

Characterization of the Essential oil and Fatty oil from Makhwaen Fruit (*Zanthoxylum rhetsa* (Roxb.) DC)

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ABSTRACT

In this work, the essential oil (EO) was isolated from the fruit of makhwaen by using hydrodistillation. Then the dried residue from hydrodistillation was further extracted with hexane to produce crude hexane extract (CHE). The NMR data confirmed the CHE as fatty oil (FO). Then FO was *trans*-esterified into fatty acid methyl esters (FAMES). The NMR data confirmed the formation of FAMES. The chemical compositions of EO and FAMES of FO were analyzed by GC-MS. The 24 compounds were identified in the EO, including 11 monoterpenes, 6 oxygenated monoterpenes, 4 sesquiterpenes, 1 hydrocarbon and 2 ketones. The four major monoterpenes from EO of makhwaen fruit were α -phellandrene (14.57%), terpinen-4-ol (9.22%), sabinene (6.57%) and α -pinene (5.92%). While the makhwaen FAMES of FO contained 5 unsaturated fatty acid methyl esters and 2 saturated fatty acid methyl esters. Interestingly, the major components of FAMES from FO contained high amount of α -linolenic acid methyl ester (50.49%), linoleic acid methyl ester (33.08%) and palmitic acid methyl ester (10.81%). Moreover, the small amount of *cis*-vaccenic acid methyl ester (0.33%) was also found. The amount of each fatty acid methyl esters found in FAMES analysis could be directed to the relative proportions of each fatty acids containing in FO. Therefore, the highest percentage of α -linolenic acid methyl ester was directly related to the highest content of α -linolenic acid (ALA, omega-3) in FO. The results showed that the makhwaen fruit could be considered as a new source of ALA. This was the first report on the study of fatty acid components from makhwaen fruit in form of FAMES.

Keywords: *Zanthoxylum rhetsa* (Roxb.) DC fruit, essential oil, fatty acids

INTRODUCTION

The scientific name for the makhwaen tree was *Zanthoxylum rhetsa* (Roxb.) DC (Syn. *Zanthoxylum limonella* (Dennst.) Alston) (Smitinand, 2014). Makhwaen was found in the northern and central parts of Thailand. The makhwaen fruit is served as a spice for favoring of traditional food and this fruit has been used as a traditional medicine (Wutithamawech, 1997). Makhwaen fruit consist of seed and pericarp. The seeds contain fatty oil, while the essential oil is concentrated in pericarp (Agarwal *et al.*, 1959). Several research groups have demonstrated the essential oil from makhwaen fruit. This essential oil has been studied in isolated guinea pig ileum, conscious mice and rat thoracic aorta (Itthipanichpong *et al.*, 2002). The extraction of essential oil from makhwaen fruits was performed by subcritical CO₂, modified methanol-subcritical CO₂, hydrodistillation and solvent extraction processes. The chemical compositions of the essential oil were identified by gas chromatography-mass spectrometry analysis (GC-MS). The major components of the essential oil have

been reported as sabinene, terpinen-4-ol, β -phellandrene and α -terpineol (Rout *et al.*, 2007). The makhwaen fruit has been used as a traditional medicine and contained high quantity of oil. Therefore, the oil extracts from various solvents have been investigated to show the herbicidal and biological activities including antimalarial and antituberculous (Charoenying *et al.*, 2008). Moreover, the isolated xanthoxylene from the crude ethyl acetate extract completely inhibited the growth of weeds with low concentration (Charoenying *et al.*, 2010).

In this work, the essential oil (EO) from dried makhwaen fruit was extracted by using hydrodistillation. Then the chemical components of EO were studied by GC-MS analysis. Furthermore, the residue from hydrodistillation of EO was dried. Then the dried residue was extracted with hexane to produce brown crude hexane extract (CHE). ^1H NMR profile of CHE was investigated as fatty oil (FO). The FO was *trans*-esterified to fatty acid methyl esters (FAMES). The chemical compositions of FAMES from FO were identified by ^1H NMR spectroscopy and GC-MS analysis. These investigations were to study the chemical compositions of EO and FAMES of FO.

