Original Article

Pharmacognostic Investigation of the Leaves of *Mentha cordifolia* and Its DNA Fingerprints

Worapan Sitthithaworn[1*], Sornkanok Vimolmangkang[2], Chuda Chittasupho[1], Damri Petcheunsakul[3] and Siriprapa Apa-adul[3]

[1] Faculty of Pharmacy, Srinakharinwirot University, Nakonnayok, 26120 Thailand

[2] Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand

[3] 5th Year Pharmacy Student, Faculty of Pharmacy, Srinakharinwirot University (at the time of research conduct)

* Corresponding author: worapan@swu.ac.th

ABSTRACT

Objective: To perform microscopic investigation of *Mentha cordifolia* leaf and determine DNA fingerprint of the plant. **Methods:** Tissues of *M. cordifolia*, *M. arvensis* var *piperascens* and *M. spicata* were investigated under light microscope. DNA marker for *M. cordifolia* was established by amplification of the internal transcribed spacer (ITS) of nuclear ribosomal RNA gene using conserved plant sequences as primers and fragmentation with the restriction enzyme *Bsm*1. **Results:** The results showed the presence of uniseriate epidermal cells covered by a fine cuticle layer, glandular trichomes of multicellular type, capitate and peltate, and non-glandular trichomes. The fragmentation pattern of ITS of nuclear ribosomal RNA gene was applied as DNA marker for discrimination of *M. cordifolia* from *M. arvensis* var *piperascens* and *M. spicata*. **Conclusion:** Pharmacognostic investigation of *M. cordifolia* leaf exhibited the characteristic of *Mentha* species and the plant is distinguishable from *M. arvensis* var *piperascens* and *M. spicata* by DNA fingerprint pattern.

Keywords: *Mentha cordifolia*, microscopic investigation, DNA marker, internal transcribed spacer

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Introduction

Mentha cordifolia is locally known in Thailand as “saranae”. It is a culinary herb and is useful in traditional medicine as stimulant, digestive aid and for reduction of flatulence. The volatile oil has been reported to exhibit antibacterial and antioxidant activities.[1] Recently the vasorelaxant activity of *M. cordifolia* extract has been found in experimentally induced hypertensive rat.[2]. As it has a potential of providing useful drugs, it is important to standardize the plant for medicinal use. In this present study, we therefore examined some

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pharmacognostic characters for the leaves of *M. cordifolia* as well as its DNA fingerprint pattern. This information could be used to prepare a monograph for the proper identification of this plant.

DNA fingerprint can be generated using various methods. In this study, we used polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). In this method, fragments of DNA are amplified from each sample with a set of specific primers and they are further examined for their nucleotide sequences to provide information for restriction enzyme cut site. Finally, DNA fragmentation pattern is generated by selected restriction enzymes.[3]

Among various DNA sequences in plants, the internal transcribed spacer (ITS) of nuclear ribosomal RNA gene (rDNA) is usually used for plant authentication. ITS is non coding region separating three ribosomal subunits (Figure 1).[4] ITS1 locates between 18S and 5.8S rDNA and ITS2 between 5.8S and 26S rDNA. The advantages of PCR-RFLP using ITS regions are that they can be amplified with universal primers because they have conserved genes on both sides and their sizes are small, under 700 bp in flowering plants.[5] They are more diverged than ribosomal DNA subunit and are sufficiently variable to allow resolution in closely related taxa.

Figure 1 The organization of plant ribosomal RNA genes. The three subunits, 18S, 5.8S and 26S are separated by internal transcribed spacers (ITS1 and ITS2).

Materials and Methods

Collection

Mentha cordifolia Lej. & Courtois was collected from the botanical garden of Faculty of Pharmacy, Srinkahrinwirot University whereas *M. arvensis* var *piperascens* Malinv. ex L. H. Bailey and *M. spicata* L. were obtained from Sirirukhachat garden, Faculty of Pharmacy, Mahidol University. Voucher specimens were deposited in the herbarium of Faculty of Pharmacy, Srinakharinwirot University, Nakonnayok, Thailand.

