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PHARMACOGNOSTIC SPECIFICATION OF MENTHA CORDIFOLIA LEAF AND STEM WITH SPECIAL REFERENCE TO ROSMARINIC ACID CONTENTS

Ploychat Chamnanthongpiwan[1], Chanida Palanuvej[2]*, and Nijsiri Ruangrungsi[1,2]

[1]Public Health Sciences Program, College of Public Health Sciences, Chulalongkorn University, Bangkok 10330, Thailand
[2]Department of Pharmacognosy, College of Pharmacy, Rungsit University, Pathum Thani, 12000, Thailand

*Corresponding author : E-mail : chanida.p@chula.ac.th
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Abstract: *Mentha cordifolia* Opiz ex Fresen.(Sa-ra-nae) is one of the popular Thai kitchen herbs. This study aimed to specify the quality parameters of *M. cordifolia* dried leaves and stems with special reference to rosmarinic acid (RA) contents. The results showed that diacytic stomata type were found in upper and lower sides of leaf. The microscopic leaf constant numbers were found to be stomatal index, trichome index, oil gland index and epidermal cell area of upper leaf was 2.85 ± 0.64, 0.45 ± 0.44, 3.09 ± 0.89 and 2128.64 ± 182.80μ m^2; of lower leaf was 19.95 ± 2.65, 0.46 ± 0.33, 5.97 ± 1.75 and 1793.52± 262.45μ m^2 respectively. The physicochemical specification demonstrated the contents of acid-insoluble ash, total ash, loss on drying and moisture in leaf should not be more than 1.98, 9.41, 7.06 and 10.50; in stem should not be more than 1.63, 8.40, 7.04, 10.01 % by weight respectively; the ethanol-soluble extractive matter, water-soluble extractive matter and volatile oil in leaf should not be less than 5.04, 15.23 and 0.36; in stem should not be less than 5.20 and 16.60 % by weight respectively. The main chemical compound in the volatile oil was piperitenone oxide (73.22%). RA quantitative analysis was made by RP-HPLC using intentsil® ODS-3 column as stationary phase and methanol: 0.2% phosphoric acid (45% : 55%) as mobile phase. RP-HPLC analysis revealed the RA content in leaf as 1.92 ± 1.27 and in stem as 0.99 ± 0.41 g/100 g by dry weight.

Keywords: *Mentha cordifolia*, Marsh mint, rosmarinic acid, RP-HPLC, crude drug, quality control

INTRODUCTION

Mentha cordifolia Opiz ex Fresen. or the synonym, *Mentha villosa* Huds. is a hybrid of *Mentha suaveolens* Ehrh. and *Mentha spicata* L. (GBIF, 2021; Tucker AO and Naczi, 2016). The common name in English is Marsh mint, Kitchen mint and in Thai is Sa-ra-nae. It is one of the popular Thai kitchen herbs due to its unique aroma and benefits to human health such as helping to relieve cold, flu, fever, motion sickness, gastrointestinal problems, asthma, muscle spasms and inflammation (Srirattanakul *et al*., 2016). It was in an ingredient the traditional herbal preparation such as Ya Lueat Ngam (GPO, 2013), which was used as a blood tonic in menstrual disorders and leucorrhea use (Chaowuttikul *et al*., 2012).

Several species in Labiatae plants are aromatic and have also yielded commercially important polyphenol substances including rosmarinic acid (RA) and other derivatives of caffeic acid (Ellis *et al*., 1970). Many *in vitro* and *in vivo* pharmacological activities of RA have been studied and reported such as antioxidative, anti-inflammatory, antiproliferative, antimutagenic, cytoprotective and immunomodulation properties. Clinical studies with RA showed atopical dermatitis-mitigating and seasonal allergic rhinoconjunctivitis effects (Amoah, 2015).