MATERIALS AND METHODS

General Experimental Procedures

The chemical components of EO and FAMES were investigated on an Agilent 19091s-433 technologies 6890N gas chromatograph interfaced to an Agilent technologies 5973N Mass spectrometer (GC-MS). Proton nuclear magnetic resonance (^1H NMR) experiment was carried out on a Bruker/Avance 400 spectrometer operating at 400 MHz with chloroform-D as solvent.

Chemicals

Hexane, methanol and sodium hydroxide (analytical grade) were purchased from RCI Labscan (Thailand). Chloroform-D (99.9%D) was purchased from Wilmad LabGlass (USA).

Plant Material

The makhwaen fruit was collected in January, 2015 from the natural habitats of Pua District, Nan province (Thailand) and the plant was identified as *Zanthoxylum rhetsa* (Roxb.) DC. A voucher specimen (No. 003565) was deposited at Department of Biology, Faculty of Science, Naresuan University.

Extraction

The air-dried makhwaen fruit (250.0 g) was placed into the hydrodistillation apparatus and distilled at 100 °C for 5 h. The 12.30 g (4.92% w/w) of golden yellow essential oil (EO) was obtained and stored in a freezer for GC-MS analysis.

The residue from hydrodistillation of EO was dried in the oven at 60 °C for 24 h to obtain 200.0 g of dried residue. Then the residue was extracted with hexane (800 mL) two times at room temperature for 7 days, each. The solution was filtered, and the filtrate was evaporated under vacuum to produce 19.27 g (9.64% w/w) of brown crude hexane extract (CHE). ^1H NMR profile of CHE was investigated as fatty oil (FO). Then the FO was *trans*-esterified to fatty acid methyl esters (FAMES). The FAMES of FO was confirmed by ^1H NMR spectroscopy and GC-MS analysis.

Transesterification process

FO (2.20 g) was dissolved in 9 mL of 1% (w/v) sodium hydroxide in methanol. The mixture was stirred at 750 rpm and heated at 60 °C for 1 h. The reaction mixture was allowed to cool to room temperature with separation of two phases. The phase of FAMES from FO (upper layer) was collected and the methanol was removed under vacuum. The FAMES of FO phase was then washed with 1:1 mixture of water and hexane. The FAMES of FO in hexane phase (upper layer) were separated and dried with sodium sulfate anhydrous (anh. Na₂SO₄). The Na₂SO₄ was filtered to provide FAMES of FO in hexane solution. The FAMES of FO solution was evaporated under vacuum to provide FAMES as golden yellow liquid (1.00 g). The chemical compositions of FAMES from FO were determined with ¹H NMR spectroscopy and GC-MS analysis.

Gas Chromatography-Mass Spectrometry analysis

The EO analysis was performed on a 0.25 mm x 30 m i.d. HP-5MS capillary column coated with a 0.25 μm film thickness. The temperature of quadrupole was 150 °C after injection. Then the column oven was programmed at 5 °C/min to 250 °C and maintained at 250°C for 10 min. Split injection was conducted with a split ratio of 40:1. Helium gas was used as the carrier gas at constant flow-rate of 1.0 mL/min. The ion source temperature was set at 230 °C. Mass spectra were obtained by electron impact ionization mode at 70 eV; mass range, 50-550 amu. The mass of each compounds was compared with those of mass spectra of Wiley7n.1 libraries.

The FAMES of FO analysis was performed on a 0.25 mm x 30 m i.d. HP-5MS capillary column coated with a 0.25 μm film thickness. The temperature of quadrupole was 150 °C after injection. Then the column oven was programmed at 5°C/min to 250 °C, and maintained at 250 °C for 5 min. Split injection was conducted with a split ratio of 20:1. Helium gas was used as the carrier gas at constant flow-rate of 1.0 mL/min. The ion source temperature was set at 230 °C. Mass spectra were obtained by electron impact ionization mode at 70 eV; mass range, 50-450 amu. The mass of each compounds was compared with those of mass spectra of Wiley7n.1 libraries.

RESULTS AND DISCUSSION

Data analysis of essential oil

The dried makhwaen fruit (250.0 g) was extracted with hydrodistillation to obtain the golden yellow essential oil (EO) (12.30 g, 4.92% w/w). The chemical components of EO were investigated by GC-MS. The gas chromatogram in figure 1 showed the relative concentrations of various compounds eluted as a function of retention time. The compounds were identified by comparison of fragmentation patterns in mass spectra with those of Wiley7n.1 libraries as shown in table 1. Totally 24 compounds were identified in the EO, including 11 monoterpene, 6 oxygenated monoterpenes, 4 sesquiterpenes, 1 hydrocarbon and 2 ketones. The major components of EO were α-phellandrene (14.57%), terpinen-4-ol (9.22%), sabinene (6.57%) and α-pinene (5.92%) at the retention time of 6.22, 10.67, 5.49 and 4.70 minutes respectively.

