Assessment of pharmacognostic specification of Cannabis sativa leaves in Thailand

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ABSTRACT

Lack of quality control can affect the safety, efficacy, and acceptability of herbal products that may lead to health problems. Cannabis sativa L. (Cannabaceae) has been widely used as an ethnomedicinal practice for its medicinal values. This study aims to establish pharmacognostic specifications of C. sativa as per standard procedures. Macroscopic-microscopic characteristics, physicochemical parameters, thin-layer chromatography (TLC) fingerprinting, and phytochemical screening of C. sativa leaves collected from various locations throughout Thailand were investigated. Leaves are palmate consists of seven leaflets with green color, margin is serrate with acuminate apex. Anomocytic stomata were found in the upper epidermis while unicellular and glandular trichomes with cystolith were found in the lower epidermis and the epidermis layer covered with cuticle. The physicochemical analysis revealed that the loss on drying (4.068 ± 0.084 %w/w) was within acceptable limits, total ash (14.360 ± 0.084%w/w), acid insoluble ash (2.726 ± 0.080%w/w), ethanol-soluble extractive (11.101 ± 0.223%w/w), water-soluble extractive (23.038 ± 0.306%w/w), and water content (7.523 ± 0.524%w/w). TLC fingerprint showed nine spots with Rf value 0.14, 0.19, 0.23, 0.29, 0.32, 0.45, 0.58, 0.70, and 0.76. Phytochemical screening of Cannabis leaves indicated the presence of phenolic compounds, flavonoids, alkaloids, diterpenes, triterpenes, and steroids. This study provided referential data for the accurate plant identity, and establishment of cannabis leaves monograph in Thailand.

Key words: Cannabis, pharmacognostic specification, physicochemical, phytochemical screening

INTRODUCTION

The demand for herbal medicines is growing more and more due to their long historical use and fewer side effects. [1,2] Cannabis sativa L is a genus of annual, dioecious, and flowering plants in the family Cannabaceae and indigenous from Central Asia, Asia, and Europe.[3] Cannabis has some medicinal properties, for example, reducing nausea and vomiting during chemotherapy, chronic pain, muscle spasms, analgesic, intoxicant, stomachic, and sedative. [4-7] In 2019, Thailand, is the first country in Southeast Asia that allow cannabis to be used for medical purposes and research. [8] As a result, this study is the first report for the standardization of C. sativa dry leaves in Thailand.

Access this article online

Quick Response Code: 
Website: www.japtr.org
DOI: 10.4103/japtr.japtr_96_22

How to cite this article: Nakkliang K, Areesantichai C, Rungsihirunrat K. Assessment of pharmacognostic specification of Cannabis sativa leaves in Thailand. J Adv Pharm Technol Res 2022;13:226-31.
As *C. sativa* has been an important source of medicinal substances, it is necessary to develop standardization to determine the quality, safety, and efficacy of cannabis materials. Herbal medicines are natural products, their phytochemical constituents depend on many factors\(^\text{[6-11]}\) and these variations could impact its efficacy profile. Recently, standard methods for quality control of herbal medicines were established. There are various parameters that should be considered; macroscopic-microscopic evaluation, physicochemical evaluation, phytochemical evaluation, toxicity, and biological activity.\(^\text{[12]}\) Lack of quality control can affect the safety, efficacy, and acceptability of herbal products that may lead to health problems. Therefore, quality control of herbal products is essential. Although in Thai herbal pharmacopoeia 2021, the monograph of the dried female flower of *C. sativa* have been prepared, but still lacked pharmacognostic specification of *C. sativa* leaves. Therefore, this study was designed to evaluate the pharmacognostic specification of *C. sativa* leaves for its authenticity, purity, and quality control of plant material for Thai traditional medicine remedies.

**MATERIALS AND METHODS**

**Plant samples**

The *C. sativa* leaves were kindly received from the Drug Dependence Research Center, College of Public Health Sciences, Chulalongkorn University. Twelve samples of *C. sativa* leaves were collected during August–December 2020 from various locations throughout Thailand. Plant samples were authenticated and deposited in voucher specimens at the College of Public Health Sciences, Chulalongkorn University, Thailand. Plant samples were washed and oven drying at 45°C–50°C.

**Determination of standardization parameters**

The standardization parameters including macroscopic-microscopic, physicochemical, thin-layer chromatography (TLC) fingerprinting, and phytochemical analysis were investigated according to the World Health Organization (WHO) guidelines.\(^\text{[14]}\)

**Macroscopic examination**

Morphological and organoleptic characteristics of *C. sativa* fresh mature leaves were analyzed by visual inspection and recorded.