The quality of crude drug including the active chemical composition is essential for medicinal efficacy. World Health Organization (WHO) is continuously emphasizing to ensure quality control of medicinal plant products by using modern techniques and applying suitable system of standardization (WHO, 2011). The aim of the present study was to evaluate pharmacognostic parameters of *M. cordifolia* leaf and stem crude drugs in Thailand and to determine their rosmarinic acid contents by high performance liquid chromatography (HPLC) for used as a tool for quality control of these Thai herbal crude drugs.

MATERIALS AND METHODS

Plant materials

Fresh *M. cordifolia* samples were collected from 15 different locations of Thailand as follows: Ratchaburi, Petchaburi, Sisaket, Lopburi, Nakhon sawan, Kanchanaburi, Suphanburi, Prachinburi, Chachoengsao, Samutprakarn, Nakhon Pathom, Bangkok, Saraburi, Nakhon ratchasima and Petchaburi. The samples were authenticated by specialist, Associate Professor Dr. Nijsiri Ruangrungsi. The herbarium specimens were prepared and deposited at College of Public Health Sciences, Chulalongkorn University. Plant samples were separated to 2 parts i.e. leaf and stem .Each sample was cleaned and dried at 45°C then ground for physicochemical and rosmarinic acid studies.

Microscopic evaluation

The anatomical section (transverse section) of midrib and stem were investigated. Fresh and mature laminae from 3 locations were investigated for leaf constant numbers. Chlorophyll was clearly removed by soaking in Haiter (containing 6% sodium hypochloride) : water (1 : 1 v/v) for 24-48 hours. The lamina section was heated in chloral hydrate : water (4 : 1 w/v) on water bath for 48 hours, then transferred to slide and observed the cells under microscope. The image was recorded using an Axio Vision software. The measurements were done for 90 fields (30 fields per location).

Stomatal number and stomatal index

The stomatal number is an average number of stomata per square millimeters (mm^2) of epidermis of the leaf. The stomatal index is the ratio of the number of stomata to the total number of ordinary epidermal cells in the same area.

$$\text{Stomatal index} = \frac{S}{E + O + T + S} \times 100$$

Trichome number and trichome index

Trichome number is an average number of trichomes or cicatrices per 1 mm^2 of epidermis. Trichome index is a percentage proportion of trichome number to all epidermal cell number in one square millimeter.

$$\text{Trichome index} = \frac{T}{E + S + O + T} \times 100$$

Oil gland number and oil gland index

Oil gland number is an average number of oil gland cells per square millimeter of epidermis. Oil gland index is a percentage proportion of oil gland number to all ordinary epidermal cell number in one square millimeter.

$$\text{Oil gland index} = \frac{O}{E + T + S + O} \times 100$$

O = Number of oil gland in a given area of the leaf

E = Number of epidermal cells in the same area of leaf

T = Number of trichome and cicatrix in the same area of leaf

S = Number of stomata in the same area of leaf

Palisade ratio

Palisade ratio is the average number of palisade cells under one epidermal cell of the leaf. The palisade cells under four continuous epidermal cells were counted the divided by four to obtain the palisade ratio.

Epidermal cell number and epidermal cell area

Epidermal cell number per square millimeters of epidermis was counted both sides of leaf. The epidermal cell area was calculated by dividing one square millimeter by the epidermal cell numbers.

Physicochemical evaluation

The physicochemical parameters of leaf and stem crude drugs from 15 sources were investigated including the amounts of acid-insoluble ash, total ash, loss on drying, moisture, extractive matters and volatile oil (Pitakpawasutthi *et al*., 2018). All samples were analyzed in triplicate. The results were represented by grand mean ± pooled standard deviation.

