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Article

Effect of genotypic and phenotypic variations on essential oil aromatic profiles of makwhaen fruits

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Abstract: In order to obtain makhwean (MK) fruit essential oil of constant aromatic profile during raw material sourcing, evaluation of relationship between genotype, phenotype and chemical profiles are necessary. Three specimens of the MK (MK1-3) distributed in Northern Thailand were genetically and morphologically compared with other *Zanthoxylum* spices known locally as mamaad (MM) and makwoung (MKO), respectively. MM was taxonomical confirmed as *Z. armatum* based on plant structure and leaf characteristic (Odd-pinnately compound leaf). MKO and MK were identified as *Z. rhetsa* and *Z. myriacanthum* using number of petals and anthers. Genetic sequencing by Internal Transcribed Spacer (ITS) sequence and Random Amplified Polymorphic DNA moreover, divided these *Zanthoxylum* spps. into three groups accordingly to their species viz., MM, MKO and MK. Essential oil of the dried fruits from these samples was extracted and analysed for physical and chemical profiles. Cluster analysis (PCA-biplot) of volatile compositions was able to separate 1) MK1 and MK3 with limonene as leading component, 2) MK2 and MKO related with sabinene and β-philandrene, 3) MM with linalool. By using odour attribute representatives, the essential oil of MKO and MK1-3 were closely related possessing fruity, woody and citrus aromas, while the MM was sweet/ floral. In summary for MK raw material sourcing, plant genotyping played the most important role to odour characteristics than growing locations, thus plant species confirmation should be first considered.

Keywords: Chemical profiles, taxonomical description, volatile compositions, *Zanthoxylum* spp.

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1. Introduction

The *Zanthoxylum* species (Rutacea) contains oil glands that yield high amount of essential oil with distinctive aroma [1]. Their fruits are known as spices for indigenous food in Asia such as *Z. piperitum* [2], *Z. armatum* [3], *Z. fagara* [4] and the extractable essential oil are used in cosmetic and pharmaceutical purposes. Commonly known as makhwaen or makhan, *Z. myriacanthum* are grown extensively in many areas in the North of Thailand viz., Pong district of Payao, Song Khwae district of Nan and in many high-altitude areas of Chiang Mai [5]. Previous studies described that *Z. myriacanthum* essential oil gives unique citrus top-note followed by woody and spice aromatic profile [5,6]. Thus, there has been demand of high-quality raw material for essential oil from food and perfumery industries. However, complaint from raw material purchaser advised that plant morphological characteristics such plant structure as well as sizes and colour of the berry clusters and aroma from different regions, are variable which makes it is difficult for quality controlling (Mrs. Anne Saget pers. Comm.). Moreover, complexity within the species remains ambiguous as such *Z. myriacanthum* is often misunderstood with *Z. limonella* [7]. Thus, there also urge to truly describe plant species.

In general, both genetic and environmental variables i.e., growing condition, light intensity, day length, temperature, altitude as well as their interactions could influence quantity and quality of the essential oils [8-10]. Identification of plant species and variety in the same genus can be accomplished by using morphological characteristics and chemical compositions [11]. However, only the use of these phenomenon may not be enough to truly describe the species. Studies with the essential oil containing plants revealed that chemical compositions and characteristics of essential oils from plants within the same genus are diverse such as those belong to *Ocimum* spp. [12] and *Zanthoxylum* spp.[13]. The use of DNA fingerprints can therefore accomplish the accurate identification of plant species [14].

ITS2 (Internal Transcribed Spacer), is a potential DNA barcode, has been reported to be an efficient barcode locus for plant identification [15-20] and classification by many plant species such as Indian *Berberis* [21], timber species of the mahogany family [22], *Dendrobium* species [23], and seed plants [24]. In addition to ITS region, RAPD analysis is an alternate method for estimating genetic diversity and relatedness in plant populations, cultivars and germplasm accessions especially in non-model plant species. There is no research work to date to fully describe genotyping differences among of raw materials for makhwaen essential oil production in relation to their physical properties and aromatic profiles as compared to those of other *Zanthoxylum* spp. This research is therefore, descriptively establish profile specification of raw materials used in makhwaen essential oil extraction industry.

2. Results and Discussions

2.1 Morphological confirmation

The morphological description of five plants specimen belonging to the *Zanthoxylum* spp. known locally as mamaad (MM), makwoung (MKO) and makwhaen (MK1-3)] were documented using plant structure, thorn, leaf type, floral structures and fresh fruit colour [25]. From our data in Table 1, plant structure of MM was of shrub and was different from that of MKO and MK1-3 (tree-like structure). Thorn of all specimens were either initiate on the trunk or branches. The same compound leaf type was noticed in the MKO and MK1-3 (even-pinnately) which were different from the MM (odd-pinately compound leaf). Petals of female structure composition were different in every species; the MM consisted of the flower with 6-9 petals while the MKO was with 4 petals and MK1-3 were with 5 petals.

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In the similar pattern, the number of anthers were different in every species 4-8 anthers for MM, 3-4 anthers for MKO and 5 anthers for MK1-3. The colour of fresh fruit was red in MM and MKO while MK1-3 gave greenish-red colour characteristics. Fruit sizes varied from 2-3 mm of the MK1-3 to 4-5 mm of the MM and the MKO was 5-7 mm, respectively. The 3 species gave brown fruit when dried with crack revealing the inner seeds. According to these specific characteristics, the scientific names of *Z. armatum*, *Z. rhetsa* and *Z. myriacanthum* are given to MM, MKO and MK specimens [25-27]. To describe the verity within the same speices, floral and fruit characteristics of MK 1-3 were compared (Table 2). The result confirmed that the MKs were those of *Z. myriacanthum* as the sepals and petals are pentamerous and male flower organs composed 5 of stamens.

According to the results from UPGMA analysis and PCA by plant characteristic and seven samples of *Zanthoxylum* spp., plant samples can be categorised into three groups including group i) MM, group ii) MKO, and group iii) MK1-3 (Figure 1 and 2). Analysis of PCA can distingue species based on plant characteristics elucidating that MKO and MK1-3 (tree) were separated from MM with plant structure characteristic (shrub). Nonetheless, MK1-3 were detached from MKO using floral characteristics (Figure 2).

