Quality evaluation of *Zanthoxylum rhetsa* fruits and seeds - a Thai traditional medicine

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*Zanthoxylum rhetsa* (Roxb.) DC or Ma-khwuang (Rutaceae family) has long been used in Thai culinary and traditional medicine. Its fruits and seeds are used for anti-diabetic, antispasmodic, diuretic and anti-inflammatory activity. Based on its medicinal usage, we have evaluated the quality parameters of *Z. rhetsa* fruits and seeds. Ten samples of *Z. rhetsa* fruits and seeds from different topographical areas, throughout Thailand, were examined following WHO guideline for quality control of medicinal plant materials. The *Z. rhetsa* fruits and seeds were evaluated by microscopic and physicochemical studies. The GC-MS study showed the major phytoconstituents of *Z. rhetsa* are sabinene (56.62%), 4-terpineol (13.82%), germacrene (10.1%), gramma-terpinene (5.5%) and alpha-terpinene (3.5%). These pharmacognostic specifications with GC-MS can be used to develop some standard parameters for judge the quality, quantity and impurity of *Z. rhetsa* crude drug future.

Keywords: GC-MS analysis, Pharmacognostic specification, Physicochemical parameters, TLC, *Zanthoxylum rhetsa*

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*Zanthoxylum rhetsa* (Roxb.) DC or Ma-khwuang (Rutaceae family) has long been used in Thai culinary and traditional medicine. Its fruits and seeds are used for anti-diabetic, antispasmodic, diuretic and anti-inflammatory activity. Based on its medicinal usage, we have evaluated the quality parameters of *Z. rhetsa* fruits and seeds. Ten samples of *Z. rhetsa* fruits and seeds from different topographical areas, throughout Thailand, were examined following WHO guideline for quality control of medicinal plant materials. The *Z. rhetsa* fruits and seeds were evaluated by microscopic and physicochemical studies. The GC-MS study showed the major phytoconstituents of *Z. rhetsa* are sabinene (56.62%), 4-terpineol (13.82%), germacrene (10.1%), gramma-terpinene (5.5%) and alpha-terpinene (3.5%). These pharmacognostic specifications with GC-MS can be used to develop some standard parameters for judge the quality, quantity and impurity of *Z. rhetsa* crude drug future.

Materials and methods

Chemical and reagent

Dichloromethane (AR grade), 99.9% ethanol (absolute denatured), toluene (AR grade), n-hexane (AR grade) and 37% hydrochloric acid (AR grade) were purchased from QRëC (New Zealand). Methanol (AR grade) and ethyl acetate (AR grade) were purchased from RCI Labscan (Bangkok, Thailand). 98/100% formic acid (AR grade) was purchased from Fisher Scientific (Loughborough, UK).
Plant materials

Fruits and seeds of *Z. rhetsa* were purchased from ten different sources from different topographical areas including Tak (Mae Ramas district), Chiang Rai (Muang district), Chiang Rai (Mae suai district), Lamphun (Muang district), Lamphun (Mae Ta district), Nan (PhuPhiang), Nan (Ruang sub-district), Nan (Pha Tub sub-district), Nan (San Tha sub-district), Nan (Sa Than sub-district). Voucher specimens were identified by comparison with the herbarium at Queen Sirikit Botanical Garden, Chiang Mai, Thailand and authenticated by Associate Professor Ruangrungsi N. The specimens were deposited at the School of Integrative Medicine, Mae Fah Luang University, Thailand.

Macroscopic and microscopic identification

The macroscopic character of authenticated samples of *Z. rhetsa* were observed and recorded while the microscopic characters were examined and recorded from the transverse section and powdered samples under a microscope for anatomical and histological character of *Z. rhetsa*, including the various cell types and their cyto-morphological content. Stomatal index and pellucid dots were evaluated for all the collected species.

