Pharmacognostic specification of *Zanthoxylum limonella* (Dennst.) Alston: Fruits and seeds in Thailand

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**Abstract**

*Zanthoxylum limonella* (Dennst.) Alston. (*Rutaceae*) or Ma-khwaen is one of the medicinal plants in Thai traditional medicine. To investigate the pharmacognostic specifications and chemical constituents of *Z. limonella* fruits and seeds. Fruits and seeds of *Z. limonella* were collected from 15 sources throughout Thailand; then examined the pharmacognostic specification following WHO guideline of quality control method for medicinal plant materials. Microscopic determination of *Z. limonella* powders demonstrated fragment of mesocarp, fragment of brown vitta, oil glands, fragment of endocarp, and endosperm containing oil globule, trichome and pale brown stone cells. Stomatal index and pellucid dots in mm$^2$ were 19.87 and 4.2 respectively. Physico-chemical parameters unveiled that loss on drying, water content, total ash, and acid-insoluble ash should be not >17.90%, 9.18%, 4.50%, and 0.60% of dried weight respectively; while ethanol, water, and hexane extractive values and volatile oil content should be not <2.24%, 2.27%, 1.57% and 9.63% of dried weight respectively. R$_f$ values of thin-layer chromatographic fingerprint of *Z. limonella* fruits and seeds ethanolic extract were 0.38, 0.45, 0.90, and 0.97 detected ultraviolet (UV) light 254 nm, 0.30, 0.44, 0.67, and 0.77 detected UV light 366 nm, and 0.24, 0.73, 0.78, and 0.93 detected 10% sulfuric acid. There are three main chemical compounds in *Z. limonella* oil including limonene (43.63%), (+)-sabinene (16.72%), and terpinen-4-ol (10.95%). The result gained from pharmacognostic specifications and chemical fingerprints could be used as standardization data of *Z. limonella* fruits and seeds to apply or provide for guarantee of quality.

**Key words:** Gas chromatography/mass spectrometry, pharmacognostic specification, physico-chemical parameter, thin-layer chromatographic fingerprint, *Zanthoxylum limonella*

**INTRODUCTION**

*Zanthoxylum limonella* (Dennst.) Alston., called “Ma-khwaen” in Thailand, belongs to the family *Rutaceae* and it is an evergreen tree. It has been used as traditional medicine including the fruits and seeds are used as spice and there are essential oils which occupies stimulation effect on reducing muscle strain and some metabolites have shown cytotoxic, antibacterial, anti-inflammatory, antifungal and anesthetic features. Limonene is one of the main compound in *Z. limonella*, which is monoterpene with possessing smell of oranges. It has been usually applied for food and cosmetic products. Gas chromatography/mass
spectrometry (GC/MS) is accepted methods for analysis of volatile constituents of herbal drugs due to its sensitivity, stability and high efficiency. However, the report on pharmacognostic assessment of Z. limonella has not been whole performed in Thailand. In this study, the fruits and seeds of Z. limonella from 15 sources throughout Thailand were inspected following WHO guideline of quality control method for medicinal plant materials in order to establish the pharmacognostic parameters and standardization supplied to guarantee quality and purity.

MATERIALS AND METHODS

Chemical and reagent
n-Hexane (AR grade), dichloromethane (AR grade), toluene (AR grade), 99.9% ethanol (absolute denatured) (AR grade) and 37% hydrochloric acid (AR grade) were purchased from QRëC (New Zealand). 98/100% formic acid (AR grade) was purchased from Fisher scientific (Loughborough, UK). Methanol (AR grade) and ethyl acetate (AR grade) were purchased from RCI Labscan (Bangkok, Thailand).

Plant materials
Fifteen samples of Z. limonella fruits and seeds were collected from different geographical areas in Thailand including; Mae Hong Son, Chiang Mai, Chiang Rai (Mae Sai District), Chiang Rai (Doi Hang Subdistrict), Chiang Rai (Wiang Chai District), Tak, Lam Pang, Uttaradit, Nan (Chetawan District), Nan (Na Muen District), Nan (Song Kawe District), Phrae (Muang District), Phrae (Song District), Lam Phun and Phayao. All sets of crude drugs were authenticated by Ruangrungsi N. Voucher specimens were deposited at the School of Health Science, Mae Fah Luang University, Thailand.

Macroscopic and microscopic identification
The macroscopic character of Z. limonella was illustrated as the drawing of the plant by the author. The anatomical and histological characters of transverse section and powder of Z. limonella fruits and seeds were examined under microscope to identify the structural features, cells, and ergastic substances of plant samples.