Table 1. Plant characteristics for taxonomical identification of collected *Zanthoxylum* spps. used in this experiment

	Common names		
Part of Plant for classification	**mamaad (MM)**	**makwoung (MKO)**	**makhwaen (MK1-3)**
Plant structure	 Shrub/*1	 Tree	 Tree
Thorn	 Thorn on tree	 Thorn on tree	 Thorn on tree
Compound leaf type			

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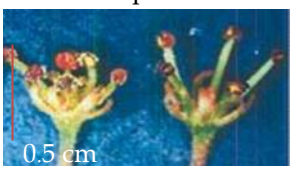
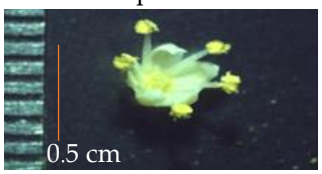
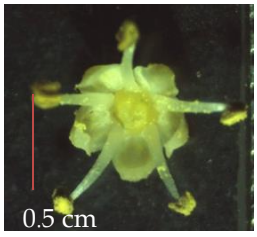
	Odd-pinately compound leaf	Even -pinately compound leaf	Even-pinately compound leaf
Female flower structure composition (petals)	 6-9 petals	 4 petals	 5 petals
Male flower structure composition (anthers)	 4-8 anthers	 3-4 anthers	 5 anthers
Fresh fruit colour	 Red*2	 Red	 Greenish red
Dry fruit colour	 Brown	 Dark brown	 Brown
Scientific name**	*Zanthozylum armatum*	*Zanthozylum rhetsa*	*Zanthozylum myriacanthum*

*1 online; https://en.wikipedia.org/wiki/*Zanthoxylum_armatum*

2 online; https://www.flickr.com/photos/helicongus/15771073680

** as confirmed the species by QSBG and as in [6].

Table 2. Floral and fruit characteristics of makhwaen collected from different locations (MK1-3)

Part of Plant for classification	**makhwaen (MK1)**	**makhwaen (MK2)**	**makhwaen (MK3)**
Female flower structure composition (petals)	 5 petals	 5 petals	 5 petals
Male flower structure composition (anthers)	 5 anthers	 5 anthers	 5 anthers

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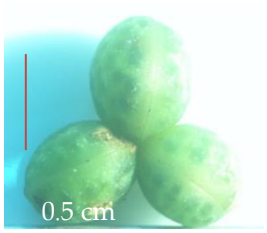
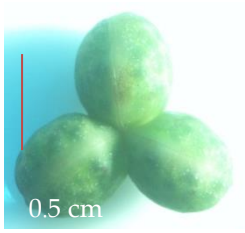
Fresh fruit structure			
	3 capsules	3 capsules	3 capsules

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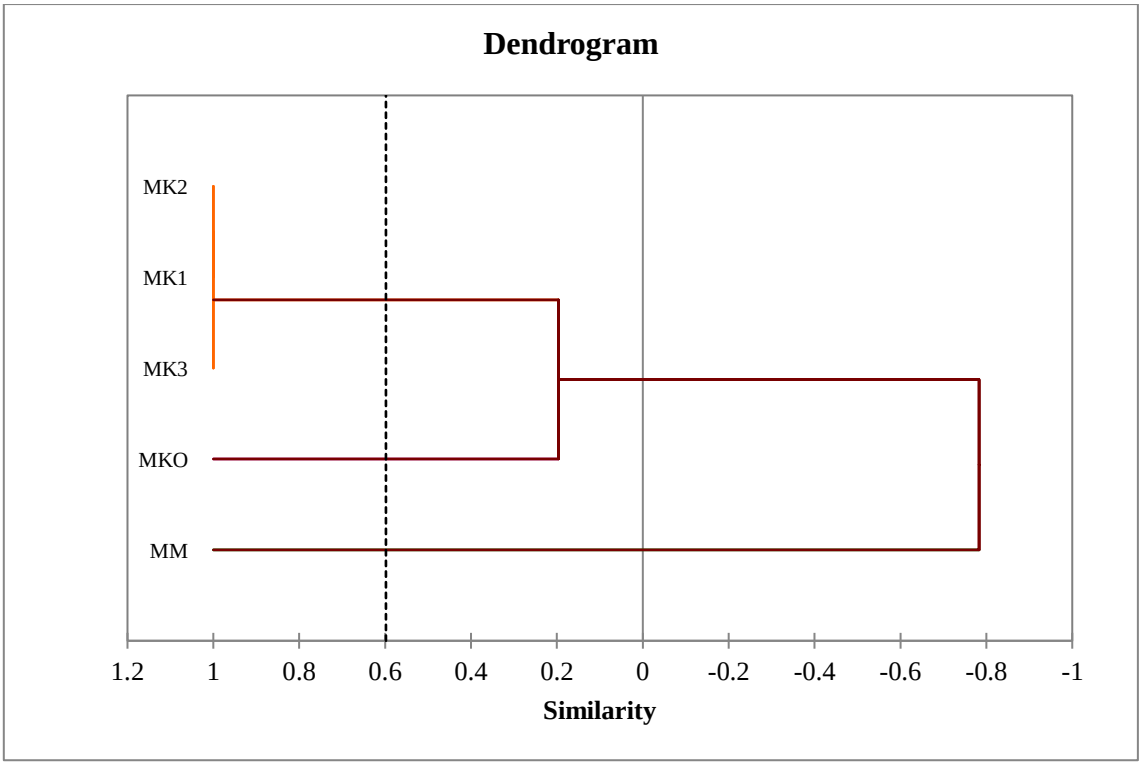

Figure 1. The dendrogram of *Zanthoxylum* spp. in North of Thailand; mamaad (MM), makwhoung (MKO), MK1 (makhwaen from Mae Tang district), MK2 (makhwaen from Mae Rim district) and MK3 (makhwaen from Song Kwae district) derived by UPGMA from the similarity matrix based on seven morphology data (plant structure, thorn, compound leaf type, petals, anthers, fresh and dry fruit colour).

