysis was y = 480.2x + 586.7 (R² = 0.9986) where y is peak area and x is concentration. The accuracy was examined from percent recovery by spiking known amount of chrysazin (10, 25 and 45 µg/ml) in a sample. The recovery values of both methods were between 90.67–99.16% (Table 2). The precision of these methods was studied in aforementioned three concentrations of spiked samples. The % RSD for repeatability and intermediate precision of TLC densitometry were 0.76 - 2.77% and 1.47 – 4.48% respectively. The %RSD for repeatability and intermediate precision of TLC image analysis were 0.78 - 3.10% and 2.46 - 3.95% respectively. LOD and LOQ were evaluated based on the standard deviation of y-intercepts and the slope of the calibration curve. The LOD and LOQ of TLC-densitometry and TLC image analysis were 0.79 and 2.39 µg/ml and 1.69 and 5.13 µg/ml, respectively. 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 v/v) was 4.44% RSD of peak area from TLC-densitometry and 3.60% RSD from TLC image analysis. Table 2 demonstrated the method validity of both methods.

Discussion and conclusion

The quality control methods are important tool in traditional medicines which serve as useful information for identification, authentication and standardization of herbal medicine[8]. The safety and efficacy of herbal...
medicine are dependent on the standardization and quality of plant materials\textsuperscript{[10]}. Macroscopic and microscopic methods can help to identify and authenticate plant materials. The microscopic characteristics of powdered \textit{X. indica} flowering heads were remarkable diagnostic characteristics of this plant part. The constant numbers from pharmacognostic evaluation could be used for quality and purity of plant materials. TLC fingerprint showed the pattern of phytochemical characteristic components.

To develop alternative TLC method for analyzing chrysazin content, TLC-densitometry and TLC image analysis using ImageJ software were performed and validated to confirm that the analytical procedure employed reliable and accurate results. Chrysazin content in dried flowering heads was 0.022 ± 0.001 % w/w by both methods. The previous phytochemical study of anthraquinones in \textit{X. indica} flowering heads showed that chrysazin was major component\textsuperscript{[3]}. In addition, chrysazin was able to be isolated from dried leaves and stems of \textit{X. semifuscata}\textsuperscript{[11]}. Chrysazin also occurs naturally in \textit{Cassia}, \textit{Aloe}, \textit{Rheum} and \textit{Rhamnus} species\textsuperscript{[5]}.

The specificity of the TLC method indicated that 430 nm is maximum absorption of chrysazin used as peak identity. This wavelength is optimal wavelength for scanning densitometer in this study that quantified chrysazin accurately. The calibration curves of both methods showed good linearity relationships ($R^2 > 0.99$). Moreover, the percent recovery exhibited an acceptable accuracy of both methods\textsuperscript{[13]}. The precision showed that repeatability and intermediate precision of the methods were satisfactory as RSD at each concentration level less than 15\%\textsuperscript{[13]}. The LOD and LOQ values from both methods displayed sufficient sensitivity of the methods. However, TLC-densitometry showed better sensitivity than TLC image analysis. The result of robustness indicated that changing mobile phase composition was not affected in both methods.

Thus, the result from method validation and paired t-test indicated that TLC image analysis is efficient, reliable and suitable technique for using in quantitative analysis of chrysazin in \textit{X. indica}. Moreover, TLC image analysis can be use as alternative method for any laboratory due to its advantages which is easy to perform, fast, inexpensive instruments. Charge-coupled device camera becomes more widely used because it is much faster and efficient than scanning densitometer\textsuperscript{[14]}. Image analysis using ImageJ software was not required sophisticated instrument and easily applicable\textsuperscript{[15]}. Besides, ImageJ software, other image analysis softwares can also be used in TLC quantitative analysis. For example, Photoshop 7.0 and Scion image software were chosen to determine the amount of three curcuminoid in \textit{Curcuma longa}. The techniques were validated and showed to be accurate and reliable method\textsuperscript{[16–17]}.

In conclusion, this research provides pharmacognostic specification and chrysazin content of \textit{X. indica} flowering heads that can be used for basic quality control and standardization of plant material. TLC image analysis method could be use as alternative method for the simultaneous analysis of chrysazin content in plant material.

### Table 2: Validity of chrysazin quantitative analysis in \textit{Xyris indica} flowering heads by TLC-densitometry and TLC image analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>TLC-densitometry</th>
<th>TLC image analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity</td>
<td>$y = 98.67x - 147.7$</td>
<td>$y = 480.2x + 586.7$</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.9997</td>
<td>0.9986</td>
</tr>
<tr>
<td>Range</td>
<td>15.0–75.0 $\mu g/ml$</td>
<td>15.0–75.0 $\mu g/ml$</td>
</tr>
<tr>
<td>Accuracy: % Recovery</td>
<td>90.67–99.16 %</td>
<td>91.87–96.00 %</td>
</tr>
<tr>
<td>Precision</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repeatability</td>
<td>0.76–2.77 %RSD</td>
<td>0.78–3.10 %RSD</td>
</tr>
<tr>
<td>Intermediate precision</td>
<td>1.47–4.48 %RSD</td>
<td>2.46–3.95 %RSD</td>
</tr>
<tr>
<td>Limit of detection (LOD)</td>
<td>0.79 $\mu g/ml$</td>
<td>1.69 $\mu g/ml$</td>
</tr>
<tr>
<td>Limit of quantitation (LOQ)</td>
<td>2.39 $\mu g/ml$</td>
<td>5.13 $\mu g/ml$</td>
</tr>
<tr>
<td>Robustness</td>
<td>4.44 %RSD</td>
<td>3.60 % RSD</td>
</tr>
</tbody>
</table>
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