Stomatal index determination
The stomatal index was calculated from 3 points in 1 mm² per point on the surface of 30 leaves by using this formula. Stomatal index = number of stomata/(number of stomata + number of epidermal cells) × 100.

Pellucid dots determination
Pellucid dots are small area that has light colored circular areas visible with the leaflet held up to light. There are ethereal oil, aromatic volatile oil secondary plant products, which glow brightly when held against the sun and the have usually found in Rutaceae.

Physico-chemical determination
Total ash, acid-insoluble ash, loss on drying, water content, volatile oil content and extractive matters values of Z. limonella fruits and seeds were performed according to WHO guideline for quality control methods for medicinal plant materials as briefly described below:

Three grams of ground sample was dried at 105°C until constant weight to determine the percent of loss on drying. Then, 3 g of ground sample was incinerated at 500°C until white to obtain the carbonless total ash. The ash was boiled with 25 ml of HCl (70 g/l); insoluble matter was incinerated again at 500°C for 5 h to obtain the percentage of acid-insoluble ash. Water content was determined by azeotropic distillation method using water-saturated toluene. Volatile content was determined using Clevenger apparatus. Determination of extractive matters was carried out with hexane, 95% ethanol and water as solvents. Five grams of ground sample was macerated with 100 ml of solvent under shaking for 6 h and standing for 18 h before filtration. Twenty milliliters of the filtrate was evaporated on a water bath and further dried at 105°C until constant weight was obtained.

Data analysis
The physico-chemical parameters were expressed as grand mean ± pooled standard deviation (SD) values (% by weight). Grand mean and pooled SD values were calculated from 15 sources throughout Thailand and each sample was done in triplicate.

Thin-layer chromatographic identification
The ethanolic extracts of Z. limonella fruits and seeds were dissolved in ethanol (10 mg/ml). Five microliter of each sample was applied into the thin-layer chromatographic (TLC) plate. Silica gel 60 F254 was used as the coating substance. The mobile phase was used a mixture of hexane, toluene, ethyl acetate, formic acid (2:5:2:1). The spots were visualized under ultraviolet (UV) light at 254 nm and 366 nm; then sprayed with detecting reagent (10% sulfuric acid in methanol) and heated at 105°C for 10 min.

Gas chromatography/mass spectrometry analysis
The mixture from 15 sources of Z. limonella oil at the concentration of 10 µl/ml in hexane was used for GC/MS analysis. GC fingerprinting was determined using Agilent 6890N GC equipped with HP-5ms capillary column (30 m × 0.25 mm, 0.25 µm film thicknesses) and Agilent 5973N MS detector. The oven temperature was ramped from 60°C to 240°C at a constant rate of 3°C/min. 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 mode at 70 eV. Mass range was of 30-300 amu. The chemical constituents of Z. limonella oil were identified by matching their mass spectra and retention indices with Wiley Mass Spectral library.
RESULTS AND DISCUSSION

Macroscopic and microscopic identification

*Z. limonella* (Dennst.) Alston is a tree with growing up to 50 m height. Bark have brown prickle, 3–5 cm × 2–4 cm. Leaves are alternate compound leaves blade, odd pinnate, lanceolate shape, 5–6 cm × 7–11 cm, edge serrulate, apex aristate, unequal base, leaf surface have scrabrous. Flowers are numerous in large, compound dichasium, polypetalous rosaceous corolla, and 5 white-yellow petals with 5 epipetalous stamens. Fruits are globose hesperidium with 1–2 mm in diameter.\[15\]

Macroscopic and microscopic evaluations were illustrated in Figures 1-6. Cytological and histological characterization demonstrated fragment of mesocarp, fragment of brown vitta, oil glands, fragment of endocarp, and endosperm containing oil globule, trichome and pale brown stone cells. It is a valuable equipment for the identification of each ingredient in *Z. limonella*. The family of *Rutaceae* has outstanding feature from other plant containing oil gland in part of plant. The most important and diagnostic character of this family was the presence of pellucid gland dots in leaves,\[16\] which *Z. limonella* had the number of pellucid dots on the average of 4.2 dots/mm\(^2\). Moreover, stomatal index was examined under a microscope with a ×40 objective and a ×6 eyepiece. The stoma of *Z. limonella* was a type of anomocytic or ranunculaceous (irregular-celled) surrounded by a varying number of cells, generally not different from those of the epidermis.\[7\] Afterwards to count number of epidermal cell for each stoma in square millimeter (mm\(^2\)), the stomatal index was 19.43.