**Microscopic examination**

The transverse section of the midrib and lamina of *C. sativa* leaves was examined under a microscope at \( \times 20 \) and \( \times 40 \). The fresh mature leaves were cut in parallel including the midrib and lamina into pieces as thin as possible using sharp razor blades by free hand sectioning, cleared with chloralhydrate, and transferred onto a slide in glycerin water for microscopic examination. Photomicrographs of different magnifications were taken and recorded with Axio Vision 4.0 V4.6.3.0 software (Carl Zeiss Imaging Solutions, Munich, Germany).

**Powder drug examination**

Shade-dried *C. sativa* material was grounded into a fine powder, mounted with water, and examined under the microscope.\(^\text{[15]}\)

**Physicochemical determination**

Physicochemical determination was investigated according to the WHO guidelines.\(^\text{[14]}\) All physicochemical parameters were performed in triplicate. The data were exhibited by mean ± standard deviation.

**Determination of loss on drying**

Weighted 3 g of the *C. sativa* dried powder in a crucible and then heated at 105°C until constant weight. The percentage of loss on drying was calculated.

**Determination of water content (Azeotropic Distillation Method)**

Weighted 50 g of *C. sativa* dried powder and placed it into the round bottom flask. Water-saturated toluene (200 ml) was added and boiled by using azeotropic apparatus. After completely distilled, allowed the receiving tube to be cooled in room temperature. Observed the water-toluene separated layers and calculated water content as the percentage of dry weight.

**Determination of total ash**

Placed 3 g of the *C. sativa* dried powder in a crucible. The sample was ignited at 500°C until white ash was obtained. The percentage of total ash was calculated.

**Determination of acid-insoluble ash**

Added 50 ml of 2N HCL to the total ash, and the mixture was boiled gently for 5 min. The insoluble matter was filtrated (ashless filter paper No. 40), then transferred to the original crucible. After drying, ignited the crucible until obtain constant weight. The residue was allowed to cool in desiccators and weighed. Calculated the percentage.

**Determination of ethanol and water extractive value**

Added 100 ml of absolute ethanol into 5 g of *C. sativa* dried powder in a closed conical flask in shaking bath for 6 h and let stand for 18 h. The extract was filtered, evaporated to dryness, and then heated until obtained constant weight. For water-soluble extraction, use water in place of ethanol.

**Determination of volatile oil content (Clevenger distillation method)**

Added water (200 ml) into 100 g *C. sativa* dried powder in the round bottom flask. After volatile oil completely separated from water, measured, and calculated volatile oil volume.

**Thin layer chromatography fingerprint**

Ethanol extract (20 mg) was dissolved in 1 ml methanol and applied (3 µl) onto the silica gel 60 F254 TLC plate
using A Linomat IV. The TLC plate was developed with a solvent system; hexane: ethyl acetate: acetic acid (4:1:0.5) and observed spots on the plate under white light, short-wavelength (254 nm), and ultraviolet light and sprayed the plate with 0.5% fast blue B salt. Rf value was calculated following this formula:

\[
Rf = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}
\]

Phytochemical screening
The phytochemical screening was performed based on the standard method to detect the presence of phenolics (ferric chloride test), alkaloids (Dragendorff’s and Wagner’s test), flavonoids (alkaline reagent test), triterpenes and steroids (Salkowski and Liebermann-Burchard test), diterpenes (copper acetate), and saponin (foam test).

RESULTS

C. sativa leaves were collected from 12 different geographic regions throughout Thailand, as shown in Table 1.

Macroscopic examination
Morphologically, the fresh mature leaf of C. sativa is compound, palmate shaped, 7–9 linear-lanceolate leaflet blades, serrate margin with acuminate apex, alternate or opposite in arrangement. The upper (adaxial) surfaces are dark green, while the lower (abaxial) are pale green with rough surfaces. Leaves are 0.2–2 cm wide, 3–15 cm long, and 2–7 cm petiole (Figure 1) and bitter test.

Microscopic examination
The upper epidermis surface revealed rectangular cells with striations, anomocytic stomata, palisade, and cicatrix. Trichomes consist of glandular trichome and unicellular trichome with cystolith found in the lower epidermis (Figure 2).

The transverse section of the midrib and lamina of C. sativa leaf (Figure 3) showed the upper and lower epidermis surface is covered by a single layer of the epidermis. The epidermis is undulating, with unicellular nonglandular trichome and glandular trichomes. The midrib was composed of collenchyma layer cell underneath the upper and lower epidermal, parenchyma containing rosette aggregate crystal. The mesophyll showed of distinct palisade layer and spongy parenchyma. The vascular bundle was surrounded by sclerenchyma tissue.

The powder microscopic examination of C. sativa leaves indicated the presence of anomocytic stoma, palisade, parenchymacell containing rosette aggregate crystal, spiral vessels, unicellular trichomes, cystolithic, and fiber, as shown in Figure 4.

Physicochemical parameters
The physicochemical parameters of C. sativa leaves collected from the different geographic regions were found to be in an acceptable range, as summarized in Table 2.