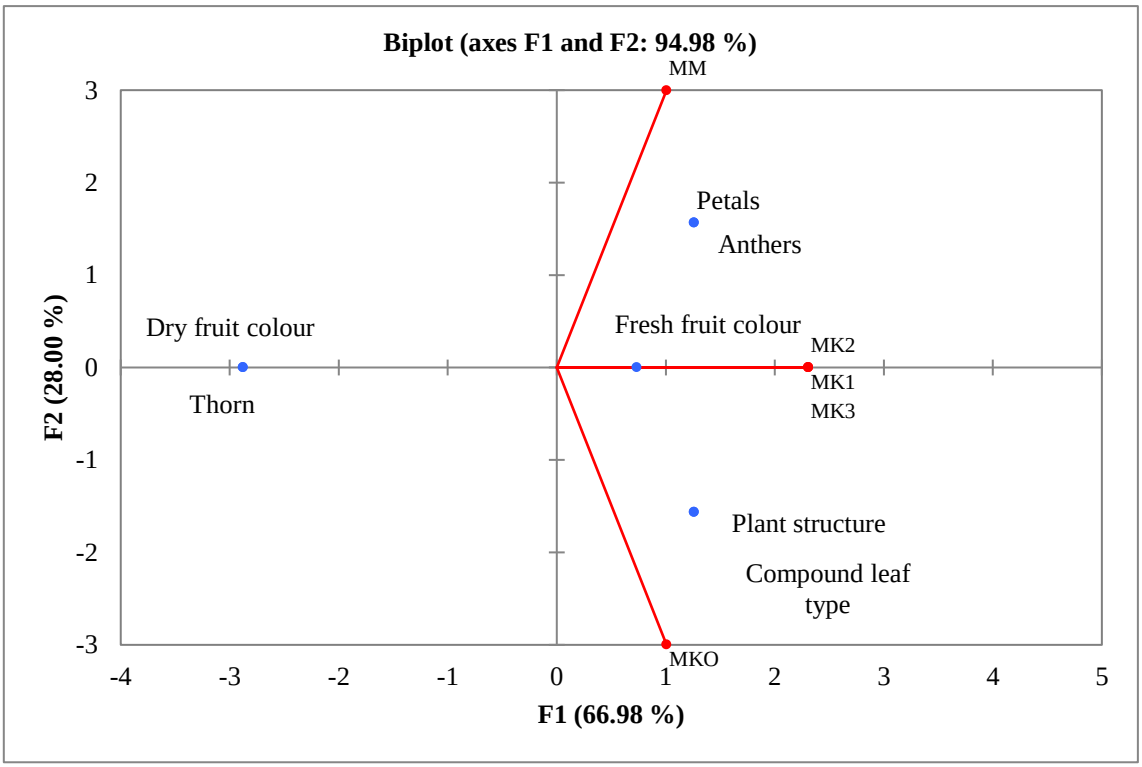

Figure 2. Principal component analysis (PCA) biplot (axes F1 and F2: 94.98%) illustrating the relationships among the parts of plant for classification different species of *Zanthoxylum*.

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Abbreviations; MM (mamaad), MKO (makwoung), MK1(makhwaen from Mae Tang district), MK2 (makhwaen from Mae Rim district) and MK3 (makhwaen from Song Kwae district).

2.2 ITS sequencing analysis

The aligned lengths of the ITS region (including both ITS1 and ITS2 regions) ranged from 596 bp for MK (*Z. myriacanthum*) to 600 bp for MM (*Z. armatum*). Among the five MK sampling (two from Mae Tang district: MK1-1 and MK1-2, one from Mae Rim: MK2 and two from Nan: MK3-1 and MK3-2), the ITS sequences were completely identical whereas thirty-nine single nucleotide polymorphism were found among MK, MKO and MM samples. The phylogenetic relationship analysis was investigated based on the total ITS region sequences. The dendrogram showed three major clades (Figure 3), the first formed among five MK samples from the three regions, second consisted of MKO while the last is MM. ITS sequence is an efficient tool for genetic identification among species however, very low efficiency for evaluation of genetic variation within species.

2.3 RAPD analysis

RAPD analysis revealed that the DNA bound only with S6, S7, S9, OPA01 and OPA04 primers. Thus, these positively responding primers were used to calculate unweighted pair group method with arithmetic mean (UPGMA). Result illustrated as dendrogram which split the *Zanthoxylum* spp. into three groups: group 1-MM, group 2 -MKO and group 3 consisting of MK1-3 as shown in Figure 4. It was clearly shown that MK1-3 were clustered as closely related species and taxonomically described as Z. *myriacanthum* while MM and MKO were genetically identified as separated species. In addition to our investigation, the experiments of plant genetic distribution of *Zanthoxylum* spp. using RAPD technique was successful to determine genetic variation of many plants of this kind including *Z. hamiltonianum, Z. nitidum, Z. oxyphyllum, Z. rhesta, Z. armatum and Z. schinifolium* [28-30]. Indeed, RAPD makers revealed significant however slightly genetic variability between *Z. myriacanthum* samples from different geographical regions in the present study.

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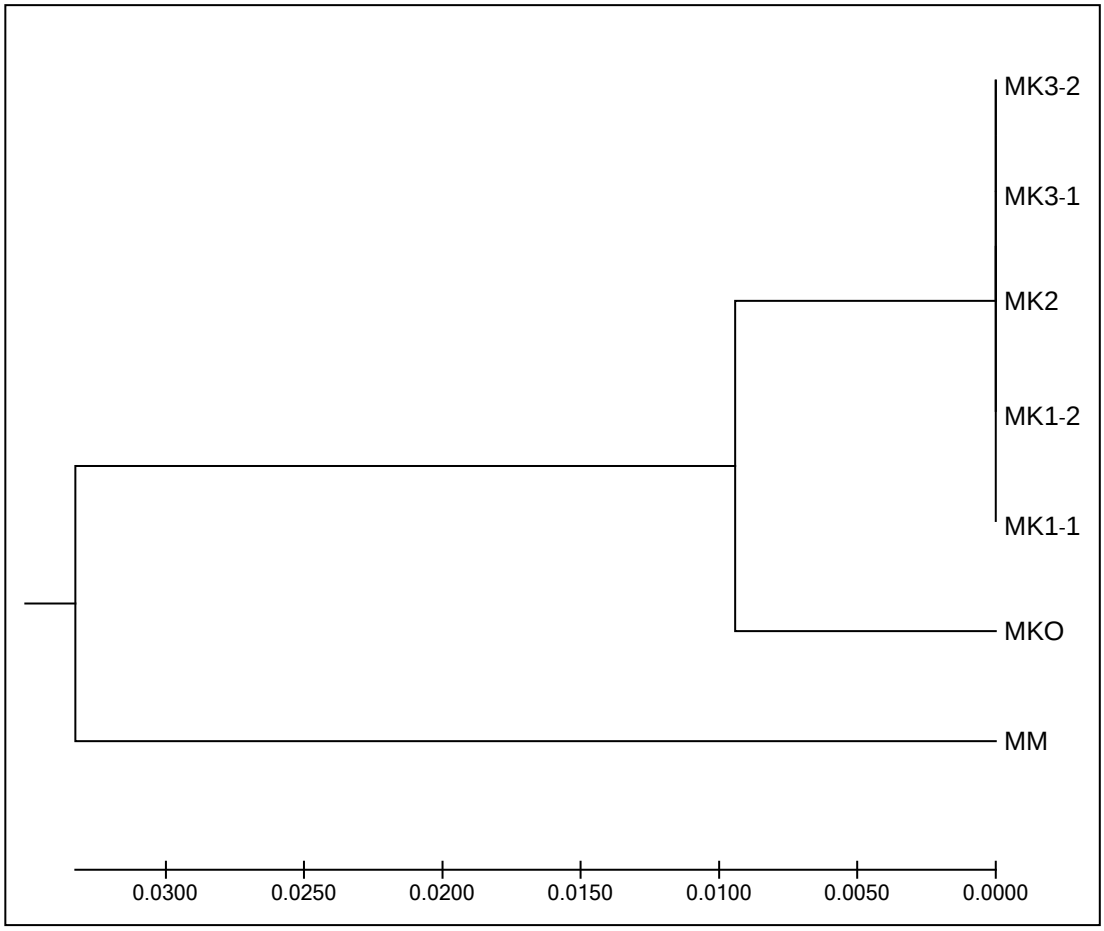

Figure 3. The dendrogram of *Zanthoxylum* spp. in North of Thailand; MM (mamaad), MKO (makwoung), MK1(makhwaen from Mae Tang district), MK2 (makhwaen from Mae Rim district) and MK3 (makhwaen from Song Kwae district) derived by UPGMA from the similarity matrix of the ITS sequence data.