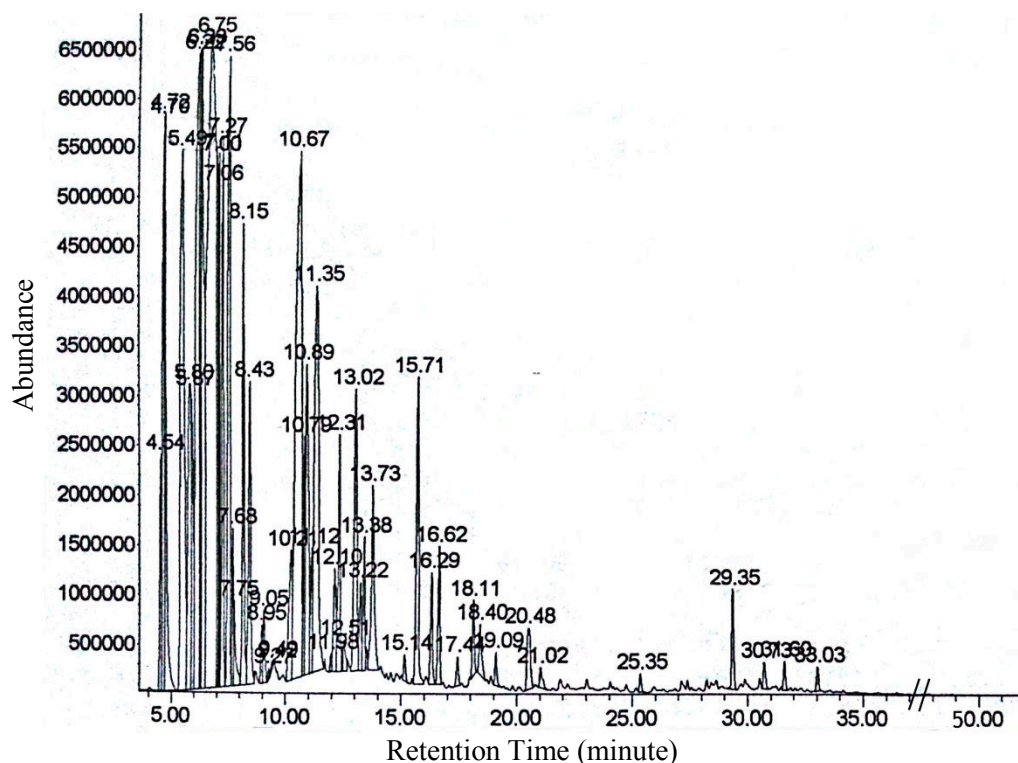


Figure 1 Gas chromatogram of the EO components from makhwaen fruit

In 2007, Rout and coworkers have reported the four major monoterpenes from EO of makhwaen pericarp including sabinene, terpinen-4-ol, β -phellandrene and α -terpineol (Rout *et al.*, 2007). The predominance of monoterpenes in EO from makhwaen pericarp was consistent with the EO from makhwaen fruit. However, the studies of biological activity of EO from the same genus of makhwaen fruit have been previously reported. The EO of *Z. alatum* fruit proved repellent to the insect *Allacophora foveicollis* and fungistatic to 24 fungi, including aflatoxin-producing strains of *Aspergillus flavus* and *A. parasiticus* (Dube *et al.*, 1989). The EO of *Z. fagara* fruit showed fungicidal activity on *Fusarium oxysporum* and *Colletotrichum acutatum* (Prieto *et al.*, 2011). The EO from the *Z. xanthoxyloides* and *Z. leprieurii* fruits were tested for antiproliferative, antimicrobial and antioxidant activities (Fogang *et al.*, 2012). The EO from the *Z. rhoifolium* fruit had an antibacterial activity (Costaa *et al.*, 2008). Therefore, the finding of EO components from makhwan fruit might lead to the study of further interesting bioactivities.