Evaluation of physicochemical characteristic

The physicochemical parameters including loss on drying, total ash, acid soluble ash, solvent extractive values, water content and volatile oil content of *Z. rhetsa* fruits and seeds were determined following the WHO guideline of the Quality control methods for medicinal plant materials. Loss on drying was estimated to measure the loss of both water and volatile matter in crude drugs. Three grams of dried powdered drug taken in a clean dried pre-weighed crucible and the crude drug was dried at 105°C. Ash values were used to detect the impurities of crude drug. The ashes value determined by total ash method was tested by burning 3 g of powdered drug in a pre-weighed crucible at 500°C for 5 h to obtain carbonless ash which was further weighted. Then 25 mL of 2N HCl was added into the remaining ash and gently boiled, filtrated and burned at 500°C for 5 h, and measured the amount. Water content was determined by Azeotropic distillation method. The sample was distilled with water saturated toluene. Volatile content of the sample was determined by using Clevenger apparatus. The solvent extractive values were determined to evaluate the amount of active constituents dissolved in the solvents. Five grams of powdered crude drug was macerated with 100 mL of solvent (hexane, 99.9% ethanol or DI water) under shaking for 6 h and standing for 18 h before filtration. Twenty milliliters of the filtrate was evaporated to dryness on a water bath and the sample was dried at 105°C. The physicochemical parameters were calculated from ten different samples as mentioned, each samples was done in triplicate.

Thin Layer Chromatographic fingerprinting

The crude extract of *Z. rhetsa* dissolved in ethanol at 10 mg/mL was subjected to TLC fingerprinting on TLC plate consisting of Silica gel 60 G. A mixture of ethyl acetate: hexane: methanol (8:1:1) was used as a mobile phase to developed chromatograms. The spots produced in daylight, under ultraviolet (UV) light, short-wave (254 nm) and long-wave (366 nm) was sprayed with specific reagent (10% sulfuric acid in methanol), heated at 105°C for 10 min and mark the center of each spot. The spots were measured and recorded to calculate Rf value.

GC-MS analysis

The chemical components of *Z. rhetsa* oil were analyzed by gas chromatography/mass spectrometry (GC/MS) in an Agilent 7890N GC equipped with HP-5ms capillary column (30 m x 0.25 mm, 0.25 µm film thicknesses) and Agilent 5973N MS detector. The oven temperature was maintained at 60°C to 240°C at a constant rate of 3°C/min; and the injection port was held at 220°C throughout the separation. The carrier gas was helium with a flow rate of 1 mL/min. MS was performed by electron ionization (EI) mode at 70 electron volts; and Mass range of 30–300 amu. The chemical constituents of *Z. rhetsa* oil were identified by matching their mass spectra and retention indices with Wiley Mass Spectral library and a book reference.

Result and discussion

Macroscopic and microscopic identification

*Z. rhetsa* (Roxb.) DC is a deciduous, aromatic tree with spreading crown up to 35 m high. Bark have brown vertical prickles 5-6 cm x 4-6 cm. Leaves are alternate with compound leaves blade, paripinnate to lanceolate shape, 3-4 cm x 5-7 cm, entire edge, attenuate-acuminate apex, unequal base, and lustrous surface. Fruits are sub-globose hesperidium, 5-7 mm in diameter, with one seed/carpel, green to brown when ripen, seeds are
hard and black, 4-6 mm in diameter. Fig. 1 showed the fruits and seeds as crude drug of *Z. rhetsa*. The anatomical and histological character on transverse section of *Z. rhetsa* fruit and seed, stem and leaf were provided on Fig. 2, Fig. 3 & Fig. 4, respectively. Fig. 5 showed powders samples including endosperm, vitta, mesocarp, trichome, sclereid, endocarp, epicarp, oil gland and testa. The results were related with the report of pharmacognostic standardization of *Z. rhetsa* in India and two reports that studied about *Z. armatum* and *Z. limonella*.

The most important diagnostic character of Rutaceae family is the presence of pellucid dots containing oil gland after examined under a microscope with 40X objective and a 6X eyepiece observed as pellucid dots and stomata on leaves. The *Z. rhetsa* revealed the number of pellucid ratio 0.7 dots/mm² and the stomata was anomocytic or ranunculaceous (irregular-celled) surrounded by varying number of cells. The stomatal index of *Z. rhetsa* was 21.18 that was in accordant with the report where the plant material was collected from India. The report revealed that stomatal index was 11.53-12.