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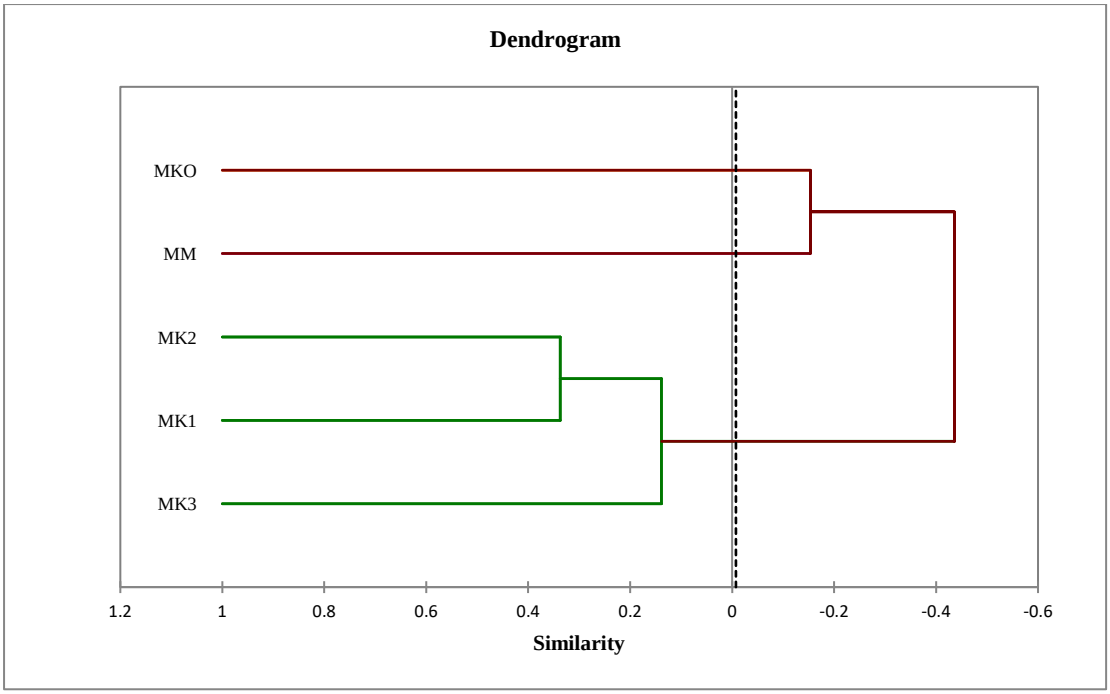

Figure 4. The dendrogram of *Zanthoxylum* spp. in North of Thailand; MM (mamaad), MKO (makwoung), MK1 (makhwaen from Mae Tang district), MK2 (makhwaen from Mae Rim district) and MK3 (makhwaen from Song Kwae district) derived by UPGMA from the similarity matrix based on 37 DNA bands obtained from five RAPD markers.

2.4 Essential oil analysis

Essential oils were extracted from dried fruits of makhwaen samples from 3 areas (MK1, MK2, and MK3), mamaad (MM) and makwoung (MKO) using hydro-distillation extraction. The extract yield varied by mean of species differentiation i.e., MK1-3 (~7%), followed by MM (~5%) and MKO (~2%). Thirty-five volatile compounds were detected using GC-MS (Table 4). Essential oil of the MKO contained linalool (7.35 μg.mL^{-1}), β-thujone (1.03 μg.mL^{-1}) and sabinene (0.44 μg.mL^{-1}), respectively. Sabinene was the key dominant substance in every *Zanthoxylum* species analysed, except for the essential oil of the MKO. This is in agreement with other works done with plants belongs to the *Zanthoxylum* spp. i.e, *Z. xanthoxyloïdes* and *Z. leprieurii* [31], *Z. rhoifolium* [32] with sabinene and limonene that represented woody and citrus aromas [33].

The chemical profiles of the essential oils from makhwaen fruits collected from different locations were variable. The major components of all samples could be described as following sequence: MK1; limonene (4.05 μg.mL^{-1}), sabinene (3.20 μg.mL^{-1}) and L-phellandrene (1.47 μg.mL^{-1}), MK2; sabinene (2.55 μg.mL^{-1}), terpinene-4-ol (2.05 μg.mL^{-1}) and β-phellandrene (1.85 μg.mL^{-1}) essential oil, MK3; limonene (6.89 μg.mL^{-1}), sabinene (3.00 μg.mL^{-1}) and β-ocimen (1.47 μg.mL^{-1}) essential oil, MM; sabinene (4.56 μg.mL^{-1}), teroinene-4-ol (4.31 μg.mL^{-1}) and γ-terpene (1.08 μg.mL^{-1}) essential oil. To this extend, environmental factors would play the important role to chemical composition of the volatiles [8,9]. The variations due to growing locations of aromatic crops were fully descriebd in chamomile (*Matricaria recutita* L.) [34], Satureja kitaibelii [35] and Myrsine leuconeura [36]. In the *Zanthoxylum spp.*, plants growing in altitude areas could affect important compositions (limonene, sabinene and linalool) viz., *Z. armatum* [3, 37-38] and *Z. alatum* [39]. It is apparent that plants used in this experiment were grown at different altitude.