Table 1 Chemical compositions analysis of EO by GC-MS

Retention Time (min)	Compounds found	Molecular Formula	Molecular weight	Peak area (%)
4.55	thujene ^a	C ₁₀ H ₁₆	136	1.24
4.70	α -pinene ^{a*}	C ₁₀ H ₁₆	136	5.92
5.49	sabinene ^{a*}	C ₁₀ H ₁₆	136	6.57
5.80	β -myrcene ^a	C ₁₀ H ₁₆	136	4.47
6.22	α -phellandrene ^{a*}	C ₁₀ H ₁₆	136	14.75
7.00	β -phellandrene ^a	C ₁₀ H ₁₆	136	1.87
7.27	β -ocimene ^a	C ₁₀ H ₁₆	136	4.02
7.56	γ -terpinene ^a	C ₁₀ H ₁₆	136	4.50
7.68	<i>trans</i> -sabinene ^a	C ₁₀ H ₁₆	136	0.54
8.15	α -terpinolene ^a	C ₁₀ H ₁₆	136	2.36
8.43	linalool ^b	C ₁₀ H ₁₈ O	154	2.02
8.95	terpinene ^a	C ₁₀ H ₁₆	136	0.27
10.67	terpinen-4-ol ^{b*}	C ₁₀ H ₁₈ O	154	9.22
10.97	cryptone ^b	C ₉ H ₁₄ O	138	0.81
10.89	α -terpineol ^b	C ₁₀ H ₁₈ O	154	2.66
12.31	carvotanacetone ^c	C ₁₀ H ₁₆ O	152	1.35
12.51	geraniol ^b	C ₁₀ H ₁₈ O	154	2.30
13.38	2-undecanone ^e	C ₁₁ H ₂₂ O	170	0.66
13.74	carvacrol ^b	C ₁₀ H ₁₄ O	150	1.54
16.62	β -caryophyllene ^c	C ₁₅ H ₂₄	204	0.79
17.43	α -humulene ^c	C ₁₅ H ₂₄	204	0.16
18.11	germacrene-D ^c	C ₁₅ H ₂₄	204	0.40
19.08	δ -cadinene ^c	C ₁₅ H ₂₄	204	0.18
21.02	cyclododecane ^d	C ₁₂ H ₂₄	168	0.15

* Major component, ^a Monoterpene, ^b Oxygenated monoterpenes,

^c Sesquiterpene, ^d Hydrocarbon, ^e Ketones

Data analysis of FO from CHE

The residue from hydrodistillation (200.0 g) was extracted with hexane to produce brown CHE 19.27 g (9.64 % w/w). ¹H NMR spectrum of CHE (figure 2A) showed CH₃, CH₂ and allylic protons of the fatty acid fragments at 0.82-2.81 ppm. The CH₂ protons of the glyceryl appeared as two doublet of doublet at 4.10-4.30 ppm. The CH proton of the glyceryl appeared as multiplet at 5.24-5.26 ppm and the vinylic (=CH) protons of the double bonds of the fatty acid chains appeared as a broad multiplet at 5.28-5.43 ppm. All of the ¹H NMR signals were in agreement with the structure of a glycerol bearing unsaturated fatty acid chains as expected for fatty oil (FO).

Data analysis of FAMES from FO

The FO from CHE (2.20 g) was *trans*-esterified into golden yellow FAMES of FO (1.00 g, 45.45% w/w) in order to increase volatility for detection by GC-MS. Then the initial supporting structure of FAMES from FO was examined by ¹H NMR spectroscopy before analyzed by GC-MS. The CH₃O protons of the methyl ester functionality appeared as singlet at 3.63 ppm. The CH₂ protons of the glyceryl

disappeared at 4.10-4.30 ppm and the CH proton of the glyceryl disappeared at 5.24-5.26 ppm (figure 2B). This evidence confirmed that the glyceryl group was replaced by CH₃O group of methanol already. These NMR data confirmed the formation of FAMEs from FO.