The essential oil composition was analyzed by a Finnigan Trace GC Ultra with DSQ Quadrupole detector. Zebron ZB-5 MS fuse silica column (30 m × 0.25 µm, 0.25 µm film thicknesses) was used as stationary phase. The oven temperature started from 60 °C up to 240 °C with the rate of 3°C/min. The carrier gas was helium with the flow rate of 1 ml/min. One microliter of *M. cordifolia* essential oil (1:100 in hexane) was injected by Finnigan Autoinjector A3000 with split ratio of 100:1. The chemical constituents were identified by matching mass spectra and retention time indices with Adams Essential Oils Mass Spectral library and NIST05 Mass Spectral library. Peak area was shown in percentage.

For the determination of TLC fingerprint, the ethanolic extract solution from maceration were dried and re-dissolved in ethanol to 10 mg/ml was applied onto the TLC plate (silica gel 60 GF_{254}). The plate was developed in a mixture of toluene: ethyl acetate: formic acid (5:4:1.2 v/v/v) and then examined under ultraviolet (UV) light (254, 365 nm). Then detected by spraying with ρ-anisaldehyde reagent.

Quantitative analysis of rosmarinic acid

Five grams of samples were exhaustively extracted with 95% ethanol (300 ml) in a Soxhlet apparatus. The prepared extract was filtered and evaporated till dryness. The extract was weighed to calculate the % yield. The extract solution was prepared by dissolving 1 mg of extract in methanol and filtered through a 0.45 µm PTFE membrane syringe filter.

The standard solution of RA was prepared in methanol (1 mg/ml) and filtered through a 0.45 µm PTFE membrane syringe filter. This stock solution was diluted serially for calibration curves (0.1, 0.2, 0.3, 0.4, 0.5 mg/ml).

HPLC system and data analysis were processed with Shimadzu LC solution software. The column temperature was maintained at 30°C and injection volume was 5 µl. Chromatographic separation was conducted using the reversed-phased C18 and coupled with C18 guard column. The sample were analyzed using 0.2% phosphoric acid in water (solvent A) and methanol (solvent B) as mobile phase. The program was set in isocratic mode at 55% solvent A for 20 minutes at flow rate of 1 ml/min. The maximum wavelength of photodiode array (PDA) detector was set at 330 nm for monitory chromatographic profile. The measurement was done in triplicate.

Method validation

Linearity, accuracy, precision, limit of detection, limit of quantitation, specificity and robustness were examined following to ICH guideline (ICH, 2005).

RESULTS AND DISCUSSION

Microscopic characteristics

Plant morphological character is very important for plant authentication. *Mentha* is a taxonomically difficult genus because of extensive hybridization, vegetative propagation, polyploidization and cultivation (Šaric-Kundalic *et al*., 2009). The leaf midrib was formed by a large vascular bundle, with xylem facing the adaxial surface and phloem facing the abaxial surface. The glandular trichomes were form on abaxial surface. The midrib cross section was shown in Figure 1. The stem presented uniseriate epidermis and it can be found one or two layers of collenchyma. The vascular bundle consisted of four main xylem points, and externally to it was found the phloem, which gives the quadrangular shape to the stem (Bezerra *et al*., 2019).