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Table 4. Chemical profiles of makhwaen, mamaad and makwoung essential oils

No.	Chemical Compounds	Descriptors	RI[ref]	Amount of Chemical (μg.mL^{-1} Essential Oil [a])				
				MK1	MK2	MK3	MM	MKO
	The amount of essential oil extractions			7.15±0.07	6.2±0.14	7.4±0.14	5.5±0.01	1.8±0.07
1	α thujene	Woody	926.00	0.15±0.01	0.11	0.04±0.01	0.18	0.05
2	α pinene	Pine	937.00	0.42±0.02	0.45	0.09±0.01	0.52	0.03
3	Sabinene[#]	Woody	942.13	3.20±0.18	2.55	3.00±08	4.56	0.44
4	2β pinene	Pine	974.42	ND	0.02	ND	0.75	0.05
5	β myrcene	Spicy	993.25	0.66±0.04	0.27	0.86±0.05	0.21	0.13
6	octanal	Citrus	999.48	0.06±0.01	0.05	0.10±0.01	0.02	ND
7	l-phellandrene	Fruity	1009.09	1.47±0.07	0.61	0.32±0.02	0.09	0.04
8	acetic acid, hexyl ester	Sweet/ Floral	1048.40	0.02±0.01	0.04	0.02±0.01	ND	ND
9	α terpinene	Citrus	1050.30	0.29±0.02	0.40	0.08±0.01	ND	ND
10	benzene, methyl(1-methylethyl)	Citrus	1018.41	0.16±0.01	0.91	0.03±0.01	0.50	0.13
11	L-limonene[#]	Citrus	1058.22	4.05±0.01	1.01	6.89±0.18	0.31	0.97
12	β phellandrene	Fruity	1047.39	1.08±0.08	1.85	0.90±0.05	0.42	ND
13	cis-ocimene	Herbal	1103.60	0.17±0.01	0.11	0.08±0.01	0.06	0.02
14	B ocimene	Herbal	1132.13	0.76±0.04	0.31	1.47±0.06	ND	0.03
15	γ terpinene	Fruity	1144.60	0.47±0.02	0.63	0.14±0.01	1.08	0.25
16	trans sabinene hydrate	Herbal	1151.52	0.06±0.01	0.00	0.06±0.01	0.39	ND
17	1-octanol	Waxy	1167.87	0.09±0.01	0.17	0.05±0.01	0.03	ND
18	α terpinolene	Fruity	1168.98	0.15±0.01	0.17	0.09±0.01	0.30	ND
19	linalool	Sweet/ Floral	1180.06	0.43±0.02	0.53	0.26±0.02	ND	7.35
20	1-terpineol	Woody	1189.20	0.07±0.01	0.11	0.09±0.07	0.21	0.08
21	terpinene-4-ol	Citrus	1210.93	1.09±0.07	2.05	0.39±0.03	4.31	0.14
22	bicyclo[3.1.0]hexan-2-one, 5-(1-methylethyl)-	Citrus	1236.01	0.03±0.01	0.06	ND	ND	ND
23	β thujone	Minty	1251.13	ND	ND	ND	ND	1.03
24	.beta. fenchyl alcohol	Pine	1263.67	0.37±0.08	0.53	0.13±0.08	0.27	0.16

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25	piperitol isomer ii	Minty	1282.32	0.02±0.02	ND	ND	0.12	ND
26	decanal	Sweet/ Floral	1282.64	0.22±0.02	0.16	0.24±0.01	0.17	ND
27	acetic acid, 2-ethylhexyl ester	Herbal	1370.61	0.28±0.01	0.48	0.25±0.02	ND	ND
28	trans-geraniol	Sweet/ Floral	1379.21	0.03±0.01	0.12	ND	ND	0.55
29	1-decanol	Sweet/ Floral	1387.81	0.03±0.03	0.07	0.04±0.01	0.06	ND
30	2-undecanone	Fruity	1391.40	0.47±0.02	0.04	0.36±0.04	ND	ND
31	geranyl acetate	Sweet/ Floral	1395.70	0.26±0.01	0.23	0.03±0.01	ND	ND
32	dodecanal	Citrus	1396.06	0.07±0.01	0.05	0.06±0.01	ND	ND
33	trans-caryophyllene	Spicy	1408.88	0.03±0.03	0.02	0.03±0.01	0.09	ND
34	germacrene-d	Woody	1423.55	0.09±0.01	ND	ND	0.03	ND
35	bicyclogermacrene	Woody	1439.00	0.02±	ND	ND	ND	ND

RI^{Ref}: Retention index from the referent [5,12]. Values are calculated as reference to internal standard toluene (0.003% w v^{-1}), makhwaen fruit, mamaad and makwhoung essential oil were analysed by GC-MS (MK1, MK2, MK3, MM and MKO). ND: not detectable. #: main components.

The PCA-biplot analysed relationship between the volatile compositions and the species revealed three clustering groups. The first group (MK1 and MK3) had the dominant limonene and linalool representing the citrus and sweet/floral aromas (Figure 5). The second cluster was of the MK2 and MKO with β-phellandrene (fruity aroma), terpinene-4-ol (citrus aroma) and sabinene (woody aroma) as distinctive compounds. The last group was MM with the dominant L-phellandrene, β-myrcene and β-ocimene indicating the fruity, spicy and herbal aromas [5]. By categorising substances according to descriptors, it was found that MM was separated from other species due to the sweet/floral scents, while MK and MKO had the dominated components that described fruity, woody and citrus (Figure 6).

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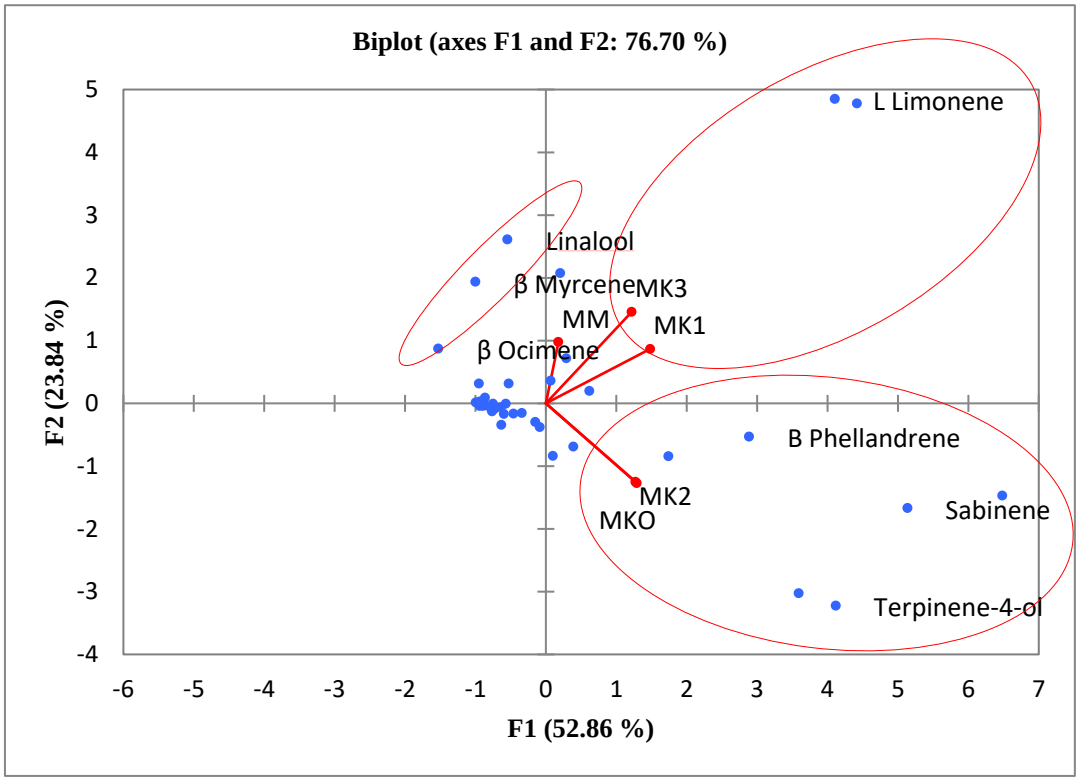

Figure 5. Principal component analysis (PCA) biplot (axes F1 and F2: 76.70%) illustrating the relationships among the chemical components and different species of *Zanthoxylum* essential oil. Abbreviations; MM (mamaad), MKO (makwoung), MK1(makhwaen from Mae Tang district), MK2 (makhwaen from Mae Rim district) and MK3 (makhwaen from Song Kwae district).