Thin-layer chromatography fingerprinting
TLC fingerprints of this extract are shown in Figure 5. TLC pattern of ethanolic extract indicated nine spots with Rf value 0.14, 0.19, 0.23, 0.29, 0.32, 0.45, 0.58, 0.70, and 0.76 using the solvent system n-hexane, ethyl acetate, acetic acid (4:1:0.5) with 0.5% Fast blue B Salt as staining reagent.

Phytochemical screening
Phytochemical screening of C. sativa ethanol extract showed the presence of alkaloids, flavonoids, phenolics, steroids, triterpenes, and diterpenes. However, saponin was not detected (Table 3).

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Table 1: The locations of 12 Cannabis sativa samples collected throughout Thailand

| Number | Sources of Cannabis sativa leaves collection | Voucher specimen (number) | Locations |
|--------|---------------------------------------------|---------------------------|-----------|
| 1      | CM-1                                        | CSL01/2020                | Northern  |
| 2      | CM-2                                        | CSL02/2020                | Northern  |
| 3      | CM-3                                        | CSL03/2020                | Northern  |
| 4      | LP-1                                        | CSL04/2020                | Northern  |
| 5      | PH-1                                        | CSL05/2020                | Northern  |
| 6      | SK-1                                        | CSL06/2020                | Northeastern |
| 7      | SK-2                                        | CSL07/2020                | Northeastern |
| 8      | SK-3                                        | CSL08/2020                | Northeastern |
| 9      | MD-1                                        | CSL09/2020                | Northeastern |
| 10     | NK-1                                        | CSL10/2020                | Northeastern |
| 11     | SR-1                                        | CSL11/2020                | Central   |
| 12     | SR-2                                        | CSL12/2020                | Central   |

CM: Chiang Mai, LP: Lampang, PH: Phrae, SK: Sakhonnakorn, MD: Mukdahan, NK: Nakhonrajchasima, SR: Saraburi

Figure 1: Leaf of Cannabis sativa
DISCUSSION

Establishing pharmacognostic standards is necessary for the evaluation of medicinal plants. According to the WHO, the organoleptic and microscopic characteristics are the starting step toward identification and evaluation of purity. Microscopic characterization is one of the diagnostic features for the identification of medicinal plants, broke of crude drugs or small fragments, and detection of adulterants, substituents, and authentic plants. Epidermal characteristics and stomata are also widely used in identification at genus and species levels. The powdered drug was assessed for its structural cell and physicochemical analysis.

Loss on drying measures the amount of volatile oil and water containing in the plant material. The loss on drying of *C. sativa* leaves powder was presented 4.068 ± 0.084%w/w. To prevent the decomposition of crude drugs, the water content of the crude drugs should be minimized ranging from 10% to 20% which is an ideal range for minimum bacterial and fungal growth. The water content was measured using the azeotropic distillation method, the result showed that it should not be higher than 7.954 ± 0.324%w/w. Total ash values of crude drug give the concept of inorganic matter and other impurities; phosphates, carbonates, silica, and silicates. Ash values of *C. Sativa* powdered leaves were found to be 14.360 ± 0.165%w/w, while acid insoluble ash was found to be 2.726 ± 0.080% w/w which indicates that crude drug is low contamination. The different extractive values were indicated the chemical composition present in crude drug and solubility in a specific solvent. The result of *C. sativa* leaves powder showed that the water extractive value (23.038 ± 0.306) was higher than the ethanol extractive values (11.101 ± 0.223%w/w), indicating the presence of water-soluble compounds contained in plant materials and their different solubility property in different solvents. In this study, the volatile oil content was not detected from...
The phytochemical screening is a qualitative determination of the class of compounds contained in the plant. Phenolics, flavonoids, steroids, triterpenoids, diterpenes, and alkaloids were presented in Cannabis sativa leaves which may be responsible for some medicinal properties. However, saponins were undetectable in the crude ethanolic extract of Cannabis sativa leaves. The findings in accordance with the previous study of Cannabis sativa leaves cultivars from China and India.\textsuperscript{[16,25]} Phytochemical examinations are helpful in quality evaluation and drug discovery. The parameters studied in the present work may be successfully used for quality evaluation and could be applied as a standard reference, quality control, monograph preparation, and assurance of crude drug.

**CONCLUSION**

The characteristics of Cannabis sativa leave according to macroscopic examination, physicochemical parameter, TLC fingerprinting and phytochemical screening can be served as a standard reference for traditional practices and used to characterize the identity and quality of medicinal plant materials.

**Acknowledgments**

This work was supported by the Students Research Support of Chulalongkorn University Scholarship. The authors appreciate all staff members of the College of Public Health Sciences, Chulalongkorn University, Thailand, for their assistance and instrument support.

**Financial support and sponsorship**

Nil.

**Conflicts of interest**

There are no conflicts of interest.

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