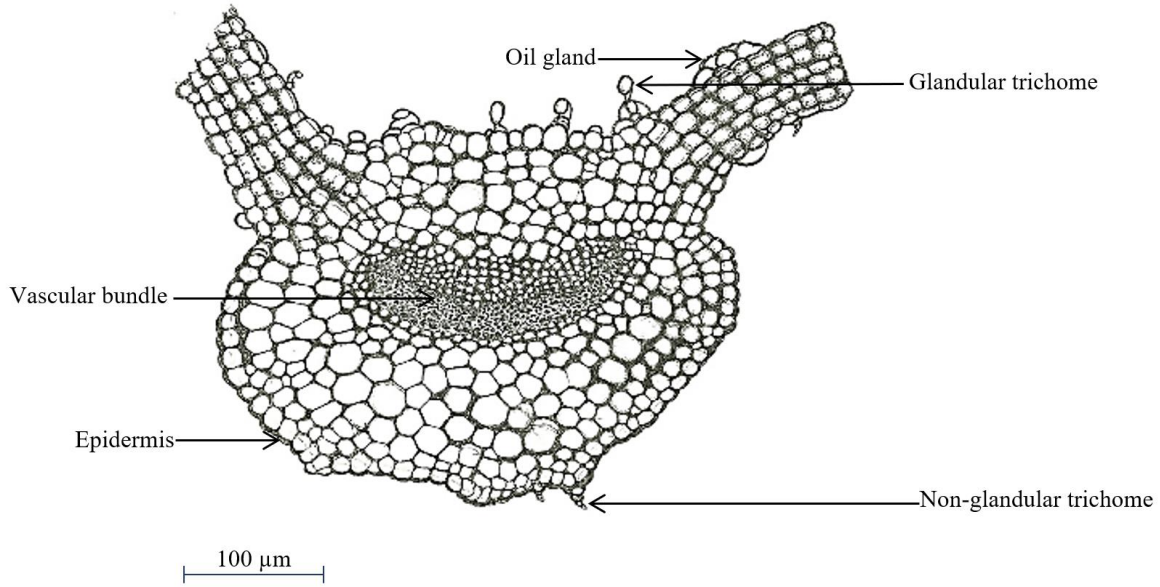

Figure 1. Midrib cross section of *M. cordifolia*

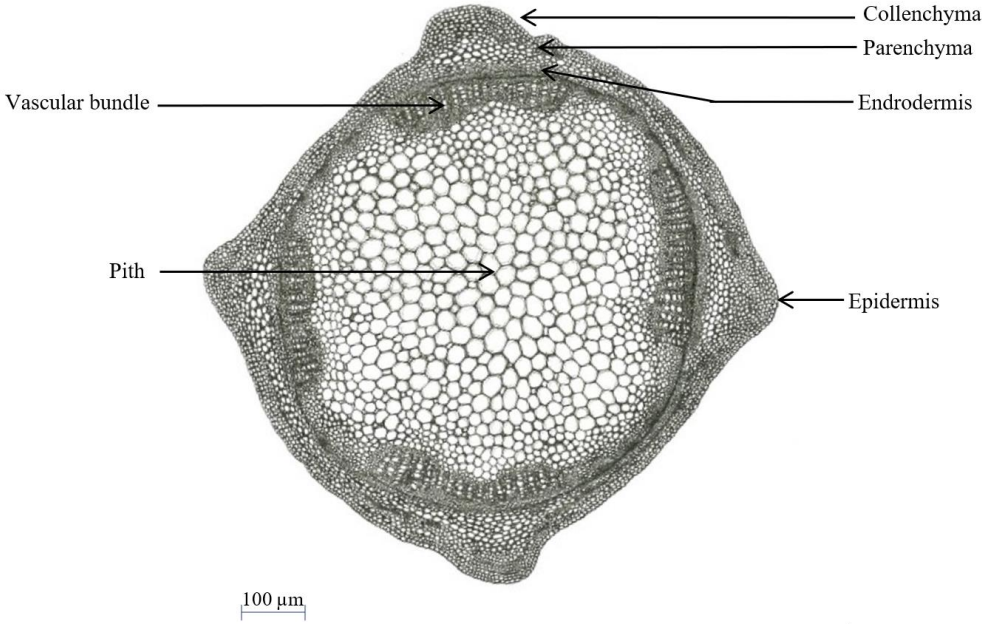

Figure 2. Stem cross section of *M. cordifolia*

Quantitation of microscopic leaf constant number is often used for medicinal plant samples. Leaf constant numbers were used to identify between some closely species which cannot differentiated by general microscopy (Evans *et al*., 2009). It could be the first step to identify the plant (Thitikornpong *et al*., 2018).

The results of leaf constant numbers consisting of stomatal number, stomatal index, epidermal cell number, epidermal cell area, oil gland number, oil gland index, trichome number, trichome index and palisade ratio were shown in Table 1.