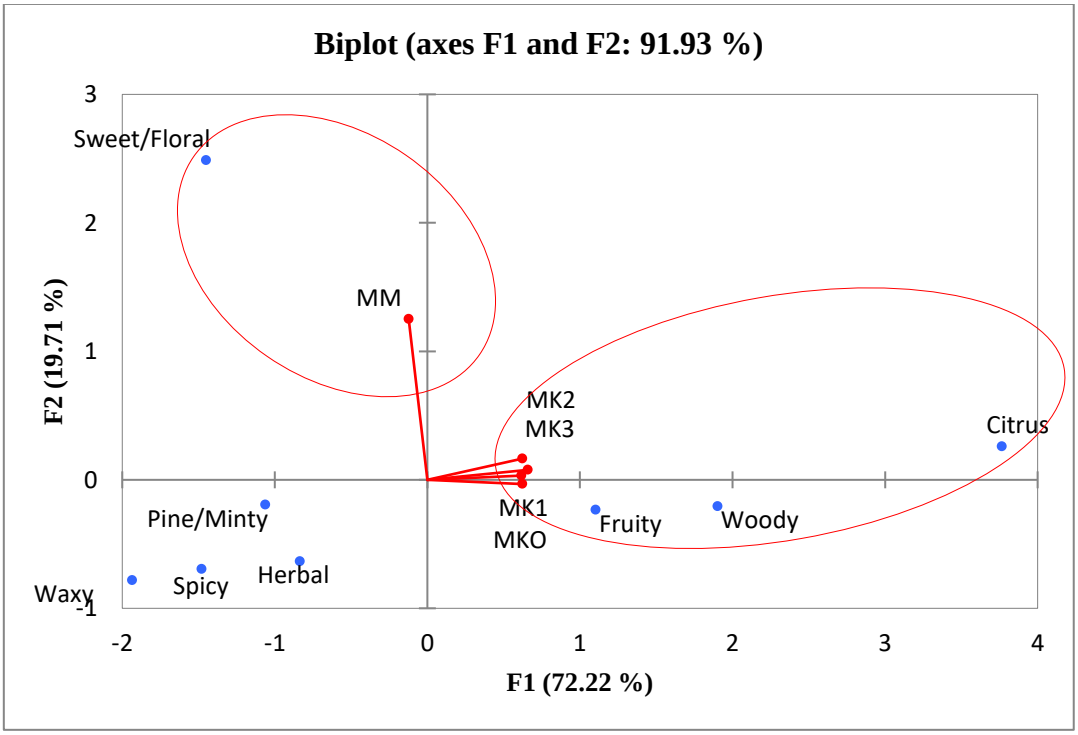

Figure 6. Principal component analysis (PCA) biplot (axes F1 and F2: 91.93%) illustrating the relationships among the odour attribute and different species of *Zanthoxylum* essential oil.

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Abbreviations; MM (mamaad), MKO (makwoung), MK1(makhwaen from Mae Tang district), MK2 (makhwaen from Mae Rim district) and MK3 (makhwaen from Song Kwae district).

2.5 FTIR analysis

Fourier-transform infrared spectroscopy (FTIR) is a technique used to obtain an infrared spectrum of absorption or emission of a solid, liquid or gas. An FTIR spectrometer simultaneously collects high-spectral-resolution data over a wide spectral range. This confers a significant advantage over a dispersive spectrometer, which measures intensity over a narrow range of wavelengths at a time. FTIR spectrum patterns have been adopted to expose volatile composition of plant essential oils using, they are found in plants such as lavender (*Lavandula officinalis*), pepper-mint (*Mentha piperita*), green douglas (*Pseudotsuga menziesii*), fir (*Abies alba*) and chicory (*Cichorium intybus*). The spectrum patterns of their Eos responded at the wave number ranges of 2,800-2,300 and 1,800-1,000 cm^{-1}) representing of free O-H bond valence and carboxylic acid broadband absorption [40,41]. Our result illustrated that the oil samples were dominated by overtones and different combinations of C-H reflection and shine occurring between 500-4,000 nm (Figure 7). FTIR spectrum scans of the three *Zanthoxylum* species essential oil (MK1-3, MM and MKO) absorbed light at wavenumber of 1,722-798 cm^{-1} and 2,967-2,926 cm^{-1}, respectively, therefore illustrating similar light transmission. EO of the MM on the other hand showed distinct spectrum characteristics from other samples (Figure 7, Table 5).

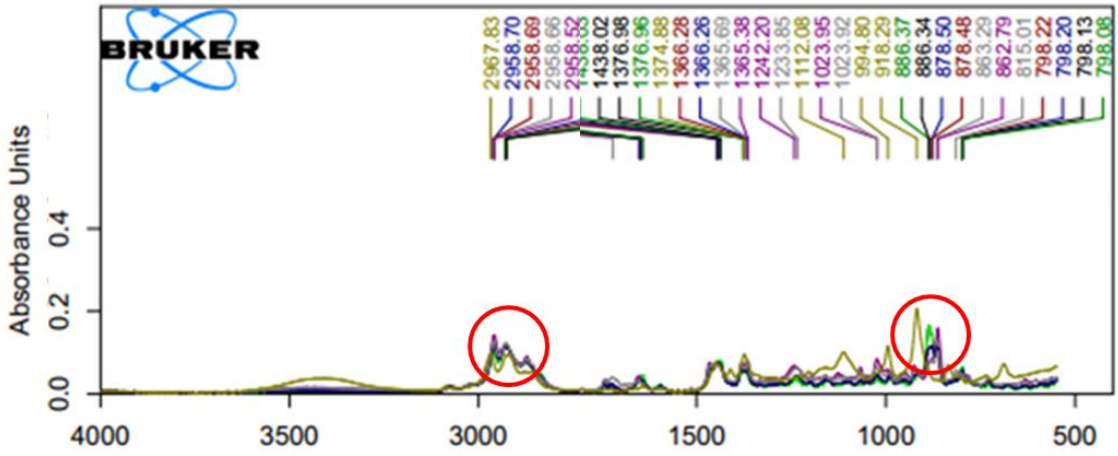

Figure 7. Fourier transform infrared spectrophotometer (FTIR) spectra of the essential oils from five different *Zanthoxylum* species. The insertion is the inset evidence of the peaks between 500-4,000 cm-1):(---) MK1, (---) MK2, (---) MK3, (---) MM- yellow an (---) MKO .Abbreviations; mamaad (MM) makwoung (MKO) MK1(makhwaen from Mae Tang district), MK2 (makhwaen from Mae Rim district) and MK3 (makhwaen from Song Kwae district).