Microscopic analysis

The leaves of *M. cordifolia*, *M. arvensis* var *piperascen* and *M. spicata* were boiled with saturated chloral hydrate solution for microscopical observation.[6]

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis

DNA was extracted from 100 mg of the fresh leaves of *M. cordifolia*, *M. arvensis* var *piperascen* and *M. spicata* using a DNeasy Plant Mini kit (Qiagen, Germany). The resulting DNAs were then used as a template for PCR. Primers 18d (5'-CAC ACC GCC CGT CGC TCC TAC CGA-3') and 28cc (5'-ACT CGC CGT TAC TAG GGG AA-3') complementary to the conserved regions of plant 18S and 26S subunits of rDNA[3] were used to amplify the region of ITS1-5.8S-ITS2 of rDNA (Figure 1). The PCR mixture contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1.5 U Taq polymerase (Invitrogen, USA) and 0.25 mM of each primer. The PCR program was hot start at 94 °C, 5 min followed by 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min for 30 cycles, and final extension at 72 °C for 10 min. After purification with a QIA quick PCR Purification Kit

(Qiagen, Germany), DNA sequencing was performed with a Big Dye Terminator Kit Version 3.1 (Applied Biosystems) using a model ABI Prism 3100 Avant Genetic Analyzer (Hitachi). DNA sequences were analyzed using CodonCode Aligner 1.6.3 (Demo version, www.codoncode.com/aligner/download.htm) and CLUSTAL W (1.83) (www.ebi.ac.uk/clustalw/). The PCR products of the obtained genes were digested with 1 unit of *Bsm*I (Biolab, New England) in 20 μL restriction buffer at 65 °C for 18 h. Restriction fragments were separated by electrophoresis in 2% agarose gel and visuailized by ethidium bromide staining.

Results and Discussion

Paradermic section of *M. cordifolia* leaf showed the presence of uniseriate epidermal cells covered by a fine cuticle layer, presenting glandular trichomes of multicellular type (capitate and peltate) and non-glandular trichomes (Figure 2 and 3). The capitate trichomes presented with a basal, a pedunculary and a great apical cell, whereas the peltate trichomes consisted of a basal cell, a short, wide and unicellular stalk cell. The peltate trichomes were dominant in relation to capitate trichomes.

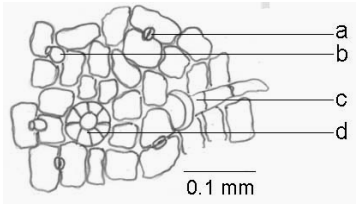

Figure 2 Epidermis of *M. cordifolia* leaf showing diacytic type of stomata (a); capitate type of multicellular glandular trichome (b); non-glandular trichome (c); and peltate type of multicellular glandular trichome (d).

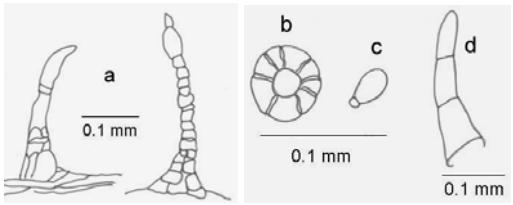

Figure 3 Glandular (a, b, c) and non-glandular (d) trichomes observed in *M. cordifolia* leaf.

Similar types of trichome also observed in *M. spicata* and *M. arvensis* var *piperascen* (Figure 4 and 5). It should be noted that trichome with a collapsed cell was observed in *M. arvensis* var *piperascen* (Figure 5). The collapsed cell type of trichome was also reported with *M.* piperita.[6] The epidermis of the leaf surface showed diacytic type of stomata (Figure 6), a characteristic stomata type for all *Mentha* species.

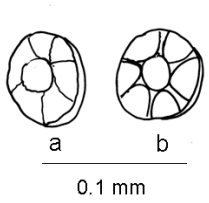

Figure 4 Peltate of multicellular glandular trichomes observed in the leaves of *M. spicata* (a) and *M. arvensis* var *piperascen* (b).

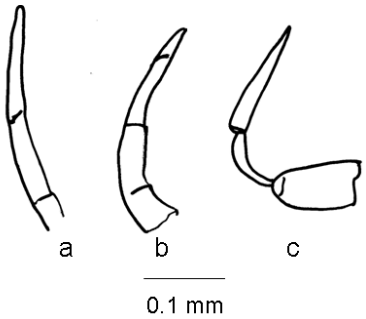

Figure 5 Glandular trichomes observed in the leaves of *M. spicata* (a); *M. arvensis* var *piperascen* (b); and trichome with a collapsed cell (c) observed in the leaves of *M. arvensis* var *piperascen*.