Table 1. Microscopic leaf constant numbers of *M. cordifolia* in Thailand

Leaf constant values	Upper epidermis		Lower epidermis	
	Min - max	**Mean ± SD**	**Min - max**	**Mean ± SD**
Epidermal cell number	408 - 588	480.71 ± 42.44	424 - 924	665.02 ± 134.53
Epidermal cell area	1724.68 – 2482.98	2128.64 ± 182.80	1370.25 – 2502.49	1793.52 ± 262.45
Stomatal number	8 - 24	14.58 ± 3.41	88 - 300	174.04 ± 54.44
Stomatal index	1.39 – 4.24	2.85 ± 0.64	12.77 – 26.22	19.08 ± 2.65
Trichome number	0 - 8	2.31 ± 2.24	0 - 12	4.00 ± 2.68
Trichome index	0 – 1.63	0.45 ± 0.44	0 – 1.95	0.46 ± 0.33
Oil gland number	8 - 24	15.69 ± 4.29	28 - 80	50.80 ± 8.84
Oil gland index	1.40 – 4.92	3.09 ± 0.89	2.56 – 9.89	5.97 ± 1.75
Palisade ratio	3.75 – 5.25	4.63 ± 0.35	-	-

The stomatal type is one of histological characteristic evaluation which used to differentiate plants species. *M. cordifolia* leaf is amphistomatous which diacytic type were found on both sides (Figure 3). The diacytic type is one of common stomata which can found in Labiatae.

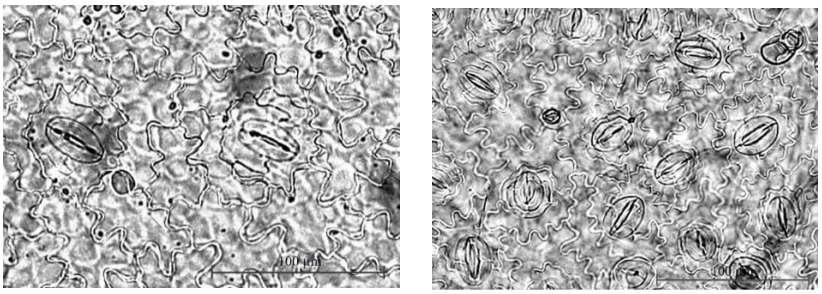

Figure 3. Upper (left) and lower (right) stomatal cells of *M. cordifolia* in Thailand

The trichomes were found both upper and lower epidermis the leaf. The uniseriate non-glandular and uniseriate glandular trichomes were found (Figure 4). The vesicle of glandular trichome may secreted the chemical defense of a plant by possessing glands such as essential oil, terpenes, gums and tannins, which in contact with predators can trigger several reactions, repelling, provoking limb immobility or even toxicity and death which exude terpenes, phenolics, alkaloids or other substances which are olfactory or gustatory repellent (Naidu and Shah, 1980).

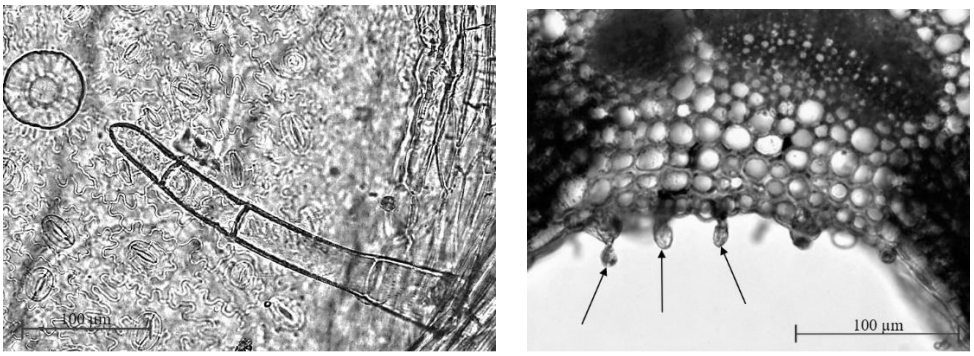

Figure 4 The uniseriate non-glandular trichome and cicatrix on the lower epidermis (left) and the uniseriate glandular trichome on the midrib (right) of *M. cordifolia* in Thailand