**Physicochemical determination**

The physicochemical parameters (% w/w) of *Z. rhetsa* fruits and seeds were demonstrated in Table 1. The loss on drying, total ashes, acid-insoluble ash and water content were found to be not more than 16.04, 11.50, 1.02 and 11.04% (w/w), respectively. Incidentally, the water, ethanol and hexane soluble extractive and volatile oils content were not less than 3.14, 3.05, 2.92 and 2.32% (w/w), respectively; as the water content should be accomplished with high volatile oil content. The physicochemical parameters differ from the report of pharmacognostic standardization of *Z. rhetsa* in India. However, the
results of this study were related with pharmacognostic standardization of *Z. limonella* in Thailand\textsuperscript{20}. The content of moisture and volatile oil are also depended on diverse factors such as climate, soil type, timing of harvest, method of harvesting and storage\textsuperscript{22}. The same herbal material growing from different areas and harvested at different times or different processing methods has an effect on amount of chemical constituent and the quality. Therefore, it is important to specify the standardization of herbal plant for control the quality. Fig. 6 demonstrated TLC fingerprint of *Z. rhetsa* fruits and seeds ethanolic extract. (I) Scanning at 254nm; (II) Scanning at 366nm & (III) Scanning at Visible light after derivatization with 10% Sulfuric acid.

Table 1 — Physicochemical specification (% by weight) of *Z. rhetsa* fruits and seeds

<table>
<thead>
<tr>
<th>Physicochemical parameters</th>
<th>Mean±SD*</th>
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<tbody>
<tr>
<td>Loss on drying</td>
<td>16.04±2.69</td>
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<tr>
<td>Total ashes</td>
<td>11.50±0.86</td>
</tr>
<tr>
<td>Acid-insoluble ashes</td>
<td>1.02±0.31</td>
</tr>
<tr>
<td>Water content</td>
<td>11.04±0.62</td>
</tr>
<tr>
<td>Volatile oils</td>
<td>2.32±0.16</td>
</tr>
<tr>
<td>Water-soluble extractive</td>
<td>3.14±0.09</td>
</tr>
<tr>
<td>Ethanol-soluble extractive</td>
<td>3.05±0.10</td>
</tr>
<tr>
<td>Hexane-soluble extractive</td>
<td>2.92±0.19</td>
</tr>
</tbody>
</table>

(*Grand Mean±Pooled SD values were calculated from ten sources and each sample was done in triplicate.*)
The pale yellow volatile oil with a good smell of *Z. rhetsa* showed a yield of 2.3% (v/w) by hydrodistillation method. The volatile content was related with other report from India (1.94%)\(^7\). The chemical composition of *Z. rhetsa* was presented in Table 3. The GC chromatogram of *Z. rhetsa* oil illustrated in Fig. 7 revealed 5 main chemical constituents as sabinene (56.62%), 4-terpineol (13.82%), germacrene (10.10%), gramma-terpinene (5.50%) and alpha-terpinene (3.50%). The chemical structures of these major compounds were shown in Fig. 8. The results were related with previous reports that monoterpene are secondary metabolites of *Zanthoxylum* species. The previous study reported that the major chemical constituents of the volatile oil of *Z. rhetsa* fruits in Thailand are sabinene\(^11\). In contrast, chemical constituents of the volatile oil of *Z. rhetsa* seed coat and pericarp from India and Jordan showed terpinen-4-ol as a major component\(^7,8\). However, in this study do not find any limonene in *Z. rhetsa* oil.
Conclusion
Several quality evaluation parameters including GC-MS profile have been proposed for the phytoconstituent analysis of a plant, similar to this report on Z. rhetsa. Here the GC-MS analysis demonstrated that Z. rhetsa oil was a rich source of sabinene. The physicochemical properties such as loss on drying, total ash, acid insoluble ash value, water content, volatile oil content and extractive values were also determined. These pharmacognostic parameters were usable to standardize the sample while the standard parameters are advantageous for assurance of the quality, quantity and impurity of Z. rhetsa fruits and seeds crude drug widely used in Thailand.

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