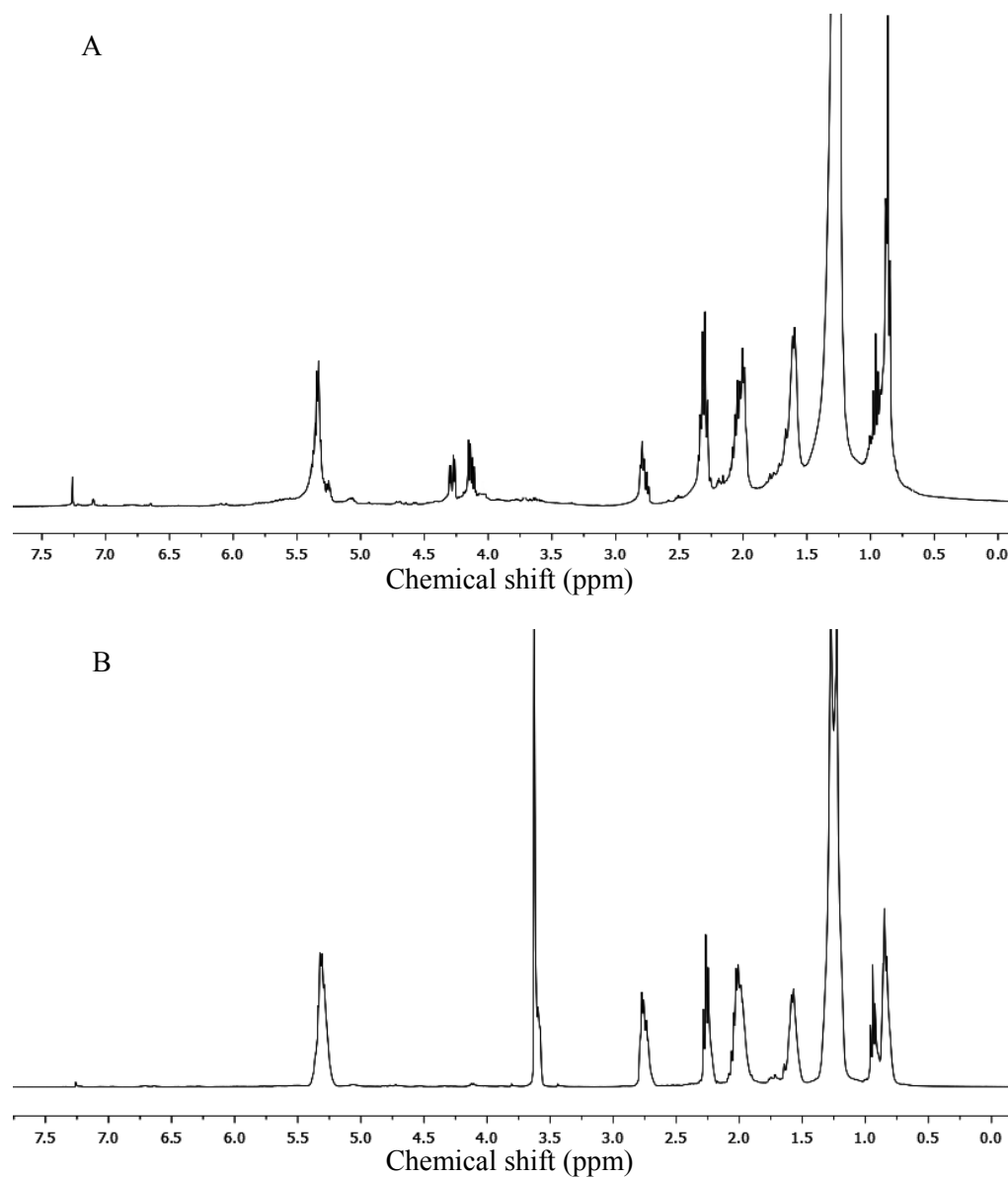


Figure 2 ¹H NMR spectra of CHE (A) and FAMEs (B) from makhwaen fruit

The chemical components of FAMEs from FO were analyzed by GC-MS. The gas chromatogram in figure 3 showed the relative concentrations of various compounds eluted as a function of retention time. The compounds were identified by comparison of fragmentation patterns in mass spectra with those of Wiley7n.1 libraries.