Essential oils are lipophilic substances produced by specialized secreting tissues called glandular trichomes. Glandular trichomes secreting essential oils are the base for the economic importance of several plant families, including the Labiatae. Anatomical taxonomy of *Mentha* specie is reported that contain both capitate and peltate glandular trichomes like other members of the Labiatae family (Maffei *et al*., 1986).

Physicochemical parameters

The quality specification due to the physicochemical parameters indicated that the contents of acid-insoluble ash, total ash, loss on drying and moisture content of leaf crude drug should not be more than 1.98, 9.41, 7.06 and 10.50, and the contents of stem should not be more than 1.63, 8.40 ,7.04 and 10.01 % of dry weight, respectively. Ash values is the one parameter useful to reflex the purity and quality of crude drugs. It's useful to determine the inorganic substance; such as Ca, Na, K, Cl etc., in plant material after complete incineration. The acid insoluble ash was evaluated by boiling the total ash with 70g/l hydrochloric acid which could evaluate aluminum and silicon. Ash value is used as the parameter indicates the adulteration, contamination or substitutions in plant crude drug. To evaluate loss on drying content by gravimetric method, both of water and volatile matter were came out, while the water content evaluation by azeotropic distillation can carry out only water which is in the plant crude drug. The moisture in the environment of storage can affect to the plant crude drug quality. In the other hand, the excessive of water content in plant crude drug can be the suitable condition for growth of bacteria, fungi or activate enzyme which induce chemical degradation or microbial contamination. Thus, the limit of moisture content should be set for stability of plant crude drug. The ethanol-soluble extractive and water-soluble extractive values of leaf should not be less than 5.04 and 15.23 % of dry weight and stem should not be less than 5.20 and 16.60 % of dry weight, respectively. The evaluation of soluble extractive matter is used to determine the amounts of active components when extract with specified solvent. The result presented that water soluble extractive value was higher than ethanol soluble extractive value which indicated the high content of polar compounds. Ethanol and water are common solvents used for traditional medicine preparation (Phumthum *et al*., 2018). The volatile oil content found only in leaf should not be less than 0.36 % of dry weight.

Table 2. Physicochemical content of *M. cordifolia* leaf and stem (% by weight) from 15 sources throughout of Thailand

Specification	Leaf	Stem
	Content (% of dry weight)	
Acid-insoluble ash	1.98 ± 0.07	1.63 ± 0.05
Total ash	9.41 ± 0.18	8.40 ± 0.18
Ethanol-soluble extractive	5.04 ± 0.18	5.20 ± 0.20
Water-soluble extractive	15.23 ± 0.42	16.60 ± 0.48
Loss on drying	7.06 ± 0.14	7.04 ± 0.14
Volatile oil content	0.36 ± 0.02	0
Water content	10.50 ± 0.41	10.01 ± 0.38

TLC fingerprint, the solvent system consisting toluene : ethyl acetate : formic acid (5 : 4 :1.2 v/v/v) and silica gel GF_{254} were demonstrated for chemical fingerprints in standardization of *M. cordifolia* leaf and stem crude drug which are shown in Figure 5.