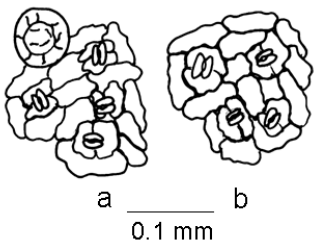

Figure 6 Epidermis of the leaves of *M. spicata* (a); and *M. arvensis* var *piperascen* (b) showing diacytic type of stomata.

In order to establish the molecular markers for authentication of *M. cordifolia*, its DNA profile was determined by direct sequencing of ITS1-5.8S-ITS2 of rDNA with subsequent comparison of the nucleotide sequence data to other selected *Mentha* species.

Nucleotide sequence of ITS1-5.8S-ITS2 of rDNA obtained from *M. cordifolia* consisted of 623 nucleotide residues whereas *M. arvensis* var *piperascen*s and *M. spicata* consisted of 622 nucleotide residues. The nucleotide sequences obtained from *M. arvensis* var *piperascens*[7] and *M. spicata*[8] were identical to those reported in EMBL/GenBank/DDBJ databank. The nucleotide sequence from *M. cordifolia* obtained from this study has been deposited in the EMBL/GenBank/DDBJ databank under the accession number of AB291546.

Sequence alignment of the three *Mentha* species resulted in identical nucleotide residues in the 5.8S gene but nucleotide variations were observed in the non-coding ITS1 and ITS2 regions (Figure 7). However, DNA sequence in the regions of ITS1 and ITS2 from *M. arvensis* var *piperascen* and *M. spicata* are identical. Thus, the polymorphism observed in *M. cordifolia* allows various restriction enzymes to create RFLP profiles characteristic to *M. cordifolia*. Restriction analysis suggested by a computer programme (webcutter 2.0, http://rna.lundberg.gu.se/cgi-bin/cutter2/cutter) revealed that the restriction sites unique to *M. cordifolia* were at nucleotide positions 47 (*Bst*DSI and *Dsa*I) and 143 (*Bsm*I, *Mva*1269I and *Bsa*MI) of the ITS1 region as well as nucleotide position 595 (*Asp*HI, *Bbv*12I, *Alw*21I, *Bsi*HKAI) of the ITS2 region.

Site of digestion is important for producing an optimum fragmentation pattern. Digestion of a 600 base pairs PCR product at either nucleotide position 47 or 595 could not produce gel pattern distinguishable from the intact PCR product. On the other hand digestion at nucleotide position 143 could result in two bands at approximately 480 and 140 bp which could obviously seen in agarose gel. Thus, PCR-RFLP analysis using *Bsm*I where digestion performed at nucleotide position 143 was applied to the PCR product.

As in Figure 8, the selected *Mentha* species were determined based upon the fragmentation pattern of ITS regions. Mint species that lacks the *Bsm*1 site exhibited the intact PCR product, with a single band at 600 bp, while *M. cordifolia* containing the *Bsm*1 site had two bands at approximately 480 and 140 bp, which corresponded to the restriction fragments.