Table 5. Wave number and functional groups of *Zanthoxylum* spp. essential oil

Name	Wave number	Type of vibration	Functional groups
MM	918.29	(=C-H) bending strong	Alkene
	994.8	(=C-H) bending strong	Alkene
	1112.08	C-O stretch strong	alcohol
	1374.88	bending variable -C-H	Alkane

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	2967.83	C-H stretch strong	CH_2 group
MKO	862.79	(=C-H) bending strong	Alkene
	1023.95	C-O stretch strong	Alcohol
	1365.38	bending variable -C-H	Alkane
	1445.39	bending variable -C-H	Alkane
	2958.52	C-H stretch strong	CH_2 group
MK1	878.5	(=C-H) bending strong	Alkene
	1366.26	bending variable -C-H	Alkane
	1445.3	bending variable -C-H	Alkane
	1650.64	(C=O) stretch	Ester
	2958.7	C-H stretch strong	CH_2 group
MK2	863.29	(=C-H) bending strong	Alkene
	1233.85	(C-O) stretch	Alcohol
	1365.69	bending variable -C-H	Alkane
	1446.33	bending variable -C-H	Alkane
	2958.66	C-H stretch strong	CH_2 group
MK3	886.37	(=C-H) bending strong	Alkene
	1376.96	bending variable -C-H	Alkane
	1438.03	bending variable -C-H	Alkane
	1643.61	(C=O) stretch	Alcohol
	2926.69	C-H stretch strong	CH_2 group

*Abbreviations; mamaad (MM) makwoung (MKO) and makhwaen (MK1-3)

3. Materials and Methods

3.1 Plant materials

Three plants specimen of the *Zanthozylum* spp. locally known as makhwaen were collected from the local orchards in 3 areas: (MK1) Papea, Mae Tang district, Chiang Mai province (19° 7' 27''N, 98° 42' 14'' E), (MK2) Pong Yang, Mae Rim district, Chiang Mai province (18° 53' 24''N, 98° 49' 53''E), (MK3) Yod, Song Kwae district, Nan province (19° 22' 37''N, 100° 35' 49''E) in September 2018. Mamaad (MM) was harvested from Ban rak Thai, Mok Champae, Muang district, Mae Hong Sorn province (19° 32' 32''N, 97° 53' 35.''E) in September 2018. Makwoung specimen (MKO) was sampled from Phichai, Muang Lampang District, Lampang province (18° 22' 11''N, 99° 35' 44''E) in September 2018 (Table 6). Based on the samples from harvest, all samples can be divided into two groups: (i) young leaves for DNA analysis and [41] fruits for essential oil analysis.

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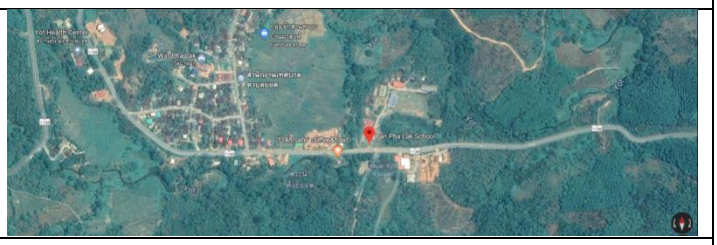
Table 6. Study site the sample collections

Location	Coordinate	Elevation (m)	Picture of area
Papea, Mae Tang district, Chiang Mai province (MK1)	19° 7' 27''N, 98° 42' 14'' E	924	
Pong Yang, Mae Rim district Chiang Mai province, (MK2)	18° 53' 24''N, 98° 49' 53''E	800	
Yod, Song Kwae district, Nan province (MK3)	19° 22' 37''N, 100° 35' 49''E	1,600	
Ban rak Thai Mok Champae, Moung district, Mae Hong Sorn province (MM)	19° 32' 32''N, 97° 53' 35.''E	1,176	
Phichai, Mueang Lampang district , Lampang province (MKO)	18° 22' 11''N, 99° 35' 44''E	294	

* The points of sampling location are presented in red pin.

** Satellite images by Google Maps

The morphological appearances of leaves, flower and fruit were recorded [25,43]. Their fruits correspondent to all specimen samples were also collected for the purpose of essential quality assessment at the mature stage and subjected to initial drying process as described in previous report [44]. Taxonomical confirmation has been done by comparison of the taxonomical descriptions from

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those of the literatures [25,26] and also confirmed by the botanist. The sample specimens were deposited at Queen Sirikit Botanic Garden (QSBG, Maerim Chiang Mai, Thailand) and the accession numbers of Trid01-05C were assigned.

3.2 Morphology relationship within species of Zanthoxylum spp.

Collected data of the part of Plant for classification were analysed. Those characters were assigned and scored as plant structure: shrub=0, tree=1; thorn: not have thorn=0, thorn on tree=1; compound leaf type: odd-pinnate=0, even-pinnate=1; number of petals: 4 petals=0, 5 petals=1, more 6 petals=2; number of anthers: 4 anthers =0, 5 anthers =1, more 6 anthers =2; fresh fruit colour: red=0, greenish-red=1 and dry fruit colour: brown=0, no brown=1. These data were analysed using cluster analysis (Dendogram and PCA-biplot) via XLstate, version 2016.

3.3 ITS and RAPD analysis

3.3.1 DNA Extraction

For the extraction of DNA, the DT-S DNA extract kit (Kurabo, Osaka, Japan) was used with modification of the CTAB extraction procedure. Young leaf tissue of 3 *Zanthozylum* spp. from 5 samples (0.5 g) were ground to powder using a mortar and pestle in the presence of liquid nitrogen and transferred to a 1.5 mL polypropylene centrifuge tubes and follow the steps of DNA extract kit. Tissue lysis buffer (MDT) 200 µL and proteinase K (EDT) 20 µL were combined and mixed together. After that, the centrifuge tubes were incubated by using the incubator at a temperature of 55 °C for an hour. At this stage, the centrifuge tubes were flipped every 15 min. Then, these tubes were centrifuged at 10,000 XG. When the process was completed, the supernatant (~200 µL) was moved to the new centrifuge tubes and added lysis buffer (LDT) 180 µL. Later, these new tubes were centrifuged with vertex for 15 sec. before they were incubated by using the incubator at a temperature of 70 °C for 10 min. A solution was moved into the new cartridge tubes and west tubes, then these tubes were aerated. After that, wash buffer (WDT) (washing buffer) 75 µL was added into the tubes. These tubes were aerated repeatedly for three times in order to elute DNA. Then, the cartridge tubes were moved into the collection tubes. At this stage, elution buffer (CDT) 50 was added and left for 30 min. After that, they were aerated repeatedly for two times. Finally, the centrifuge tubes were tested and stored at a temperature of -20 °C.