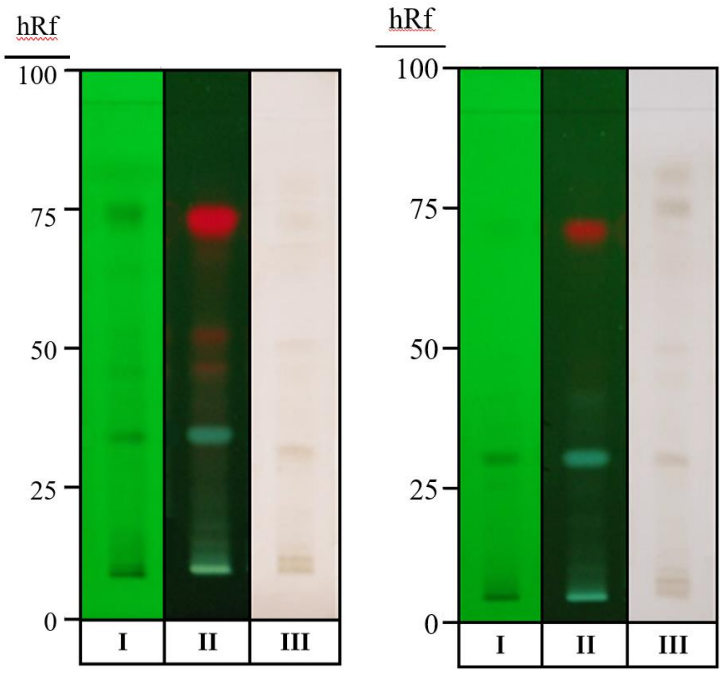

Figure 5. TLC fingerprint of *M. cordifolia* leaf (left) and stem (right) ethanolic extracts

For GC/MS analysis, the chemical constituents of *M. cordifolia* essential oil in this study were almost related to the previous study which reported the main chemical constituent in *M. cordifolia* leaf essential oil as piperitenone oxide (73.22%). The previous study also reported that the peperitenone oxide content was also be the highest in arial part essential oil *M. cordifolia* (69%) of Turkey (Başer *et al*., 2012).

Table 3. Chemical constituent in *M. cordifolia* leaf volatile oil in Thailand

Compound name	RT (min)	Peak area (%)*	Kovat's index
α-Pinene	6.20	0.74 ± 0.19	939
Sabinene	7.40	0.25 ± 0.20	975
β-Pinene	7.59	0.89 ± 0.23	979
Myrcene	7.88	0.75 ± 0.37	990
3-Octano	8.21	0.30 ± 0.24	991
Sylvestrene	9.37	2.16 ± 1.20	1030
1,8-Cineole	9.55	2.04 ± 0.61	1031
p-Cymenene	11.81	0.12 ± 0.47	1091
3-Octanol acetate	12.93	0.15 ± 0.19	1123
z-isopropyldenecyclohexanone	13.72	0.27 ± 0.24	-
Borneol	15.36	0.04 ± 0.12	1169
p-Cymen-9-ol	16.04	1.12 ± 0.54	1024
Coahuilensol, methyl ether	17.24	0.95 ± 0.40	1221
Carvone	18.53	1.61 ± 3.06	1249
Nonanal <dimethyl acetal>	19.19	0.03 ± 0.11	1279
Dihydroedulan I	20.51	0.14 ± 0.20	-
Piperitenone	22.65	0.53 ± 0.22	1343
Eugenol	23.12	0.04 ± 0.17	1359
Piperitenone oxide	23.58	73.22 ± 7.62	1368
Cinerolon	24.99	4.74 ± 1.70	-
(E)-Caryophyllene	25.79	0.87 ± 0.19	1419
(E)-β-Farnesene	27.18	0.02 ± 0.05	1456
cis-Muurola-4(14),5-diene	27.56	0.37 ± 0.13	1466
γ-cadinene	28.34	2.72 ± 0.56	1513
trans-Calamenene	29.94	0.39 ± 0.32	1522
chlorothymol	31.88	5.15 ± 4.63	1486
α-Cadinol	35.20	0.15 ± 0.20	1654