Physico-chemical parameters

The physico-chemical evaluation (% by weight) of *Z. limonella* fruits and seeds was demonstrated in Table 1. The loss on drying, water content, total ash and acid-insoluble ash values should be not >17.90, 9.18, 4.50 and 0.60% w/w respectively. The value of loss on drying was high (17.90% w/w) due to the contents of volatile oil (9.63% w/w) and water (9.18% w/w). The British Pharmacopoeia 2011 revealed that a determination of...
water content should be carried out for herbal drugs with high volatile oil content.\textsuperscript{[17]} Incidentally, the determination of hexane, 95% ethanol and water-soluble extractive values, and volatile oils content should be not <1.57, 2.24, 2.27, and 9.63% w/w respectively. However, the high range of maximum and minimum of volatile oil content may be due to age and geographical area of each \textit{Z. limonella} source.

**Thin-layer chromatographic fingerprint**

TLC fingerprint was demonstrated in Figure 7; TLC image unveiled well-defined bands led to evaluate the \( R_f \) values in Table 2.

**Gas chromatography/mass spectrometry analysis**

The \textit{Z. limonella} oil consists of three main chemical compounds including limonene (43.63%), (+)-sabinene (16.72%), and terpinen-4-ol (10.95%) which was related with previous studies.\textsuperscript{[4,5]} GC fingerprinting of \textit{Z. limonella} oil was illustrated in Figure 8. The chemical constituents of \textit{Z. limonella} oil was demonstrated in Table 3. GC/MS fingerprint was strongly recommend for quality control of herbal drugs to authenticate the herbal part used.\textsuperscript{[6]}

![Figure 5: Powder of \textit{Zanthoxylum limonella}; (1) vitta, (2) endosperm, (3) endocarp, (4) oil gland, (5) mesocarp, (6) trichome, (7) pale brown stone cells](image)

![Figure 6: Leaf of \textit{Zanthoxylum limonella} under microscope; (1) pullucid dots, (2) stomata](image)

![Figure 7: Thin-layer chromatographic fingerprint of \textit{Zanthoxylum limonella} fruits and seeds ethanolic extract with hexane:toluene:ethyl acetate:formic acid (2:5:2:1) as mobile phase; I: detection under ultraviolet light 254 nm, II: detection under ultraviolet light 366 nm, III: detection with 10% sulfuric acid](image)

![Figure 8: Gas chromatography fingerprinting of \textit{Zanthoxylum limonella} oil](image)

**Table 1: Physico-chemical parameters (% by weight) of \textit{Zanthoxylum limonella} fruits and seeds**

| Content (% by weight)                  | Mean±SD\*                  | Minimum–maximum | \( n \) |
|---------------------------------------|----------------------------|-----------------|---------|
| Loss on drying                        | 17.90±3.21                | 13.32–22.50     | 15      |
| Water content                         | 9.18±1.20                 | 7.73–11.98      | 15      |
| Volatile oils                         | 9.63±5.01                 | 1.87–18.93      | 15      |
| Water-soluble extractive             | 2.27±0.68                 | 1.14–3.45       | 15      |
| Ethanol-soluble extractive           | 2.24±0.58                 | 1.41–3.74       | 15      |
| Hexane-soluble extractive            | 1.57±0.80                 | 0.71–4.13       | 15      |
| Total ashes                           | 4.50±0.44                 | 3.93–5.43       | 15      |
| Acid-insoluble ashes                 | 0.60±0.06                 | 0.45–0.71       | 15      |

\*Grand mean±pooled SD values were calculated from 15 sources throughout Thailand and each sample was done in triplicate. SD: Standard deviation
CONCLUSION

Total ash, acid-insoluble ash, loss on drying, water content, and extractive matters values of *Z. limonella* fruits and seeds are not different between 15 sources. Limonene, (+)-sabinene, and terpinen-4-ol are three major chemical compounds in *Z. limonella* oil. Moreover these compounds can be used as markers in *Z. limonella* oil. Therefore, the pharmacognostic specifications and GC/MS fingerprint were able to set as the standard parameter to be beneficial for quality control and authentication of the *Z. limonella* fruits and seeds crude drug in Thailand.

Acknowledgment

The authors are grateful to Mae Fah Luang University for funding the research and providing laboratory facilities throughout the work.

Financial support and sponsorship

School of Health Science, Mae Fah Luang University.

Conflicts of interest

There are no conflicts of interest.

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