After extraction, total DNA was quantified using nano-drop spectrophotometer (ND-1000, spectrophotometer). For re-quantification, the extracted DNA was run on 1.5% agarose gel electrophoresis using 1× TBE buffer at 5-8 V mL^{-1} for 30 min and visualised under BLook LED transilluminator (Genedirex, Taiwan) by staining with MaestroSafe TM (Maestrogen, USA). The DNA solution was diluted with sterile distilled water (DI) to a concentration of 10 $ng.\mu L^{-1}$ for PCR analysis and kept in -20 °C until use [43].

3.3.2 ITS sequence

The ITS2 sequences were amplified using the following pair of universal primers, ITS5-ITS4 (including both ITS1 and ITS2 regions), ITS5 GGAAGTAAAAGTCGTAACAAGG and ITS4 TCCTCCGCTTATTGATATGC. Each 50 µL reaction contained 5 µL 10X PCR buffer, 2.5 µL 2.5mM MgCI2, 0.4 µL 0.2mM Deoxyribonucleotides (dNTP), 5 µL of each primer (10ng/ µL), 0.4 µL 0.5U Taq DNA polymerase (Thermo scientific), 40 µL ddH2O and 5 µL genomic DNA (50ng/ µL). The amplification consisted of 94 ºC/ 2 min, followed by 40 cycles of 94 ºC/ 45 s, 50 ºC/ 45 s, and 72 ºC/ 1

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min, and ending with 72 ºC for 5 min for final extension. Amplified products were genotyped using 1.5% agarose gel electrophoresis. Then they were staining with MaestroSafeTM Nucleic Acid Stains (MAESTROGEN, Taiwan) and visualized under UV transilluminator (BioDoc-It2 imaging systems, Analytik Jena US) before samples were send to sequencing at Macrogen, Inc. (South Korea).

3.3.3 RAPD-PCR protocols

For RAPD analysis of the genomic DNA, 10-base primers from Operon Technologies (Alameda, USA) and UBC (University of British Columbia, Canada) were chosen (Table 3.2). A total of nine primers were screened. The polymerase chain reaction (PCR) was adjusted to 10 μL^{-1} containing 8 μL^{-1} of OnePCRTM Plus (Genedirex, Taiwan), 1 μL^{-1} of 1 µM RAPD primer and 1 μL^{-1} of 10 ng genomic DNA. All the reactions were carried out on a Flexcycler2 thermal cycler (Analytik Jena, Germany) using the following profile: 1 cycle, 94 °C, 4 min; 40 cycles, 95 °C, 30 s; 37 °C, 30 s; 72 °C, 60 s; 1 cycle, 72 °C, 10 min. The sample was separated in a 1.5% agarose gel in 1× TBE buffer. The samples were run at 70 V for 120 min. The gels were then visualised using the BLooK LED transilluminator (Genedirex, Taiwan).

3.3.4 Dendrogram analysis

The banding pattern for each primer was scored as diallelic (1 = band present, 0 = band absent), and stored in an Excel (Microsoft) spreadsheet file in the form of a binary matrix. In order to assess the genetic differentiation between the five samples accessions, ten RAPD markers were analysed using the statistical package XLSTAT version 2016 software. The coefficients of genetic similarity for all the pair-wise comparisons were computed using the Jaccard's coefficient of similarity, and then the distance matrix was subjected to cluster analysis by using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) to produce a dendrogram.

Table 7. Sequence of RAPD primers

Primer	Sequence	References
S6	TGCTCTGCCCC	-
S7	GGTGACGCAG	-
S9	GCGTCGAGGG	-
OPA01	CAGGCCCTTC	-
OPA04	AATCGGGCTG	-
OPN05	AGGGGTCTTG	[45]
OPN06	GAGACGCACA	[45]
OPN07	CAGCCCAGAG	[45]

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OPN13	AGCGTCACTC	[46]

3.4 Essential oil analysis

3.4.1 Essential oil extraction

The essential oil was extracted by hydro-distillation for 4 h, from 100 g of dried fruits in 600 mL of DI water in a 2 L flask Clevenger-type apparatus. The oil was dried over anhydrous sodium sulphate and was kept at 4 °C until analysis (usually within 3 days). The extraction was repeated twice and yield (mean value) was reported as percentage of essential oil from dry plant material [47].

Gas Chromatography-Mass Spectrometry (GC–MS) analysis was performed on Bruker-scion 436 GC a Rxi 5Sil MS (30 m × 0.25 mm; 0.25 μm film thickness). Temperature program includes oven temperature held for 2 min at 60 °C and was enhanced to 150 °C with 3 °C min^{-1}. Then, temperature enhancement was programmed up to 270 °C at 5 °C min^{-1} and held at this temperature for 15 min. Other operating conditions include: carrier gas was Helium with a flow rate of 1.1 mL min^{-1}; injector and detector temperatures were 300 °C, and split ratio, 1:50. Mass spectra (MS) were taken at 70 eV. The mass spectra and retention indices of essential oil components were identified by comparison to MS computer library (NIST 05.L and NIST 98.L) [48].

3.4.2 Fourier transforms infrared spectrophotometer (FTIR) analysis

The FTIR spectrometer used was Bruker model ALPHA II, Daimond ATR (Hamburg, Germany) and operating at the basic of 500-4,000 wavenumber for averaging 47 scans per spectrum [41].

3.5 Statistical analysis

The data was statistically analysed using a comparison of the means of yield for essential oils evaluated by Tukey Multiple Comparison's test test at 95% confidential level [49]. A Principle Component Analysis (PCA) was used to identify the main sources of systematic variation in the chemical compounds data using XLstat software version 2016 [5].

4. Conclusions

Even though large number of secondary metabolites interfere DNA sequencing, morphological description is adequate for differentiation of plant belonging to this genus. The locally known makhwaen were taxonomically and genetically confirmed as *Z. myriacanthum*. Base up on the principal component evaluation, mamaad essential oil was described to have different aroma characteristic as compared to the rest of *Zanthoxylum* spp. analysed. The essential oils of makhwaen from Nan and Chiang Mai are similar in terms of quantity and characteristics of the chemical compositions. For example, limonene and sabinene represent the aroma of citrus and woody.

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