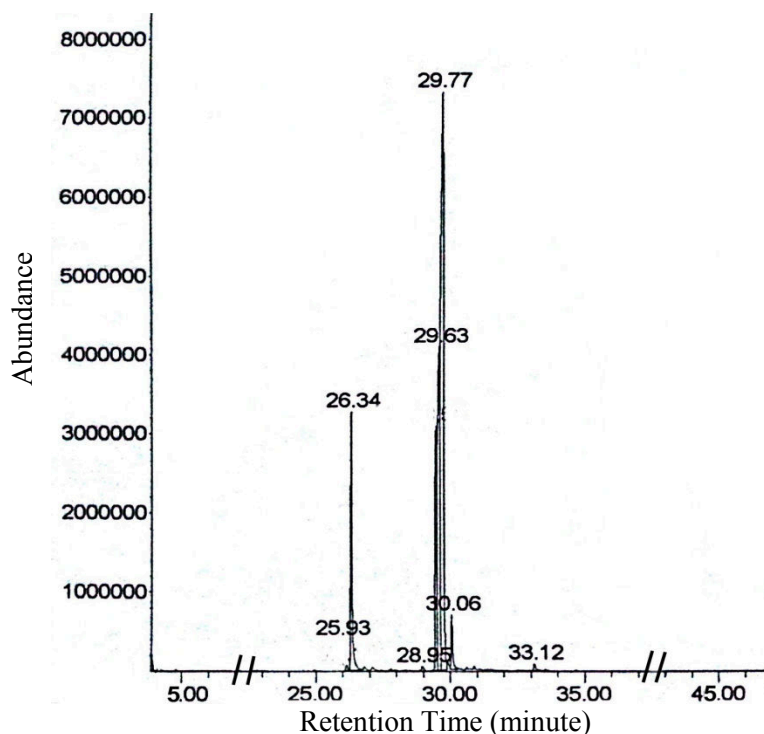


Figure 3 Gas chromatogram of the FAMES from makhwaen fruit

The chemical components of FAMES from FO (Table 2) contained 5 unsaturated fatty acid methyl esters, including α -linolenic acid methyl ester 50.49%, linoleic acid methyl ester 33.08%, palmitoleic acid methyl ester 1.52%, *cis*-gondoic acid methyl ester 0.40% and *cis*-vaccenic acid methyl ester 0.33% at the retention time 29.77, 29.63, 25.93, 33.12 and 28.95 minutes respectively. The 2 saturated fatty acid methyl esters were also found including palmitic acid methyl ester 10.81% and stearic acid methyl ester 2.27% at the retention time 26.34 and 30.06 minutes respectively. Interestingly, α -linolenic acid methyl ester, linoleic acid methyl ester and palmitic acid methyl ester were the major of fatty acid methyl ester components from FAMES. Moreover, small amount of *cis*-vaccenic acid methyl ester was also found. However, these FAMES components were esters version of fatty acids. The relative concentrations of fatty acids from oil samples were measured as their corresponding methyl esters. Therefore, the high percentages of α -linolenic acid methyl ester, linoleic acid methyl ester, palmitic acid methyl ester and small percentage of *cis*-vaccenic acid methyl ester found in FAMES analysis identified the relatively high concentrations of α -linolenic acid (ALA), linoleic acid (LA), palmitic acid and small concentration of *cis*-vaccenic acid containing in FO of makhwaen fruit. The findings were the first report on the study of fatty acid components from makhwaen fruit oil.

Table 2 Chemical compositions analysis of FAMES by GC-MS

Retention Time (min)	Compounds found	Molecular Formula	Molecular weight	Peak Area (%)
25.93	(Z)-9-hexadecenoic acid methyl ester (palmitoleic acid methyl ester) ^u	C ₁₇ H ₃₂ O ₂	268	1.52
26.34	Hexadecanoic acid methyl ester (palmitic acid methyl ester) ^s	C ₁₇ H ₃₄ O ₂	270	10.81
28.95	(Z)-11-octadecenoic acid methyl ester (<i>cis</i> -vaccenic acid methyl ester) ^u	C ₁₉ H ₃₆ O ₂	296	0.33
29.63	(Z,Z)-9, 12-octadecadienoic acid methyl ester (linoleic acid methyl ester) ^u	C ₁₉ H ₃₄ O ₂	294	33.08
29.77	(Z,Z,Z)-9, 12, 15-octadecatrienoic acid methyl ester (α -linolenic acid methyl ester) ^u	C ₁₉ H ₃₂ O ₂	292	50.49
30.06	Octadecanoic acid methyl ester (stearic acid methyl ester) ^s	C ₁₉ H ₃₈ O ₂	298	2.27
33.12	(Z)-11-eicosenoic acid methyl ester (<i>cis</i> -gondoic acid methyl ester) ^u	C ₂₁ H ₄₀ O ₂	324	0.40