*The percentage of peak area calculated from 15 locations by GC/MS

Quantitative analysis of rosmarinic acid

The quality control of medicinal plant material also requires the determination of the phytochemical compound for ensuring the quality reliability of natural product obtained from plant sourced (Mukherjee *et al*, 2002). RP-HPLC is suitable simplicity, versatility, and scope to handle compound of a diverse polarity and molecular mass such as plant secondary plant metabolites. Rosmarinic acid is a phenolic compound containing conjugated double bonds which have strong UV absorbtion; thus, PDA detector is suitable detector for analysis. The reverse phase HPLC column is widely used to separate phenolic compounds in plant extracts. Octadecilsilane (ODS or simply C_{18}) column is preferred for polar compound analysis. Many natural product materials contain significant level of strongly binding components, such as chlorophyll and other endogenous materials that may in the long term compromise the performance of analytical columns. Therefore, the guard columns will significantly protect the lifespan of the analytical columns. (Boligon and Athayde, 2014). The chromatographic condition optimization as mobile phase, gradient elution procedure, flow rate, column temperature and wavelength detection were performed the good separation. Formic acid,

phosphoric acid and acetic acid were usually employed to the aqueous phase to enhance the resolution, restrain the ionization and reduced the peak tailing of compounds. The most suitable mobile phase following previous study showed good resolution and symmetric peak shape were performed by two parts as solvent A ;0.2% phosphoric acid in water and solvent B ; methanol with isocratic program. The condition of solvent system used in this study was practically consistent with the previous study (Chaowuttikul *et al*., 2012). The mainly component of *M. cordifolia*; RA is a hydroxycinnamic acid derivatives, are synthesized by shikimate partway. The HPLC results demonstrated that RA contents of *M. cordifolia* leaf and stem crude drugs in Thailand were 1.92 ± 1.27 and 0.99 ± 0.41g/100g respectively (Table 4). The validity of the analysis was shown in Table 5.

Table 4. RA content of *M. cordifolia* leaf and stem from 15 different sources throughout Thailand

No.	Sources	Rosmarinic acid content (g/100 g dry weight)	
		Leaf	Stem
1	Ratchaburi	0.84	0.88
2	Petchabun	0.73	0.87
3	Si sa ket	1.43	0.38
4	Lopburi	0.96	0.83
5	Nakhon Sawan	2.26	1.31
6	Kanchanaburi	1.57	0.29
7	Suphanburi	3.56	1.82
8	Prachinburi	5.57	1.08
9	Chachoengsao	2.19	1.60
10	Samutprakan	0.83	0.65
11	Nakornpathom	1.51	1.07
12	Bangkok	1.21	1.22
13	Saraburi	2.47	0.83
14	Nakhon Ratchasima	1.57	1.07
15	Petchaburi	2.06	1.92
	Average	1.92	0.99
	SD	1.27	0.41

Table 5. Method validation parameter of RA of *M. cordifolia* in Thailand

Validation parameter	Leaf	Stem
Linearity	y=15,443.98x-105,319.77 (r^2 = 1.00)	
Limit of detection (mg/ml)	0.006	
Limit of quantitation (mg/ml)	0.019	
Accuracy (%recovery; low, medium, high)	101.3, 110.7, 101.7	99.2, 104.8, 100.1
Precision (%RSD; low, medium, high)		
Repeatability precision	1.91, 0.66, 0.68	1.14, 0.55, 0.51
Intermediate precision	0.42, 0.97, 0.59	0.26, 0.50, 0.75
Robustness (%RSD; peak area, retention time)	0.48, 3.40	0.95, 3.35
Specificity (Peak purity index)	1.00	1.00

CONCLUSION

Pharmacognostic specifications of *M. cordifolia* leaf and stem crude drugs in Thailand were established. This present study indicated that piperitenone oxide was found as major component of the essential oil from *M. cordifolia* dried leaves in Thailand. The RP-HPLC with PDA detector was developed for quantitative of rosmarinic acid in *M. cordifolia* dried leaves and stems. The rosmarinic acid contents in *M. cordifolia* dried leaves and stems from various locations in Thailand were revealed which could be used for reference to its chemical marker.

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