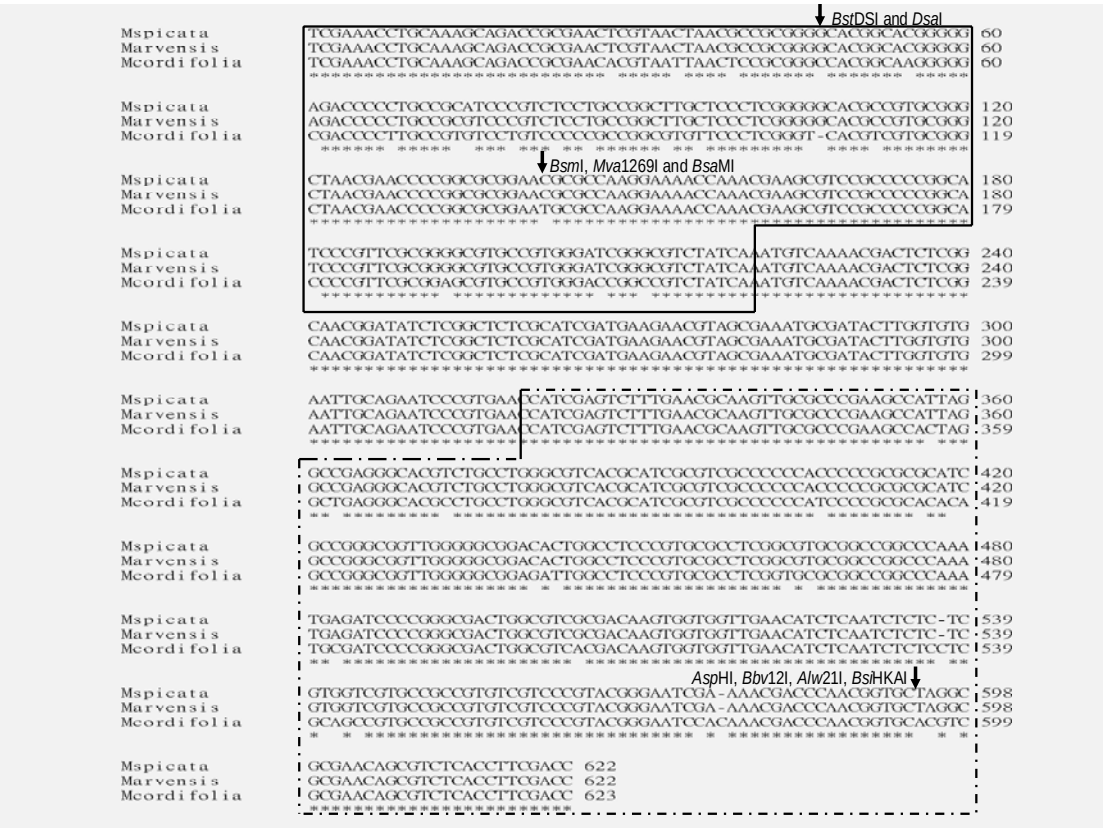

Figure 7 The DNA sequences of *M. spicata*, *M. arvensis* var *piperascens* and *M. cordifolia* in the ITS1-5.8S-ITS2 region. ITS1 and ITS2 are boxed in solid line and dash line, respectively. Arrows indicate restriction site presented only in *M. cordifolia*. An asterisk (*) indicates consensus base. A hyphen (-) indicates a gap.

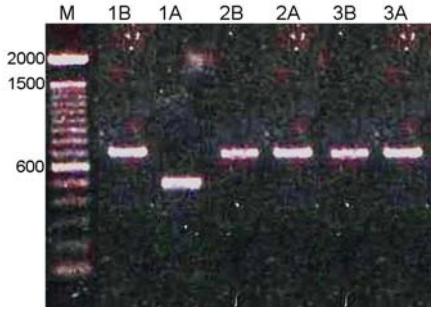

Figure 8 The PCR-RFLP patterns of the ITS1-5.8S-ITS2 region for of *M. cordifolia* (1), *M. arvensis* var *piperascens* (2) and *M. spicata* (3).

Note: B = intact PCR product; A = *Bsm*I digested PCR product. Molecular (M) sizes are in bp.

Conclusion

Microscopic investigation of *M. cordifolia* leaves revealed the presentation of glandular trichomes of multicellular type, capitate and peltate, which have been regarded as the characteristic of *Mentha* species[6]. Recently, DNA markers have become a popular means for identification and authentication of medicinal plant materials as DNA is less affected by age, physiological condition of samples and environmental factors.[9] In this study, DNA profile of *M. cordifolia* has been established by a comparative study to DNA profiles of *M. arvensis* var *piperascens* and *M. spicata* which are widely used as flavour in food and drug industries. Amplification of

the ITS gene and fragmentation with the restriction enzyme *Bsm*1 could result in DNA pattern that is useful in discrimination of *M. cordifolia* from *M. arvensis* var *piperascens* and *M. spicata*. Due to the variations of *Mentha* species, it should be noted that comparison of DNA profiles with mints other than *M. arvensis* var *piperascens* and *M. spicata* should be performed to ensure the result.

Acknowledgement

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