^u Unsaturated fatty acid, ^s Saturated fatty acid

The α -linolenic acid (ALA) is well known as an essential omega-3 polyunsaturated fatty acid and it was metabolized to provide eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). EPA and DHA are important for growth, health, brain development and reproduction. Generally, soybean oil (4-11%), rapeseed oil (5-12%) and flaxseed oil (30-60%) were main sources of ALA from plants (Devick, 2009), while fish oils, egg oil, krill oil and squid oils were main sources of ALA from animals (Wikipedia, 2015). This evidence showed that there were a few sources of ALA from plants and animals. Therefore, the finding of high ALA quantity in makhwaen fruit indicated that this plant was an important source of ALA. Furthermore, the ALA had an anti-inflammatory, hypocholesterolemic, cancer preventive, hepatoprotective, nematocide, insectifuge, anti-histaminic, anti-arthritis, anti-coronary, anti-eczemic, anti-acne, 5-alpha reductase inhibitor and anti-androgenic activities (Devi and Muthu, 2014).

In addition, the linoleic acid (LA) is also well known as an essential omega-6 polyunsaturated fatty acid and it was metabolized to provide EPA. LA has been recognized as a flavoring agent in food (JECFA, 2003). Usually, LA was found in almond seed oil (7-30%), borage seed oil (38%), peanut seed oil (13-43%), sesame seed oil (35-50%), cottonseed oil (33-58%), maize embryo oil (34-62%), soybean oil (44-62%), sunflower seed oil (50-70%), evening primrose seed oil (65-80%) and fish

oil (Devick, 2009). Interestingly, the relative major contents of ALA and LA in makhwaen fruit oil were found. Therefore, makhwaen fruit could be considered as a good source for ALA and LA.

Palmitic acid is the most widely distributed of the saturated fatty acids. Generally, almond seed oil (4-9%), sesame seed oil (7-12%), peanut seed oil (7-16%), olive fruit oil (8-21%), theobroma kernel oils (26%) and cottonseed oil (17-29%) were main sources of palmitic acid from plants, while pork lard (25%), suet of sheep and cow (27%) and butter of milk (29%) (Devick, 2009) were main sources of palmitic acid from animals. In a previous report, Harada and coworkers found the antitumor activity of palmitic acid (Harada *et al.*, 2002).

Furthermore, the *cis*-vaccenic acid is an omega-7 fatty acid. This substance is commonly found in animal fats and butter. In addition, the *cis*-vaccenic acid had an anticarcinogenic activity (Lock *et al.*, 2004).

CONCLUSIONS

The essential oil (EO) was hydrodistilled from the fruit of makhwaen. The four major monoterpenes from EO were α -phellandrene (14.57%), terpinen-4-ol (9.22%), sabinene (6.57%) and α -pinene (5.92%). Therefore, the monoterpenes were predominant in the EO from makhwaen fruit.

The dried makhwaen fruit residue from hydrodistillation was further extracted with hexane to provide the crude hexane extract (CHE). The NMR data confirmed the CHE as fatty oil (FO). Then the FO was *trans*-esterified into FAMES. The chemical components of FAMES were analyzed by GC-MS. The major components of FAMES from FO were high amount of α -linolenic acid methyl ester (50.49%), linoleic acid methyl ester (33.08%) and palmitic acid methyl ester (10.81%). Moreover, the small amount of *cis*-vaccenic acid methyl ester (0.33%) was also found. The percentages of each fatty acid methyl esters were analyzed to estimate the relative amount of fatty acids in FO. Therefore, the high percentages of α -linolenic acid methyl ester and linoleic acid methyl ester were related to the high amounts of α -linolenic acid (ALA, omega-3) and linoleic acid (LA, omega-6). These were noteworthy that makhwaen fruit gave a considerable yield of oil. Furthermore, makhwaen fruit could be a good source of omega-3 and omega-6 fatty acids, nutritional applications and pharmaceutical industries. Interestingly, this was the first report on the study of fatty acid components from makhwaen fruit in form of FAMES.

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